

EFFECT OF CYTOCHROME P-450 INDUCTION ON THE  
METABOLISM AND TOXICITY OF OCHRATOXIN A

CENTRE FOR NEWFOUNDLAND STUDIES

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# Effect of Cytochrome P-450 Induction on The Metabolism and Toxicity of Ochratoxin A

by

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## ABSTRACT

The role of cytochrome P-450 in the stimulation of lipid peroxidation induced by the mycotoxin ochratoxin A (OTA) has been investigated. Purified cytochrome P-450 (IIB1) could effectively replace EDTA in stimulating lipid peroxidation in a reconstituted system consisting of phospholipid vesicles, NADPH-cytochrome P-450 reductase,  $\text{Fe}^{3+}$ , EDTA and NADPH, suggesting that it could mediate the transfer of electrons from NADPH to  $\text{Fe}^{3+}$ . Microsomes isolated from livers of cobalt protoporphyrin IX-treated rats (in which cytochrome P-450 was depleted) underwent OTA-dependent lipid peroxidation much more slowly than control microsomes.

The role of cytochrome P-450 in OTA metabolism was also investigated. To determine which cytochrome P-450 isoforms are involved in the metabolism of OTA, we used different cytochrome P-450 inducers to induce the major isoforms of cytochrome P-450 in the rat liver. Microsomes from these livers were used to investigate their effect on OTA metabolism. Pretreatment of rats with pregnenolone-16 $\alpha$ -carbonitrile (PCN), phenobarbital (PB), 3-methyl cholanthrene (3MC), and isosafrole (ISF) greatly induced 4(R)-4-OH-OTA formation; 4(S)-4-OH-OTA formation was also induced after pretreatment with PB, PCN, 3MC and ISF. INH pretreatment primarily induced the 4(S) isomer formation. The formation of the 4(R) and 4(S) isomers showed significant differences with respect to pH optima, effect of antioxidants and iron chelators. The 4(R) isomer formation showed a pH optimum of 6.0 using microsomes from rats treated with 3MC and ISF, and 6.5 using microsomes from rats treated with PB and PCN and was not inhibited by antioxidants or iron chelators. In contrast, both the 4(S) isomer formation and lipid peroxidation showed a pH optimum of 7.0 - 7.5 and both activities were highly sensitive to inhibition by antioxidants and iron chelators. Lipid peroxides were not involved in the 4(S) isomer formation since addition of linoleic acid hydroperoxide to microsomes did not give rise to the 4(S) isomer. Cytochrome P-450 appeared to be essential since other hemoproteins such as horseradish

peroxidase and hemoglobin were ineffective in metabolizing OTA. Microsomes from rats pretreated with Co-protoporphyrin IX resulted in no metabolism of ochratoxin A. 7-Ethoxy- and 7-pentoxoresorufin assays showed specificity towards cytochromes P-450 induced by 3MC (IA1/IA2) and PB (IIB1) respectively. Also, metyrapone (inhibitor of cytochrome P-450 IIB1) preferentially inhibited OTA metabolism by microsomes from rats treated with PB, and  $\alpha$ -naphthoflavone (inhibitor of cytochrome P-450 IA1/IA2) preferentially inhibited OTA metabolism by microsomes from 3MC and ISF treated rats. Monoclonal antibodies (MAbs) 1-7-1 (against P-450 IA1/IA2) and 2-66-3 (against P-450 IIB1) showed preferential inhibition of OTA metabolism by microsomes from 3MC and PB treated rats respectively.

Excretion of renal enzymes in urine is a sensitive non-invasive index of renal damage. Therefore, we examined the effect of cytochrome P-450 induction on the excretion of alkaline phosphatase and  $\gamma$ -glutamyl transferase ( $\gamma$ -GT) in a PB-treated rat group and a control rat group (both groups received OTA). At the fourth day of OTA treatment higher enzyme levels were found in the control group compared to the PB group. The results provide evidence for the stimulatory role of cytochrome P-450 in OTA-induced lipid peroxidation. Also, our data suggest that 4(R)-4-OH-OTA is formed by normal mixed function oxidation but that 4(S)-4-OH-OTA formation may involve free iron. Also, our results show that the major cytochrome P-450 isoforms catalyzing OTA hydroxylation are IA1/IA2 (3MC and ISF), IIB1 (PB) and IIIA1/IIIA2 (PCN). The results from *in vivo* experiments suggest a protective effect of PB against OTA toxicity (through inducing cytochrome P-450, thus metabolizing more OTA and excreting it faster from the body compared to controls). This was also evident from *in vivo* experiments of [ $^3$ H]OTA distribution. Therefore, OTA hydroxylation is more likely to be a detoxification pathway.

To my parents,  
my wife  
and my daughter

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## LIST OF ABBREVIATIONS

BEN,	Balkan endemic nephropathy
BHA,	butylated hydroxyanisole
BHT,	Butylated hydroxytoluene
BNF,	$\beta$ -naphthoflavone
BPS,	bathophenanthrolinedisulfonic acid
C,	control (no treatment)
CHAPS,	3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate
CHP,	cumene hydroperoxide
CLF,	clofibrate
DPPD,	N,N-diphenyl-1,4-phenylenediamine
DTT,	dithiothreitol
FMN,	flavin mononucleotide
Fp,	NADPH-cytochrome P-450 reductase
HRP,	horseradish peroxidase
INH,	isoniazid
ISF,	isosafrole
LAHP,	linoleic acid hydroperoxide
MAb,	monoclonal antibody
3MC,	3-methylcholanthrene
MDA,	malondialdehyde
OTA,	ochratoxin A
PB,	sodium phenobarbital
PCN,	pregnenolone-16 $\alpha$ -carbonitrile
SOD,	superoxide dismutase

TBA,	2-thiobarbituric acid
TCA,	trichloroacetic acid.
v/v,	volume/volume
w/v,	weight/volume

**NOTE** - For the sake of brevity, in some tables and figures, the term INH microsomes, PCN microsomes or Co-heme microsomes. This is meant to imply that the microsomes were isolated from the livers of INH-, PCN- or cobalt protoporphyrin IX (Co-heme)-pretreated rats and not that the microsomes contained INH, PCN or Co-heme.

# CHAPTER 1

## INTRODUCTION

### 1.1 OCHRATOXIN A

#### 1.1.1 Natural Occurrence and human exposure

Ochratoxin A (OTA) is a mycotoxin produced by some species of the fungal genera Aspergillus and Penicillium. It was first reported in 1965 by De Scott (1965) as the toxic metabolite in a culture medium of Aspergillus ochraceus willh. OTA was chemically characterized by Van der Merwe et al. (1965a,b) and Steyn and Holzapel (1967). It consists of a 5'-chloro-8-hydroxy-3,4-dihydro-3-methyl isocoumarin moiety linked by an amide bond to L-β-phenylalanine (Figure 1). The toxigenic molds known to produce the toxin are the following species:

Aspergillus species

A. ochraceus

A. ostianus

A. melleus

A. petrakii

A. sclerotium

A. sulphureus

Penicillium species

P. viridicatum

P. cyclopium

P. commune

P. palitans

P. purpurescens

P. variable

The occurrence of OTA in food and feed is widespread (Krogh and Nesheim, 1982). It is present as a contaminant in plant products, especially, cereals, beans and peanuts (Kurata, 1978). OTA is also found in meat, dried fish (Ueno, 1985a) as well as in the kidneys, liver and blood of slaughtered pigs (Hult et al., 1980). Scott et al. (1972) detected OTA in concentrations of up to 27 µg/gm in 18 out of 29 samples of heated grain from Saskatchewan farms in Canada. In a recent analysis of 1200 blood samples obtained from pigs slaughtered in Western Canada, Marquardt et al. (1988) found that 76% of the samples had detectable levels of OTA and 11.3% had OTA levels of > 10 ng/mL with the

highest being 229 ng/mL. The World Health Organization (WHO) recently reviewed OTA, and in their report they summarized the occurrence of OTA in food and animal feed of plant origin around the world (WHO, 1990). Among the highest levels of OTA were those samples listed in Table 1.

Residues of OTA are not generally found in ruminants, because OTA is cleaved in their forestomachs by protozoan and bacterial enzymes (Galtier and Alvinerie, 1976; Hult et al., 1976); the non-toxic hydrolytic cleavage product is ochratoxin a (ochratoxin A without the phenylalanine moiety). When two milking cows were fed a ration containing 317-1125 µg OTA/kg body weight for 11 weeks, a residue of 5 µg OTA/kg was found in the kidneys of one of the animals but not in any other tissue or the milk. Ochratoxin a was not found in any tissue (Shreeve et al., 1979). Residues of OTA have been detected in a number of tissues in single-stomached animals such as pigs (Hult et al., 1980). OTA is also present in the blood because it binds to serum albumin (Chu, 1971; Galtier, 1974). The incidence of OTA in meat and meat products has been reviewed by Marquardt et al. (1990) (Table 2).

Human exposure occurs through the direct consumption of contaminated food (mainly cereals) and/or meat from animals that retain OTA in their tissues after being fed contaminated feed (see diagram, Figure 2). OTA levels in humans were reported in a review by Kuiper-Goodman and Scott (1989) (Table 3).

### 1.1.2 Stability of OTA and effects of food processing

OTA is moderately stable, even on heating, in such food as wheat, pork and beans. However, levels of OTA added to ground green coffee beans were reduced 87% on roasting for 20 min at 200°C (Levi et al., 1974). Tsubouchi et al. (1987) found that after inoculation with *A. ochraceus*, roasting green coffee beans for 20 min at 200°C reduced the concentration of OTA by a maximum of only 12% in whole beans and 18-22% in ground beans. They concluded that the mode of OTA contamination was the reason for this greater

heat resistance. Cleaning and milling of wheat and barley did not remove OTA in naturally contaminated samples, and levels in flour and bran were the same (Chelkowski et al., 1981). There was, however, a marked reduction of OTA levels in pearled barley compared to those in bran (Chelkowski et al., 1981). Cooking of polished wheat by an Egyptian method destroyed only 6% of added OTA, and during cooking of faba beans by two methods the average destruction was 16% and 20% (El-Banna and Scott, 1984).

