AN ABSTRACT OF THE THESIS OF

Sue Sun Wong for the degree of Master of Science in Toxicology presented on September 1, 1989.

Title: In Vitro Metabolism of Pyridine and Chlorpyrifos by Rainbow Trout Liver Microsomes

Abstract approved: Redacted for Privacy

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The focus of this study is the biotransformation of pyridine and chlorpyrifos by rainbow trout liver microsomal enzymes *in vitro*, including optimum incubation conditions, the effect of reactions by cofactors, and the effect of inhibitors and inducers.

Microsomes from untreated and β-naphthoflavone (BNF)-induced trout liver were used to determine the biotransformation of pyridine *in vitro*. The BNF-induced microsomes which had a 2-fold increased P-450 content also showed a 2-fold increased rate of pyridine-N-oxidation compared with the control microsomes. The optimum condition for pyridine-N-oxidase activity was pH 8.5 with more than 2 mg of liver microsomes and a substrate concentration above 4 mM using a 30 min incubation. The K_m and V_{max} value for the pyridine-N-oxidation reaction by control trout liver microsomes was 3.3 mM and 0.212 nmol/min mg under optimum condition and 7.3 mM and 0.067 nmol/min mg at physiological pH. Rabbit antibodies raised against rat NADPH-cytochrome P-450 reductase decreased the

N-oxidase activity to around 80% in both microsomes. P-450 LM_2 IgG had no effect on pyridine-N-oxidase activity whereas LM_{4b} IgG cause 40% inhibition at 60 mg IgG/nmol P-450 concentration. Pyridine-N-oxidation was completely inhibited by n-octyamine and 1-benzyl-imidazole; partial inhibition occured with thiourea and metyrapone, but methimazole had no effect.

The organophosphorous insecticide chlorpyrifos was oxidatively activated to chlorpyrifos oxon and detoxified to 3,5,6-trichloro-2-pyridinol (TCP) by trout liver microsomes. These reactions required NADPH, and were insensitive to pH in the range 7 to 8.5. Kinetic analyses determined the K_m's for formation of chlorpyrifos oxon and TCP by untreated microsomes to be 113 μM and 59 μM, respectively. The V_{max}'s for the same reactions were 0.12 nmol/mg/min and 0.22 nmol/mg/min, respectively. BNF pretreated microsomes had K_m's for formation of chlorpyrifos oxon and TCP of 22 μM and 73 μM, respectively, with V_{max}'s of 0.10 nmol/mg/min and 0.30 nmol/mg/min, respectively. Both reactions were inhibited by cytochrome P-450 substrates and FMO substrates had no effect. However, antibodies against NADPH-cytochrome P-450 reductase and trout cytochrome P-450 LM_2 had little or no effect on inhibition of these reaction. The P-450 LM_{4b} IgG produced 60% inhibition at 40 mg IgG/nmol P-450 content. The rate of hydrolysis of chlorpyrifos oxon to TCP by trout liver microsomes was very low. The K_m's for BNF-induced and control microsomes were 1.58 mM and 1.69 mM, with V_{max}'s of 0.341 nmol/min/mg and 0.389 nmol/min/mg, respectively. Chlorpyrifos oxon hydrolysis by trout microsomes seemed to be a nonenzymatic reaction since it was heat-stable, not stimulated by

calcium ions and was not deactivated by disodium ethylenediamine tetraacetate (EDTA). None of the inhibitors tested in chlorpyrifos metabolism had effect on hydrolysis of chlorpyrifos oxon.

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by

Sue Sun Wong

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of
Master of Science

Completed September 1, 1989

Commencement June 1990

APPROVED:

Redacted for Privacy

Professor of Agricultural Chemistry in charge of major

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Date thesis is presented September 1, 1989

Thesis typed by Sue Sun Wong

TABLE OF CONTENTS

INTRODUCTION	1
Biotransformation of chemicals in fish	3
Pyridine	9
Chlorpyrifos	15
CHAPTER I	
The *In Vitro* Metabolism of Pyridine by Rainbow Trout Liver Microsomes	27
Abstract	28
Introduction	29
Materials and methods	31
Results	36
Discussion	41
CHAPTER II	
The *In Vitro* Metabolism of Chlorpyrifos by Rainbow Trout Liver Microsomes	70
Abstract	71
Introduction	73
Materials and methods	75
Results	80
Discussion	85
CONCLUSIONS	111
BIBLIOGRAPHY	113
APPENDIX A: GC/MS Identification of Pyridine Metabolites	126

LIST OF FIGURES

Figure		Page
1	The structure of pyridine and its biological quaternization reactions	22
2	The proposed metabolite pathway of chlorpyrifos by mouse liver microsomes	25
3	HPLC chromatograms and superimposition of radioactivity of pyridine and its possible metabolites analyzed by μ-Partisil 10-SCX column	49
4	HPLC chromatograms and superimposition of radioactivity of pyridine and its possible metabolites analyzed by μ-Zorbax 10 ODS column	51
5	Time course of pyridine N-oxidation by incubation with rainbow trout liver microsomes	53
6	Effect of protein concentration on pyridine N-oxidation by rainbow trout liver microsomes	56
7	The effect of pyridine concentrations on pyridine N-oxide formation by trout liver microsomes	58
8	Relation of pH and pyridine N-oxidation by rainbow trout liver microsomes	60
9	The difference of pyridine-N-oxidation between optimum pH and physiological pH by tout liver microsomes	62
10	Inhibition of trout liver microsomal pyridine N-oxidation with rabbit antibodies against purified trout cytochrome P-450 LM_2 and LM_{4b}	66
11	HPLC chromatograms of ^{14}C-chlorpyrifos and metabolite standards and a ^{14}C-chlorpyrifos microsomal incubation extract on a μ-Porasil column	90
12	HPLC chromatograms of chlorpyrifos, chlorpyrifos oxon and TCP by a μ-Zorbax SIL column	92
13	Time course of chlorpyrifos biotransformation with a rainbow trout liver microsomal incubation	94
14	Effect of microsomal protein concentrations on the biotrnsformation of chlorpyrifos by rainbow trout liver microsomal incubation	96

LIST OF FIGURES

Figure		Page
15	Effect of pH on the biotransformation of chlorpyrifos by rainbow trout liver microsomes	98
16	Double reciprocal plots for the hydrolysis of chlorpyrifos oxon by trout liver microsomes	101
17	Inhibition of chlorpyrifos metabolism by trout liver microsomes with rabbit antibodies against purified trout cytochrome P-450 LM_2 and LM_{4b}	105
18	The proposed metabolite pathway of chlorpyrifos by rainbow trout liver microsomes	108
19	Mass spectra of pyridine and pyridine-N-oxide	128
20	Mass spectra of 2-OH pyridine and 4-OH pyridine	130
21	Mass spectra of 3-OH pyridine and 3-OH pyridine-N-oxide	132
22	GLC chromatograms of incubation extract from the incubation of pyridine with trout liver microsomes	134
23	Mass spectrum of pyridine recovered from incubation extract samples	136
24	Mass spectrum of pyridine-N-oxide recovered from incubation extract samples	138

LIST OF TABLES

Table		Page
1	Induction of hepatic microsomal monooxygenase activities in rainbow trout	20
2	Comparison of LC_{50} and LD_{50} of bluegill and rat exposed to pesticides	21
3	Quaternization reactions at nitrogen in pyridine	24
4	The effect of BNF pretreatment on rainbow trout liver microsomes	47
5	The retention time of pyridine and its possible metabolites from different HPLC column systems	48
6	The effect of incubation time on the K_m and V_{max} values of pyridine N-oxidation	55
7	The effect of pH on the K_m and V_{max} values of pyridine N-oxidation	64
8	The effect of chemical inhibitors on pyridine N-oxidation by rainbow trout liver microsomes	65
9	The effect of ethanol and pyridine pretreated rainbow trout liver microsomes	68
10	The K_m and V_{max} value of pyridine N-oxidation in various animal liver microsomes *in vitro*	69
11	The K_m's and V_{max}'s of chlorpyrifos metabolites formed by incubation with rainbow trout liver microsomes	100
12	The hydrolysis of chlorpyrifos oxon by boiled liver microsomes from untreated trout	103
13	The effect of inhibitors on the biotransformation of chlorpyrifos and chlorpyrifos oxon by rinbow trout liver microsomes	104
14	Metabolism of chlorpyrifos and chlorpyrifos oxon by trout liver microsomes *in vitro*	107
15	Comparison of K_m's and V_{max}'s of chlorpyrifos and chlorpyrifos oxon metabolized by mouse and trout liver microsomes	110

In Vitro Metabolism of Pyridine and Chlorpyrifos by Rainbow Trout Liver Microsomes

INTRODUCTION

Compounds with nitrogen-containing heterocyclic rings are wildly distributed in nature including endogenous compounds in microorganisms, plants and animal tissues, and also synthetic drugs and pesticides. The metabolic routes of these compounds have received increasing attention during the past decade in order to understand their biological fate. The metabolic reaction of these compounds in animals including oxidation, hydroxylation, reduction, N-oxidation and conjugation. Microsomal oxidation enzymes such as cytochrome P-450 monooxygenase (P-450) and flavin-containing monooxygenase (FMO) play a major role in the metabolism of these nitrogen-containing heterocyclic compounds.

Fish as well as mammals have many enzymes that biotransform xenobiotics. In general, fish enzymes have lower activity and specific contents than those of their mammalian counterparts. Some chemicals are more toxic to fish than mammals. The greater susceptibility of fish to poisoning by chemicals compared with that of mammals may be caused by a more efficient route of absorption (e.g. highly efficiency gill uptake), or by greater target organ sensitivity, or most importantly, by decreased rates of metabolism

and elimination, or by a combination of these factors.

The focus of this research is on the biotransformation of two aromatic heterocyclic nitrogen-containing compounds by trout liver microsomal enzymes. The first chemical to be studied, pyridine, because of its simple structure, has often been used as a model compound for investigating the occurrence and enzymology of each of these metabolite pathway. The second chemical to be investigated is chlorpyrifos, the only insecticide containing pyridiyl structure which has been widely used for thirty years. The biotransformation activity of rinbow trout liver microsomal enzymes on these two compounds, especially under optimum condition for in vitro testing, and the effect of cofactors, inhibitors and inducers will be examined in this study. The background review of pyridine, chlorpyrifos, and biotransformation of chemicals in fish are described below.

BIOTRANSFORMATION OF CHEMICALS IN FISH

Fish can absorb chemicals directly from water as well as by ingesting contaminated food. The toxicity of chemicals to fish depends on the route of absorption, target organ sensitivity, metabolism pathways and rate of excretion. The main uptake of chemicals by fish from water is by passive absorption via the gills and the skin (McKim et al., 1985). After absorption, chemicals are metabolized within the animals and either accumulated in the tissue or terminated by excretion via the bile, urine, feces or gill (Adamson and Sieber, 1974).

Biotransformation of chemicals by enzyme systems of fish, as in other organisms, is important to the toxicity of the chemicals (Buhler and Williams, 1988; Kleinow et al., 1987; Lech and Bend, 1980). The major enzyme systems involved in the biotransformation of chemicals can be classified as Phase I and Phase II reactions. Phase I reactions introduce polar groups into the molecule through oxidative, hydrolytic and reductase processes; Phase II reactions involved conjugation of a chemical or its Phase I metabolites with polar cellular constituents such as glucuronic acid, sulfate or glutathione to form highly water-soluble and easily excreted metabolites (James, 1987). Phase I and Phase II biotransformation reactions work together in a sequential manner to metabolize xenobiotics. Most of xenobiotics are biotransformed to less toxic

metabolites, but some become carcinogenic by biotransformation (Jefcoate, 1983).

The first phase of xenobiotic metabolism by fish consists of reactions in which the xenobiotics undergoes reduction, oxidation or hydrolysis or a combination of these reactions. The enzymes responsible for nitroreduction and azoreduction exist in several species of fish (Adamson, 1967; Buhler and Rasmusson, 1968a). Hydrolysis of xenobiotics is confined to esters and amides and is of particular importance in the detoxication of organophosphate and pyrethroids insecticides. The inability of fish to rapidly hydrolyze pyrethroids (i.e. low content and activity of hydroxy-esterase) may result in a overall low rate of detoxification (Bradbury et al., 1986; Edwards et al., 1987a, 1987b; Glickman et al., 1981; Kao et al., 1985). Oxidation can occurs by various pathways including epoxidation, deamination,aromatic and aliphatic hydroxylation, O-dealkylation, N-dealkylation, N-oxidation, and desulfoxidation (Buhler and Rasmusson, 1968b). These reactions constitute the most important category of Phase I reactions.

The most important enzymes involved in Phase I reactions are the cytochrome P-450 mixed function oxidase (MFO) systems (P-450)(EC 1.14.14.1) and flavin-containing monooxygenase (FMO)(EC 1.14.13.8) which are both located in endoplasmic reticulum and are associated with microsomal enzymes. Epoxide hydrolases and esterases are other important Phase I enzymes. The most important Phase II enzymes are UDP-glucuronyl transferases, sulfotransferases and glutathione transferases. The highest enzyme activity for

transformation of xenobiotics of fish is in the liver, therefore, the liver microsomal enzymes, P-450 and FMO, become the most interested enzymes for metabolism studies.

The cytochrome P-450 dependent monooxygenase of fish species resemble the mammalian enzymes with regard to their ability to catalyze the oxidation of a great variety of lipophilic xenobiotic compounds, including many environmental contaminants such as pesticides and polycyclic aromatic hydrocarbons (PAHs). Highest MFO activity and P-450 content in fish is in liver microsomes; significant MFO activity and P-450 is also found in the intestine and kidney (Funari et al., 1987; James et al., 1977; Stegeman and Kloepper-Sams, 1987).

P-450-Dependent monooxygenase activity of fish can be induced by PAHs, certain coplanar polychlorinated biphenyls (PCBs), polybrominated biphenyls (PBBs), β-naphthoflavone (BNF), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), and isosafrole but not by phenobarbital-type agents such as phenobarbital (PB), kepone, and myrex (Bend et al., 1978; Elcombe and Lech, 1978, 1979; Elsele et al., 1984; James and Bend, 1980; Kleinow et al., 1986; Stegeman et al., 1981; Voldicnik et al., 1981). Table 1 showed the induction of hepatic microsomal MFO activities in rainbow trout (Binder et al., 1984). The induction of cytochrome P-450 in fish may significantly change the rate of biotransformation through altering both the metabolism and toxicity of xenobiotic chemicals. For example, the LC_{50} values for rotenone in rainbow trout was decreased by piperonyl butoxide (PBO) exposure and increased by

BNF administration (Erickson et al., 1986). BNF is also a potent inhibitor of aflatoxin B_1 (AFB_1) hepatocarcinogenesis in trout by inducing MFO activity and altering AFB_1 metabolism to favor of detoxication (Bailey et al., 1984; Williams and Buhler, 1983a). The inducer may be the pollutant in the water that affected the physiological status of the fish. High MFO activity and impaired reproductive success was found in fish collected in polluted sites (Lee, 1988).

Cytochrome P-450 has many subfamilies (Nebert and Gonzalez, 1987), and the isozymes can be induced by different compounds (Papkinson et al., 1988). Multiple P-450 isozymes have been described for several fish species, each possessing somewhat different catalytic properties (Ahokas et al., 1977; Stegeman and Klopper-Sams, 1987). The biotransformation products of certain chemicals have been related to specific P-450 isozymes, and the formation of these products can be influenced by induction (Elcombe and Lech, 1979). The two major isozymes, P-450 LM_2 and LM_4, have been isolated and purified from the liver and kidney of rainbow trout (Williams and Buhler, 1982, 1983b; Williams et al., 1986), and other P-450 isozymes have been isolated from the marine fish (Klotz et al., 1983, 1986). In rainbow trout P-450 LM_2 is a major constitutive isozyme which is active towards lauric acid and is very effective at activating AFB_1 to the carcinogenic and DNA-binding metabolite, AFB_1-2,3 epoxide (Williams and Buhler, 1983a, 1984). Trout P-450 LM_2 is not induced following exposure to BNF or PCBs. By contract, trout P-450 LM_4, which is barely detectable in

untreated fish, is induced by BNF, PCBs and 3-methylcholanthrene (3-MC), and has high benzo[a]pyrene hydroxylase activity (Williams and Buhler, 1982, 1983b, 1984).

Flavin-containing monooxygenase is the major enzyme catalyzing the oxidation of lipophilic xenobiotics especially organic nitrogen and sulfur compounds (Tynes and Hodgson, 1985; Ziegler, 1984). Recently research has found that the enzyme exists not only in endoplasmic reticulum in the tissue of liver, lung, kidney and brain in mammalian animals (Tynes and Hodgson, 1985a, 1985b; Ziegler, 1980, 1988), but also in the postmitochondrial fraction and microsomes of the digestive grand in marine mussel (Kurelec, 1987; Kurelec et al., 1986) and in the gill, intestine, liver, kidney of rainbow trout (Schlenk, 1989). More than one specificity for the enzyme have been identified and more chemicals have been found to be oxidized by this enzyme. Compounds undergoing FMO-mediated reaction include primary arylamines, secondary amines, hydrazines, thiocarbamates, thioamides, sulfides disulfides and thiols (Hajjar and Hodgson, 1980, 1982; Ziegler, 1980, 1984, 1988; Ziegler et al., 1980).

Cytochrome P-450 and FMO have many common characteristics, including location in the endoplasmic reticulum, NADPH and oxygen required for activity, and similar broad substrate specificity. Selective inhibitors, positive effectors or immunochemical cytochrome P-450 reductase antibodies were used to distinguish the contribution of these two enzyme on xenobiotic metabolism (Tynes and Hodgson, 1983, 1984).

Although fish and mammals share many common enzyme content and activities, the toxicity of many chemicals, especially pesticides, are quite different between these two groups of animals. For example, parathion is toxic to both fish and mice whereas methyl parathion is less toxic to fish than to mice. Chlorpyrifos and fenpropathrin, however, are more toxic to fish than to mice (Table 2). Except the different route of absorption and target organ sensitivity, the amount and activity difference of biotransformation enzymes which catalyze desulfuration, ester hydrolysis and conjugate formation in these two animals are the major factor of the toxicity (Benke et al., 1974; Edwards et al., 1986, 1987a; Glickman and Lech, 1981, 1982; Maemura and Omura, 1983; Melancon et al., 1985).

PYRIDINE

Pyridine (structure as in Fig. 1 (I)), which was discovered during the years 1845-51, is found in a variety of industrial and laboratory settings. It is used as a solvent in drug manufacture, a reagent in industrial processes where hydrochloric acid is evolved , and in solution phosgenation processes for making polycarbonate resins. Pyridine also serves as an intermediate in manufacture of antihistamines and anti-infectives (Reinhardt and Brittelli, 1981).

The chemical behavior of pyridine, in particular its great stability and general similarity to benzene, could be explained by a cyclic formula (Kekul'e structure). The pyridine base (pK_a = 5.2) is a stable colorless liquid with characteristic unpleasant smell, completely water miscible resulting from hydrogen binding with nitrogen atoms, stable to acid and alkali , and is not oxidized by either chromic acid or fuming nitric acid. Pyridine is also a very good solvent. Nearly all classes of organic compounds are soluble in pyridine (Sidgwick, 1966).

Pyridine has relatively low acute toxicity; the acute oral LD_{50} for rats or mice is 0.8-1.6 g/kg. Pyridine can be absorbed from the gastrointestinal tract, intraperitoneal cavity and lungs. Its major actions are local irritation and nervous system depression with narcosis. Effective doses by any route produce

weakness, ataxia, unconsciousness, and salivation, but convulsions are uncommon. With exposure to pyridine vapors, there are symptoms of moderate mucous membrane irritation. Injection of 20 mg pyridine into chicken eggs caused typical muscular hypoplasia of the legs and, on rare occasions, abnormality of the facial skeleton and neck vertebrae. Pyridine incorporated into a choline- and casein-deficient diet of rats caused chronic liver and kidney damage with fatty changes and fibrosis (Reinhardt and Brittelli, 1981). Pyridine partially inhibits the potassium conductance of the nervous system, but pyridine derivatives such as 4-OH pyridine and 4-amino pyridine induced transmitter release in the nervous system (Montoya et al., 1984; Pelhate and Sattelle, 1982).

Pyridine has often been used as a model compound for studying the enzymology of the azoheterocyclic metabolite pathway (Namboodiri, 1987). The metabolic options available to nitrogen heterocyclics include: (a) reactions at a tertiary heteroaromatic ring nitrogen leading to a greater formal positive charge on nitrogen, i.e. quaternization, for example, N-protonation, N-methylation, N-oxygenation and N-glucuronidation - all such reactions may be technically defined as "N-oxidation" (Fig. 1 (II)); (b) ring oxidative reactions at carbon leading to hydroxylated products and in some cases to ring cleavage; and (c) metabolic reactions at the electronegative heteroatoms (Caldwell, 1982; Damani, 1985).

The pyridyl nitrogen is a likely target for electrophilic enzymatic oxidation because of its relatively high electro-

negativity and its unshared pair of electrons. Electron density calculations show that electrophilic attack in pyridine is favored at C-3 whereas nucleophilic attack occurs preferentially at C-2 (Damani and Crooks, 1982). C-Oxidation (ring hydroxylation) does not appear to be an important in vivo route of metabolism for pyridine, since most of the dose is eliminated either as the N-oxide or as the N-methyl pyridinium (Table 3) (Damani, 1985).

N-Oxidation of nitrogen-containing organic compounds varies by species, age, pH optimum, inducers and inhibitors (Gorrod, 1973). Pyridine N-oxide was a quantitatively important metabolite of pyridine. It is a major product formed when pyridine undergo cytochrome P-450 mediated oxidation in vitro and in vivo (Blaauboer and Paine, 1980; D'Souza et al., 1980; Gorrod and Damini, 1979a, 1979b, 1980). The amount of N-oxidation products excreted varies widely with the animal species and sex. The percentage of the dose excreted in the urine as pyridine N-oxide tested in six different animal species ranged from 10% in rats to about 40% in mice and guinea pigs in the first 24 hours after administration (Gorrod and Damini, 1980). The activity of pyridine-N-oxidase resides mainly in the hepatic and pulmonary microsomal fractions of the rabbits and guinea pigs (Gorrod and Damini, 1979a).