### 1.1.3 Toxicity

OTA is known to be highly toxic to animals (Van der Merwe et al., 1965 a; Purchase and Theron, 1968; Peckham et al., 1971 and Szczech et al., 1973). The main pathological changes associated with OTA toxicity are primarily damage to the kidney and to a lesser extent damage to the liver (Harwig, 1974 and Krogh, 1978). Alterations in a variety of biochemical and immunological parameters have also been observed following OTA administration (Berndt et al., 1980; Meisner and Meisner, 1981; Haubeck et al., 1981; and Kane et al., 1986). The changes in renal function in OTA-exposed rats and pigs are characterized by polyuria, glucosuria, proteinuria and an increase in blood urea nitrogen, and a decrease in urine osmolarity and glomerular filtration rate (Berndt et al., 1980). Kane et al. (1986) observed a good correlation between the increase in urinary excretion of  $\gamma$ -glutamyl transferase, alkaline phosphatase and leucine aminopeptidase and a decrease in renal activities of these same enzymes within a week of the oral administration of 145  $\mu$ g OTA/kg body weight for 12 weeks.

An inhibition of renal gluconeogenesis was also observed in kidney-cortex slices from rats which had been fed with 2 mg OTA/kg body weight for 2 days (Meisner and Selanik, 1979) most probably because renal phosphoenolpyruvate carboxykinase was inhibited (Meisner and Meisner, 1981). After the daily administration of OTA (5 mg/kg) for 3 days to rats, Suzuki et al. (1975) observed a 60% decrease in hepatic glycogen levels

and a concomitant increase in serum glucose, and blood and liver lactate levels. Later, Subramanian et al. (1989) showed that OTA drastically depleted liver glycogen, increased blood glucose level and lowered serum insulin level in rats. They concluded that OTA is diabetogenic in nature, and exerts its effect by reducing the level of insulin, thereby suppressing glycogenesis and enhancing glycogenolysis. Administration of OTA to mice inhibited protein synthesis, the degree of inhibition 5 hr after administration of 1 mg OTA/kg body weight being 26% in liver, 68% in kidney and 75% in spleen (Creppy et al., 1984). Phenylalanine (100 mg/kg body weight) injected together with OTA (10 mg/kg body weight) prevented the inhibition of protein synthesis in all of these organs (Creppy et al., 1984). OTA is thought to inhibit protein synthesis through its competition with phenylalanine in the reaction catalyzed by phenylalanyl t-RNA synthetase (Creppy et al., 1984). Interestingly, babies born with phenylketonuria are less susceptible to OTA toxicity because of their elevated level of phenylalanine (Woolf, 1986). OTA was shown to inhibit rat liver mitochondrial respiration by acting as a competitive inhibitor of carrier proteins located in the inner mitochondrial membrane (Meisner and Chan, 1974; Wei et al., 1985). The LD<sub>50</sub> values of OTA in various species are listed in Table 4 (WHO, 1990).

#### **1.1.4 Teratogenicity**

OTA is a potent teratogen in mice, rats and hamsters. OTA injected intraperitoneally in pregnant mice at 5 mg/kg body weight on one of gestation days 7-12 resulted in increased prenatal mortality, decreased fetal weight, and various malformations, including exencephaly and anomalies of the eyes, face, digits and tail (Hayes et al., 1974). When rats were treated orally with OTA at 0.25, 0.50, 0.75, 1, 2, 4 or 8 mg/kg body weight on gestation days 6-15, maternal toxicity was not observed below 4 mg/kg body weight, but an increased incidence of fetal resorptions was observed above 0.50 mg/kg body weight (Brown et al., 1976). Subcutaneous administration of OTA (1.75 mg/kg

body weight) on gestation days 5-7 resulted in the highest number of malformations, including hydrocephaly, omphalocele and anophthalmia, as well as a shift in position of the oesophagus (Mayura et al., 1982). Increased prenatal mortality and malformations, including hydrocephaly, micrognathia, and heart defects were observed in hamsters injected intraperitoneally with OTA at doses of 5-20 mg/kg body weight on one of gestation days 7-9 (Hood et al., 1976).

### 1.1.5 Immunotoxicity

In chickens fed 2-4 ppm OTA in the diet for 20 days there was a depression of IgG, IgA and IgM in lymphoid tissues and serum (Dwivedi and Burns, 1984). OTA in the concentration range studied (20-1667  $\mu\text{mol/L}$ ) caused a 47-50% inhibition of macrophage migration (Klinkert et al., 1981); this effect could be prevented by the simultaneous addition of phenylalanine to the medium. In Balb/c mice, a dose of OTA as low as 0.005  $\mu\text{g/kg}$  was able to suppress the immune response to sheep red blood cells (Haubeck et al., 1981). In the standard plaque counting assay for the estimation of antibody-producing spleen lymphocytes OTA (1  $\mu\text{g/kg}$  body weight) given once intraperitoneally to Balb/c mice, 8-12 weeks of age, had an immunosuppressive effect on both IgM and IgG responses to a single injection of sheep red blood cells (Creppy et al., 1982). The immunosuppressive effects of OTA could be prevented by the intraperitoneal administration of phenylalanine at 10  $\mu\text{g/kg}$  body weight (Haubeck et al., 1981 and Creppy et al., 1982). Thus, the immunosuppressive action of OTA could be due to its action on protein synthesis. When female B6C3F1 mice were administered ochratoxin A 6 times during 12 days in amounts of 0.34, 6.7, or 13.4 mg/kg body weight (total dosage), the natural killer cell activity was inhibited at all dose levels, and the growth of transplantable tumour cells was increased without affecting T-cell or macrophage-mediated antitumour activity. Ochratoxin B did not influence immune function at 13.4 mg/kg body weight administered 6

times during 6 days (Luster et al., 1987). The inhibition by OTA of natural killer cell activity appeared to be caused by reduced production of basal interferon. Because natural killer cells are involved in the destruction of tumour cells, the ability of OTA to modulate the activity of these cells might contribute to its capacity to induce renal and hepatic carcinomas. Lea et al. (1989) reported that OTA has a potent inhibitory effect both at the level of B and T lymphocytes.

#### 1.1.6 Carcinogenicity and mutagenicity

Kanisawa and Suzuki (1978) reported renal and hepatic tumors in male ddY mice after they were fed a diet containing 40 mg OTA/kg for 45 weeks. Dietary feeding of OTA (40 mg/kg diet for 20 months) has also been shown to induce renal adenomas and hepatocellular carcinomas in mice (Bendele et al., 1985). It was quoted that ochratoxin A is one of the most potent toxic carcinogens ever tested by the National Toxicology Program (Dietrich and Swenberg, 1993). In that extensive carcinogenicity study by the National Toxicology Program, OTA administered by gastric intubation in corn oil at levels of 70 and 210 µg/kg body weight/day for 24 months induced high incidences of renal cell carcinoma of a clearly malignant nature in rats (Boorman, 1988). OTA has also been associated with Balkan endemic nephropathy (BEN), a disease that affects residents of certain regions of Bulgaria, Romania and Yugoslavia where OTA contaminates their crops (Krogh, 1974). In these infected areas, OTA was isolated from the sera of residents and those who had high OTA levels also (often) had urinary tract tumors and BEN (Hult et al., 1982a,b and Petkova-Bocharova et al., 1988). Creppy et al. (1985) showed that OTA administration induced single strand breaks in DNA in liver, kidney and spleen, and they suggested that OTA is weakly genotoxic to mammalian cells. OTA has been tested for mutagenicity in a variety of *Salmonella typhimurium* strains, with and without metabolic activation, and results were negative (Wehner et al., 1978; Bartsch et al., 1980). Recently, after

incubation with cultured hepatocytes for 24 hours, OTA was shown to be mutagenic to *Salmonella typhimurium* in a cell-free culture medium (Hennig et al., 1991).

#### **1.1.7 Absorption, distribution and metabolism**

Small intestine is thought to be the primary site of ochratoxin A absorption. When OTA was injected into the lumen of the stomach, the small intestine, cecum or colon of male Wistar rats, the highest absorption was in the proximal jejunum (Kumagai and Aibara, 1982). When OTA was given orally to mice, the site of highest absorption was the duodenum (Lee et al., 1984). In the latter study, immunohistochemical staining revealed that the highest concentration of OTA was in the intestine with decreasing levels in the kidney and liver (Lee et al., 1984).

Galtier et al. (1979) studied the pharmacokinetic profile of ochratoxin A using [ $^{14}\text{C}$ ]OTA injected intravenously to rats. Six hours after injection, 24% of the administered dose was in the plasma, 12% in muscle, 11.1% in skin, 2.63% in the liver and 0.64% in the kidneys. Per gram of tissue, the concentration of OTA was higher in the kidneys than in the liver. The stomach, small intestine, cecum and large intestine contained 0.34%, 2.14%, 0.66% and 0.57% of the administered dose, respectively. The contents of the stomach, small intestine, cecum and large intestine contained 0.33%, 4.02%, 4.02% and 1.87% of the administered dose respectively.

A number of studies have shown that, in the presence of NADPH, OTA is metabolized by liver microsomes from several species (rat, pig and human) primarily to 4(R)-4-OH-OTA, and to a smaller extent to the epimeric 4(S)-4-OH-OTA (Stormer et al., 1981, 1983; Ueno, 1985; Oster et al., 1991; Hietanen et al., 1986). OTA metabolism has also been shown to be induced by PB and/or 3MC in Wistar rats (Stormer and Pedersen, 1980), DA and Lewis rats (Hietanen et al., 1986) and in rats of unidentified strain (Jeno, 1985).

### 1.1.8 Mechanisms of ochratoxin A toxicity

The mechanism by which ochratoxin A exerts its toxic effects is not known yet. In a recent review by Marquardt and Frohlich (1992), three potential mechanisms of ochratoxicosis were recognized which involved effects of OTA on the enzymes involved in phenylalanine metabolism, effects on lipid peroxidation, and effects on mitochondrial function.

Ochratoxin A affects DNA, RNA, and protein synthesis in many different organisms (Meisner and Meisner, 1981; Creppy et al., 1983a,b, 1986; Meisner et al., 1983; Roschenthaler et al., 1984; Meisner and Krogh, 1986; Meisner and Polsinelli, 1986), presumably due to an effect by the phenylalanine moiety of OTA. Administration of OTA to mice inhibited protein synthesis; the degree of inhibition 5 hr after administration of 1 mg OTA/kg body weight was 26% in liver, 68% in kidney and 75% in spleen (Creppy et al., 1984). Phenylalanine (100 mg/kg body weight) injected together with ochratoxin A (10 mg/kg body weight) prevented the inhibition of protein synthesis in all of these organs (Creppy et al., 1984). OTA is thought to inhibit protein synthesis through competition with phenylalanine in the reaction catalyzed by phenylalanyl t-RNA synthetase. OTA also inhibited phenylalanine hydroxylase (Creppy et al., 1990). Ochratoxin A (OTA without the phenylalanine moiety), in contrast, was not effective, which indicates the importance of phenylalanine for the inhibition. The blockage of phenylalanine hydroxylase activity could produce phenylketonuria-like effects, including an enhanced production of phenylpyruvate and phenylacetate (Creppy et al., 1990)

Rahimtula et al. (1988) have demonstrated that when administered to rats *in vivo* or when added to liver or kidney microsomes *in vitro*, OTA greatly enhanced lipid peroxidation (Rahimtula et al., 1988). In a subsequent study (Omar et al., 1990) using a reconstituted microsomal lipid peroxidation system consisting of microsomal phospholipid, the flavoprotein NADPH cytochrome P-450 reductase (Fp) and iron ions, we demonstrated

that OTA induced lipid peroxidation by chelating  $\text{Fe}^{3+}$ , that the resulting OTA- $\text{Fe}^{3+}$  chelate was readily reducible by the flavoprotein NADPH cytochrome P-450 reductase to the OTA- $\text{Fe}^{2+}$  complex. The latter in the presence of oxygen provided the active species that initiated lipid peroxidation. Using electron spin resonance, the production of the extremely toxic hydroxyl radicals by the  $\text{Fe}^{3+}$  complex of OTA in the presence of the NADPH-cytochrome P-450 reductase system was also demonstrated (Hasinoff et al., 1990). Khan et al. (1989) have shown that OTA-stimulated lipid peroxidation is also accompanied by the leakage of calcium from calcium-loaded microsomes. Lipid peroxidation may be an important process in hepatotoxicity, which results in structural changes in the cell membrane sufficient to allow an influx of extracellular calcium to cause changes in metabolic activity within the cell (Orrenius and Bellomo, 1986). Aleo et al. (1991) have reported that OTA stimulated lipid peroxidation in isolated rat renal proximal tubules. Because mitochondrial toxicity was not changed by the iron chelator desferal, they suggested that OTA toxicity in the proximal tubules (indicated by increased lactate dehydrogenase release and decreased ATP production) was primarily related to inhibition of mitochondrial function and not to iron-mediated lipid peroxidation. OTA toxicity to the mitochondria is discussed further below. The latter study, however, did not conclusively rule out the importance of lipid peroxidation, and the role of lipid peroxidation in the toxicity of OTA remains to be determined.

Ochratoxin A has been shown to inhibit the respiration of whole mitochondria by acting as a competitive inhibitor of carrier proteins located in the inner mitochondrial membrane (Meisner and Chan, 1974; Wei et al., 1985). Meisner (1976) has shown that the uptake of OTA by mitochondria resulted in intramitochondrial ATP depletion. He also showed that OTA inhibited intramitochondrial phosphate transport, resulting in deterioration of the mitochondria. The failure of OTA to deplete ATP in rat hepatocytes

observed by Creppy et al. (1990), and the ability of  $O\alpha$ , which is non-toxic, to inhibit mitochondrial respiration more effectively than OTA (Moore and Truelove, 1970) would suggest that mitochondrial effects probably did not contribute to the toxic effects of OTA.

## 1.2 LIPID PEROXIDATION

### 1.2.1 Definition

In eukaryotes, membrane fluidity is maintained by the incorporation of polyunsaturated fatty acid (PUFA) chains into membrane lipids. Most of these PUFA chains occur on the 2-C position of the glycerol moiety of phospholipids, particularly phosphatidylcholine and phosphatidylethanolamine, although some also occur in neutral lipids. In the membranes of rat liver microsomes, the most abundant PUFAs (expressed as percentage of total fatty acids) are: arachidonic acid (17%), linoleic acid (10%), docosahexanoic acid (5%) and linolenic acid (5%) (Horton and Fairhurst, 1987). In these acids the presence of an adjacent double bond weakens the carbon-hydrogen bonds. These allylic hydrogens can be abstracted by reactive species containing one or more unpaired electrons (free radicals). The lipid radical thus formed will then react with molecular oxygen, and the ensuing chain reaction results in the breakdown of the PUFA. This reaction sequence is known as **lipid peroxidation**. Lipid peroxidation propagates by reaction between a radical molecule and a non-radical molecule and terminates when two radicals react with each other. The reactions of lipid peroxidation may be categorized into three main steps: 1) initiation, 2) propagation and 3) termination (Figure 3).

### 1.2.2 Measurement

The products of lipid peroxidation include lipid epoxides, hydroperoxides, epoxy alcohols, and the short-chain compounds such as malondialdehyde (MDA), ethane, pentane, and 4-hydroxy alkenals (Dahle et al., 1962; Dillard et al., 1977; Hafeman and Hoekstra, 1977; Benedetti et al., 1980). Lipid peroxidation has been measured by the detection of conjugated dienes formed during the early phase of the peroxidation reaction sequence (Corongiu and Milia, 1983; Recknagel and Glende, 1984), and less commonly

by measurement of lipid hydroperoxides (Catheart et al., 1983; Pryor and Castle, 1984). The most common procedures are based on the measurement of the products of lipid hydroperoxide breakdown such as malondialdehyde (MDA). This is the most widely used method because of its simplicity and sensitivity, and MDA has been commonly detected by the thiobarbituric acid (TBA) reaction (Niehaus and Samuelsson, 1968; May and McCay, 1968; Frankel and Neff, 1983; Csallany et al., 1984). In addition, lipid peroxidation has been assayed recently by the evolution of short-chain alkanes (ethane and pentane) both *in vivo* and *in vitro* (Lawrence and Cohen, 1982; Kappus and Muliawan, 1982; Kunert and Tappel, 1983; Muller and Sies, 1984).

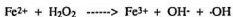
### 1.2.3 Enzymatic lipid peroxidation systems

Enzymatically induced microsomal lipid peroxidation was first described by Hochstein and Ernster in 1963, who demonstrated the requirement for reduced nicotinamide adenine dinucleotide phosphate (NADPH) and adenosine 5'-diphosphate (ADP) and the enzymatic nature of the process (Hochstein and Ernster, 1963). In a subsequent study, they showed the necessity for iron (Hochstein et al., 1964) which had been a contaminant of their original ADP solutions. Later, Pederson and Aust (1972) characterized the enzymatic nature of lipid peroxidation further by demonstrating that NADPH-cytochrome P-450 reductase (Fp) was the enzyme linking NADPH oxidation to the ADP-Fe<sup>3+</sup>-dependent peroxidation of microsomal membranes (Pederson and Aust, 1972). They also developed a reconstituted lipid peroxidation system consisting of phospholipid vesicles (liposomes), purified Fp, ferric chelates and NADPH. In their reconstituted system, a second ferric chelate, EDTA-Fe<sup>3+</sup> (in addition to ADP-Fe<sup>3+</sup>) was also required (Pederson and Aust, 1972). Therefore, they suggested that there may be a microsomal component(s) that directly reduces ADP-Fe<sup>3+</sup> (for which EDTA-Fe<sup>3+</sup> can

substitute) in the reconstituted lipid peroxidation system. Later, Morehouse and Aust (1988) demonstrated that when cytochrome P-450 was incorporated into phospholipid vesicles, EDTA-Fe<sup>3+</sup> was not required suggesting that cytochrome P-450 may be the endogenous component replacing EDTA-Fe<sup>3+</sup>.

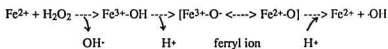
#### 1.2.4 Initiation

Lipid peroxidation is sometimes a major cause of cellular injury in organisms subjected to oxidative stress (reviewed Comporti, 1985; Sies, 1985; Poli et al., 1987). Surprisingly little is known about the chemistry of initiation of peroxidation in membrane systems such as liposomes or microsomes, and the nature of the free radical species responsible for the initiation of iron-dependent lipid peroxidation has been the subject of considerable debate. The principal candidates suggested for this role are the hydroxyl radical ( $\cdot\text{OH}$ ) and the ferrous dioxygen complex ( $\text{Fe}^{2+}\text{--O}_2$ , perferryl ion). The hydroxyl radical is an extremely reactive species, reacting very rapidly with most organic molecules. Hydroxyl radicals can be formed via the Fenton reaction as follows:



The superoxide radical ( $\text{O}_2^{\cdot-}$ ) is produced at a number of intracellular sites (Fridovich, 1974; Mishin et al., 1976; Chance et al., 1979) and  $\text{H}_2\text{O}_2$  can then be formed readily from the nonenzymic or superoxide dismutase-catalyzed dismutation of  $\text{O}_2^{\cdot-}$  (Chance et al., 1979). Subsequently, chelated iron can yield  $\cdot\text{OH}$  in the Fenton reaction as above. An alternative hypothesis is that ferrous ion, in undergoing autoxidation to ferric ion, passes through  $\text{Fe}^{2+}\text{--O}_2$  and  $\text{Fe}^{3+}\text{--O}_2^{\cdot-}$  as intermediates. Upon the discovery of ADP-Fe<sup>3+</sup>-initiated microsomal lipid peroxidation, Hochstein and Ernster (1964) postulated the

involvement of an  $\text{ADP-Fe}^{2+}\text{---O}_2$  complex. Aust's group has also pursued the idea that an ADP-ferrous dioxygen complex initiates lipid peroxidation (Svingen et al., 1978; Aust and Svingen, 1982). The involvement of a ferrous dioxygen complex has been criticized on the grounds that the complex is insufficiently reactive towards PUFA (Halliwell and Gutteridge, 1985). The highly reactive hydroxyl radical can often be detected in microsomal or liposomal lipid peroxidation systems (Gutteridge, 1982; Morehouse et al., 1983; Gutteridge, 1984; Belouqui and Cederbaum, 1986). The hydroxyl radical is known to be capable of initiating lipid peroxidation by abstracting a hydrogen atom from fatty acids (Barber and Thomas, 1978; O'Connell and Garner, 1983).  $\text{H}_2\text{O}_2$ -degrading enzymes or scavengers of  $\cdot\text{OH}$ , however, rarely inhibit iron-dependent peroxidation in microsomal or liposomal systems (Gutteridge, 1982; Morehouse et al., 1983; Gutteridge, 1984; Belouqui and Cederbaum, 1986). It has been proposed that the ferryl ion (see below) is the true Fenton reagent, rather than  $\cdot\text{OH}$ , and this would not be scavenged by conventional  $\cdot\text{OH}$  scavengers (Fee, 1981; Koppenol, 1981).



Superoxide radicals may play a minor role in initiating lipid peroxidation under conditions in which they act to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  (Gutteridge 1977; Tien et al., 1981). Recently, Minotti and Aust (Minotti and Aust, 1987a and b) have proposed that a specific  $\text{Fe}^{2+}\text{---O}_2\text{---Fe}^{3+}$  complex, or at least a 1:1 ratio of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$ , acts as an initiator of peroxidation in liposomal and microsomal systems, but some doubts have been raised about this complex as a specific initiator of peroxidation. Attempts to isolate such a complex have failed (Minotti and Aust, 1987a; Aust, 1988). The  $\text{Fe}^{2+}/\text{Fe}^{3+}$  ratios required for maximal

stimulation of peroxidation have been reported to vary from 1:1 to 1:7 in different experiments (Braugher et al., 1986), perhaps suggesting that a specific stoichiometric complex is not required. It must be concluded that the identity of the initiating species of lipid peroxidation produced by ferrous iron is still an open question.