N-methylation of pyridine was first reported in dog in 1887 by His and this route has proven to be the very important route of metabolism for pyridine in vivo (D'Souza et al., 1980; Okuda, 1959a, 1959b; Shaker et al., 1982). Pyridine methylation was also found to be species and sex dependent. Methylation was

extensive (20-40% dose) in the cat, guinea pig, gerbil, rabbit and hamster but was low (5-12% dose) in rat, mouse and man (D'Souza et al., 1980). Pyridine N-methyltransferase activity resides mainly in the pulmonary cytosolic fraction, and to a lesser extent in the hepatic and renal cytosolic fractions of rabbit tissues. Pyridyl N-glucuronide was not detected when several animal species were examined (Damini, 1985).

Results of induction, inhibition and activation studies of pyridine metabolisms revealed that the reaction was mediated by a PB-inducible cytochrome P-450 system but was not affected by 3-MC or BNF inducers in mammals (Gorrod and Damini, 1979b, 1980; Kim et al., 1988). 2-Diethylaminoethyl-2,2-diphenylvalerate (SKF-525A), 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) and carbon monoxide caused partial inhibition whereas n-octylamine and 1-naphthylurea completely inhibited N-oxidation of pyridine _in vitro_ (Gorrord and Damini, 1979b, 1980).

Pyridine also inhibited the P-450 catalyzed drug metabolism by noncompetitive inhibition of N,N-dimethylaniline (DMA) and p-nitroanisole (PNA) demethylation _in vitro_ and decreased hexobarbital metabolism _in vivo_. Chronic administration of pyridine to rabbits resulted in increased hepatic microsomal cytochrome P-450 content but did not change FMO content and activity. In the rabbit pyridine-induced P-450 has further been identified as P-450 LM_{3a} isozyme with induction of different forms exhibiting elevated catalytic activations toward pyridine, N-nitrosodimethylamine, alcohols and aniline (Kaul and Novak, 1987;

Kim et al., 1988).

Pyridine, cannot be N-oxygenated by pure hog liver FMO (Ziegler, 1980) but this oxidation is catalyzed by cytochrome P-450 (Gorrod, 1982). Pyridines in which the 3-substituent was -H, $-CH_3$, $-C_2H_5$, -Cl, -Br, -F, $-COCH_3$, -CN, $-CONH_2$ and $-CON(C_2H_5)_2$ were shown to be N-oxidized by microsomal fractions; however, no N-oxides were detected as metabolites when the 3-substituent was $-NH_2$, $-NHCOCH_3$, -COOH, -OH, or $-CH_2OH$, and 2-substituted or 2,6-disubstituted pyridines have not been reported to form N-oxides (Danami and Crooks, 1982). These differences may caused by differences in the pK_a's, hybridization states of electrons or stereochemical factors (Gorrord, 1973, 1985; Hlavica, 1982).

Although metabolism of pyridine has been studied since 1887, many questions regarding the biotransformation of pyridine remain unanswered. One of the difficulties of analysis is based on the distinct structure and physical-chemical properties of pyridine and its derivatives, e.g. its high vapor pressure, ionized form and thermo-unstability (Hlavica, 1982). Paper chromatography, TLC, and GLC methods have been used to separate the metabolites from <u>in vivo</u> or <u>in vitro</u> experiment (Damini et al., 1978; Gorrod, 1985; Gorrod and Damini, 1978; Matkovics et al., 1975; Okuda 1959a, 1959b). These studies have recently been stimulated owing to the development of new, highly sensitive techniques. HPLC with a combination of cation-exchange and reversed-phase columns is now commonly used for this purpose (Blaauboer and Paine, 1980; Shaker et al., 1982).

No reports are available describing the enzyme system mediating the metabolism of pyridine in fish or other aquatic organisms.

CHLORPYRIFOS

Chlorpyrifos [0,0-diethyl-0-(3,4,6-trichloro-2-pyridyl) phosphothioate] is the only phosphate insecticide which contains a pyridine ring. This broad spectrum insecticide registered for control of mosquito and agricultural pests including bertha armyworms, cutworms, root maggots and chinch bugs has been used for over 30 years in various crops around the world.

Although chlorpyrifos is a pyridine-containing insecticide, the physical and chemical properties are quite different from pyridine. Chlorpyrifos is only slightly soluble in distilled water (0.4 mg/L at 23°C) but very soluble in organic solvents. It is strongly lipophilic (log P = 4.81) and tends to readily partition from water to organic matter (K_{OC} = 13,600). According to its air to water partition coefficient (8.9 x 10^{-4} at 25°C), chlorpyrifos is slightly volatile from water. The half-life of chlorpyrifos in aqueous solution is affected by pH, metal ions, temperature and light intensity, yielding 3,5,6-trichloro-2-pyridinol (TCP) as its hydrolysis products (Marshall and Robert, 1978).

The toxicity of chlorpyrifos is by inhibition of acetylcholinesterase in animals. This action causes accumulation of the neural transmitter molecule acetylcholine at nerve junctions and in turn, disruption of normal transmission of nerve impulses, resulting in death (Eto, 1977).

Chlorpyrifos, as other organothiophosphate insecticides,

must be biotransformed to its oxygen analogues (chlorpyrifos oxon) to show its biological activity (Neal and Halpert, 1982; Ohkawa, 1982). Most of these oxidation reactions were catalyzed by P-450 (i.e. parathion, methyl parathion and diazinon) but some were by FMO (i.e. fonofos) (Hajjar and Hodgson, 1982; Halpert et al., 1980; Kulkarni and Hodgson, 1984). In vitro tests with housefly brain cholinesterase (ChE), chlorpyrifos caused little or no inhibition whereas chlorpyrifos oxon was a potent ChE inhibitor. Other chlorpyrifos metabolites including TCP, 3,5,6-trichloro-2-pyridyl phosphate, ethyl O-3,5,6-trichloro-2-pyridyl phosphate and ethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate, all shown to have no effect on ChE activities (Smith et al. 1967). In vivo tests, however, demonstrated that chlorpyrifos and its dechlorinated derivatives were potent ChE inhibitors (Hutacharern and Knowles, 1974, 1975). Chlorpyrifos and TCP are also active as uncouplers of respiratory chain phosphorylation (Abo-Khatwa and Hollingworth, 1974).

Biotransformation of chlorpyrifos in vivo showed that the only metabolites identified in the body tissue of rat were chlorpyrifos, TCP, and occasionally very small amounts of chlorpyrifos oxon. The principal metabolites that have been identified in the urine and feces of rats exposed to chlorpyrifos are TCP, diethyl phosphorothioic acid, diethyl phosphoric acid, and trace amounts of chlorpyrifos. The chlorpyrifos oxon has not been detected (Smith et al., 1967). TCP is the only metabolite found in human urine after oral and dermal administration of chlorpyrifos (Nolan et al.,

1984).

Branson and Wass (1970) demonstrated that chlorpyrifos acted as a substrate for rat liver microsomal enzymes requires NADPH and TCP was the major metabolite. Chlorpyrifos could be biotransformed to form detectable chlorpyrifos oxon and TCP by mouse hepatic microsomes in the presence of EDTA as an esterase inhibitor. This reaction required NADPH and was inhibited by carbon monoxide. Chlorpyrifos oxon can be hydrolyzed to TCP by esterase in a reaction that is NADPH independent, activated by calcium and inhibited by EDTA. The hydrolysis of chlorpyrifos oxon is also catalyzed by the nonenzymatic phosphorylation of certain hepatic proteins. The proposed metabolite pathway of chlorpyrifos by mouse liver microsomes is shown in Fig. 2 (Sultotas and Murphy, 1983a, 1983b). Chlorpyrifos oxon is also a competitive inhibitor of paraoxonase present in mice microsomes and in human serum (Furlong et al., 1988; Sultatus and Murphy, 1983b). The chlorpyrifos oxonase activity in human serum has been used recently to distinguish the genetic type of paraoxonase polymorphism (Furlong et al., 1988). Pretreatment of mice with PB resulted in an increase in the oxidative activation and detoxification of chlorpyrifos _in vitro_, and also increased hydrolysis of chlorpyrifos oxon by liver microsomes whereas pretreatment with BNF gave the opposite results as with PB-pretreatment. The mortality of BNF treated mice caused by chlorpyrifos is higher than control while PB-treatment gave a lower mortality rate compared with the control group (Sultatus et al., 1984).

Chlorpyrifos is less toxic to mammals but highly toxic to fish, compared with some organophosphothioate insecticides such as parathion, fonofos, and methyl parathion. The reported 24-96 hr LC_{50} value for a number of salt-and freshwater species based on static bioassays ranged from 4 to 550 μg/L in water (Clark et al., 1985; Marshall and Robert, 1978). Chlorpyrifos has been used directly in ponds for mosquito control. The distribution of the pesticide and its effects on fish were thus a matter of concern. Chlorpyrifos distribution in an actual field study was predicted to be 11.4% of chlorpyrifos was evaporated, 11% metabolized by fish and 76% hydrolyzed (Neely and Blau, 1977). The chlorpyrifos residues are high in bottom sediments after pond application because the pesticide is highly lipophilic and strongly adsorbed to organic matter. Bottom-dwelling fish, such as channel catfish and black crappie, can, therefore, accumulate high concentrations of chlorpyrifos after 2 to 3 weeks posttreatment (Mulla et al., 1973, 1979). Chlorpyrifos has been investigated as a cause of toxicity to fish and aquatic invertebrates in ponds by direct toxicity or indirectly by reducing dissolved oxygen content and increasing free carbon dioxide in natural waters (Hurlbert et al., 1970, 1972; Mani and Konar, 1988). Continuous exposure of fish in low chlorpyrifos concentration (0.5 μg/l) reduced fish weight in an early life-stage test (Goodman et al., 1985).

Smith et al. (1966) investigated the metabolism of ^{14}C-labelled chlorpyrifos in goldfish subjected to the chemical in a static-flow model ecosystem. Their studies reveal that

chlorpyrifos is rapidly absorbed from the water and degraded by goldfish. Five metabolites were identified by TLC and paper chromatography as: chlorpyrifos, ethyl O-3,5,6-trichloro-2-pyridyl phosphothioate, ethyl O-3,5,6-trichloro-2-pyridyl phosphate, 3,5,6-trichloro-2-pyridyl phosphate, and TCP. Chlorpyrifos oxon was not detected by chromatographic analyses nor by fly-brain ChE assays of various fish homogenate fractions. The identified metabolites were shown not to be significant inhibitors of fly-brain ChE. Metcalf (1974) also reported the distribution of chlorpyrifos and its degradation products in mosquitofish exposed for 3 days in a model ecosystem which had been treated with ^{14}C-chlorpyrifos 30 days prior to introduction of the fish. Chlorpyrifos and TCP were the major products identified. Again, chlorpyrifos oxon was not found. Thus, in fish, as in mammals and birds, detoxification of chlorpyrifos appears to proceed by hydrolysis to TCP, much of which is then eliminated.

Low biotransformation activity towards chlorpyrifos may play a role in the high acute toxicity of the insecticide towards fish as compared to that seen in mammals. No reports have appeared on the *in vitro* metabolism of chlorpyrifos by fish.

Table 1. Induction of Hepatic Microsomal Monooxygenase Activities in Rainbow Trout[a]

Treatment[b]	Dose (mg/kg)	EMD[c]	BeND[d]	AHH[e]	ECOD[f]	EROD[g]
Corn oil	--	100	100	100	100	100
PB	65	81	--	104	64	65
TCB	150	--	101	--	467	5281
TCDD	0.012	--	89	--	2892	1414
BNF	100	88	--	4081	1178	4455
PCBs	150	105	49	1300	509	1460

a. Data from Binder et al. (1984) is expressed as percent of corn oil controls.

b. The compounds used as inducers are phenobarbital(PB), 3,4,3',4'-tetrachlorobiphenyl (TCB), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), β-naphthoflavone (BNF), and polychlorobiphenols, Arochlor 1254 (PCBs)

c. Ethylmorphine N-demethylase (EMD)

d. Benzphetamine N-demethylase (BeND)

e. Aryl hydrocarbon hydroxylase (AHH)

f. Ethoxycoumarin O-deethylase (ECOD)

g. Ethoxyresorufin O-deethylase (EROD)

Table 2. Comparison of LC_{50} and LD_{50} of Bluegill and Rat Exposed to Pesticides

Pesticide	Bluegill LC_{50} (96 hr, $\mu g/l$)	Rat Oral LD_{50} (mg/kg bw)
Parathion	95[a]	7.6[b]
Methyl parathion	1900[a]	10.1[b]
Chlorpyrifos	3.6[b]	166 [b]
Fenpropathrin	2.0[c]	107 [c]

[a]. Data cited from Benke et al., 1974

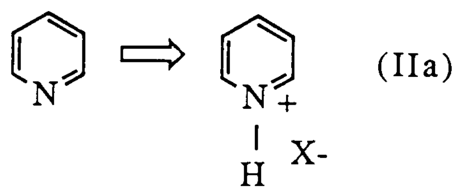
[b]. Data cited from Marshall and Roberts, 1978

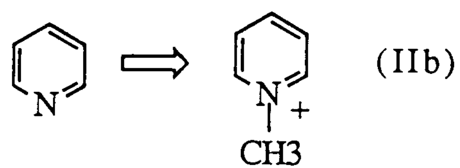
[c]. Data cited from Smith and Stratton, 1986

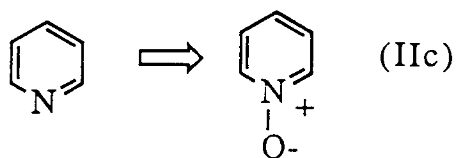
Fig. 1. The structure of pyridine and its biological quaternization reactions.

Pyridine (I), Pyridine protonation (IIa), Pyridine N-oxygenation (IIb), Pyridine N-methylation (IIc), and Pyridine N-glucuronidation (IId).

Fig. 1.







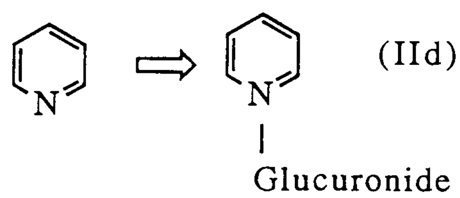


Table 3. Quaternization Reactions at Nitrogen in Pyridine

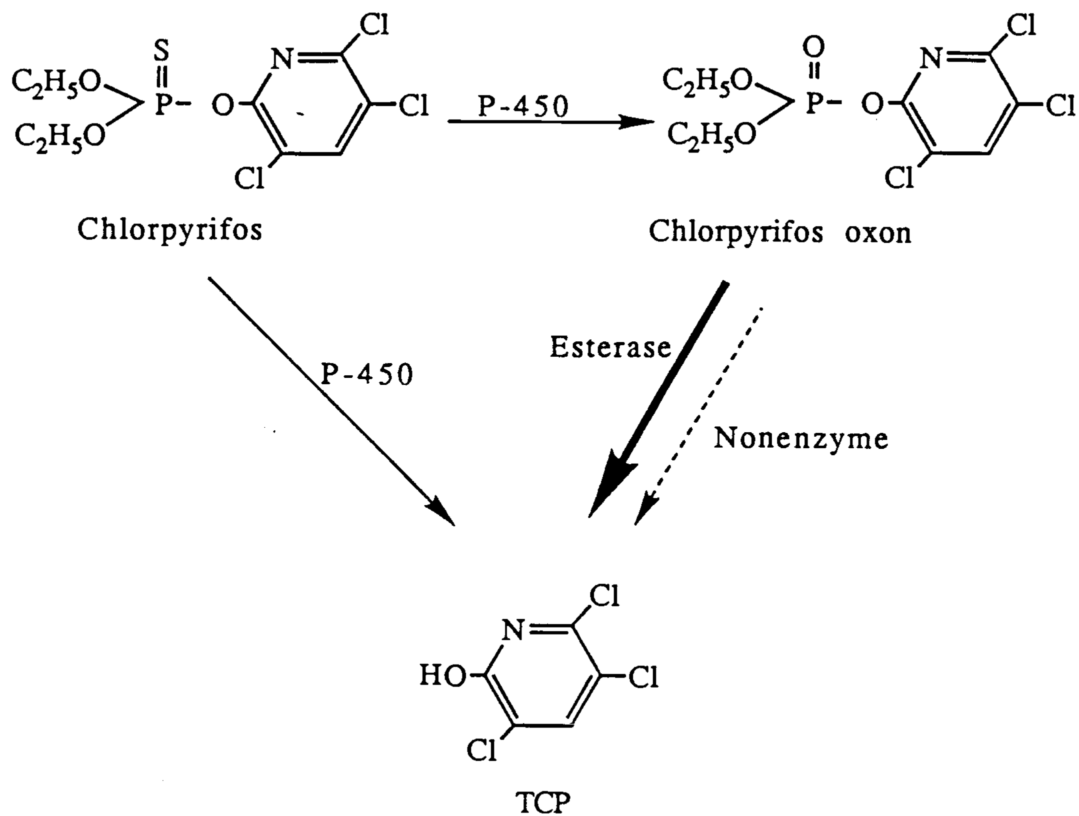
	% Dose excreted in urine (0-72 hr) as: (dose 5 mg/kg body weight)		
Species	N-methyl-pyridinium	Pyridine N-oxide	Pyridine N-glucuronide
Hamster	49.6 ± 10.5	13.5 ± 3.3	<5
Guinea pig	31.7 ± 4.3	9.9 ± 7.0	<5
Rabbit	12.6 ± 4.8	13.2 ± 4.8	<5
Rat	8.1 ± 0.3	11.0 ± 1.6	<5
Mouse	6.3 ± 1.0	15.0 ± 3.0	<5

Fig. 2. The proposed metabolite pathway of chlorpyrifos by mouse liver microsomes

Chlorpyrifos is metabolized to chlorpyrifos oxon and TCP by mouse liver microsomes. P-450 catalyzed chlorpyrifos desulfuration and deesterification; chlorpyrifos oxon further metabolize to TCP by esterase (major pathway) and nonenzymatic phorsphoration (minor pathway).

Reference from Sultatos and Murphy, 1983b

Fig 2.



The *In Vitro* Metabolism of Pyridine by Rainbow Trout Liver Microsomes

by

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ABSTRACT

Microsomes from untreated and β-naphthoflavone (BNF) induced trout liver were used to determine the biotransformation of pyridine *in vitro*. The BNF-induced microsomes which had a 2-fold increased P-450 content also showed a 2-fold increased rate of pyridine-N-oxidation compared with the control microsomes. The optimum condition for pyridine-N-oxidase activity was pH 8.5 with more than 2 mg of liver microsomes and a substrate concentration above 4 mM using a 30 min incubation. The K_m and V_{max} value for the pyridine N-oxidation reaction by control trout liver microsomes was 3.3 mM and 0.21 nmol/mg/min under optimum condition and 7.3 mM and 0.07 mol/mg/min at physiological pH condition. Ethanol and pyridine pretreated rainbow trout liver microsomes had no significant difference on comparing the protein content or P-450 content or pyridine-N-oxidation activity.

Rabbit antibodies raised against rat NADPH-cytochrome P-450 reductase decreased the activity to around 80% in both microsomes. Antibodies prepared against purified trout P-450_s were used in additional studies. P-450 LM_2 IgG had no effect on pyridine-N-oxidase activity whereas LM_{4b} IgG caused 40% inhibition at 60 mg IgG/nmol P-450 concentration. Pyridine-N-oxidation was completely inhibited by n-octyamine and 1-benzylimidazole, partial inhibited by thiourea and metyrapone, and methimazole had no effect.

INTRODUCTION

Compounds classified as heteroaromatic tertiary amines are wildly distributed in nature. N-oxidation seemed to be a major pathway in the electrophilic enzyme metabolism of these compounds because of their relatively high electronegativity. Pharmacological or toxicological activity of organic nitrogen compounds is always changed by N-oxidation (Hlavica, 1982; Bickel, 1969).

Pyridine with its simple structure has often been used as a model compound for studying the occurrence and enzymology of each of these metabolic pathways. Pyridine shows a drift of electrons from the ring carbons to the electronegative nitrogen. Therefore, chemical quaternization reactions readily take place at the nitrogen atom, including protonation, N-oxide formation, N-methylation and N-glucuronidation (Damini, 1985).

Pyridine has been reported to undergo cytochrome P-450 mediated N-oxidation and hydroxylation in mammals (Gorrord and Damimi, 1979a, 1979b, 1980; Blaauboer and Paine, 1980; D'Souza et al., 1980; Kim et al., 1988). The activity of pyridine-N-oxidase resides mainly in the hepatic and pulmonary microsomal fractions of the rabbits and guinea pigs (Gorrod and Damani, 1979a) and the pyridine-N-oxidation was mediated by a PB-inducible cytochrome P-450 system but 3-MC or BNF induction had no effect (Gorrod and Damani, 1979b, 1980; Kim et al., 1988). 2-Diethylaminoethyl-2,2-

diphenylvalerate (SKF-525A), 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) and carbon monoxide caused partial inhibition whereas N-octylamine and 1-naphthylurea completely inhibited N-oxidation of pyridine in vitro (Gorrord and Damini, 1979b, 1980).

No reports are available describing the enzyme system mediating the metabolism of pyridine in fish or other aquatic organism. Since fish enzymes have properties comparable to those of mammals by induction activity and metabolites activity, it is of interest to study pyridine metabolites by fish enzyme systems. The purpose of this research is an investigation of the in vitro biotransformation of pyridine by rainbow trout liver microsomal enzymes with and without β-naphthoflavone pretreatment; establishing optimum incubation conditions; and investigating the effects of inhibitors and inducers on the reaction.

MATERIALS AND METHODS

Materials

Analytical Standards

Pyridine [ring-2,6-^{14}C] 50 μCi (specific activity 7.3 mCi/mmole; 95% purity) was purchased from ICN Radiochemicals Co. Pyridine anhydrous (>99%), pyridine N-oxide (95%), 2-hydroxypyridine (97%), 3-hydroxypyridine (98%), 4-hydorxypyridine (95%), and 3-hydroxy-pyridine-N-oxide (99%) were purchased from Aldrich Co., Milwaukie, WI.

Microsomes preparation buffers

The homogenization buffer A contained 0.1 M Tris-acetate (pH 7.4), 0.1 M KCl, 1 mM disodium ethylenediamine tetraacetate (EDTA) and 0.1 mM phenylsulfonyl fluoride (PMSF). Buffer B (for washing) contained 0.1 M potassium pyrophosphate (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.1 mM PMSF. Buffer C (for storage) contained 0.1 M potassium phosphate (pH 7.25), 20% glycerol and 0.1 mM PMSF.

Antibodies

Rabbit polyclonal antibodies raised against rat NADPH-cytochrome P-450 reductase (reductase IgG) and rabbit antibodies

raised against purified trout P-450 LM_2 (LM_2 IgG) were prepared in our lab previously (Williams and Buhler, 1983b); and rabbit antibodies raised against purified trout P-450 LM_{4b} (LM_{4b} IgG) was from the laboratory of Dr. D.E. Williams.