### 1.2.5 Cellular toxicity

Membrane lipid peroxidation is an important part of oxidative tissue injury and can be an effect as well as a cause of reactions culminating in cytotoxicity (Halliwell and Gutteridge, 1986). The peroxidative breakdown of PUFA has been implicated in the pathogenesis of many types of injury and especially in the hepatic damage induced by several toxic substances. Among these toxic substances are the haloalkanes, carbon tetrachloride (Comporti, 1965; Slater, 1972), trichlorobromomethane (Slater, 1972; Slater, 1982), chloroform (Ekstrom and Hogberg, 1980), 1,2-dibromomethane (Albano et al., 1984) and halothane (Tomasi et al., 1983). In addition, paracetamol (Wendel and Feuerstein, 1981), bromobenzene (Casini et al., 1985), iron (Halliwell, 1981), bipyridyl compounds (Sandy et al., 1986), allyl alcohol (Jaeschke et al., 1987) and in some instances, ethanol (Dianzani and Torrielli, 1981; Sies, 1985; Dianzani, 1985) have been shown to stimulate lipid peroxidation. The peroxidation of PUFA within biological membranes results in a complex series of biochemical and biophysical events which lead to inactivation of enzymatic functions in several subcellular organelles (Comporti, 1965; Slater, 1972; Dianzani and Ugazio, 1978; Vladimirov et al., 1980; Recknagel et al., 1982). These alterations include changes in the physical properties of the lipid bilayer, reactions between acylperoxyl radicals and membrane proteins, and the formation of reactive products originating from the degradation of peroxidized fatty acids (Dianzani and Ugazio, 1978; Vladimirov et al., 1980; Recknagel et al., 1982).

The stimulation of lipid peroxidation in either artificial membranes of liposomes or

in subcellular organelles has been shown to increase membrane rigidity (Curtis et al., 1984; Bruch and Thayer, 1983). Such a loss of fluidity does not seem to be dependent upon an increase in the ratio between cholesterol and phospholipids (Curtis et al., 1984), but is rather an effect of the formation of cross-linking between acyl chains (Eichenberg et al., 1982) and of the depletion of long chain PUFA (Curtis et al., 1984). In addition to the changes in fluidity, lipid peroxidation causes an increase in the ionic permeability, and affects the surface potentials of the membranes (Vladimirov et al., 1980). In the liver, the membranes of the mitochondria and endoplasmic reticulum contain unsaturated fatty acids in high proportion, and therefore are vulnerable to peroxidative attack. At the same time they contain enzymes of the electron transport systems which make them capable of producing free radical species (Comporti, 1965; Slater, 1972; Vladimirov et al., 1980; Recknagel et al., 1982). The consequences for the cell of lipid peroxidation reactions and products are many. Microsomal membranes undergoing peroxidation *in vitro* show fragmentation and turbidity changes, destruction of cytochrome P-450 (Hogberg et al., 1973), and loss of latency and activity of glucose-6-phosphate and UDP-glucuronyl transferase (Hogberg et al., 1973; Ferrali et al., 1980; de Groot et al., 1985). The plasma membrane  $\text{Ca}^{2+}$ -ATPase is inactivated because of the oxidation of essential sulfhydryl groups in the enzyme (Jones et al., 1983), resulting in defective control of cytosolic calcium. Ribosomes become detached from the endoplasmic reticulum during lipid peroxidation (Palmer et al., 1978). In mitochondria, peroxidation causes membrane swelling, deterioration of electron transport, and organelle lysis (Hunter et al., 1963; Narabayashi et al., 1982). Lipid peroxidation of lysosomes causes lysis and enzyme release (Wills and Wilkinson, 1966; Fong et al., 1973), and the erythrocyte plasma membrane responds in a similar manner (Brownlee et al., 1977).

Like OTA, cephaloridine, a beta-lactam antibiotic of the cephalosporin type, causes renal injury in man and in animals, affecting the proximal tubules (Atkinson et al., 1966;

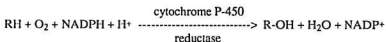
Silverblatt et al., 1970). Of the several biochemical mechanisms proposed to explain the nephrotoxic effects of cephaloridine (Tune and Fravert, 1980; Kuo et al., 1983; Goldstein et al., 1986), the most recent hypothesis suggests an involvement of lipid peroxidation (Kuo et al., 1983; Cojocel et al., 1985a). Furthermore, recent studies showed that the formation of cephaloridine-induced reactive oxygen species and peroxidation of renal cortical membrane lipids was inhibited by radical scavengers and antioxidants (Cojocel, 1985b; Goldstein et al., 1986).

### 1.3 Cytochrome P-450

#### 1.3.1 Background and role in xenobiotic metabolism

The carbon monoxide-binding pigments of liver microsomes are hemoproteins that have a characteristic absorption maximum near 450 nm, and hence are called cytochromes P-450. The cytochromes P-450 enzymes comprise a family of hemoproteins with an identical prosthetic group (a thiolate-bound heme), but widely different apoprotein structures which are responsible for the different substrate specificities of reactions in which they are involved. Many families of cytochrome P-450 have been described including ten in mammals, two in insects, one in snail, one in plants and six in bacteria (Gonzalez, 1992). The ten mammalian cytochrome P-450 families can be functionally subdivided into two major classes; those involved in synthesis of steroids and bile acids (families 7, 11, 17, 19, 21 and 27) and those that primarily metabolize xenobiotics (families 1, 2, 3 and 4) (Gonzalez, 1992). Xenobiotic-metabolizing cytochromes P-450 probably evolved from the cytochromes P-450 involved in steroid synthesis, and then began to oxidatively degrade dietary chemicals which were easily eliminated from animals only after they were converted to more hydrophilic derivatives. The cytochromes P-450 are terminal oxidoreductases of the microsomal, nuclear membrane, mitochondrial and soluble monooxygenases. Microsomal cytochromes P-450 catalyze the metabolism of a wide variety of endogenous and exogenous compounds including drugs, steroids, prostaglandins, chemical carcinogens and other xenobiotics (Guengerich, 1990; Wislocki et al., 1980). They catalyze the incorporation of one atom of molecular oxygen into the substrate to give a product, while the other oxygen atom is reduced by two electrons to give water. Although these enzymes play an important role in the detoxification of many drugs, chemical carcinogens, and other toxic agents, they are also responsible for catalyzing the metabolic activation of some compounds to highly reactive intermediates which then react

with critical cellular macromolecules and initiate toxic and carcinogenic events (Guengerich, 1991). The critical role these enzymes play, in the metabolic activation and detoxification of a wide variety of carcinogens and other toxic agents, makes them of particular importance in light of human exposure to these compounds in the environment. The microsomal cytochrome P-450-dependent mixed function oxidase enzyme system is composed of NADPH-cytochrome P-450 reductase, phospholipids, and cytochromes P-450, and for catalytic activity it exhibits an absolute requirement for NADPH and  $O_2$  (Guengerich, 1990 and 1991; Wislocki et al., 1980).



The catalytic cycle for cytochrome P-450-catalyzed reactions is thought to consist of at least six discrete reactions (Hollenberg, 1992) (see Figure 4). The first step involves binding of the substrate to the ferric form of the enzyme. The second step involves the transfer of one electron from the NADPH-cytochrome P-450 reductase to the iron of the ferric cytochrome P-450 enzyme to give a ferrous enzyme-substrate complex. The reduced cytochrome P-450-substrate complex then binds  $O_2$  to form a ferrous enzyme- $O_2$ -substrate ternary complex with the  $O_2$  bound to the iron. The addition of a second electron to this ternary complex by the reductase results in the formation of an iron peroxo species, best represented as  $Fe^{3+}O_2^{2-}$ . The next step involves cleavage of the oxygen-oxygen bond. One of the oxygens is released with the uptake of two protons, resulting in the formation of water. The retained oxygen remains associated with the heme iron as an activated oxygen. The activated oxygen atom associated with the iron is then inserted into the substrate, resulting in a two-electron oxidation of the substrate to the alcohol (ie. hydroxylated

substrate).

### 1.3.2 Induction

Some cytochrome P-450 isozymes are expressed constitutively, while others are expressed only in response to specific chemical stimuli (see Table 5). Examples of such chemical stimuli (inducers) are polycyclic aromatic compounds (e.g., 3-methylcholanthrene), barbiturates (e.g. phenobarbital), and steroids (e.g. pregnenolone 16 $\alpha$ -carbonitrile) (more inducers and references to them are given in the Methods section 2.5.1). After induction, the activity of particular isoforms increases and the concentration of total cytochrome P-450 may increase. Elevated levels of cytochrome P-450 are the result of multiple mechanisms (Monostory et al., 1992). The major mechanism is increased transcription of cytochrome P-450 genes (higher levels of specific mRNAs are detectable soon after treatment with some inducers of cytochrome P-450). A second mechanism is message stabilization and enhanced transport of mRNA from the nucleus to the cytoplasm so that mRNA levels at the site of protein synthesis are increased. A third mechanism is stabilization of cytochrome P-450 protein and/or mRNA (Monostory et al., 1992). The fact that induction is produced by a large variety of xenobiotics and is mediated by multiple mechanisms suggests that the cytochrome P-450 system can provide increased protection for organisms in potentially hostile chemical environments. Since cytochrome P-450 induction is a characteristic response to chemicals of various living organisms ranging from microorganisms to man, it seems to be an adaptive mechanism for organisms to increase the chance for survival.

### 1.3.3 Inhibition

The catalytic cycle of cytochrome P-450 (see Figure 4) traverses three main steps that are particularly vulnerable to inhibition: 1) the binding of substrates, 2) the binding of

molecular oxygen subsequent to the first electron transfer, and 3) the catalytic step in which the substrate is actually oxidized (Ortiz de Montellano and Reich, 1986). Cytochrome P-450 inhibitors can be divided into three mechanistically differentiable categories: 1) agents that bind reversibly, 2) agents that form quasi-irreversible complexes with the heme iron atom, and 3) agents that bind irreversibly to the protein or the prosthetic heme group (Ortiz de Montellano and Reich, 1986). On the whole, inhibitors that interfere in the catalytic cycle prior to the actual oxidative event are reversible inhibitors, whereas agents that act during or subsequent to the oxygen transfer step are generally irreversible.

### **1.3.3a Reversible Inhibitors:**

Inhibitors that compete reversibly with substrates for occupancy of the active site include substances that bind to its hydrophobic domain, that coordinate to the prosthetic heme iron atom, or that participate in specific hydrogen bonding or ionic interactions with specific active-site residues (Testa and Jenner, 1981) include the following:

- i. Coordinators to ferric heme - e.g. cyanide (Kitada et al., 1977).
- ii. Coordinators to ferrous heme - e.g. carbon monoxide (Hanson et al., 1976).
- iii. Heme coordinators and lipophilic binders - e.g. metyrapone (Dominguez and Samuels, 1963).

### **1.3.3b Catalysis-dependent inhibition:**

Several classes of inhibitors are known to be catalytically activated by the enzyme to transient species that irreversibly or quasi-irreversibly inhibit the enzyme. These mechanism-based (catalysis-dependent) inhibitors are potentially more enzyme-specific than reversible inhibitors because: 1) the inhibitor must first bind to the ferric enzyme, 2) the inhibitor must then be catalytically activated and therefore must be acceptable as a substrate, and finally, 3) the reactive species produced irreversibly alters the enzyme and renders it inactive (Ortiz de Montellano and Reich, 1986). Examples include compounds

which undergo or cause:

- i. Covalent binding to the protein - e.g. carbon disulfide (Bond and De Matteis, 1969).
- ii. Quasi-irreversible coordination to the prosthetic heme - e.g. alkyl and aromatic amines (Testa and Jenner, 1981; Franklin, 1977).
- iii. Covalent binding to the prosthetic heme - e.g. dihydropyridines and dihydroquinolines (heme alkylation) (Ortiz de Montellano and Correia, 1983; Gayarthri and Padmanaban, 1974).
- iv. Heme degradation - e.g. carbon tetrachloride (Guzelian and Swisher, 1979).

### Objectives of this work

Recently, using a reconstituted microsomal lipid peroxidation system, we demonstrated that OTA induced lipid peroxidation by chelating  $\text{Fe}^{3+}$  ions, and that the resulting complex was more readily reducible by the flavoprotein NADPH cytochrome P-450 reductase to the OTA- $\text{Fe}^{2+}$  complex which, in the presence of oxygen, provided the active species that initiated lipid peroxidation (Omar et al., 1990). Cytochrome P-450 has been shown to play an important role in stimulating lipid peroxidation in ADP- $\text{Fe}^{3+}$  system (Morehouse and Aust, 1988).

A number of studies have shown that OTA is metabolized by liver microsomes from several species (rat, pig and human) primarily to 4(R)-4-OH-OTA and to a smaller extent to the epimeric 4(S)-4-OH-OTA in the presence of NADPH (Stormer et al., 1981, 1983; Ueno, 1985; Creppy et al., 1991; Hietanen et al., 1986). OTA metabolism has also been shown to be induced by PB and/or 3MC in rats (Stormer and Pedersen, 1980; Ueno, 1985; Hietanen et al., 1986). In most of these studies, the effects of these cytochrome P-450 inducers were not examined under the same experimental conditions. In addition, the effects of other important cytochrome P-450 inducers were never investigated.

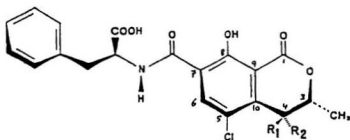
#### The objectives of my study were:

- i) To investigate the possible role of cytochrome P-450 in OTA-induced lipid peroxidation.
- ii) To characterize which cytochrome P-450 isoforms are capable of metabolizing OTA through the use of a variety of inducers, specific inhibitors and monoclonal antibodies.
- iii) To examine the effect of cytochrome P-450 induction on OTA-induced toxicity in

rats.

- iv) To examine the effect of cytochrome P-450 induction on OTA absorption and distribution in rats.

**Figure 1**      **Structures of ochratoxin A and its metabolites.**



Ochratoxin A (OTA)

$R_1=R_2=H$

4 (R)-4-OH-OTA

$R_1=OH, R_2=H$

4 (S)-4-OH-OTA

$R_1=H, R_2=OH$

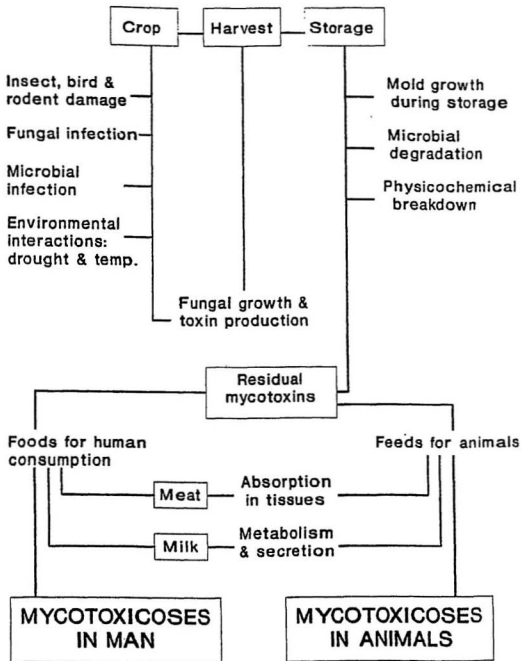
Table 1. Occurrence of OTA in food and animal feed of plant origin (adapted from WHO, 1990).

Commodity	Country	Samples analyzed	% Cont.	OTA range (ug/kg)	Reference
Wheat, hay	Canada	95	7.4	30-6,000	Prior, 1976
Wheat, oats, barley, rye	Canada	32	56.3	30-27,000	Scott et al., 1972
Mix. feed	Canada	51	7.8	48-5,900	Abramson et al., 1983
Maize	Yugoslavia	191	25.7	45-5,125	Balzer et al., 1977
Mix. feed	Australia	25	4	70,000	Connole et al., 1981
Flour	U.K.	7	28.5	490-2,900	Osborne, 1980
Maize	U.K.	29	37.9	50-500	Ministry of Agriculture, 1980
Cornflour	U.K.	13	30.8	50-500	" " "
Soya bean	U.K.	25	36	50-500	" " "
Cocoa beans (raw)	U.K.	56	17.9	100-500	" " "
Barley	Czechoslovakia	48	2.1	3,800	Vesela et al., 1978
Coffee beans	U.S.A.	267	7.1	20-360	Levi et al., 1974
wheat	U.S.A.	577	3.8	5-115	Shottwell et al., 1976
Beans	Sweden	71	8.5	10-442	Akustrand and Josefsson, 1979
Maize	France	924	3.9	15-200	Galtier et al., 1977b

**Table 2. The incidence of OTA in meat and meat products (adapted from Marquardt et al., 1990).**

Product	Country	Samples analyzed	% Cont.	OTA range (ug/kg)	Reference
Kidney					
Swine	Hungary	122	39	2-100	Jelinek et al., 1989
Meats					
Ham	Yugoslavia	206	29	40-70	" " "
Bacon			19	37-200	" " "
Kulen			13	10-460	" " "
Sausage			12	10-920	" " "
Blood					
Swine	Canada	1200	0.5	100-229	Marquardt et al., 1988
			4.1	20-229	
			72	5-20	

**Figure 2**      **Factors influencing the occurrence of mycotoxins in human food and animal feed** (redrawn from Smith and Moss, 1985).



**Table 3. OTA levels in humans** (adapted from Kuiper-Goodman and Scott, 1989).

Sample	Country	Incidence	OTA levels (ug/kg or /L)	Reference
Blood serum (from patients with urinary tract tumors/BEN)				
	Bulgaria	26%	Up to 35 (mean 20)	Petkova-Bocharova et al., 1988
Blood serum (from non-endemic areas)				
	Bulgaria	7.7	mean 10	" " "
Blood serum	Germany	173/306	0.1-14.4	Bauer and Gareis, 1987
Kidneys	Germany	3/46	0.1-0.3	" " "
Milk	Germany	4/36	0.02-0.03	" " "
Blood serum	Poland	9/216	1.3-4.8	Golinski and Grabarkiewicz-Szczesna, 1985
Blood serum (from village with endemic nephropathy)				
	Yugoslavia	25/420	1-40	Hult et al., 1982 a,b
Blood serum (from non-endemic village)				
	Yugoslavia	17/219	1-10	" " "

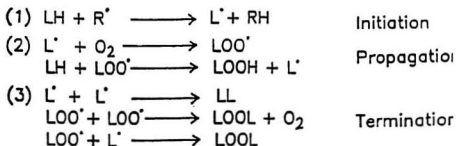
**Table 4. LD<sub>50</sub> of OTA in different species.**

<b>Species</b>	<b>Route of administration</b>	<b>LD<sub>50</sub> (mg/kg)*</b>
Rat (male)	oral	28.0
Rat (female)	oral	21.4
Rat (male)	i.p.	12.6
Rat (female)	i.p.	14.3
Rat	i.v.	12.7
Mouse	oral	46.0
Mouse	i.p.	22.0
Mouse	i.v.	25.7
Dog	oral	0.2
Guinea Pig (m)	oral	9.1
Guinea Pig (f)	oral	8.1
Chicken	oral	3.3
Turkey	oral	5.9
Rainbow Trout	i.p.	4.7

\* Adapted from WHO, 1990 and MSDS, Sigma Chemical Co., 1989.

**Figure 3**      **Simplified reactions of the process of lipid peroxidation**  
(Gutteridge, 1988).

## Lipid peroxidation



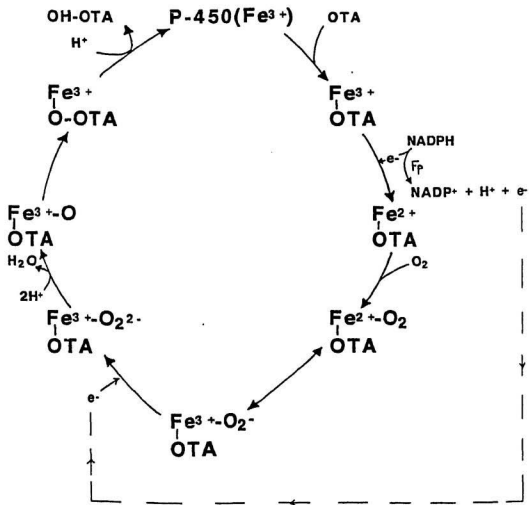
$\text{R}^{\bullet}$  = a free radical

$\text{LH}$  = lipid undergoing lipid peroxidation

$\text{L}^{\bullet}$  = lipid radical

$\text{LOO}^{\bullet}$  = lipid peroxy radical

**Figure 4**      **The catalytic cycle of cytochrome P-450-catalyzed reactions and OTA hydroxylation.**



**Table 5 . Regulation of P-450 gene expression** (Adapted from Soucek and Gut., 1992).

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CYP	Regulation of gene expression
<hr/>	
IA1	Inducible (all tissues) - 3MC, ISF, BNF: transcriptional activation + mRNA stabilization.
IA2	Constitutive (liver): transcriptional activation + mRNA stabilization. Inducible (all tissues) - 3MC, ISF, BNF: transcriptional activation + mRNA stabilization.
IIB1	Constitutive (lung, testis) - age, strain, colony, growth hormone: transcriptional activation. Inducible (all tissues) - PB, starvation: transcriptional activation + mRNA stabilization.
IIE1	Constitutive (liver, kidney) - age, growth hormone: transcriptional activation; diabetes, starvation: transcriptional activation + mRNA stabilization. Inducible (liver, lung) - INH, EtOH: protein stabilization.
IIIA1	Inducible - PCN, DEX, hypertension: transcriptional activation + mRNA stabilization.
IIIA2	Constitutive (liver, intestine) - age, sex (m), growth hormone, diabetes, starvation: transcriptional activation. Inducible - PB: activation.
IVA1	Constitutive (liver, kidney): transcriptional activation. Inducible - Clofibrate, starvation, transcriptional activation.