Experimental Procedure

Microsomes preparation

One year old rainbow trout (Oncorhynchus mykiss formerly named Salmo gairdneri) with average body weight 80 to 100 g were used for liver microsomes preparation. BNF-induced microsomes were prepared from duplicate lots of fish (30 per tank) and fed either the control diet or diet containing 500 ppm BNF for 14 days and then starving for 2 days before sacrifice. Ethanol and pyridine induced trout liver microsomes were prepared by using 3 groups (20 per group) of fish. The first group was treated by i.p. injection of 100% ethanol (0.5 ml per fish); second group was treated with an i.p. injection of 0.5 ml pyridine in aqueous solution (50 mg/kg body weight); and the third group was given i.p. injections of 0.5 ml distilled water. These three groups of fish were sacrificed 66 hours after treatment. The fishes were killed by electric shock and the liver excised and weighed. Livers were placed in ice-cold buffer A, separated to 4 groups (6 to 8 fish livers per group), minced, rinsed and homogenized in 3 volumes of buffer A using a motor driver Potter-Elvehjem-type glass-teflon homogenizer. Each liver homogenate was centrifuged at 10,000 g for 30 min at 4°C, and

the supernatant was decanted and centrifuged at 100,000 g for 90 min. The microsomal pellet obtained was resuspended in buffer B and centrifuged again at 10,000 g for 60 min. The washed pellet was suspended in buffer C to a concentration equivalent to a wet weight of liver per milliliter of suspension. All microsomes were storage at -80°C before use.

Microsomal protein content was determined by the colorimetric method of Lowry et al. (1951) using a bovine serum albumin standard. Cytochrome P-450 content was measured by the method of Estabrook et al. (1972) using an extinction coefficient of 91 mM^{-1} cm^{-1}.

Incubation Conditions

Pyridine 5 mM (nonradioactive or ring-2,6-^{14}C-pyridine 1 μCi) was used as a substrate for the microsomal incubations. Each incubation mixtures consisted of the following: Tris buffer (pH 8.5, 0.1 M), NADPH 0.5 mM, microsomes 2.5 mg and make to a final volume of 1 ml with distilled water. The reaction was started by adding substrate, incubated for 30 min at 25°C in a shaking water bath at 120 rpm and terminated by immediately placing in the ice water.

Metabolites Analysis Procedure

Incubation mixtures were centrifuge at 20,000g for 30 min at 4°C and the resulting suspension then removed from the centrifuge tube. The suspension was directly injected (50-100 μl) into a high

performance liquid chromatograph (HPLC) for analysis.

Spectra-Physics SP 8700 HPLC equipped with a Beckman 153 UV detector set at 254 nm was used to determine non-radioactive samples. A Shimadzu LC-6A HPLC equipped with a Beckman 171 isotope detector combine with SPD-6AV UV-visible spectrum detector (set at 254 nm) was used to analyze samples containing radioactivity. Two analytical systems were used to separate the potential *in vitro* metabolites of pyridine using modified conditions from Shaker et al.(1982). A Whatman Partisil-10 SCX microparticle column (4.6 mm x 25 cm) with mobile phase of ammonium acetate buffer (0.3 M, pH 3.7) and a flow rate 1.0 ml/min was used to determine the formation of pyridine-N-oxide and 3-OH pyridine. A DuPont Zorbax ODS microparticle column (4.6 mm x 25 cm) with a mobile phase of 0.1 M phosphate buffer (pH 7.4) contained 5% methanol and a flow rate of 1.0 ml/min was used to confirm identification of the metabolite products. The latter conditions also were used to separate ^{14}C-pyridine metabolites.

Immunological Studies

Inhibition of trout liver microsomal pyridine-N-oxidation activity was examined with P-450 reductase IgG, LM_2 IgG and LM_{4b} IgG. Various amounts of IgG were preincubated with microsomal protein for 10 min at 25^oC, the remaining components of the incubation mixtures were added and the reaction was run at 25^oC for 40 min. Pyridine-N-oxide was extracted and determined by the analytical methods described above.

Kinetic Analyses

Kinetic parameters were determined by Lineweaver-Burke reciprocal plot (Segel, 1975). Substrate concentration [S] is plotted as 1/[S] on a horizontal axis, while corresponding velocity, v, is plotted as 1/v on the vertical ordinate. The slope $= K_m/V_{max}$ and the intercept on the 1/v axis $= 1/V_{max}$, when $1/v = 0$, $1/[S] = -1/K_m$.

RESULTS

The effect of BNF pretreatment of rainbow trout liver microsomes

The protein concentration and cytochrome P-450 content of BNF-induced microsomes compared with untreated microsomes are listed in Table 4. BNF-pretreatment of rainbow trout had no effects on microsomal protein content but increased cytochrome P-450 content by about 72% compared with control microsomes.

Metabolites separation by HPLC

The retention time and detection limits for pyridine, pyridine-N-oxide, 3-hydroxypyridine, 2-hydroxypyridine, 4-hydroxypyridine and 3-hydroxypyridine-N-oxide eluted from two different columns and mobile systems are listed in Table 5. The HPLC chromatogram of authentic pyridine metabolites and the separation of ^{14}C-pyridine incubation extracts are shown in Fig.3 and Fig.4. An ion exchange column with an acid buffer can separate most of the metabolites except 2-OH pyridine and 4-OH pyridine which are eluted as a single peak. The incubation extract contained a broad UV absorption peak (primarily resulting from the addition of NADPH) which interfered with the identification of 3-OH pyridine-N-oxide and 2- or 4-OH-pyridine. A similar interference was seen with impurities from the radioactivity pyridine standard

when detected by the isotope detector (Fig.3(B)). A reverse phase column (Zorbax ODS) can separate all the metabolites but it requires more than 50 min to elute pyridine from the column. This was too long for routine analysis. However, the reverse phase column system can avoid the interference of impurities from radioactivity pyridine and also can seperate the hydroxypyridines in 2- or 3- or 4- position (Fig.4). In this study, pyridine-N-oxide was the only metabolites that was detected either by using radiolabelled substrate or nonradioactive pyridine as a substrate. Conformation of the identity of possible metabolites by GC-MS are shown in Appendix 1.

Effect of factors on trout liver pyridine-N-oxidation

Effect by incubation time:

Fig.5 shows the time course of pyridine-N-oxide formation by trout liver microsomes. The metabolite formation rates became linear after 15 min and increased for 45 min but decreased by 60 min. A comparison of the kinetic data for different incubation times with the K_m and V_{max} values is presented in Table 6. At 15 min, control and BNF-induced microsomes had similar K_m and V_{max} values while at 30 min BNF-induced microsomes showed a 2-fold decrease in the K_m value along with a similar increase the V_{max} value.

Effect of microsomal concentration:

Microsomal concentration from 0.5 mg/ml to 11 mg/ml were used to find the effect on pyridine-N-oxidase activity. The result showed that untreated trout microsomes gave a better linear correlation than BNF induced microsomes where different microsomal concentration were compared (Fig. 6). In general, microsomes above 2 mg per ml gave linear activity.

Effect by pyridine concentration:

Pyridine concentration from 1 mM to 10 mM were used to study the effect of substrate concentration on pyridine-N-oxide formation. Fig. 7 shows the pyridine-N-oxidase activity present according to the Michaelis-Menton equation. The result showed that saturation of pyridine-N-oxidation occurred at a 4 mM pyridine concentration with both control and BNF-induced trout microsomes.

Effect by various buffer and pH values :

Tris-HCl buffer with pH range from 7.2 to pH 11.5, and potassium phosphate buffer with pH 7.2 to pH 10.0 were used to evaluate the effect of pH on the microsomal enzyme activity for pyridine N-oxidation. The pH value of potassium phosphate buffer changed after incubation at pH values higher than 8.2, as did the Tris buffer when initial pH values were higher than 9.5. The buffer pH and post-incubation pH versus pyridine N-oxide formation is shown in Fig. 8. The optimum pH value for pyridine-N-oxidation was 8.5 in both buffer system.

Lineweaver-Burke plots of pyridine-N-oxidation in control

and BNF-induced trout microsomes at pH 8.5 and pH 7.4 showed that different pH incubation condition also affect the kinetic study results of the pyridine-N-oxidation (Fig. 9). The activity of pyridine-N-oxidase at optimum pH condition (pH 8.5) was 2 to 3 fold greater than that seen at physiological pH condition (pH = 7.4) (Table 7).

Effect of chemical inhibitors on trout liver microsomal pyridine-N-oxidation

n-Octylamine, 1-benzylimidazole, metyrapone, thiourea and methimazole at concentrations of 1 mM were added separately as inhibitors to evaluate their effect on the N-oxidation of pyridine. BNF-induced microsomes and untreated microsomes gave similar results (Table 8). Addition of n-octylamine to trout liver microsomes almost completely inhibited pyridine-N-oxidation, as did 1-benzylimidazole. Thiourea reduced the activity by about 70%, metyrapone showed partial inhibition (50%) and methimazole had almost no effect on control trout microsomes.

Immunochemical effect of pyridine-N-oxidation

P-450 reductase IgG at concentrations of 10 mg and 20 mg/mg protein incubated with trout microsomal protein diminished pyridine N-oxidation. The pyridine N-oxidation in untreated trout liver microsomes preincubated with P-450 reductase IgG showed decreased activity of 72.5% and 93.3% at 10 mg IgG/mg protein and 20 mg IgG/mg protein, respectively, and BNF-induced microsomes had

decreased activity of 75.5% and 77.4%, respectively.

Rabbit antibodies raised against trout P-450 LM_2 and LM_{4b} isozymes were utilized to determine the contribution of these isozymes to pyridine N-oxidation. P-450 IgG was incubated with control and BNF-induced microsomes at ratios of 20, 40, and 60 mg/nmol P-450 content (Fig. 10). LM_2 IgG has no effect on both BNF-induced or untreated microsomes whereas LM_{4b} IgG has 40% deactivation of pyridine-N-oxidation when pre-incubated at 60 mg IgG/nmol P-450 content microsomal protein.

<u>The effect of ethanol and pyridine pretreatment to rainbow trout liver microsomes and their pyridine-N-oxidation activities</u>

The protein concentration, cytochrome P-450 content, and the formation of pyridine-N-oxide were compared in microsomes from ethanol, pyridine and water pretreated trout (Table 9). Although there was no statistically significant difference of these three treatments on comparing the protein content or P-450 content or pyridine-N-oxidation activity, the ethanol induced fish incurred partial mortality (3 from 20) during 66 hours treatment periods and also showed a higher variation in pyridine oxidation activity.

DISCUSSION

Enzyme activity in the metabolism of xenobiotics is varied by induction or inhibition, temperature, enzyme concentration, pH, and substrate concentration. The amount of pyridine N-oxidation excreted varies widely with the animal species, sex, and several factors when tested in mammals.

A number of studies have demonstrated that fish possess an active cytochrome P-450-dependent mixed function oxidase system (Lech and Bend, 1980). Fish microsomal P-450 enzymes can be induced by 3-MC, BNF, TCDD, PCB but PB pretreatment has no effect. Different routes of induction also induced different amount of P-450 isozymes in trout (Elcombe and Lech, 1979; Stegeman et al., 1981; Elsele et al., 1984). In our study, trout treated with 500 ppm BNF-containing diet for 14 days increased P-450 content about 2-fold higher than control trout liver microsomes without any changes in protein content. This results are similar to those of Williams et al. (1984).

In order to observe the possibility of pyridine metabolites other than pyridine-N-oxide, two HPLC systems were used to separate and double check the results. Although the detection limit of all metabolites analyzed by these two systems were very low, pyridine-N-oxide is the only metabolites that has been found in this study. Mammalian studies gave similar results, with 40% pyridine N-

methylation and 60% N-oxidation metabolites being detected in urine *in vivo* (Gorrod and Damini, 1979a; Shaker et al., 1982), but upon microsomal incubation less than 5% of pyridine was metabolized and pyridine-N-oxide was the only metabolite detected (Blaauboer and Paine, 1980).

Various factors that effect the formation of pyridine N-oxide have been studied in this investigation. Different incubation times affected the amount of pyridine-N-oxide formation. Enzyme activity became linear after 15 min and continued until 45 min. However, different K_m and V_{max} values were found between 15 min and longer incubation time. Mammalian tests showed that the rate of pyridine-N-oxidation was linear up to 10 min (Gorrod and Damini, 1979a; Blaauboer and Paine, 1980). With trout liver microsomes pyridine concentration was saturated at 4 mM as determined by pyridine-N-oxide formation. The same saturated pyridine concentration was found in rat and pig liver microsomal incubations (Gorrod and Damini, 1979a; Blaauboer and Paine, 1980).

In liver microsomes, two major monooxygenases, cytochrome P-450 and flavin-containing monooxygenase all catalyze oxidation reaction, especially with amino groups, and both required NADPH and oxygen for activity (Ziegler and Poulsen, 1978). Therefore, it is not easy to characterize the relative contribution of these two enzyme systems for xenobiotic oxidation. Optimum pH, heat-stability, inhibitor, effector, and antibodies are been used for separate the activity of these two compounds on the oxidation of certain substrates (Tynes and Hodgson, 1983, 1984; Williams et al.,

1984).

The optimum pH for cytochrome P-450 is around pH 7.25 (Sato and Omura, 1978) whereas that for FMO was pH 8.0 to 9.2 (Ziegler, 1980; Tyne and Hodgson, 1984). But some N-oxidation reaction catalyzed by P-450 also have high optimum pH, such as pH 8.4 for the N-oxidation of 2-aminofluorene by visceral mass microsomes (Schlenk, 1989) and pH 8.0 for the N-oxidation of pyridine by rat liver microsomes (Gorrod and Damini, 1979a). In this study, pyridine-N-oxidation in rainbow trout microsomes had an optimum pH of 8.5. Comparison of the activity of pyridine-N-oxidase at optimum pH (pH 8.5) and physiological pH (pH 7.4) showed that at the physiological pH, the affinity and efficiency of the pyridine-N-oxidase is 3 times lower than that at pH 8.5.

P-450 and FMO have different modes of action, especially with regard to electron transfer procedures (Jansson and Schenkman, 1977; Vermillion et al., 1981). The ability to inhibit the microsomal reduction of cytochrome c by P-450 reductase IgG closely corresponds to the ability to inhibit the microsomal reaction of P-450. The specific activity of trout liver microsomal P-450 reductase is 0.30 μmol/min/mg protein, and this enzyme is inhibited by anti-rat P-450 reductase antibodies and not induced by many P-450 inducers (Williams et al., 1983). The rat-reductase IgG in this study gave about 80% inhibition of pyridine-N-oxidation at the highest antibody concentration proving that P-450 has a major role in pyridine metabolism by trout liver microsomes. However, based on the antibody test, P-450 LM_2 isozyme, the major constitutive

isozyme, does not have any contribution towards pyridine-N-oxidation in trout liver microsomes. P-450 LM_{4b}, the major BNF-induced isozyme, on the basis of the antibody incubation experiment, had partial pyridine-N-oxidation activity.

Recent studies shown that the ethanol-induced P-450 isozyme (rabbit LM_{3a} and rat P-450_j) had high activity for pyridine-N-oxidation in rabbit and rat, respectively, and that this isozyme was also pyridine-inducible (Kim et al., 1988). In our study, fish were very susceptible to the ethanol toxicity. Trout treated with 1-2 ml of 100 % ethanol by i. p. injection had a high mortality within a week (data not shown), 0.5 ml i.p. injection also cause 15% mortality after 66 hours treatment. However, trout were able to survive i.p.treatment of pyridine at 50 mg/kg body weight dose for 3 days. Nevertheless, microsomes from pyridine or ethanol pretreated trout showed no significant difference in protein or P-450 content or in pyridine-N-oxidation activity .

Chemical inhibitors are frequently used to study the mechanism of enzyme activity (Netter, 1982, 1987). n-Octylamine, a P-450 substrate which can produce a distinct type II P-450 spectrum (Hodgson, 1974), has been used as a positive effector of FMO. The amine caused 100% inhibition of pyridine-N-oxidation in hamster (Gorrod and Damini, 1979b) and also completely inhibited pyridine N-oxidation by trout liver microsomes. 1-Benzylimidazole, a more selective cytochrome P-450 inhibitor which has no effect on FMO catalyzed reaction (Ziegler, 1980), gave a high inhibition (95%) of pyridine N-oxidation in this study. Metyrapone, one of the

pyridinyl-containing compounds, is a specific substrate for cytochrome P-450 (not for P-448), apparently acting by blocking the oxygen binding site (Hildebrandt, 1972; Ioannides and Parke, 1987), had a species-dependent inhibitor activity in some tests. For example, it is an inhibitor of benzo[a]pyrene hydroxylase in rodent liver microsomes but has no effect on benzo[a]pyrene hydroxylase and lauric acid (ω-1) hydroxylase activity in rainbow trout (Elcombe and Lech, 1979; Williams et al., 1984). In our study, metyrapone showed a partial inhibition of pyridine-N-oxidation in rainbow trout liver microsomes. Methimazole and thiourea are sulfur-containing compounds both known to be the substrates for the FMO catalyzed reaction (Poulsen et al., 1979; Sabourin and Hodgson, 1984). Both compounds have different inhibitor activity on pyridine-N-oxidation by rainbow trout liver microsomes, with methimazole having no effect on pyridine oxidation whereas thiourea had a 70% inhibitory effect.

All these studies concluded that the optimal incubation condition of pyridine with rainbow trout liver microsomes at 25°C is at pH 8.5 with incubation for 30 min at a substrate concentration of more than 4 mM and a liver microsomal protein concentration more than 2 mg. Liver microsomes from BNF-pretreated trout showed an increase the pyridine-N-oxidase activity with a corresponding 1.6-fold lowering of the K_m value and a 1.3-fold increase in the V_{max} value. Microsomes from ethanol or pyridine pretreated trout did not show any significant effect on pyridine-N-oxidation. Comparison of pyridine-N-oxide formation by different

animal liver microsomes (Table 10) showed that the trout microsomes has lower ability to metabolize pyridine than did mammals (rat and mouse). Based on the effects of antibodies and inhibitors, P-450 seems to play a major role in catalyzing pyridine-N-oxidation in rainbow trout liver microsomes.

Table 4. The Effect of BNF Pretreatment on Rainbow Trout Liver Microsomes[a]

Pretreatment	Protein content (mg/ml)	Cytochrome P-450 (nmol/mg protein)
BNF[b]	11.21 ± 0.55	0.630 ± 0.031[c]
Control	12.70 ± 2.17	0.368 ± 0.013

[a] Each value represents the mean + SD of 4 groups of 6 to 8 animal pooled liver microsomal preparation.

[b] Rainbow trout were pretreated with daily feeding of 500 ppm BNF-containing diet for 14 days prior to termination.

[c] Mean statistically different from control group ($p<0.05$).

Table 5. The Retention Time of Pyridine and Its Possible Metabolites from Different HPLC Column Systems[a]

	Retention time (min)		Detection[d]
Compound	System I[b]	System II[c]	limit(nmole)
Pyridine	10.4	>50	0.042
Pyridine N-oxide	5.5	12.1	0.015
2-OH pyridine	3.5	10.9	0.220
3-OH pyridine	4.2	13.3	0.180
4-OH pyridine	3.5	5.5	0.009
3-OH pyridine-N-oxide	3.0	3.4	0.005

[a] HPLC systems were performed as described in Material and Methods.

[b] System I:μ-Partisil 10-SCX column with a mobile phase of 0.3 M ammonium acetate buffer (pH 3.7) at a flow rate of 1.0 ml/min.

[c] System II: μ-Zorbax 10 ODS column with a mobile phase of 0.1 mM potassium phosphate buffer (pH 7.4) containing 5% methanol at a flow rate of 1.0 ml/min.

[d] Detection limits are calculated based on greater than 3-fold higher than signal/noise.

Fig. 3. HPLC chromatograms and superimposition of radioactivity of pyridine and its possible metabolites analyzed by μ-Partisil 10-SCX column.

A. HPLC chromatograms of a standard mixture containing 3-OH pyridine-N-oxide (a), 2-OH pyridine or 4-OH pyridine (b), 3-OH pyridine (c), pyridine-N-oxide (d), and pyridine (e). Superimposition of radioactivity of ring-2,6-^{14}C pyridine standard (▨).

B. HPLC chromatograms and superimposition of radioactivity of an incubation extract following incubation of pyridine with trout liver microsomes under optimal conditions.

HPLC condition were μ-Partisil 10-SCX column with mobile phase 0.3 M ammonium acetate buffer (pH 3.7) with flow rate of 1.0 ml/min.

Fig. 3

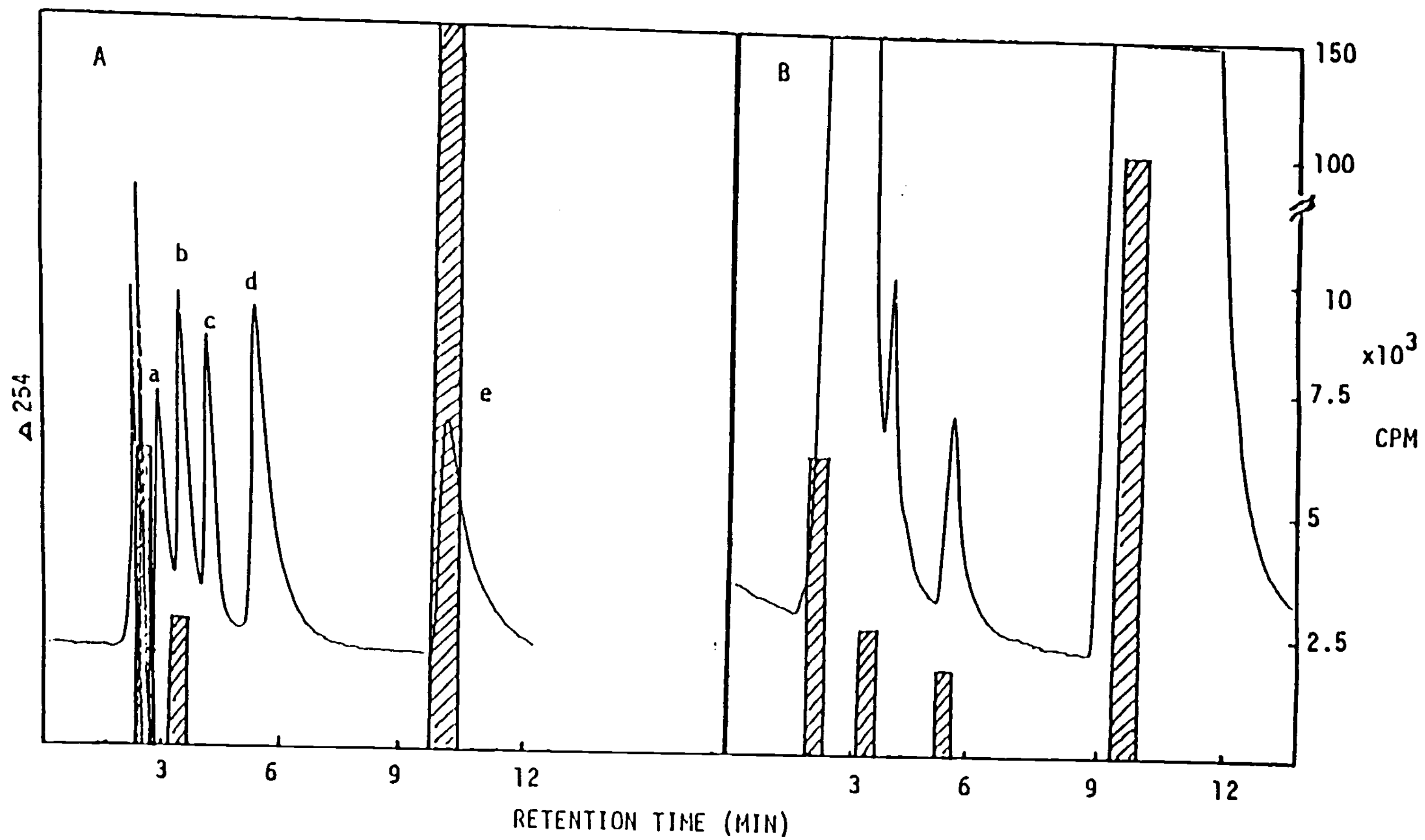

Fig. 4. HPLC chromatograms and superimposition of radioactivity of pyridine and its possible metabolites analyzed by μ-Zorbax 10 ODS column.

A. HPLC chromatograms of a standard mixture containing 3-OH pyridine-N-oxide (a), 4-OH pyridine (b), 2-OH pyridine (c), pyridine-N-oxide (d), and 3-OH pyridine (e). Superimposition of radioactivity of ring-2,6-^{14}C pyridine standard (▨).

B. HPLC chromatograms and superimposition of radioactivity of an incubation extract following incubation of pyridine with trout liver microsomes under optimal conditions.

HPLC condition were μ-Zorbax 10 ODS column with mobile phase potassium phosphate buffer 0.1 mM (pH 7.4) with flow rate of 1.0 ml/min.

Fig. 4

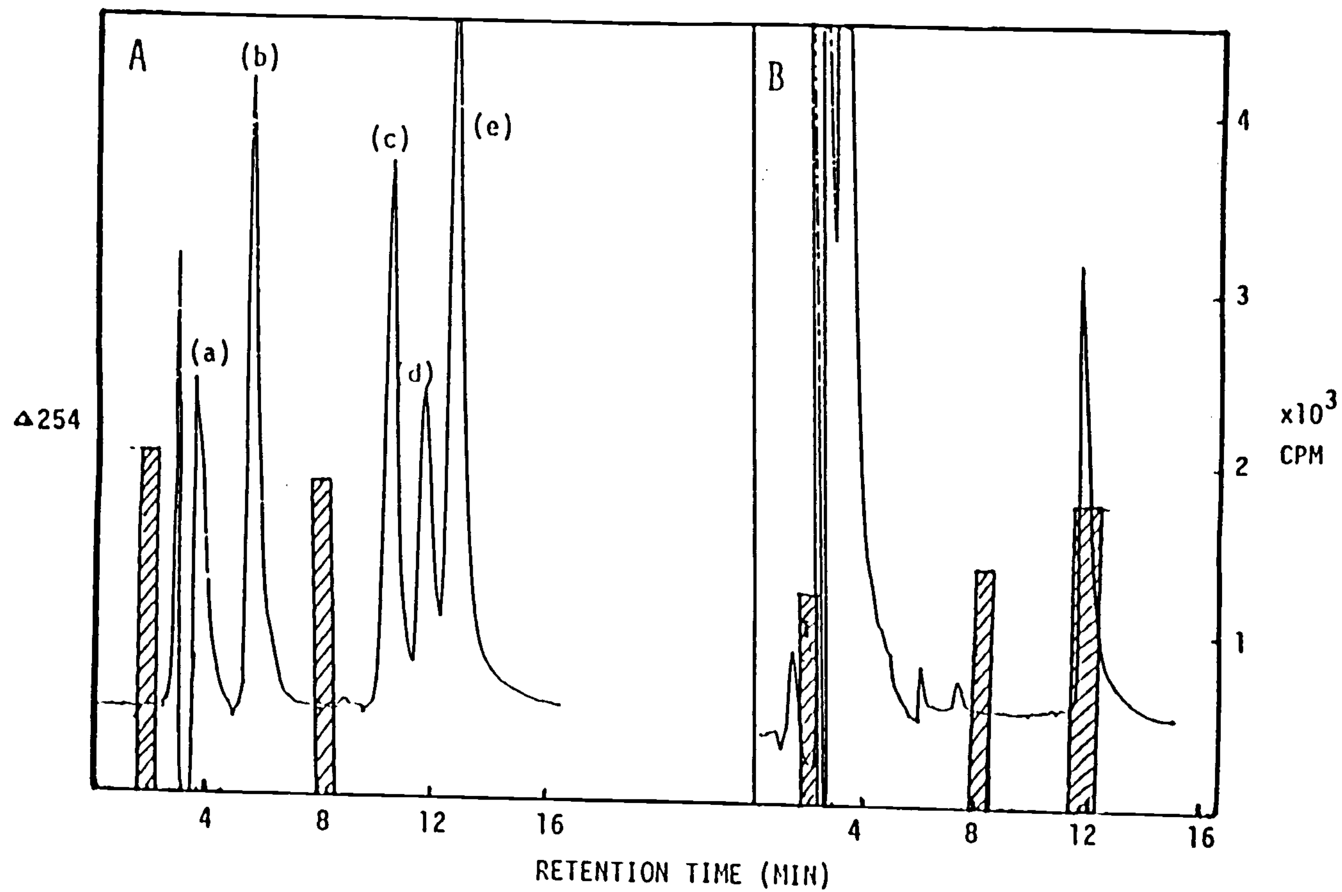

Fig. 5 . Time course of pyridine N-oxidation by incubation with rainbow trout microsomes.

Control(-□-) and BNF-induced (-◆-) trout microsomes were incubated with pyridine for 15, 30, 45 and 60 min.

The incubation condition were: trout liver microsomes 2.5 mg/ml, pyridine 5 mM, Tris buffer 0.1 M (pH 8.5), NADPH 0.5 mM and incubate at 25°C.

Fig. 5.

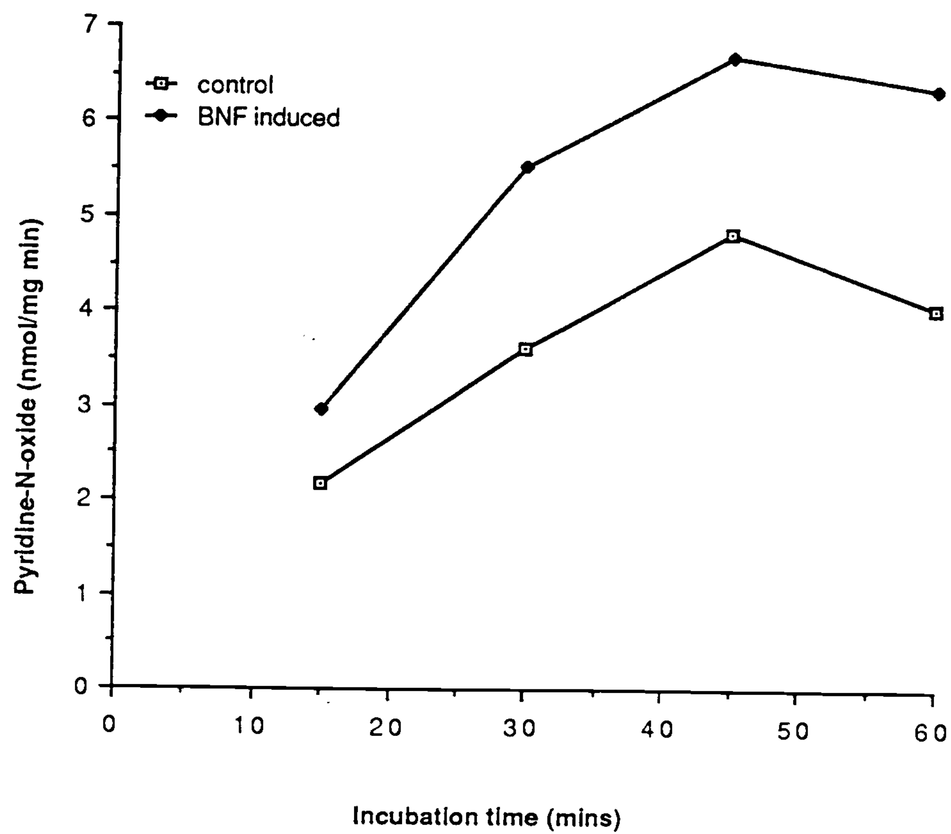

Table 6. The Effect of Incubation Time on the K_m and V_{max} Values for Pyridine N-oxidation

Time	K_m (mM)		V_{max} (nmol/min mg)	
(min)	Control	BNF	Control	BNF
15	3.80	3.66	0.286	0.356
30	3.29	1.52	0.212	0.310
45	2.12	1.77	0.156	0.229
60	2.62	1.54	0.119	0.173

The incubation condition were : trout liver microsomes 2.5 mg/ml, pyridine 5 mM, NADPH 0.5 mM, and Tris-buffer 0.1 M (pH 8.5).

Fig. 6. Effect of protein concentration on pyridine N-oxidation by rainbow trout liver microsomes.

Control microsomes (◆) with concentrations of 1-11 mg/ml and BNF-induced microsomes (▣) with concentrations of 1-9 mg/ml were used in the incubation condition with Tris-buffer (pH 8.5), pyridine 5 mM, NADPH 0.5 mM, and incubation at 25°C for 30 min.

Fig. 6.

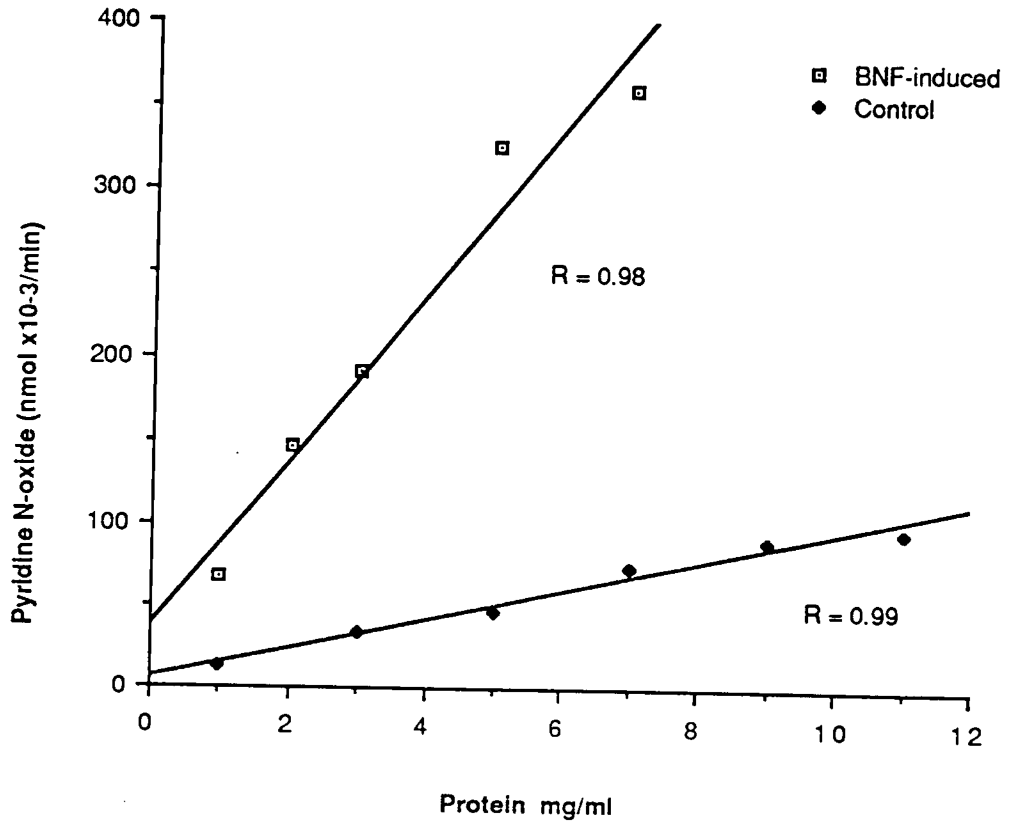

Fig. 7. The effect of pyridine concentrations on pyridine -N-oxide formation by trout liver microsomes.

Control microsomes (•) and BNF-induced microsomes (□) were incubated with 1-10 mM pyridine concentrations.

The incubation condition were : Tris-buffer 0.1 M (pH 8.5), trout liver microsomes 2.5 mg/ml, NADPH 0.5 mM, and incubated at 25°C for 30 min.

fig 7.

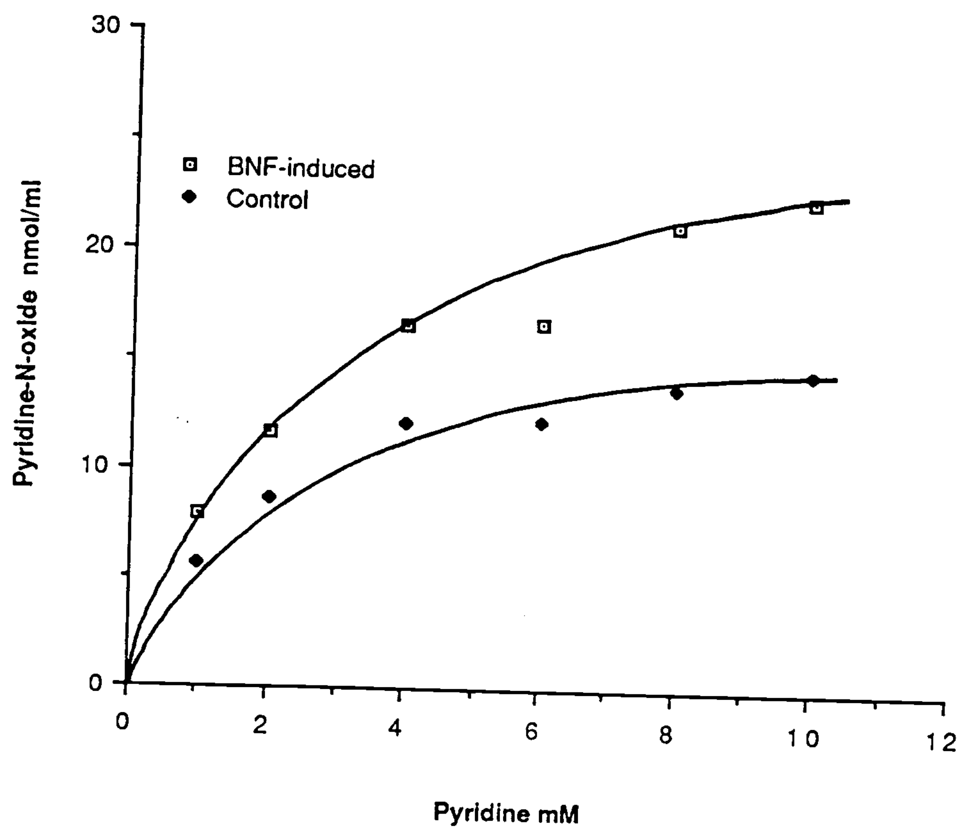

Fig. 8. Relation of pH and pyridine N-oxidation by rainbow trout liver microsomes.

Potassium phosphate buffer (-□-), potassium phosphate nominal buffer (■), Tris-HCl buffer (-◇-), and Tris-HCl nominal buffer (-◆-) were compared with amount of pyridine-N-oxide formation.

The incubation conditions were buffer 0.1 M, BNF-induced trout microsomes 2.5 mg, pyridine 5 mM, NADPH 0.5 mM and incubated at 25°C for 30 min.

Fig. 8.

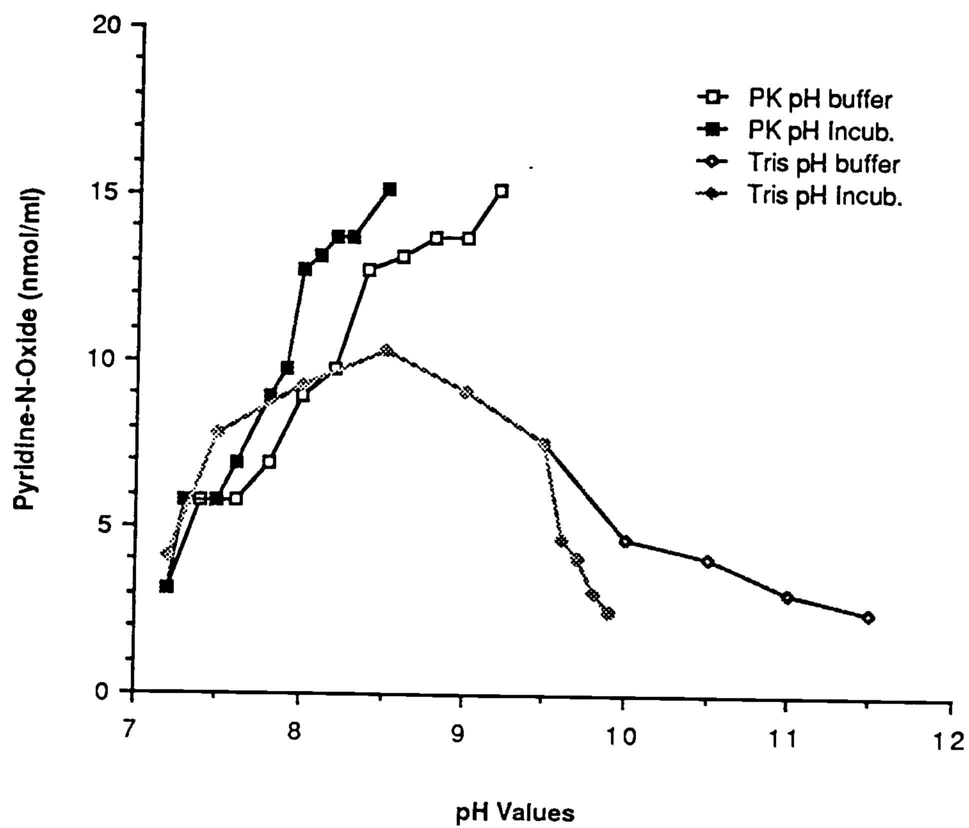

Fig. 9. The difference of pyridine-N-oxidation between optimum pH and physiological pH by trout liver microsomes.

Lineweaver-Burk reciprocal plots of pyridine-N-oxidation by incubated pyridine with control trout liver microsomes in pH 7.4(□), and pH 8.5(■); and incubated with BNF-induced trout liver microsomes in pH 7.4(◆), and pH 8.5 (◆).
The incubation conditions were Tris buffer 0.1 M, microsomes 2.5 mg/ml, pyridine 5 mM, NADPH 0.5 mM and incubation at 25°C for 30 min. The values represent the means of duplicates.

Fig. 9.

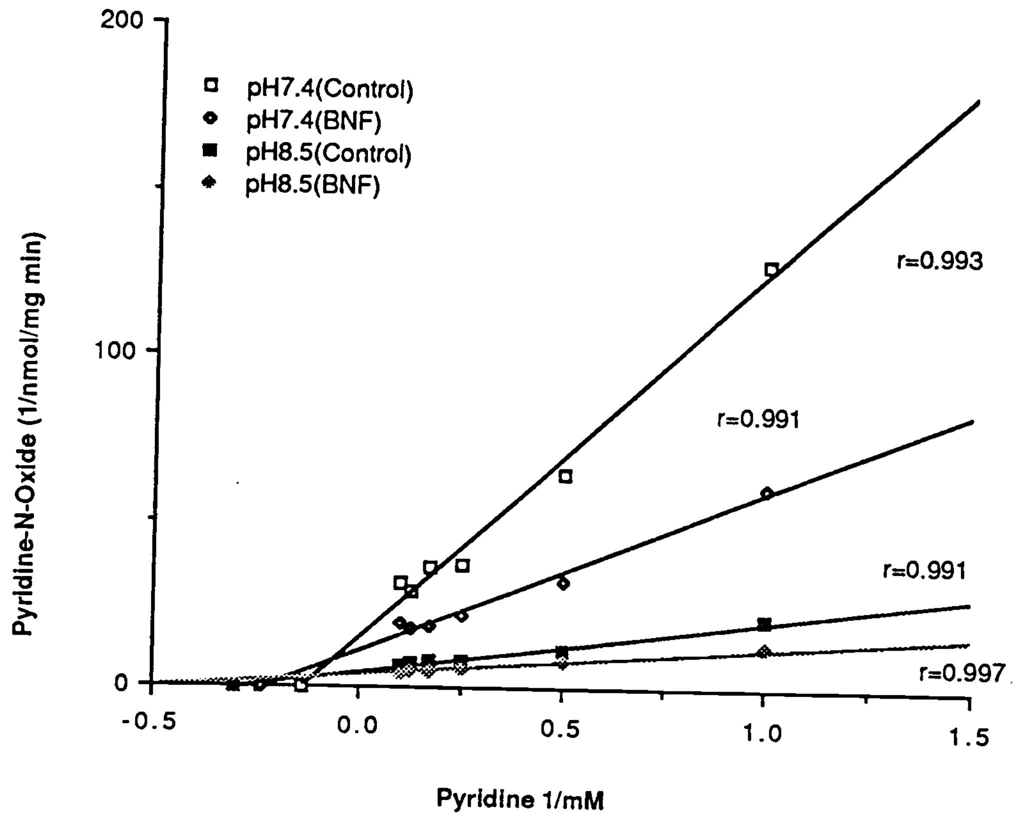

Table 7. The Effect of pH on the K_m and V_{max} Values of Pyridine N-oxidation

	K_m(mM)		V_{max}(nmol/min mg)	
Treatment	pH 7.4	pH 8.5	pH 7.4	pH 8.5
Control	7.3	3.3	0.067	0.212
BNF-induced	4.2	1.5	0.088	0.310

Incubation condition were: Tris-buffer 0.1mM, microsomes 2.5 mg/ml, pyridine 5 mM, NADPH 0.5 mM, and incubated 30 min.