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## CHAPTER 2

### MATERIALS AND METHODS

**2.1 Chemicals** - Ascorbate, bathophenanthroline disulfonic acid, butylated hydroxyanisole, catalase, clofibrate,  $\text{FeCl}_3$ ,  $\text{FeSO}_4$ ,  $\text{H}_2\text{O}_2$ , isocitric acid, isocitric dehydrogenase, isoniazid, isosafrole, lipoxigenase, mannitol, 3-methylcolanthrene, metyrapone, NADPH, 6-naphthoflavone, ochratoxin A, phenobarbital, pregnenolone-16 $\alpha$ -carbonitrile, superoxide dismutase and 2-thiobarbituric acid were purchased from Sigma Chemical Co. (St. Louis, MO). [ $^3\text{H}$ ]Ochratoxin A was obtained from Amersham, U.K. and further purified by thin layer chromatography (TLC). 7-Ethoxyresorufin, 7-pentoxyresorufin and resorufin were purchased from Pierce (Rockford, IL). Co-protoporphyrin IX was obtained from Porphyrin Products (Logan, UT, USA). Sephadex G-50 was obtained from Pharmacia (Toronto, Canada). All other chemicals were of the highest grade commercially available.

**2.2 TLC purification of [ $^3\text{H}$ ]OTA** - Two mL of [ $^3\text{H}$ ]Ochratoxin A was used, the solvent was evaporated under a stream of nitrogen and the residue was redissolved in methanol. The whole sample was applied as a streak along the lower part (the origin) of a thin layer chromatography (TLC) plate (KSF silica gel, layer thickness 250  $\mu$ , Whatman). Standard OTA was applied as a spot. The chromatogram was developed with benzene : acetic acid (4 : 1, v/v), and after evaporation of solvent examined under U.V. light. The silica gel with the band corresponding to OTA was scraped off (removed), and OTA eluted with methanol. The solution contained 83% of the total radioactivity. The latter OTA from the first chromatography was further purified on a second TLC plate following the same steps described above and the resulting OTA solution from the second chromatography contained 92% of the total radioactivity.

**2.3 Preparation of ochratoxin A** - OTA was purchased from Sigma chemical Co., but was also produced in the lab according to the procedures described in my Master's thesis (Omar, 1990). The UV and fluorescence spectra of OTA are given in Figures 5 and 6. The purity of the prepared toxin was checked by TLC (Table 6) and HPLC (Figure 7). The identity of the toxin was checked by NMR (Figure 8). OTA concentration was calculated using the absorption coefficient at 332 nm of  $6330 \text{ cm}^{-1} \text{ M}^{-1}$  (Pohland et al., 1982).

**2.4 Animals** - Male Sprague-Dawley rats (200-250 g), obtained from Canadian Breeding Farms, Halifax, Nova Scotia, were used in all experiments. Animals were maintained on a 12 hr light and dark cycle and had free access to standard laboratory chow and water.

## **2.5 Treatments of rats for *in vitro* experiments**

**2.5.1 Treatments with cytochrome P-450 inducers** - The various treatments ( $n = 4/\text{treatment}$ ) are listed in Table 7. Isoniazid was given as a 0.1% solution in drinking water for 10 days and rats were sacrificed on day 11 (Ryan et al., 1985). PCN (100 mg/kg body weight in 1 mL of 1% Tween 80) was administered by gastric intubation once daily for 4 days and rats were sacrificed 24 hr after the last dose (Graves et al., 1987). PB was given as a 0.1% solution in drinking water for 5 days and rats were sacrificed at the end of that time period (Graves et al., 1987). 3MC (20 mg/kg body weight in corn oil) was administered intraperitoneally once each day for 3 successive days, and rats were sacrificed 24 hr after the last dose (Guengerich et al., 1982a). Clofibrate was administered by gastric intubation (200 mg/kg in 1 mL sucrose syrup containing 1% gum arabic) once each day for 5 consecutive days, and rats were sacrificed 24 hr after the last dose (Fournel et al., 1987). Isosafrole (120 mg/kg body weight in corn oil) was given intraperitoneally once each day

for 3 successive days, and rats were sacrificed 24 hr after the last dose (Fisher et al., 1981).

### **2.5.2 Treatments with Cobalt protoporphyrin IX for microsomal**

**preparations** - Rats received 2 doses each of 50  $\mu\text{mol/kg}$  body weight of cobalt protoporphyrin IX (Co-heme) 9 days and 2 days prior to killing, while control rats received the vehicle saline at the same time as the treated rats (Drummond and Kappas, 1982). Co-heme (24.8 mg) was dissolved in 0.4 mL of 0.1 M NaOH, the pH was adjusted to 7.4 with HCl and the solution was made up to 4 mL with normal saline (final concentration of Co-heme, 10 mM). The freshly prepared Co-heme was administered subcutaneously to rats at a dose of 0.5 mL/100 gm body weight. After sacrifice, liver microsomes showed 20% of cytochrome P-450 content of controls.

### **2.6 Treatments of rats for *In vivo* studies -**

#### **2.6.1 Inducing cytochrome P-450 -** Four rats were used per treatment group.

Control rats had free access to drinking water and standard lab chow for 5 days, whereas PB treated rats were given 0.1% PB in drinking water for five days (as the control rats, PB treated rats also had free access to the drinking water with PB and standard lab chow) (Graves et al., 1987). Animals were maintained on a 12 hr light and dark cycle. On the fifth day, all animals (8 rats) were placed individually in metabolic cages and 24 hr urines were collected (for a base line). On the sixth day, all rats received OTA by gastric intubation (0.5 mg/kg body weight in 50 mM sodium bicarbonate, about 0.5 mL); this treatment of OTA was given for five successive days. Twenty four hour urines were collected every day until one day after the last OTA treatment. After sacrifice, liver microsomes from PB treated rats showed a 2.5 fold increase in cytochrome P-450 contents as compared to control microsomes.

**2.6.2 [ $^3\text{H}$ ]OTA experiments** - Rats were divided into two groups ( $n = 4/\text{treatment}$ ), one group received drinking water whereas the other received water containing 0.1% PB for five days before OTA treatment. All animals had free access to food (standard lab chow). After the five days, both groups were treated with [ $^3\text{H}$ ]OTA (288  $\mu\text{g}/\text{kg}$  body weight in 50 mM  $\text{NaHCO}_3$  once by gastric intubation). Rats were kept in metabolic cages for 6 hr to collect urine.

**2.7 Preparation of liver microsomes** - Liver microsomes were isolated by differential centrifugation of liver homogenates as described earlier (Rahimtula et al., 1979). Liver from each rat was excised and placed in ice-cold 0.1 M potassium phosphate buffer, pH 7.4. The liver was chopped into pieces, homogenized in 3 parts (by volume) of the above buffer to one part liver (wet weight) using a polytron homogenizer (Brinkman Instruments), and the homogenate was centrifuged at 10,000  $\times g$  for 10 min. Following filtration of the resulting supernatant through cheesecloth, microsomes were isolated from the 10,000  $\times g$  supernatant by ultracentrifugation at 110,000  $\times g$  for 60 min. The microsomal pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.4 and again centrifuged at 110,000  $\times g$  for 60 min. The final microsomal pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.4 and stored at  $-70^\circ\text{C}$  until used. Protein was measured by the Lowry method (Lowry et al., 1951). Cytochrome P-450 levels were measured as described by Omura and Sato (1964) (see below, section 2.9). NADPH-cytochrome P-450 reductase (Fp) was assayed as described by Lake (1987) using cytochrome *c* as the electron acceptor (explained below, section 2.11).

**2.8 Cytochrome P-450 purification** - Cytochrome P-450 was purified from liver microsomes isolated from PB-pretreated rats as described by Guengerich (1982). All steps

were carried out at 4°C. Microsomes were suspended to 2 mg protein/mL in 0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol, 1 mM EDTA, and 20 µM BHT. Sodium cholate (recrystallized from 50% aqueous ethanol) was added dropwise (from a separatory funnel) to the stirring suspension over 20 min to give a final concentration of 0.6% (weight/volume, w/v). After stirring for an additional 30 min, the clarified solution was centrifuged at 100,000 x g for 1 hr. An amount of the supernatant equivalent to 2,000 nmoles of cytochrome P-450 was applied at a flow rate of 1 mL/min to an  $\omega$ -amino-octyl agarose column (2.5 x 50 cm) previously equilibrated with 300 mL of 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 20% glycerol, and 0.6% (w/v) sodium cholate. The cytochrome P-450, a reddish brown protein, was bound to the top one-third of the column. The column was washed with 800 mL of 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 20% glycerol (volume/volume, v/v) and 0.42% (w/v) sodium cholate. Cytochrome P-450 was eluted using about 1,500 mL of 0.1 M potassium phosphate buffer containing 1 mM EDTA, 20% glycerol, 0.33% (w/v) sodium cholate and 0.06% (w/v) Renex 690 (ICI Americas Inc., WA, U.S.A.). The eluted fractions were monitored for cytochrome P-450 by measuring the absorption at 417 nm ( $A_{417}$ ). The  $A_{417}$  peak fractions were pooled and concentrated to about 50 mL using an Amicon ultrafiltration apparatus and a PM-30 membrane. The concentrated solution was dialyzed against 1 L of a 20% glycerol-0.1 mM EDTA solution (about 3 hr) and then versus 1 L of 10 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA, 20% glycerol, 0.1% (w/v) Lubrol PX and 0.2% (w/v) sodium cholate (not recrystallized) (about 3 hr).