Table 8. The Effect of Chemical Inhibitors on Pyridine N-oxidation by Rainbow Trout Liver Microsomes[a]

Inhibitor[b]	% Activity	
	Control	BNF-induced
n-Octylamine	2.7	2.8
1-Benzylimidazole	5.8	6.0
Thiourea	28.0	29.6
Metyrapone	60.0	50.0
Methimazole	97.8	80.0

[a] Pyridine N-oxidation was assayed by measuring the formation of pyridine-N-oxide. Results are percentage activity remaining compared to without inhibitor added treatment. The values represent the means of duplicates.

[b] Concentration of inhibitors are 1 mM.

Incubation conditions are: Tris buffer (pH 8.5) 0.1 M, trout liver microsomes 2.5 mg/ml, NADPH 0.5 mM, and pyridine 5 mM. Inhibitor with microsomes are incubate 20 min before adding pyridine and then incubated for another 30 min.

Fig. 10. Inhibition of trout liver microsomal pyridine-N-oxidation with rabbit antibodies against purified trout cytochromes P-450 LM_2 and LM_{4b}.

Untreated trout liver microsomes 0.134 mg or BNF-induced trout microsomes 0.082 mg were preincubated with 20, 40 and 60 mg IgG per nmol P-450 trout liver microsomal protein for 10 min at 25^oC. The remaining components were then added, the reaction was run at 25^oC for 40 min, and the formation of pyridine-N-oxide was analyzed as described under Experimental procedures. Percentage activity of pyridine-N-formation was presented by comparing with non-antibody preincubated treatment. Values are means of duplicates: LM_2 IgG preincubate with control microsomes (□), and BNF-induced microsomes (▨); LM_{4b} IgG preincubate with BNF-induced microsome (▩).

fig 10.

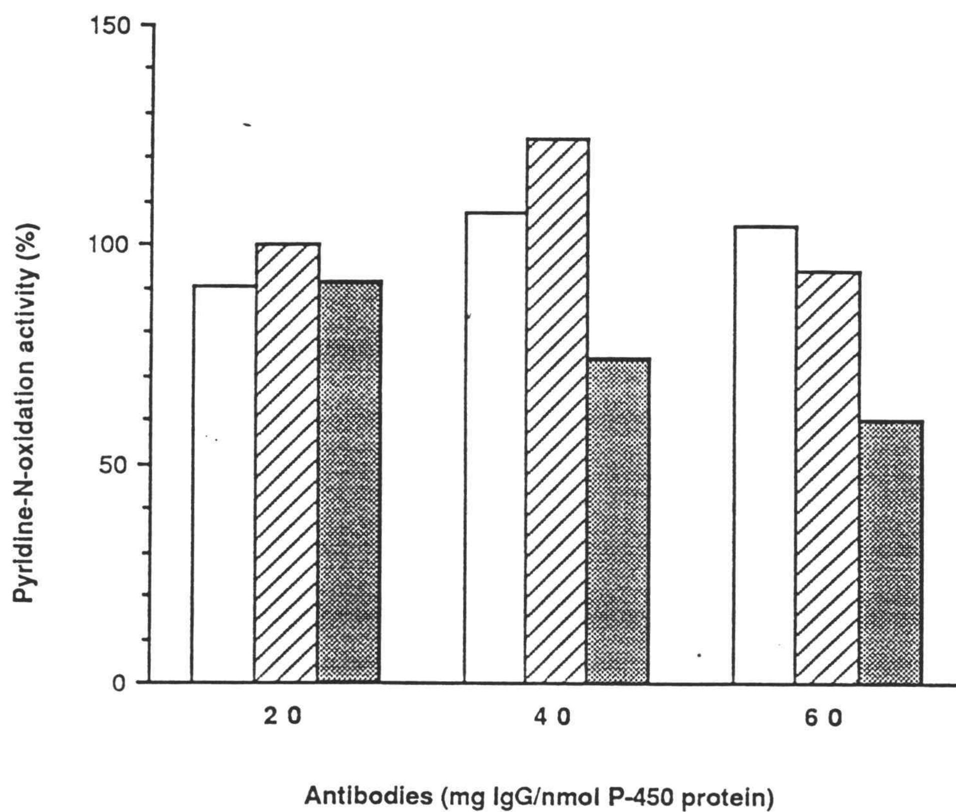

Table 9. The Effect of Ethanol and Pyridine Pretreated Rainbow Trout Liver Microsomes[a]

Pre-treatment[b]	Protein content (mg/ml)	P-450 content (nmol/mg protein)	Pyridine-N-oxide formation(nmol)
Dist.water	9.24 ± 2.30	0.388 ± 0.028	11.52 ± 3.81
Ethanol	11.45 ± 3.16	0.312 ± 0.086	8.03 ± 2.66
Pyridine	9.80 ± 2.46	0.369 ± 0.039	11.37 ± 1.66

[a] The value represent the mean ± SD of pooled liver microsomal preparations from 5 groups of 4 animal pretreated with distilled water or pyridine, and pooled liver microsomal preparations from 4 groups of 4 animals pretreated with ethanol. t-test ($p<0.05$) for statistic calculation.

[b] Fish (20 per group) were i.p. injected with 0.5 ml disttiled water, 0.5 ml 100% ethanol, or 0.5 ml pyridine in aqueous solution (50 mg/kg body weight). The microsomes were prepared 66 hrs post-treatment.

Table 10. The K_m and V_{max} Values of Pyridine N-Oxidation in Various Animal Liver Microsomes *In Vitro*[a]

Treatment	K_m (mM)	V_{max} (nmol/min mg protein)
Trout(control)	7.3	0.067
Trout(BNF-induced)	4.2	0.088
Mouse[b]	0.9	0.20
Rat[b]	0.7	0.28
Rat(control)[c]	0.6	0.17
Rat(PB-induced)[c]	0.9	0.40
Rat(BNF-induced)[c]	1.4	0.24

[a] The trout microsomes were incubated at 25°C , pH 7.4; rat and mouse microsomes were incubated at 37°C, pH 7.5.

[b] Data cited from Gorrod et al. 1979a.

[c] Data cited from Kim et al. 1988.

The *In Vitro* Metabolism of Chlorpyrifos by Rainbow Trout Liver Microsomes

by

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ABSTRACT

In vitro study of the biotransformation of chlorpyrifos [O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl) phosporothioate] by rainbow trout hepatic microsomes showed that chlorpyrifos was oxidatively activated to chlorpyrifos oxon and detoxified to 3,5,6-trichloro-2-pyridinol (TCP). These reactions required NADPH, and were insensitive to pH in the range 7 to 8.5. Kinetic analyses determined the K_m's for formation of chlorpyrifos oxon (desulfuration) and TCP (deesterification) by untreated microsomes to be 113 μM and 59 μM, respectively. The V_{max}'s for the same reactions were 0.12 nmol/mg/min and 0.22 nmol/mg/min, respectively. β-Naphthoflavone (BNF) pretreated microsomes gave K_m's for formation of chlorpyrifos oxon and TCP of 22 μM and 73 μM, respectively, with V_{max}'s of 0.10 nmol/mg/min and 0.30 nmol/mg/min, respectively. Both reactions were inhibited by cytochrome P-450 substrates and FMO substrates had no effect. However, rabbit antibodies raised against NADPH-cytochrome P-450 reductase and trout cytochrome P-450 LM_2 had little and no effect on these reaction. The P-450 LM_{4b} IgG, however, gave 60% inhibition at 40 mg IgG/nmol P-450 content. The rate of hydrolysis of chlorpyrifos oxon to TCP by trout liver microsomes was very low. The K_m's for BNF-induced and control microsomes were 1.58 mM and 1.69 mM, respectively, with V_{max}'s of 0.341 nmol/min/mg and 0.389

nmol/min/mg, respectively. Chlorpyrifos oxon hydrolysis by trout microsomes seemed to be a nonenzymatic reaction since it was heat-stable, not stimulated by calcium ions and was not deactivated by disodium ethylenediamine tetraacetate (EDTA). None of the inhibitors tested in chlorpyrifos metabolism affected the hydrolysis of chlorpyrifos oxon.

INTRODUCTION

Pesticides enter aquatic environments through intentional application, aerial drift or runoff from application or accidental release, and then become rapidly distributed in the environment, especially through the action of water (Nimmo, 1985). Chlorpyrifos, a broad spectrum insecticide, is also applied directly to water for mosquito control. This compound is very toxic to fish and had been investigated as a cause of toxicity to fish and aquatic invertebrates in ponds (Macek et al., 1972).

In vitro study of biotransformation of chlorpyrifos in mouse liver microsomes revealed that chlorpyrifos is oxidized to chlorpyrifos oxon and deesterified to 3,5,6-trichloro-2-pyridol (TCP) by cytochrome P-450; the cholinesterase inhibitor chlorpyrifos oxon is detoxified by hydrolysis to TCP by tissue esterases (Sultatos and Murphy, 1983a, 1983b). PB-pretreated mice showed an increase in chlorpyrifos metabolism by liver microsomes and further decreased pesticide-induced mortalities whereas BNF-induced mice gave the opposite result (Sultatos et al., 1984). The mouse liver contained very high of chlorpyrifos oxon hydrolysis esterase activity so the chlorpyrifos could be completely metabolized to TCP before leaving the liver (Sultatos et al., 1985).

The acute toxicity of chlorpyrifos is more than 50,000 times

higher in fish than in the rat. Because this pesticide must be bioactivated to the oxygen analoge (chlorpyrifos oxon) in order to show potent acetylcholinesterase inhibition activity, the different activation and detoxification activities of mammalian and fish livers may contribute in part to the differences in acute toxicities between mammals and fish.

The purpose of this study is to use untreated and BNF-pretreated rainbow trout liver microsomes, incubated with chlorpyrifos or chlorpyrifos oxon, to conduct a kinetic analysis of the reaction rates for both pesticide activation and detoxification, and, using inhibitors and antibodies, to characterize the contribution of the biotransforming enzymes on the metabolism of chlorpyrifos by trout liver microsomes.

MATERIAL AND METHODS

Materials

Analytical Standards

Chlorpyrifos [ring-2,6-^{14}C-O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)phosphorothioate] 50 μCi (specific activity 15.7 mCi/mmole; 94% purity), chlorpyrifos (>99%), chlorpyrifos oxon [O,O-diethyl-O-(3,5,6-trichloro-2-pyridyl)phosphorate] (>99%) and 3,5,6-trichloro-pyridinol (TCP) (>99%) were obtained as gifts from the Dow Chemical Company, Midland, MI.

Reagent and Solvents

β-Naphthoflavone (BNF) (90-95%) and nicotinamide adenine dinucleotide phosphate, reduced (NADPH)(94%) were purchased from Sigma Chemical Co.; and dichloromethane (HPLC grade), acetonitrile (HPLC grade) were purchased from J.T. Baker Co.

Microsomal Preparation Buffers

Homoginization buffer A contained 0.1 M Tris-acetate (pH 7.4), 0.1 M KCl, 1 mM disodium ethylenediamine tetraacetate (EDTA) and 0.1 mM phenylsulfonyl fluoride (PMSF). Buffer B (for washing) contained 0.1 M potassium pyrophosphate (pH 7.4), 1 mM EDTA, 1 mM dithio-threitol (DTT) and 0.1 mM PMSF. Buffer C (for storage) contained 0.1 M potassium phosphate (pH 7.25), 20% glycerol and 0.1 mM PMSF.

Antibodies

Rabbit polyclonal antibodies raised against rat NADPH-cytochrome P-450 reductase (reductase IgG) and rabbit antibodies raised against purified trout P-450 LM_2 (LM_2 IgG) were prepared in our lab previously; and rabbit antibodies raised against purified trout P-450 LM_{4b} (LM_{4b} IgG) was from the laboratory of Dr. D.E. Williams.

Experimental Procedure

Microsomes preparation

One year old rainbow trout (Oncorhynchus mykiss) with average body weight 80 to 100 g were used for preparation of the liver microsomes. Duplicate lots of fish were put into tanks (30 per tank) and fed either the control diet or a diet containing 500 ppm BNF for 14 days and then starving for 2 days before sacrifice. The fishes were killed by electric shock and the liver excised and weighed. Livers were placed in ice-cold buffer A, separated to 4 groups (6 to 8 fish livers per group), minced, rinsed and homogenized in 3 volumes of buffer A using a motor driver Potter-Elvehjem-type glass-teflon homogenizer. Each liver homogenate was centrifuged at 10,000 g for 30 min at $4^{o}C$, and the supernatant was decanted and centrifuged at 100,000 g for 90 min. The microsomal pellet obtained was resuspended in buffer B and centrifuged again at 10,000 g for 60 min. The washed pellet was suspended in buffer

C to a concentration equivalent to a wet weight of liver per milliliter of suspension. All microsomes were storage at -80°C before use.

Microsomal protein content was determined by the colorimetric method of Lowry et al. (1951) using a bovine serum albumin standard. Cytochrome P-450 content was measured by the method of Estabrook et al. (1972) using an extinction coefficient of 91 mM^{-1} cm^{-1}.

Incubation Conditions

Chlorpyrifos (nonradioactive or ring-2,6-^{14}C labelled) 10 μM or chlorpyrifos oxon 200 mM were used as substrates in the microsomal incubations. Each incubation mixtures consisted of the following: Tris buffer (pH 7.4, 0.1 M), NADPH 0.5 mM , microsomes 2 mg and made to a final volume of 1 ml with distilled water. The reaction was started by adding substrate and incubated for 30 min at 25°C in a shaking water bath at 120 rpm and terminated by immediately placing in the ice water.

Metabolites Analysis Procedure

The incubation mixtures were mixed with 3 ml ethyl acetate. the tubes were mixed 30 sec on a vortex mixer, and then centrifuged at 1,000 g for 10 min. The ethyl acetate was pipetted into other vials, and the aqueous phases were again extracted with 2 ml ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness under gaseous nitrogen. The extracts were diluted to 1 ml

with methylene chloride and injected (50 to 100 μl) into a high performance liquid chromatograph (HPLC).

A Spectra-Physics SP 8700 HPLC equipped with a Beckman 153 UV detector set at 280 nm was used to analyse non-radioactive samples. A Shimadzu LC-6A HPLC equipped with a Beckman 171 isotope detector combine with SPD-6AV UV-visible spectrum detector (set at 280 nm) was employed for the analysis of samples containing radioactivity. The HPLC conditions used to separate chlorpyrifos and its metabolites involved use of either a Waters μ-Porasil (4.6 mm x 25 cm) column or a DuPont Zorbax SIL (4.6 mm x 25 cm) column eluted with methylene chloride, acetonitrile, glacial acetic acid (95:5:0.02) as mobile phase. The flow rate was set at 1 ml/min. Since ^{14}C-TCP was a trace contaminant of the radiolabelled chlorpyrifos standard, the amount of ^{14}C-TCP formed by microsomal extract was quantitative corrected by subtracting the amount of ^{14}C-TCP from non-microsomal incubation extract.

<u>Immunological studies</u>

Inhibition of chlorpyrifos desulfuration and hydrolysis by trout liver microsomes in the presence of antibodies (P-450 reductase IgG, LM_2 IgG, and LM_{4b} IgG) was examined. Various amounts of IgG were preincubated with microsomal protein for 10 min at 25^oC, the remaining components of the incubation mixtures were added and the reaction was run at 25^oC for 40 min. Chlorpyrifos oxon and TCP were extracted and determined by the analytical methods described above.

<u>Kinetic Analyses</u>

Kinetic parameters were determined by Lineweaver-Burk reciprocal plot (Segel, 1975). Substrate concentration [S] was plotted as 1/[S] on a horizontal axis, while corresponding velocity, v, was plotted as 1/v on the vertical ordinate. The slope = K_m/V_{max} and the intercept on the 1/v axis is $1/V_{max}$, when 1/v = 0, 1/[S] = $-1/K_m$.

RESULTS

The effect of BNF pretreatment of rainbow trout liver microsomes

Liver microsomes from BNF pretreated rainbow trout used in this study had protein and cytochrome P-450 contents of 11.21 mg/ml and 0.630 nmol/mg protein, respectively. The untreated trout liver microsomes had contents of 12.7 mg/ml protein and 0.368 nmol/mg protein P-450. BNF pretreatment of trout, as others have shown (Elcombe and Lech, 1979; Williams et al., 1984), can induce P-450 content by 2-fold but caused no changes in the protein content of the liver microsomes.

Metabolites separation by HPLC

HPLC condition used to separate chlorpyrifos and two major metabolites were from Saltatos and Murphy (1983a). The retention time of chlorpyrifos, chlorpyrifos oxon and TCP with a μ-Porasil column were 3.3, 9.0 and 10.7 min, respectively (Fig. 11). The Zorbax SIL column with similar physical properties as μ-Porasil column had same efficiency and gave a better separation with a shorter equilibrium time (Fig. 12). Acetic acid (0.02%) in the mobile phase was necessary to elute the TCP. By using μ-Porasil column, pre-equilibration with the mobile phase for 30 column volumes was required to keep the retention time of chlorpyrifos

oxon constant. Chlorpyrifos oxon and TCP were the only metabolites that had been found either by radiolabelled or nonradioactive chlorpyrifos as a substrate.

Effect of cofactors on chlorpyrifos metabolites in vitro

The time course of chlorpyrifos oxon and TCP formation by trout liver microsomal incubation revealed that chlorpyrifos metabolite production increased for 40 min and then remained constant to 60 min (Fig. 13).

There was a linear correlation between the microsomal protein concentration (from 1 mg/ml to 6 mg/ml) and the yield of chlorpyrifos metabolites formed during incubation (Fig. 14). Examination of the influence of pH on the desulfuration and hydrolysis of chlorpyrifos showed that these reactions were not pH-sensitive when incubated with Tris-buffer between pH 7.0 to 8.5 (Fig. 15). Incubation with boiled microsomes or omission of NADPH completely eliminated chlorpyrifos metabolite formation (data not shown). Addition of 1 to 3 mM EDTA to the incubation mixtures had no effect on the formation of chlorpyrifos oxon and TCP (data not shown).

Kinetic analysis of chlorpyrifos metabolism by rainbow trout liver microsomes

Chlorpyrifos (10 μM to 200 μM) was used as a substrate to study the rate of biotransformation by trout liver microsomes. The K_m and V_{max} for the desulfuration and hydrolysis of chlorpyrifos by

liver microsomes from untreated or BNF-pretreated trout is summarized in Table 11. The BNF-induced microsomes had 2-fold higher rate of desulfuration (i.e. activation) than did untreated microsomes while untreated microsomes had slightly higher hydrolysis activity (i.e. detoxification) than did BNF-induced microsomes. Total biotransformation activity (activation and detoxification) for chlorpyrifos with microsomes from BNF pretreated trout showed a lower K_m but a comparable V_{max} value compared to those obtained with microsomes from the untreated controls.

<u>Hydrolysis of chlorpyrifos oxon by rainbow trout liver microsomes</u>

Chlorpyrifos oxon (1.0 mM to 8.0 mM) was used as a microsomal substrate to study the hydrolytic activity of trout liver. Kinetic analysis showed that there was no difference between microsomes from BNF-pretreated trout and from untreated fish microsomes (Fig. 16). The K_m and V_{max} values obtained for the hydrolysis of chlorpyrifos oxon was much higher than those found for the metabolism of chlorpyrifos itself.

The hydrolysis reaction for chlorpyrifos oxon was NADPH independent, but hydrolysis also occurred with boiled microsomes (Table 12). The additions of 3 mM EDTA to the incubation mixture decreased by 33% this activity whereas 2 mM $CaCl_2$ had no effect on the rate of hydrolysis (data not shown).

Effect of chemical inhibitors on the metabolism of chlorpyrifos and chlorpyrifos oxon by trout liver microsomes

n-Octylamine, 1-benzylimidazole, metyrapone, thiourea and methimazole at a 1 mM concentration were added separately as inhibitors to evaluate their effects on the metabolism of chlorpyrifos and chlorpyrifos oxon by trout liver microsomes. Microsomes from BNF-induced and control animals gave similar results (Table 13). Addition of 1-benzylimidazole to trout liver microsomes completely inhibited chlorpyrifos metabolism while metyrapone and n-octylamine gave a partial inhibition. Methimazole and thiourea had no effect. All of the inhibitors, however, had no effect (or in some cases, a positive effector response) on the hydrolysis of chlorpyrifos oxon.

Effect of antibodies on the metabolism of chlorpyrifos

Antibodies towards P-450 reductase (10 mg and 20 mg) per mg of trout microsomal protein diminished chlorpyrifos desulfuration and hydrolysis of chlorpyrifos thus these reactions seem to involve P-450. Desulfuration of the pesticide by untreated trout liver microsomes preincubated with P-450 reductase IgG still had 71.4 % and 77.8% of the original activity at 10 mg IgG/mg protein and 20 mg IgG/mg protein, respectively, comparing with nonimmune IgG. With BNF-induced microsomes, activity remained at 89.5% and 81.2% at 10 and 20 mg IgG/mg microsomal protein, respectively. Deesterification activity in the all treatment remained above 90% activity compared with nonimmune IgG treatment.

P-450 isozyme IgG at 8, 20, and 40 mg per nmol P-450 protein content was used in a study of the contribution of trout P-450 LM_2 and LM_{4b} isozymes in the metabolism of chlorpyrifos. The results are shown in Fig. 17. LM_2 IgG had no effect where both BNF-induced and control microsomes were employed whereas LM_{4b} IgG gave about a 60% deactivation of the chlorpyrifos desulfuration and hydrolysis reactions upon preincubation of microsomes at 40 mg IgG/nmol P-450 protein content.

DISCUSSION

Although counterparts for most enzyme activities in mammals can be found in fish, the activities and contents of the fish enzymes is generally lower than in mammals (Gregus et al., 1983). The contents and activities of esterases which mediate synthetic pyrethroids hydrolysis was lower in fish liver than in rat and this has been established as the major cause of the high toxicity of synthetic pyrethroids to fish (Edwards et al., 1986, 1987a; Glickman and Lech, 1981, 1982; Glickman et al., 1982; Khan et al., 1977).

Chlorpyrifos, as other phosphorothioate insecticides, must be activated by mixed-function oxidase to show its toxicity. Neverthless, the role of liver biotransformation in mediating the high acute toxicity of the pesticide to fish is unclear. The enzymes in trout liver microsomes have properties comparable to those of mammals based on their responsiveness to inducers and their ability to catalyze a variety of biotransformation reactions. It is of importance to understand the ratio of enzyme activities in the activation and detoxification of chlorpyrifos by trout liver microsomes.

In vitro study of chlorpyrifos biotransformation by trout liver microsomes showed that chlorpyrifos can be metabolized to chlorpyrifos oxon and TCP under the same incubation conditions.

These reactions both required NADPH, and were linear with regard to microsome concentration, and have broad optimal pH profiles. As in the mouse liver microsome study, chlorpyrifos becomes saturated at very low concentration (< 0.2 mM). EDTA, a chelating agent which significantly inhibited the ester hydrolysis of chlorpyrifos oxon to TCP with rat liver microsomes (Sultatos and Murphy, 1983a, 1983b), however, did not change the formation of TCP with trout microsomes. BNF-induced trout microsomes had 2-fold greater chlorpyrifos desulfuration activity compared to control microsomes. However, this does not necessary mean that the acute toxicity of chlorpyrifos would be increased by BNF-pretreatment of fish. For example, PB-pretreatment of mice induced hepatic microsomal activation of methylparathion to methylparaoxon *in vitro* and increased the clearance of methyl parathion by perfused mouse livers but also decrease the acute toxicity *in vivo* (Sultatos, 1987)

Preincubation of trout microsomes with different chemical inhibitors gave differential inhibition on chlorpyrifos desulfuration and deesterification *in vitro*. The P-450 substrates, 1-benzylimidazole, metyrapone and n-octylamine yield 100% to 60% inhibition whereas two FMO substrates (thiourea and methimazole) only slightly inhibited the reactions. These results suggested that the activation and detoxification of chlorpyrifos by trout liver microsomes is primary meadiated via P-450 rather than FMO.

Variation in the ratio of desulfuration and deesterification between BNF-induced and control microsomes indicated that these

reactions may be catalyzed by separate enzyme systems. Immunochemical tests were used to further confirm the contribution of P-450 and the P-450 isozymes on the metabolism of chlorpyrifos by trout liver microsomes. NADPH-cytochrome P-450 reductase is associated with electron transfer in cytochrome P-450 catalyzed reactions (Miwa et al., 1979). NADPH-cytochrome P-450 reductase is apparently not species specific (Gurumurthyl and Mannering, 1985), and trout NADPH-cytochrome c reductase is inhibited by antibodies raised against the rat enzyme (Williams et al., 1983). Surprisingly, the present studies showed that rat-reductase IgG produced less than a 30% reduction in chlorpyrifos oxon formation and less than 10% reduction in TCP formation at concentrations of 20 mg IgG per mg trout microsomal protein. Microsomes from BNF-pretreated trout gave less inhibition by reductase IgG of chlorpyrifos activation and detoxification comparing to that seem with untreated microsomes. The low deactivation activity of P-450 LM_2 IgG observed in this study showed that P-450 LM_2 isozyme was not a major enzyme involved in the metabolism of chlorpyrifos by trout liver microsomes.

Many organophosphates are hydrolyzed by A-esterases in mammals (Aldridge and Reiner, 1972). A-esterases are mainly localized in the cytosol, but the microsomal fraction also has esterase activity (Cammer and Hollingworth, 1976; Neal, 1967; Whitehouse and Ecobichon, 1976). The esterases which hydrolyse paraoxon, DDVP, and chlorpyrifos oxon are found in human serum and mouse liver microsomes (Furlong et al., 1988; Traverso et al.,

1989). Furthermore, multiple forms of esterase isozymes have been isolated from different mammalian liver microsomes and shown to have different substrate specificities (Kao et al., 1985; Leinweber, 1987). Some isozymes of esterases in rat liver microsomes can be induced by PB (Robbi and Beaufay, 1983). Chlorpyrifos oxon in mouse liver microsomes is hydrolyzed very quickly by a calcium-dependent A-esterase, The enzyme requires an active SH-group, and is inhibited by magnesium, manganese, p-chloromercuribenzoate (PCMB) and EDTA (Sultotos et al., 1985). Some organophosphorus compounds can be metabolized by nonenzymatic reactions (O'Brien, 1960). The hydrolysis of chlorpyrifos oxon also catalyzed by nonenzymatic phosphorylation of certain hepatic proteins (Sultotos and Murphy, 1983b). In trout liver microsomes, the situation is quite different. Hydrolysis of chlorpyrifos oxon by trout liver microsomes is substrate unsaturated and the conversion rate is much lower than that of other chlorpyrifos metabolites (Table 14). This reaction is heat-stable, not induced by BNF-pretreatment, not stimulate by calcium, not inhibited by P-450 or FMO inhibitors tested in this study and partially inhibited by EDTA. Chlorpyrifos hydrolysis by trout liver microsomes may be catalyzed by nonenzymatic phosphorylation of certain liver proteins because microsomes are necessary to catalyzed the reaction. Although trout liver microsomes have paraoxon esterase activity (Melancon and Lech, 1979), it is becoming apparent from recent work in several laboratories that hepatic microsomal esterases posses a high degree of specificity with their substrates (Leinweber, 1987).

Paraoxon and chlorpyrifos oxon hydrolysis thus may be catalyzed by different esterases either in mouse liver microsomes or in human blood serum (Sultatos and Murphy, 1983b; Furlong et al., 1988). Studies on chlorpyrifos metabolism by fish in vivo failed to show chlorpyrifos oxon residues in tissues or urine, but tissue acetylcholinease activity was significantly inhibited (Smith et al., 1966). Our study showed that chlorpyrifos can be converted to detectable level of chlorpyrifos oxon and TCP by trout liver microsomal incubation via P-450 mediated reaction, and chlorpyrifos oxon may be hydrolyzed to TCP by nonenzymatic reaction with very low activity. The proposed metabolism pathway for chlorpyrifos with trout liver microsomes is shown in Fig. 18. The high toxicity of chlorpyrifos to fish and undetectable chlorpyrifos oxon within fish tissue may be explained by the rapid hydrolysis of chlorpyrifos oxon by esterase in the soluble fraction of liver tissues or possibly by the biotrans-location and hydrolysis of the oxon in other tissues.

Comparison of the kinetic parameters for trout and mouse liver microsomal metabolism of chlorpyrifos in vitro (Table 15) shows that trout liver have lower desulfuration and deesterifi-cation activities with chlorpyrifos and almost a thousand times lower hydrolysis activity for chlorpyrifos oxon than mouse liver microsomes. The greater capacity of mouse hepatic microsomes to detoxify chlorpyrifos oxon compared to fish liver microsomes may contribute to the major differences in acute toxicities of chlorpyrifos between mammals and fish.

Fig. 11. HPLC chromatograms of ^{14}C-chlorpyrifos and metabolite standards and a ^{14}C-chlorpyrifos microsomal incubation extract on a μ-Porasil column.

HPLC chromatograms showing UV absorption of: (A) a standard mixture containing chlorpyrifos(a), 3,5,6-trichloro-pyridinol (TCP) (b) and chlorpyrifos oxon (c); and (B) an incubation extract following incubation of ^{14}C-chlorpyrifos with trout liver microsomes under optimal conditions. Radioactivity (▨) from the ^{14}C-chlorpyrifos standard (A) and an incubation extract following incubation of radiolabelled chlorpyrifos with trout liver microsomes under optimal conditions (B) is also shown on these chromatograms.
Samples were analyzed by a μ-Porasil column. The retention time of chlorpyrifos, TCP and chlorpyrifos oxon were 3.3, 9.0 and 10.7 min, respectively.

Fig. 11

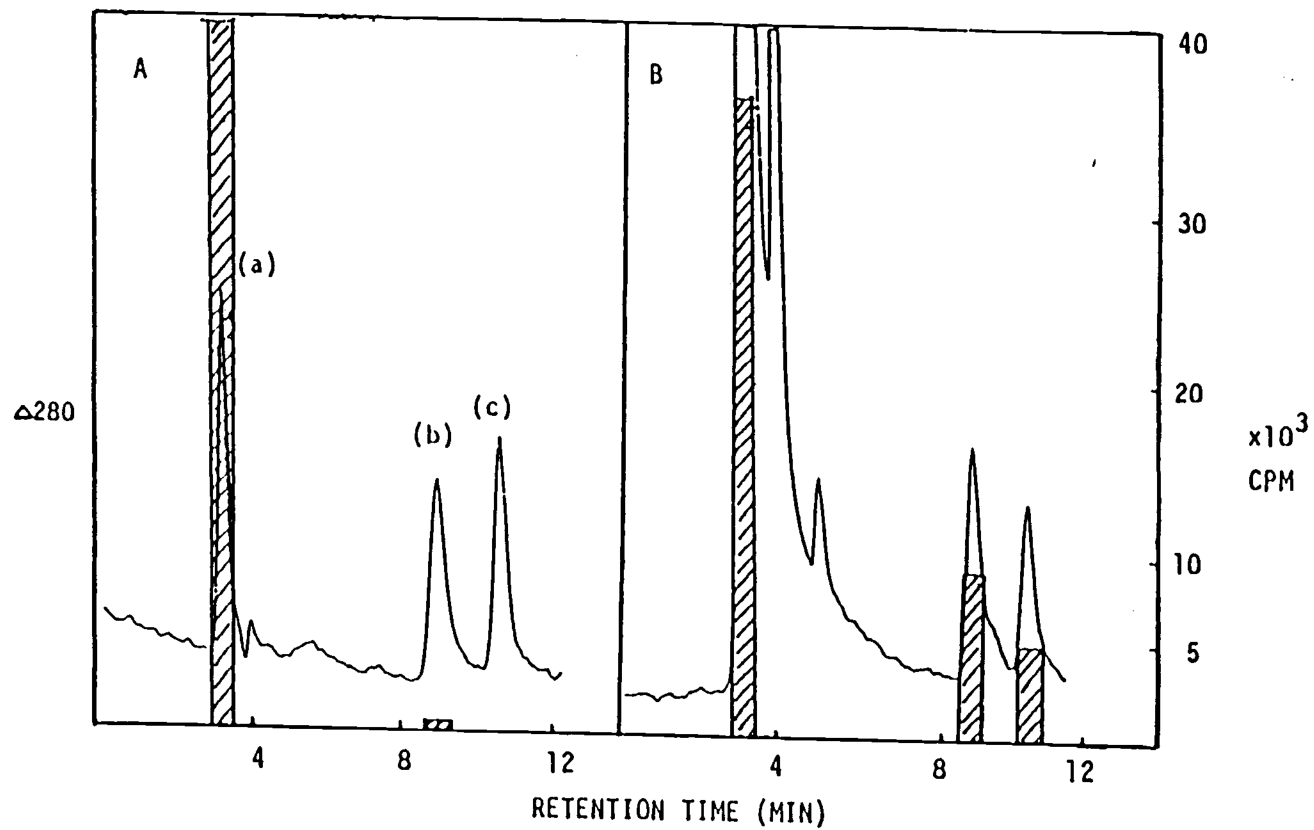

Fig. 12. HPLC chromatograms of chlorpyrifos, chlorpyrifos oxon and TCP by a μ-Zorbax SIL column.

HPLC chromatograms showing UV absorption of (A) a standard mixture containing chlorpyrifos (a), 3,5,6-trichloro-pyridinol (TCP) (b), and chlorpyrifos oxon (c). And (B) an incubation extract following incubation of chlorpyrifos with trout liver microsomes under optimal conditions. Samples were analyzed by a μ-Zorbax SIL column. The retention time of chlorpyrifos, TCP and chlorpyrifos oxon were 2.8, 6.8 and 9.8 min, respectively.

Fig. 12

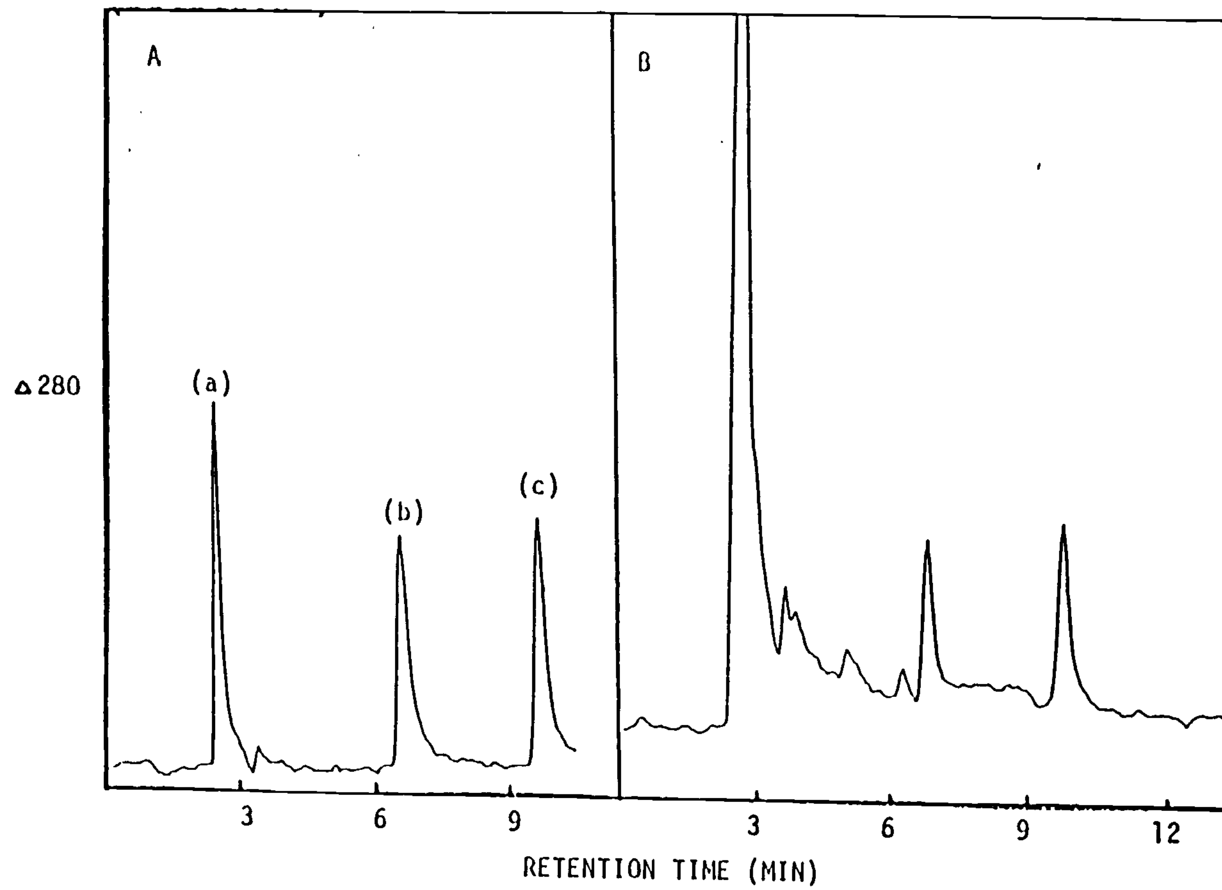

Fig. 13 Time course of chlorpyrifos biotransformation with a rainbow trout liver microsomal incubation.

Formation of chlorpyrifos oxon (-□-) and 3,5,6-trichloro-2-pyridinol (-◆-), and the sum of these two metabolites (-■-) formed by incubation of chlorpyrifos with untreated trout liver microsomes for 10, 20, 30, 40, 50, and 60 min.

Incubation condition were Tris-HCl buffer 0.1 M (pH 7.4), microsomes 2 mg/ml, chlorpyrifos 10 μM, NADPH 0.5 mM and incubation at 25°C for the indicated times.

Fig. 13.

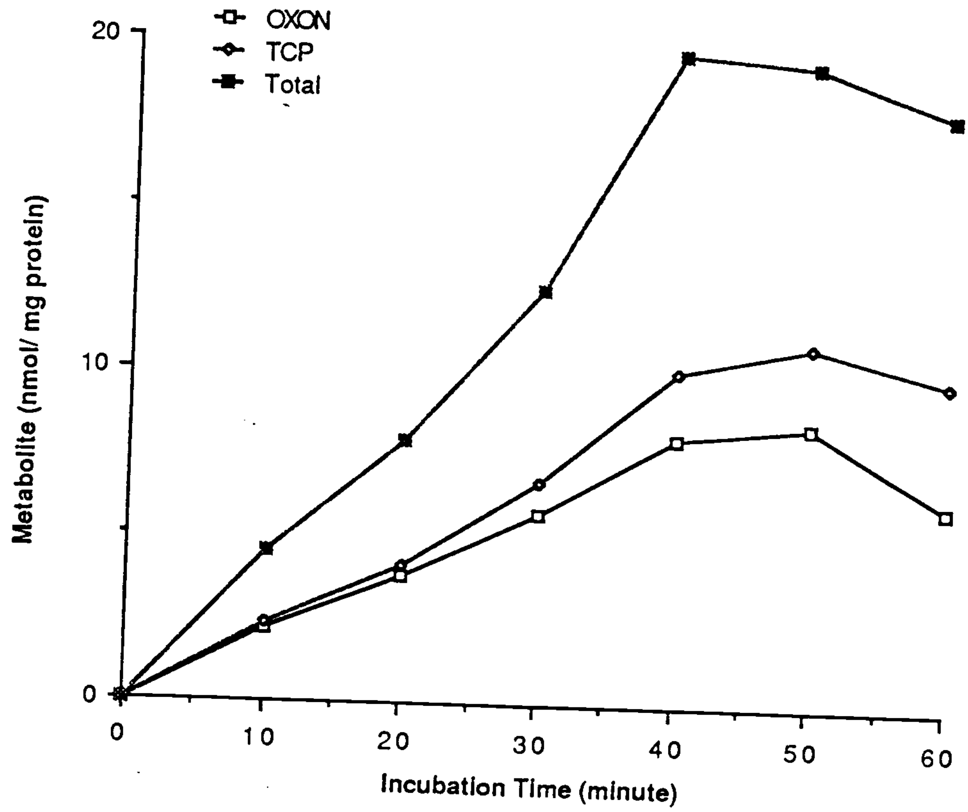

Fig. 14. Effect of microsomal protein concentrations on the biotransformation of chlorpyrifos by rainbow trout liver microsomes.

Formation of chlorpyrifos oxon (◻) and 3,5,6-trichloro-2-pyridinol (◆), and the sum of these two metabolites (■) formed by chlorpyrifos incubation with untreated trout liver microsomes at protein concentrations of 1 - 6 mg/ml.

Incubation conditions were Tris-HCl buffer 0.1 M (pH 7.4), chlorpyrifos 10 μM, NADPH 0.5 mM and incubation at 25°C for 30 min.

Fig. 14.

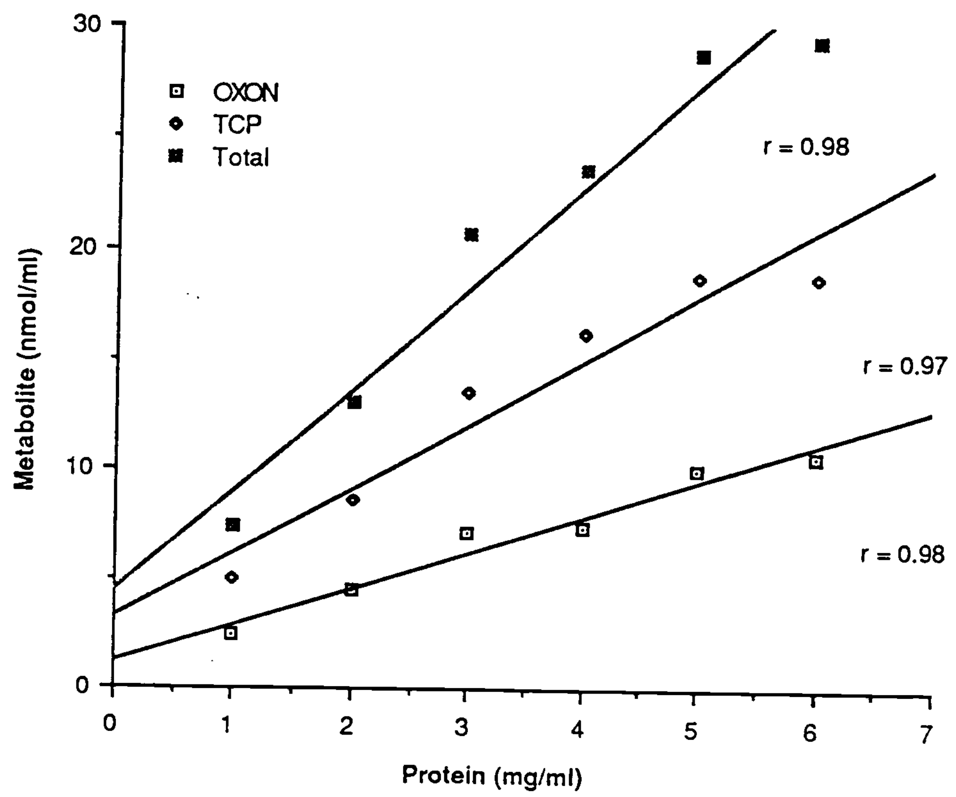

Fig. 15. Effect of pH on the biotransformation of chlorpyrifos by rainbow trout liver microsomes.

Formation of chlorpyrifos oxon (-□-) and 3,5,6-trichloro-2-pyridinol (-◆-), and the sum of these two metabolites (-■-) formed by chlorpyrifos incubation with untreated trout liver microsomes at pH 7.0, 7.5, 8.0, 8.5, 9.0 and 9.5.

Incubation condition were Tris-HCl buffer 0.1 microsomes 2 mg, chlorpyrifos 10 μM, NADPH 0.5 mM and incubation at 25°C for 30 min.

Fig. 15.