The cytochrome P-450 was further purified by DEAE cellulose chromatography at room temperature (about 22°C). The dialyzed cytochrome P-450 solution was applied to a 2.5 x 50 cm column of Pharmacia DEAE-Sepharcel previously equilibrated with 1 L of 10

mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA, 20% glycerol, 0.1% (w/v) Lubrol PX and 0.2% (w/v) sodium cholate (not recrystallized). The column was washed with 700 mL of the same buffer in which the concentration of NaCl was increased linearly to 0.25 M. The last major  $A_{417}$  peak contains the bulk of the cytochrome P-450. The peak fractions were pooled and concentrated to about 20 mL with an Amicon ultrafiltration apparatus using a PM-30 membrane. The concentrated solution was stirred with Bio-Beads SM-2 (Bio-Rad Labs; 0.2 gm/mg protein) for 3 hr to remove excess detergent and then filtered through glass wool. Finally, the enzyme preparation (cytochrome P-450 IIB1) was dialyzed overnight against 50 volumes of 10 mM Tris-acetate buffer (pH 7.4) containing 0.1 mM EDTA and 20% glycerol. The dialyzed enzyme was stored in aliquots (0.25 mL each) at  $-80^{\circ}\text{C}$ . SDS-polyacrylamide gel electrophoresis revealed the presence of a single major protein band with a very minor band underneath.

**2.9 Cytochrome P-450 assay** - Cytochrome P-450 concentration was measured spectrophotometrically as described by Omura and Sato (1964) using an extinction coefficient of  $91\text{ cm}^{-1}\text{ mM}^{-1}$ . Briefly, 6 mg microsomal protein or 0.6 mg protein of the cytochrome P-450 preparation was suspended in 6 mL of 0.1 M potassium phosphate buffer (pH 7.4) and a few small crystals of sodium dithionite were added to it. After mixing, the contents were divided between 2 matched cuvettes (3 mL in each) and scanned between 500-400 nm (scan speed 120 nm/min, chart speed 30 mm/min) to obtain a baseline. The sample cuvette was bubbled for about 40 sec to 1 min with CO gas and then re-scanned to obtain the characteristic peak for the cytochrome P-450 at 450 nm.

My cytochrome P-450 preparation contained 8.25 nmol/mg protein which was lower than expected, but it showed a complete absence of NADPH cytochrome P-450 reductase activity (Fp). Morehouse and Aust, 1988 satisfactorily used a cytochrome P-450

preparation which contained 10 nmol/mg protein. Also, Laethem et al. (1992) purified cytochrome P-450 with specific activity of 8.4 nmol/mg protein. Usually, some of the enzyme activity is lost during the purification and also some heme-degradation occurs with the use of detergents. Attempts to restore cytochrome P-450 by addition of heme has not met with success so far.

**2.10 NADPH-cytochrome P-450 reductase purification** - The flavoprotein NADPH-cytochrome P-450 reductase (Fp) was purified from liver microsomes isolated from PB-pretreated rats essentially as described by Ardies et al. (1987). The following procedures were all performed at 4°C. Microsomes (50 mg protein/mL) were diluted to 10 mg protein/mL with 100 mM Tris-HCl buffer (pH 7.7) containing 1.0 mM EDTA, 1.0 mM dithiothreitol (DTT), 20 µM BHT, 5 µM flavin mononucleotide (FMN), and 30% glycerol (buffer A). CHAPS (3-[(cholamidopropyl)-dimethylammonio]-1-propanesulfonate) was prepared as a 20% (w/v) solution in water, and neutralized to pH 7.4 with dilute KOH. Immediately prior to use, CHAPS was diluted 1:1 (v/v) with buffer A. The latter solution of 10% CHAPS (in buffer A) was added to the microsomes slowly dropwise with stirring to a final concentration of 1% and the mixture was stirred for 30 min. Then, a 1.5% solution of protamine sulfate was added dropwise to a final concentration of 0.07% (w/v). After stirring for an additional 20 min, the mixture was centrifuged at 110,000 x g for 60 min. The supernatant was removed, and the resulting gray-colored, tightly packed pellet was resuspended with the aid of a Teflon/glass homogenizer at a protein concentration of 50 mg/mL in buffer A; protein was measured by the Lowry method (Lowry et al., 1951). A 10% (w/v) solution of sodium cholate in water was then added dropwise with stirring to a final detergent : protein ratio of 3 mg/mg. Ten minutes later, a 20% (v/v) solution of Lubrol PX (Sigma Chemical Co., MO, USA) in water was added dropwise to a final concentration of 0.5% (w/v) and the mixture was stirred for an additional 30 min. The

detergent-treated fraction was centrifuged at 110,000 x g for 60 min, and the resulting supernatant was applied directly to a 2',5'-ADP agarose column (2.5 x 4.0 cm) at a flow rate of 1 mL/min. The affinity column had been equilibrated previously with 100 mM phosphate buffer (pH 7.7) containing 0.4% cholate, 0.1 mM EDTA, 0.1 mM DTT, 20  $\mu$ M BHT, 5  $\mu$ M FMN, and 20% glycerol. Once loaded, the column was washed with 15 column volumes of equilibration buffer, 12 column volumes of 100 mM phosphate buffer (pH 7.7) containing 1.0% (v/v) CHAPS, 0.5% (v/v) Lubrol PX, 0.1 mM EDTA, 0.1 mM DTT, 20  $\mu$ M BHT, 5  $\mu$ M FMN, and 20% (v/v) glycerol, and again with 15 column volumes of equilibration buffer. All washes were performed at a flow rate of a 1 mL/min. Fp was then eluted from the affinity resin with a small volume (about 25 mL) of equilibration buffer to which 10 mM NADP<sup>+</sup> had been added (flow rate = 0.5 mL/min). The peak Fp-containing fractions (detected by measuring Fp in fractions as described below, section 2.11) were pooled and dialyzed twice against 2 L of 50 mM phosphate buffer (pH 7.4) containing 0.1 mM DTT and 20% (v/v) glycerol for a total of 28 hr. The purified Fp was then stored in small aliquots (0.5 mL each) at -80°C. The purified Fp showed a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

**2.11 NADPH-cytochrome P-450 reductase assay** - The enzyme was assayed as described by Lake (1987) using cytochrome *c* as the electron acceptor. Briefly, 1 mL of 0.125 mM cytochrome *c* solution in 0.1 M phosphate buffer, pH 7.0, and 0.2 mL of 15 mM KCN in water were pipetted into each of two matched 3 mL spectrophotometer cuvettes. Ten microliters of Fp was pipetted into each cuvette and phosphate buffer was added to the test and reference cuvette contents to bring the volumes up to 2.4 and 2.5 mL, respectively. After mixing, the cuvettes were placed in the spectrophotometer (thermostatted to 22°C), and 3 min later the reaction was initiated by adding 0.1 mL of 10

mM NADPH to the test cuvette only. The contents were again mixed and the increase in absorbance with time was recorded at 550 nm. Using an extinction coefficient for the reduced cytochrome *c* at 550 nm of  $0.021 \text{ cm}^{-1} \mu\text{M}^{-1}$ , the specific activity of the flavoprotein was calculated to be 18,000 units (or nmol)/min/mg protein (Lake, 1987). One unit of enzyme activity is defined as that amount which catalyzes the reduction of 1 nmol cytochrome *c*/min.

**2.12 Fortification of microsomes from Co-heme treated rats with Fp** - Co-heme drastically depleted cytochrome P-450 (> 80% depletion of cytochrome P-450 content compared to controls). NADPH-cytochrome P-450 reductase (Fp) was also depleted (about 75% depletion). To avoid the possible effect of reduced Fp, Co-heme microsomes were fortified by incubating them with purified Fp. Microsomes (2 mg protein) were incubated with Fp (640 nmol) in a little microfuge tube (about 150  $\mu\text{L}$  capacity) for 1 hr at 22°C and then centrifuged at  $110,000 \times g$  for 5 min to collect microsomes. The microsomes were resuspended in buffer and assayed for Fp content as described above. Fortified microsomes were found to have a Fp content of 70 nmol/mg protein as compared to 20 nmol/mg protein in non-fortified microsomes.

**2.13 Preparation of phospholipid vesicles** - Total lipid was extracted from untreated rat liver microsomes by the method of Folch et al. (1956) with care being taken to flush all solvents with nitrogen and to perform all operations under nitrogen at 0 - 4°C to minimize auto-oxidation of polyunsaturated lipids. The extracted lipid in chloroform : methanol (2 : 1) was stored in aliquots under nitrogen at -80°C. Total lipid phosphorus was determined as described by Bartlett (1959). Phospholipid vesicles were prepared fresh daily by sonication of the extracted lipid under anaerobic conditions as described by

Pederson et al. (1973). Briefly, an aliquot of the phospholipid solution was evaporated to dryness in a plastic tube under nitrogen, and nitrogen-saturated Tris-HCl buffer (0.25 M, pH 6.8) was added to give a final lipid phosphorus concentration of 10  $\mu\text{mol/mL}$ . The tube was flushed with nitrogen, capped, and placed in a glass beaker filled with a mixture of ice and water. Phospholipid vesicles were obtained by placing the probe of a Branson sonifier (model W185) in the beaker and applying a power of 50 W for 5 min.

## **2.14 Lipid peroxidation assays (incubation conditions) -**

**2.14.1 Reconstituted system incubations** - Unless otherwise specified, incubations were carried out in duplicate at 37°C in 0.25 M Tris-HCl buffer/0.25 M NaCl (pH 6.8) and contained per mL: phospholipid vesicles (1  $\mu\text{mol P}$ ), 3.2 U Fp, 110 nmol  $\text{Fe}^{3+}$ , 500 nmol OTA, and 200 nmol NADPH. Cytochrome P-450 IIB1 or EDTA, when included, were added at the indicated concentrations (see Table 8).

**2.14.2 Microsomal incubations** - Incubations were carried out at 37°C in 0.1 M potassium phosphate buffer (pH 7.4 or different pHs as indicated in the figure legends) and contained per mL: 2 mg microsomal protein (from either control animals, those induced, those treated with Co-heme, or those treated with Co-heme and microsomes fortified with Fp), 125 nmol OTA and 1 mM NADPH.

Lipid peroxidation was estimated by measuring MDA levels (Rahimtula et al., 1988). For this purpose, 0.5 mL of 30% TCA and 50  $\mu\text{L}$  of BHT (2% in ethanol) were added to 0.5 mL of each incubation. Finally, 0.5 mL of 50 mM TBA was added and the mixtures were placed in a boiling water bath for 15 min. After centrifugation for 5 min at 2000 rpm (bench top centrifuge), the absorbance of the MDA-TBA complex in the supernatant was read at 535 nm ( $E_{535} = 156 \text{ mM}^{-1}\text{cm}^{-1}$ ) (Jordan and Schenkman, 1982).