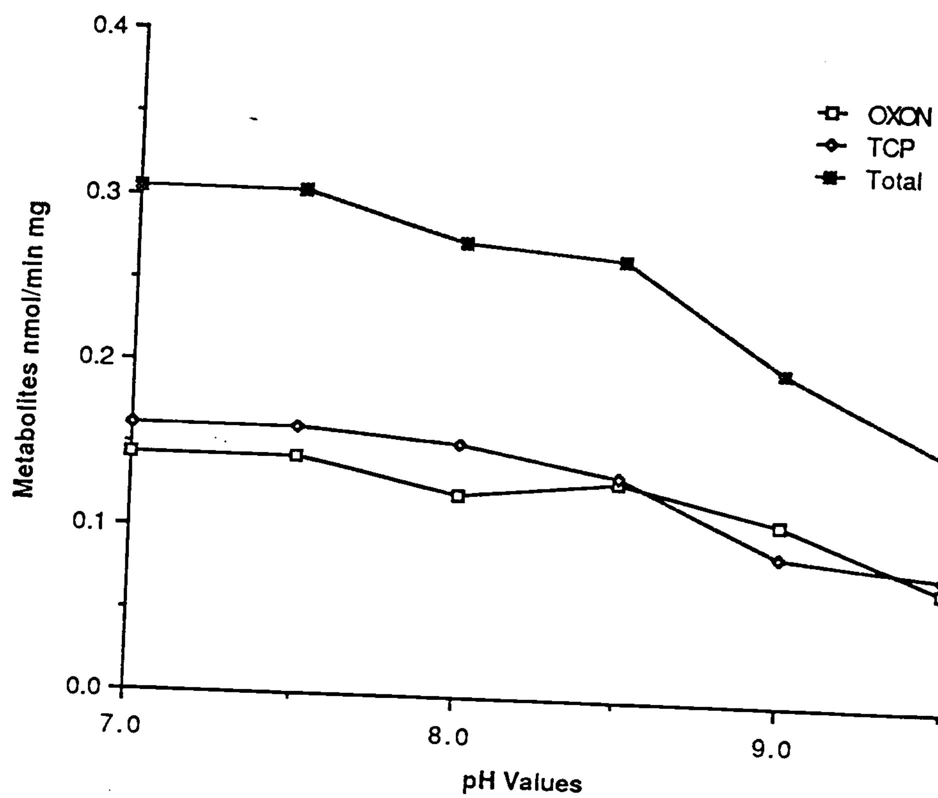

Table 11. The K_m's and V_{max}'s for Chlorpyrifos Metabolites Formed by Incubation with Rainbow Trout Liver Microsomes

	K_m (μM)		V_{max} (nmol/min/mg protein)	
Metabolites[a]	BNF	Control	BNF	Control
CPF Oxon	22	113	0.10	0.12
3,5,6-TCP	73	59	0.30	0.22
Oxon + TCP	45	77	0.37	0.36

[a] Metabolites determined were chlorpyrifos oxon (CPF Oxon), 3,5,6-trichloro-2-pyridinol (TCP), and the sum of chlorpyrifos oxon and TCP (Oxon + TCP).

Incubation condition were: Tris-buffer 0.1 M (pH 7.4), 2 mg/ml of microsomes, chlorpyrifos 10 μM, NADPH 0.5 mM, and incubation at 25°C for 30 min.

Fig. 16. Double reciprocal plots for the hydrolysis of chlorpyrifos oxon by trout liver microsomes.

Microsomes from BNF-pretreated trout liver (▣) and microsomes from untreated trout liver (◆) were used for determining the K_m and V_{max} values.

The incubation mixture contained 2 mg microsomes, 1 - 8 mM chlorpyrifos oxon, 0.5 mM NADPH, in Tris-HCl buffer 0.1 M (pH 7.4) and incubation at 25°C for 30 min. Values shown represent the means of duplicates.

Fig. 16.

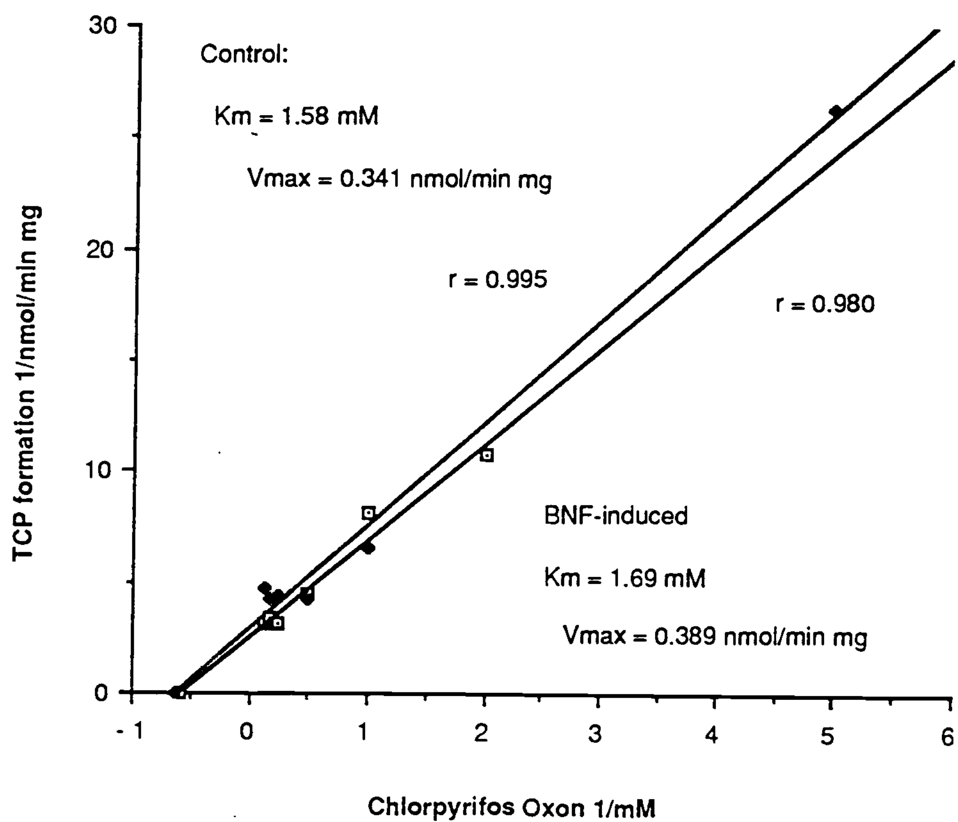

Table 12. The Hydrolysis of Chlorpyrifos Oxon by Boiled Liver Microsomes from Untreated Trout

Boiled microsomes (mg)	Chlorpyrifos oxon (mM)	TCP[a] (nmol/flask)
0	2	0
1	2	3.6
2	2	11.0 (13.8)
4	2	13.2 (13.6)
2	1	3.6 (9.0)
2	2	10.4 (13.0)
2	4	18.6 (18.6)

[a] The data in parenthesis indicated the formation of TCP by non-boiled microsomes in same incubation conditions.

Table 13. The Effect of Inhibitors on the Biotransformation of Chlorpyrifos and Chlorpyrifos Oxon by Rainbow Trout Hepatic Microsomes (% activity) [a]

	Chlorpyrifos				Chlorpyrifos Oxon
	Oxon		TCP		TCP
Inhibitors[b]	Control	BNF	Control	BNF	Control
1-Benzyl-imidazole	0.0	0.0	0.0	0.0	143.6
Metyrapone	29.4	38.6	27.6	30.0	91.0
n-Octylamine	39.2	44.6	37.9	51.7	91.0
Thiourea	105.9	92.8	81.0	83.3	110.9
Methimidazole	115.6	106.0	89.6	97.6	101.9

[a] Results are percentage activity remaining compared to without inhibitor added treatment.

[b] Concentration of inhibitors are 1 mM.

Fig. 17. Inhibition of chlorpyrifos metabolism by trout liver microsomes with rabbit antibodies against purified trout cytochromes P-450 LM_2 and LM_{4b}.

Antibody concentrations of 8, 20 and 40 mg IgG per nmol P-450 trout liver microsomal protein were preincubated with microsomes from untreated trout liver (0.134 mg) or microsomes from BNF-induced trout liver (0.082 mg) for 10 min at 25^oC. The remaining components of the microsomal incubation mixture were added, the reaction was run at 25^oC for 40 min, and the formation of chlorpyrifos oxon (A) and TCP (B) then analyzed as described under experimental procedures.

Values are presented by percentage activity of formation of metabolites comparing with microsomes without preincubating with antibodies. The values are means of duplicates: LM_2 IgG preincubated with control microsomes (□),and BNF-induced microsomes (▨); LM_{4b} IgG preincubate with BNF-induced micorosmes (▩).

Fig. 17.

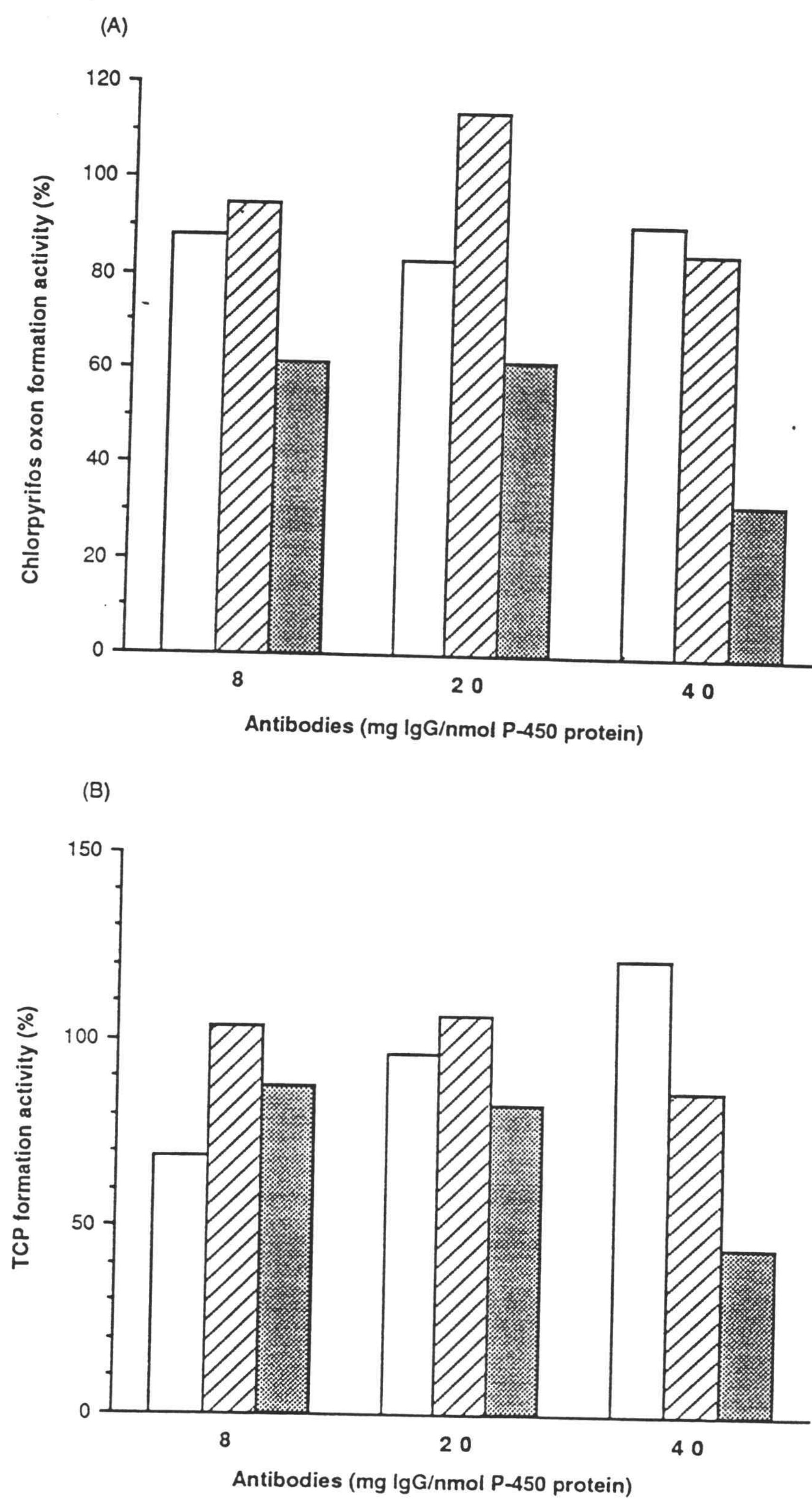

Table 14. Metabolism of Chlorpyrifos and Chlorpyrifos Oxon by Trout Liver Microsomes *in vitro*

		Conversion(%)			
	Conc.	Oxon		TCP	
Substrate	(mM)	Control	BNF	Control	BNF
Chlorpyrifos	0.2	5.68	5.79	14.66	12.38
	0.1	8.80	9.94	14.70	13.65
	0.05	9.43	12.13	26.79	23.63
	0.01	13.00	31.11	46.10	36.33
Chlorpyrifos-Oxon	8.0	---	---	0.16	0.23
	6.0	---	---	0.23	0.29
	4.0	---	---	0.34	0.47
	2.0	---	---	0.69	0.65
	1.0	---	---	0.90	0.72

Fig. 18. The proposed metabolite pathway of chlorpyrifos with rainbow trout liver microsomes.

Biotransformation of chlorpyrifos by rainbow trout liver microsomes by major (==>) P-450 mediated desulfuration pathway to form chlorpyrifos oxon and deesterification to form TCP. The minor (-->) pathway of chlorpyrifos oxon hydrolysis to TCP is catalyzed by nonenzymatic reaction.

Fig.18

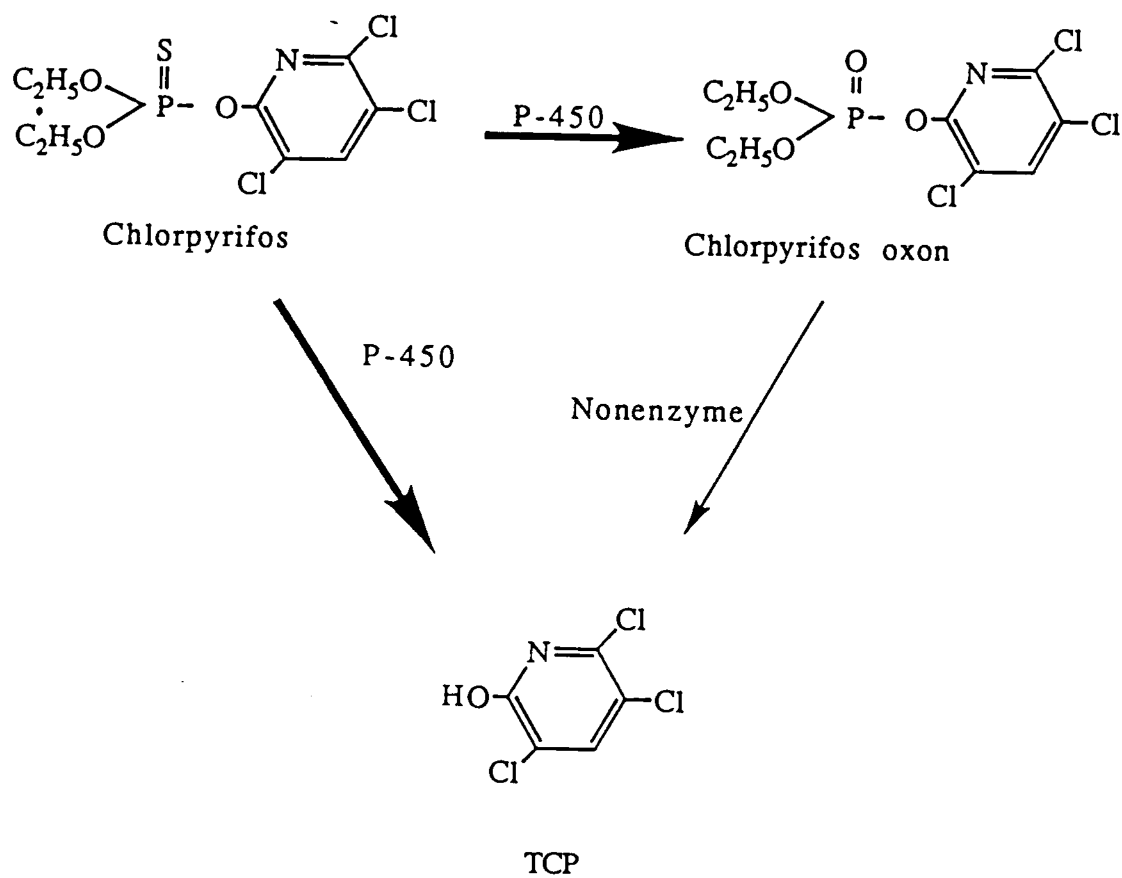

Table 15. Comparasion of K_m's and V_{max}'s of Chlorpyrifos and Chlorpyrifos Oxon Metabolized by Mouse and Trout Liver Microsome[a]

		K_m (mM)		V_{max}(nmol/100mgliver)	
Substrate	Product	Trout	Mice	Trout	Mice
CPF	CPF Oxon	0.113	0.021[b]	0.17	3.9[b]
CPF	TCP	0.059	0.016[b]	0.37	8.1[b]
CPF Oxon	TCP	1.58	1.87 [c]	0.57	2720.8[c]

[a] The abbreviation used on column substrate and product are: chlorpyrifos (CPF), chlorpyrifos oxon (CPF Oxon), and 3,5,6-trichloro-2-pyridinol (TCP).

[b] Data cited from Sultatos and Murphy (1983a).

[c] Data cited from Sultatos and Murphy (1983b).

CONCLUSIONS

Fish can metabolize xenobiotics by biotransformation with enzyme systems that share many similarities and activities with their mammalian counterparts. Therefore, fish are commonly used as animal models for research on the metabolism of toxic substances both _in_ _vivo_ or _in_ _vitro_.

Pyridine is a model compound for studing the enzymology of the heterocyclic compounds metabolism pathway. The biotransformation of pyridine by rainbow trout liver microsomal enzymes _in_ _vitro_ shows that pyridine-N-oxide is the major product of pyridine metabolism. The optimum condition for this reaction are at 25°C pH 8.5, more than 2 mg liver microsomes and a saturated substrate concentration of 4 mM. Cytochrome P-450 in trout liver microsomes seems to mediate pyridine-N-oxidation. Liver microsomes from BNF-induced trout gave a 2-fold increase in pyridine-N-oxidation activity.

Chlorpyrifos is a broad spectrum insecticide and also directly apply to the aqueous environment for mosquito control. This pesticide is very toxic to fish. Chlorpyrifos is oxidatively activated to chlorpyrifos oxon and detoxified to TCP by cytochrome P-450 system in trout liver microsomes. Liver microsomes from BNF-induced trout gave a 5-fold increase in activation but this pretreatment had no effect on detoxification. The rate of

hydrolysis activity of chlorpyrifos oxon to TCP is very low in trout liver mirosomes and this reaction seems to be catalyzed by nonenzymatic reactions. The low hydrolysis activity for chlorpyrifos oxon in trout liver microsomes compared to that found in mouse liver microsomes may contribute to the major differences in acute toxicities of chlorpyrifos between mammals and fish.

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APPENDIX

Appendix A: GC/MS identification of pyridine metabolites

Extraction of pyridine metabolites from incubation mixtures

Pyridine was incubated with control trout liver microsomes under optimum conditions and extracted as described before. Exract from 5 samples were combined (each sample took 0.6 ml), added 1 g sodium chloride and 0.2 ml 1.0 N sodium hydroxide solution, and extracted with two 5-ml volumes of dichloromethane. The combined dichloromethane extract was collected in evaporating tubes and concentrated to 20 μl. Aliquats (5 μl) were injected into the GC/MS.

Gas chromatography-mass spectrometry(GC/MS)

A Finigan 4000 mass spectrometer linked to a Varian 3400 gas-liquid chromatography, with a capillary column DBWAX (30 m) was used in this study. The oven temperature was set to 50 to 250 ^{o}C with a 20°C/min gradient. The ionization potential was 70 eV.

Results

The MS spectrum of pyridine and its possible metabolites pyridine-N-oxide, 2-OH pyridine, 3-OH pyridine, 4-OH pyridine, and 3-OH pyridine-N-oxide were shown in Figs. 19, 20 and 21. GC chromatograms of the incubation extract was shown in Fig. 22. The peak at retention time 5.45 min was identified by MS as pyridine

peak at retention time 5.45 min was identified by MS as pyridine and that at retention time 15.36 min was identified as pyridine-N-oxide (Figs. 23 and 24). Injection of mixed standards showed that 2-OH pyridine and 4-OH pyridine had same retention time under this GC condition.

Fig. 19. Mass spectra of pyridine and pyridine-N-oxide.

Mass spectra of pyridine (A) and pyridine-N-oxide (B) by using GC/MS with GC capillary column of DBWAX 30 m.

Fig.19

A

B

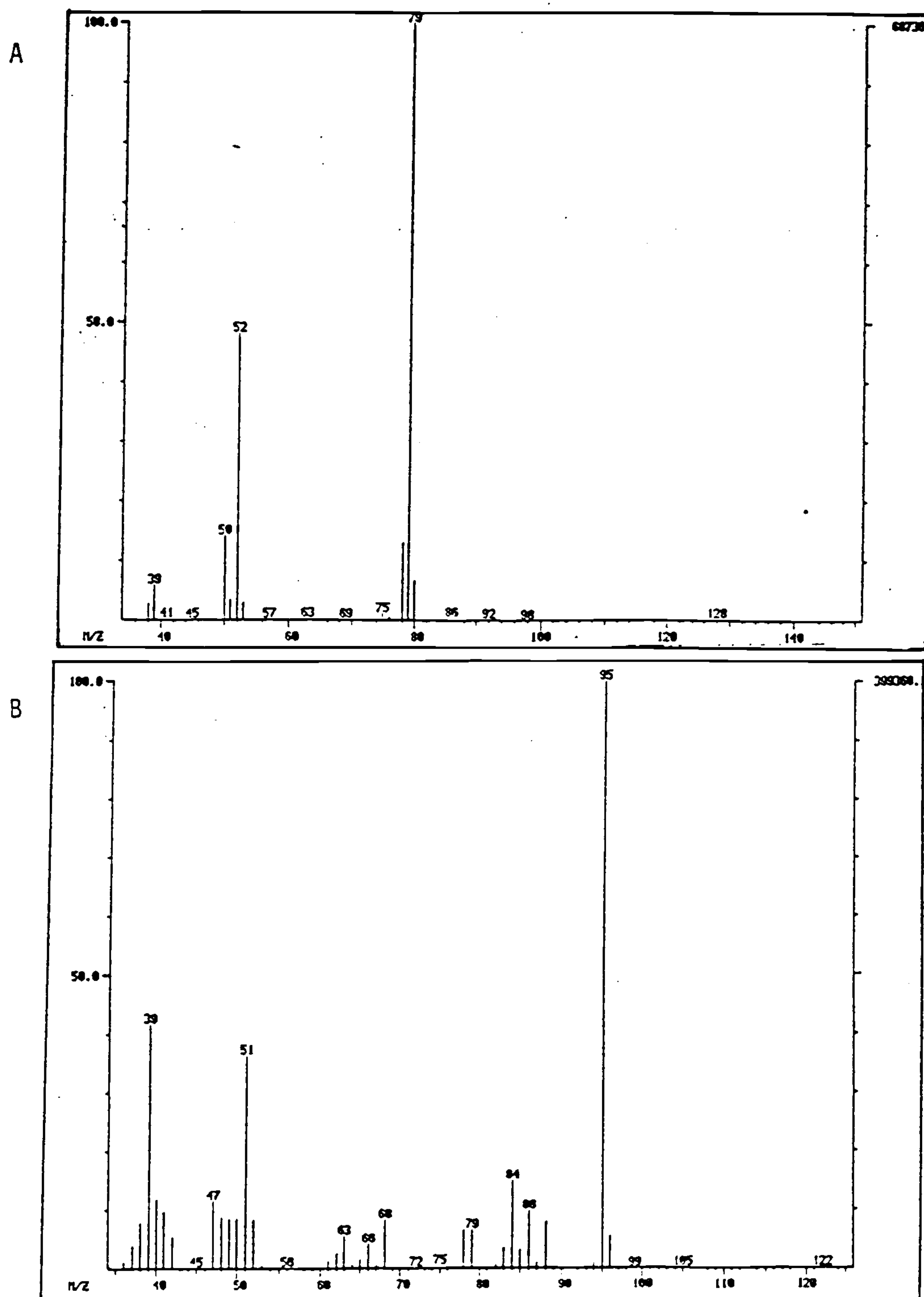


Fig. 20. Mass spectra of 2-OH pyridine and 4-OH pyridine.

Mass spectra of 2-OH pyridine (A) and 4-OH pyridine (B) by using GC/MS with GC capillary column of DBWAX 30 m.

Fig. 20

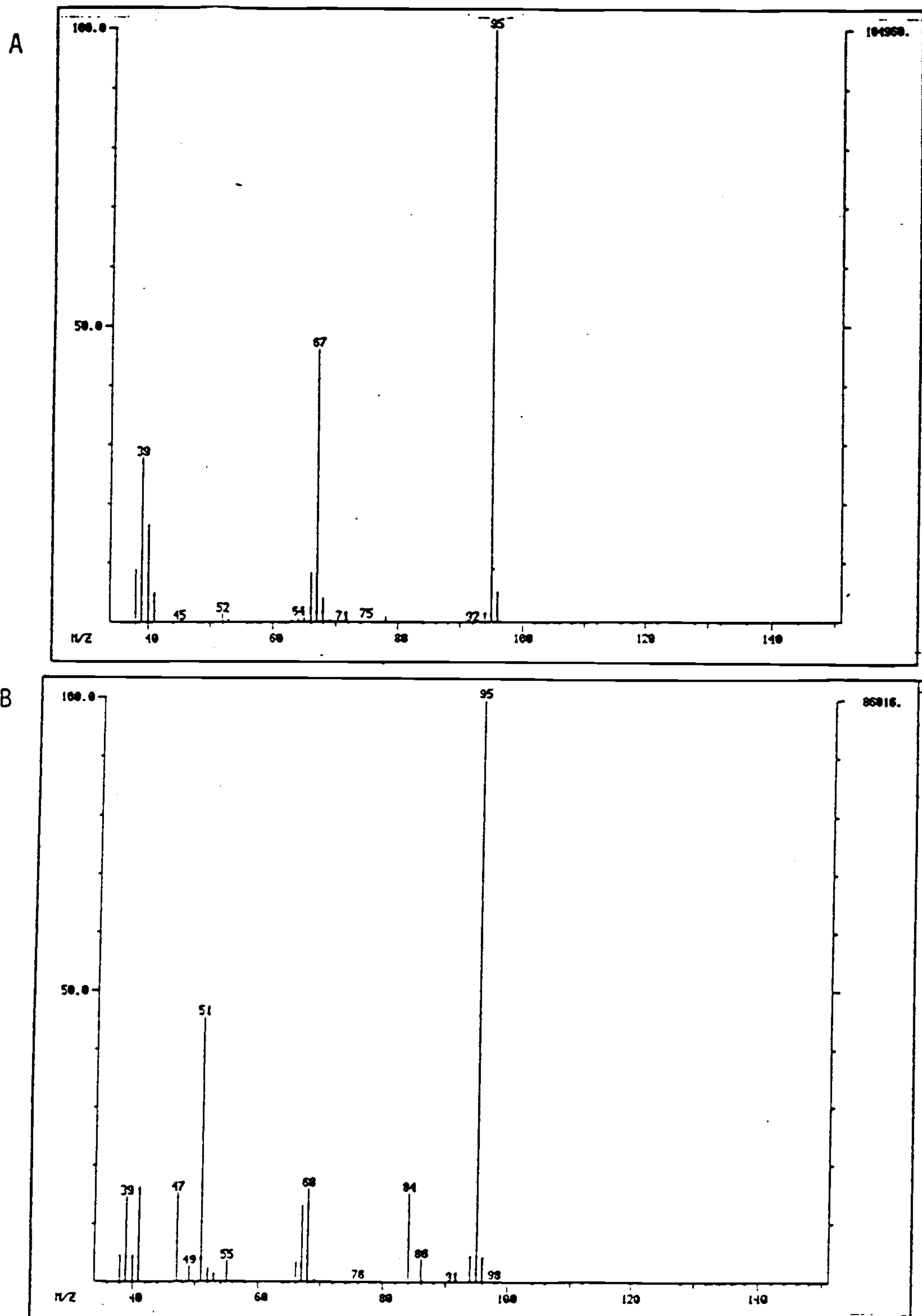
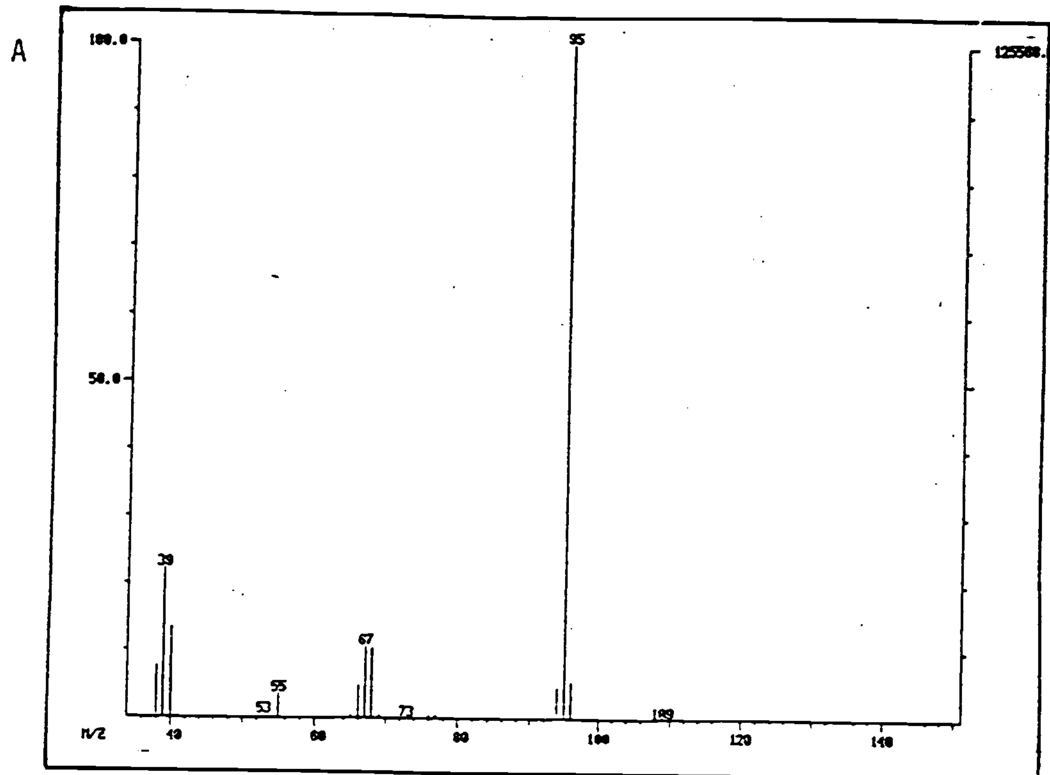


Fig. 21. Mass spectra of 3-OH pyridine and
3-OH pyridine-N-oxide.

Mass spectra of 3-OH pyridine (A) and
3-OH pyridine-N-oxide (B) by using GC/MS
with GC capillary column of DBWAX 30 m.

Fig. 21

A



B

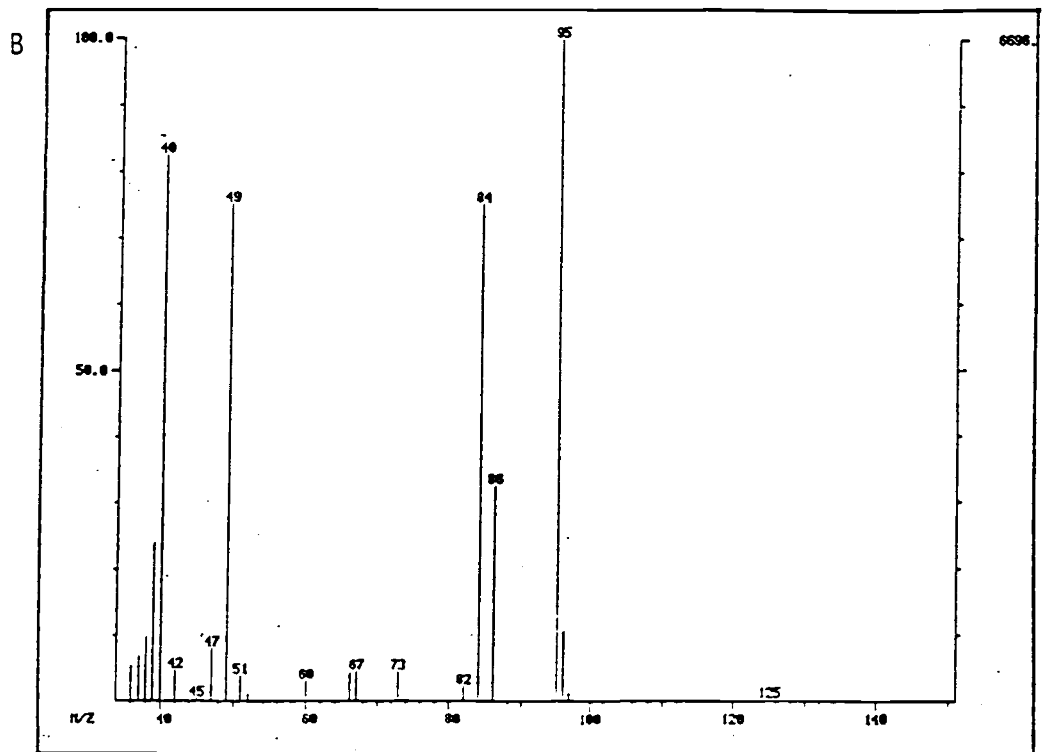


Fig. 22. GLC chromatograms of incubation extract from the incubation of pyridine with trout microsomes.

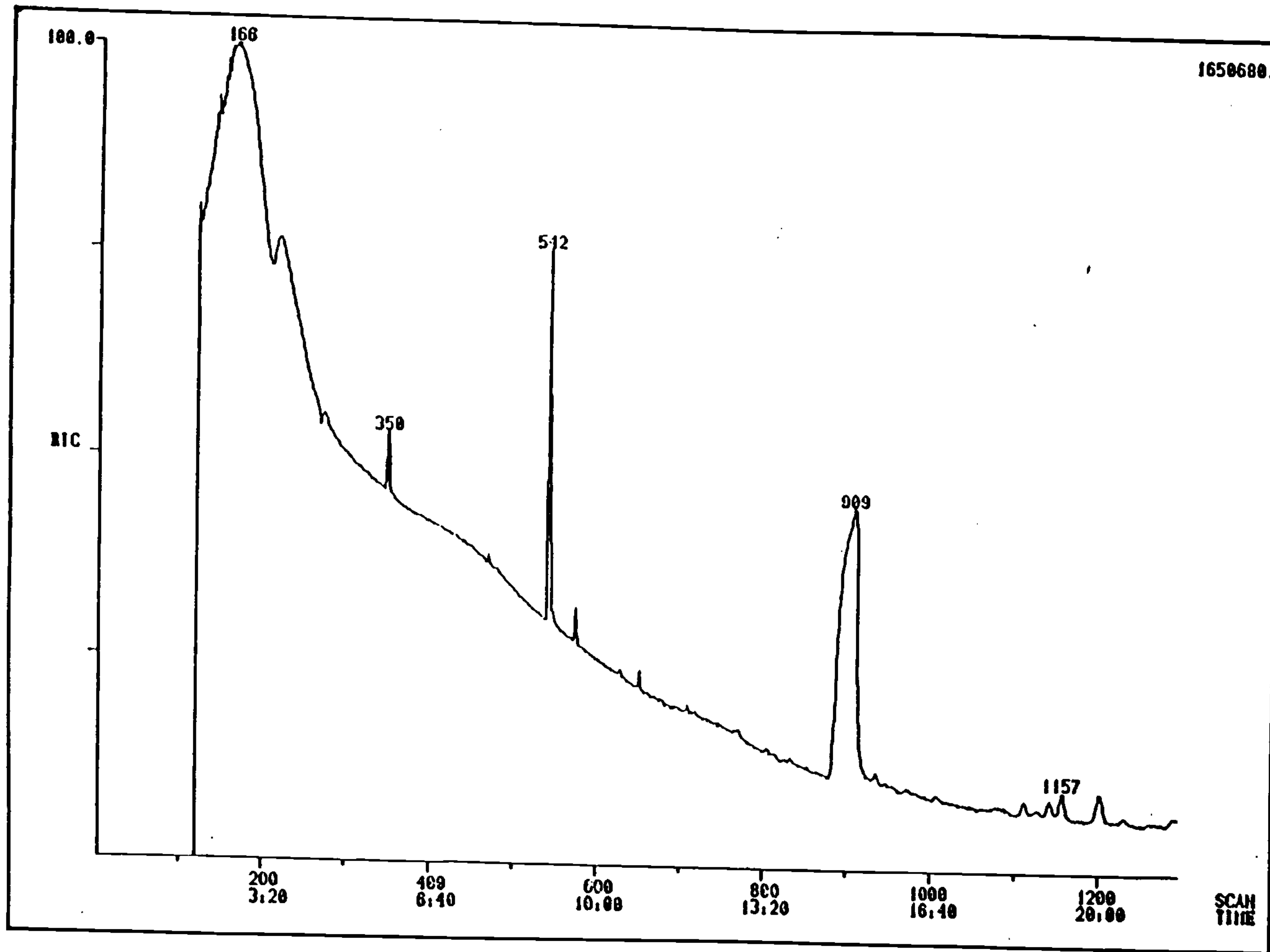


Fig. 22

Fig. 23. Mass spectrum of pyridine recovered from incubation extract samples.

Incubation extract samples analyzed by GC/MS, the MS spectra of peak at retention time of 5.45 min as identified as pyridine.

Fig. 23

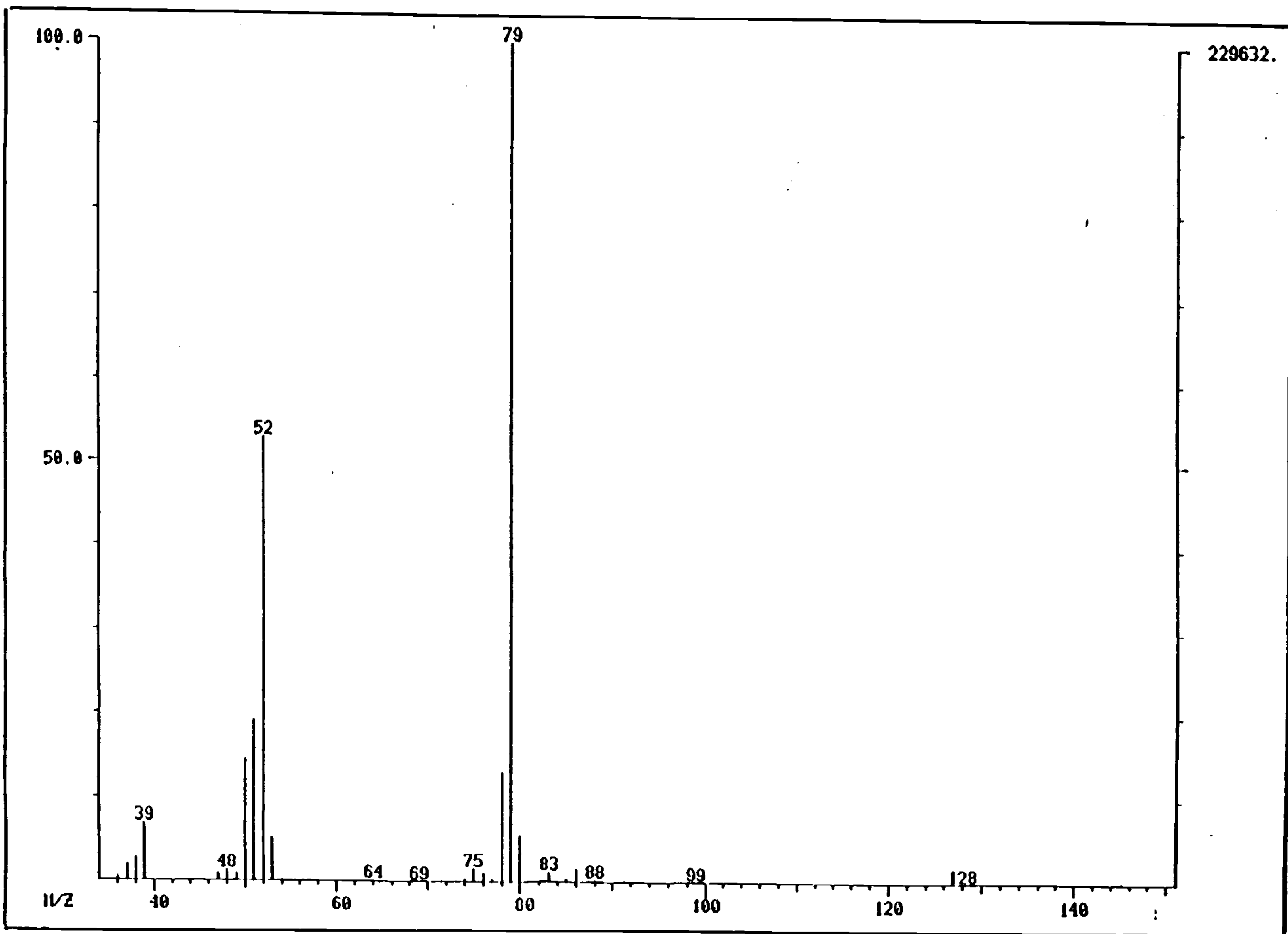


Fig. 24. Mass spectrum of pyridine-N-oxide recovered from incubation extract samples.

Incubation extract samples analyzed by GC/MS, the MS spectra of peak at retention time of 15.36 min as identified as pyridine-N-oxide.

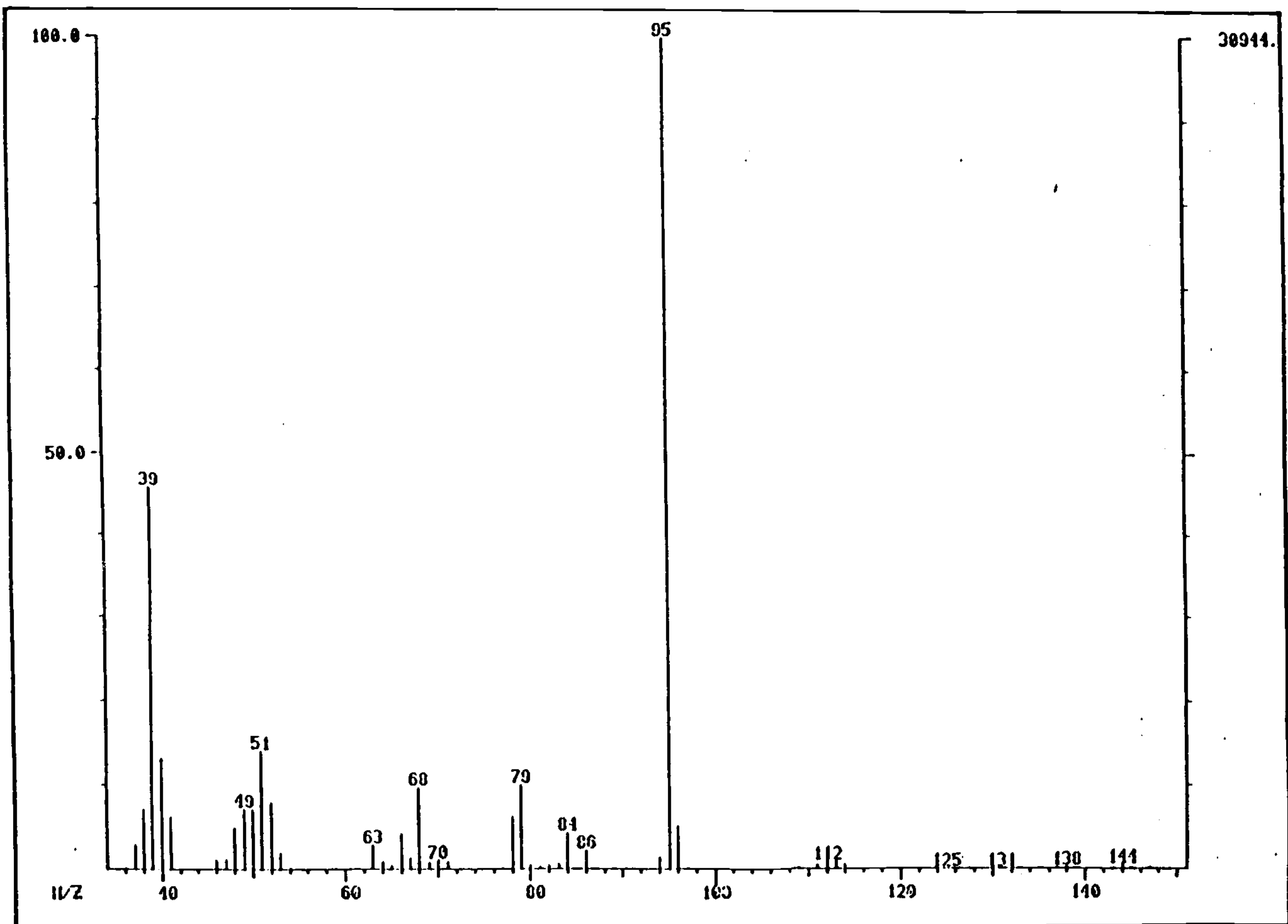


Fig. 24