## 2.15 Metabolic studies

**2.15.1 Metabolism of OTA *In vitro*** - Unless otherwise indicated, incubations were carried out in duplicate at 37°C for 30 min in 0.1 M potassium phosphate buffer (pH 7.4) and contained in a total volume of 1 mL: 2 mg microsomal protein, 125 nmol OTA and a NADPH-regenerating system (consisting of 0.4  $\mu$ mol NADP<sup>+</sup>, 5  $\mu$ mol MgCl<sub>2</sub>, 5  $\mu$ mol DL-isocitrate and 0.65 units of isocitric dehydrogenase). Catalase (800 units), SOD (35 units), mannitol (11 mM), BHA (10  $\mu$ M), DPPD (10  $\mu$ M), Desferal (50  $\mu$ M) or BPS (100  $\mu$ M), when included, were added prior to initiating the reaction with NADPH. At the end of 30 min, a 0.5 mL aliquot from each incubation was withdrawn for measurement of OTA metabolites and the remaining 0.5 mL was used to measure lipid peroxidation. Time-course studies were carried out in an identical manner except that the incubation volume was 10 mL.

Incubations with hemoproteins and hydroperoxides were carried out in duplicate in 0.5 mL of 0.1 M potassium phosphate buffer (pH 7.4) and contained (i) OTA (62.5 nmol), (ii) hemoprotein - microsomes from PCN treated rats (1 mg protein; 1.2 nmol cytochrome P-450) or Hb (640  $\mu$ g; 10 nmol) or hematin (2.5 nmol) or HRP (400  $\mu$ g; 10 nmol) and (iii) hydroperoxide - CHP (0.5 mM) or H<sub>2</sub>O<sub>2</sub> (5 mM) or LAHP (65  $\mu$ M). Incubations were terminated after 30 min and analyzed for OTA metabolites as described below. LAHP was prepared from linoleic acid and lipoxygenase as described earlier (O'Brien, 1969).

## 2.15.2 Effect of monoclonal antibodies on OTA metabolism *in vitro*

Monoclonal antibodies (MAbs) were obtained from Dr. H. Gelboin, U.S. National Cancer Institute, Lab of Molecular Carcinogenesis, Bethesda, MD. Clones 1-7-1 (against cytochrome P-450 IA1/IA2) and 2-66-3 (against cytochrome P-450 IIB1/IIB2) were used in my studies. Rabbit IgG was used to determine any non-specific reaction. Microsomes

were preincubated with MABs in buffer at room temperature 30 min (Nakajima et al., 1991) prior to initiating the OTA metabolism assay at 37°C by addition of a NADPH-regenerating system and substrate (OTA).

**2.15.3 Analysis of OTA metabolites formed in *in vitro* studies** - Aliquots (0.5 mL) were removed from incubations, and the reaction was terminated by the addition of 1 M HCl (0.1 mL) and saturated NaCl (0.5 mL). The mixtures were extracted with chloroform (2 x 2 mL), and the two chloroform extracts from each incubation were combined and dried under nitrogen. The residues were dissolved in 500 µL of methanol and 50 µL of each sample was analyzed by HPLC (Perkin Elmer, Series 4) on a Partisil 10 ODS-2 column (0.45 cm x 25 cm) using a solvent system consisting of (i) a mixture of acetonitrile : methanol (1 : 1, v/v) 60% and (ii) 5 mM sodium acetate : acetic acid (500 : 14, v/v) 40%. The flow rate was 1.5 mL/min. Ochratoxin A and its metabolites were detected fluorimetrically using light at 340 nm for excitation and measuring the emission at 465 nm. 4(S)-4-OH-OTA, 4(R)-4OH-OTA and OTA eluted at 4.2, 5.1 and 10.1 min respectively. Metabolites were identified and quantitated using standards generously provided by Dr. M. Castegnaro, IARC, Lyon, France.

**2.16 Ethoxy- and pentoxyresorufin O-dealkylation assays** - EROD/PROD assays were carried out as described by Burke et al., 1985. Incubations were carried out at 37°C for 10 min in 0.1 M potassium phosphate buffer (pH 7.8) and contained per mL 50 µg microsomal protein, 1.6 mg BSA, 5 µM substrate (ethoxy- or pentoxyresorufin) and a NADPH-regenerating system (consisting of 0.4 µmol NADP<sup>+</sup>, 5 µmol MgCl<sub>2</sub>, 5 µmol DL-isocitrate and 0.65 units of isocitric dehydrogenase). The incubation volume was 1.25 mL. The reaction was stopped by the addition of 2.5 mL methanol (total volume 3.75), the mixture was centrifuged for 3 min (bench top centrifuge, at 2,000 rpm), and the

fluorescence due to the formation of resorufin in the clear supernatant was measured at excitation wavelength of 350 nm and emission wavelength of 585 nm, excitation/emission slits 5/3 nm respectively (Instrument: Perkin-Elmer LS-5 spectrofluorimeter). Standard curve of resorufin was used for calculations.

**2.17 p-Amino hippurate (PAH) transport** - Three animal groups were used for this experiment: control rats, rats treated with OTA, and PB-pretreated rats treated with OTA. The latter two groups were rats used to study urinary enzymes ( $n = 4$ ). PAH transport was measured *in vitro* as described by Berndt and Hayes (1979). Briefly, after animal sacrifice by cervical dislocation, kidneys were removed rapidly and placed in cold Krebs-Ringer phosphate buffer pH 7.4. The renal slices (0.25 - 0.40 mm) were prepared freehand and stored in the same Krebs-Ringer phosphate buffer until used. All incubations were performed in a shaking water bath at 25°C in an atmosphere of 95% oxygen/5% CO<sub>2</sub> and were initiated within 30 min of the preparation of the slices. 100 - 150 mg of tissues from all groups were incubated for 10 and 30 min in 2 mL of Krebs-Ringer phosphate buffer after the addition of [<sup>14</sup>C]PAH (2 µCi, 50 µM; ICN, Canada). At the end of the incubation, tissues were removed from the buffer, blotted, and homogenized in 1 mL of distilled water. 0.5 mL of the homogenate was solubilized in 4.5 mL NCS-tissue solubilizer (Amersham), and 1 mL of the solution was counted for radioactivity after the addition of scintillation liquid (Scintiverse). Also, 1 mL of the bathing solution was used to count its radioactivity. The uptake (transport) of PAH is presented as slice/medium (S/M) ratio, i.e., the radioactivity/gm of tissue divided by the radioactivity/mL of bathing solution.

## **2.18 *In vivo* studies -**

### **2.18.1 Urinary enzyme experiments -**

**2.18.1a Preparation of urine samples -** Samples (whole 24 hr urines) were centrifuged for 5 min at 4,000 rpm. The clear supernatants were removed carefully and used for analysis.

**2.18.1b Gel filtration of urine samples -** This was done according to the method described by Werner et al. (1969). Eight glass columns with small dead space (inner diameter 1 cm, height 30 cm) were each filled to a height of about 18 cm with hydrated Sephadex G-50, giving a gel bed of about 14 cm<sup>3</sup> in each column. Separation was performed at room temperature, and physiological saline (0.154 M NaCl) was used as eluant. Each urine sample (3 mL) was washed into the column with 1 mL saline followed by another 1 mL of saline. The liquid emerging from the column up to that time (5 mL) was discarded. Saline was placed on each column, and 6 mL of eluates was collected for enzyme analysis. The columns were finally filled and rinsed with saline to prepare them for reuse.

**2.18.1c Measurement of urinary enzymes -** Alkaline phosphatase and  $\gamma$ -glutamyl transferase activities were measured using kits (kits numbers 104-LL and 545-A respectively) from Sigma Chemical Co. (St. Louis, MO). To avoid experimental variations due to different urine volumes, the enzyme activities were expressed as units/mg creatinine (creatinine kit number 555-A, Sigma Chemical Co.).

**2.18.2 [<sup>3</sup>H]OTA experiments -** Rats were anaesthetized with diethyl ether (6 hr after OTA treatment), and blood was withdrawn from the abdominal aorta. After sacrifice, kidneys, liver, stomach, small intestine and caecum were excised, and the small intestine (Int.) was divided into four equal parts by length (Int.1 to Int.4). Gut segments [stomach, small intestine (Int.1, Int.2, Int.3 and Int.4), and cecum] were rinsed twice with 1 mL portions of 0.5% sodium taurocholate in normal saline and then twice with 1 mL portions

of 50 mM sodium bicarbonate to ensure complete removal of all contents which was kept for extraction. Tissues were homogenized in water using a polytron. Tissue homogenates and contents were acidified to pH 2 and extracted three times with chloroform (5 mL lots). After drying the combined extracts under nitrogen, samples were redissolved in 400  $\mu$ L methanol, and 200  $\mu$ L samples were counted for radioactivity. Blood samples were allowed to stand at room temperature for at least 1 hr and then centrifuged to obtain serum. Calculation of total serum volume was done assuming the blood volume (mL) is given by the numerical value of 7% of a rat's mass (in gm) and the serum volume to be 55% of blood volume. Samples of urine and serum (50 - 100  $\mu$ L) were also counted for radioactivity.

### **2.19 Spectrophotometric/spectrofluorometric measurements -**

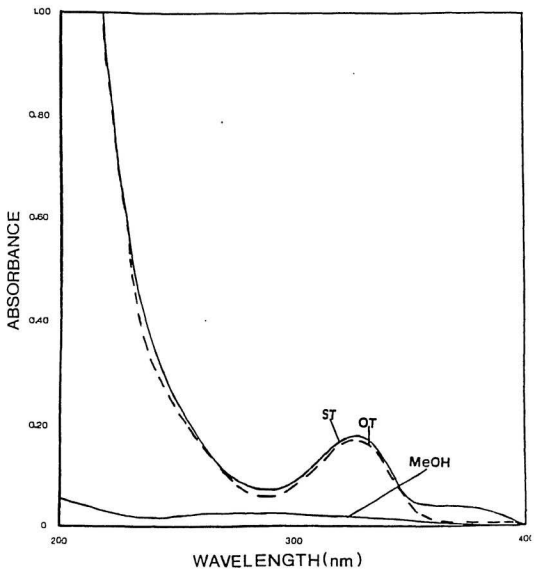
Spectrophotometric measurements were conducted on a Perkin-Elmer Lambda 3B double beam spectrophotometer in cells with a 1 cm light path. Fluorescence measurements were made in a Perkin-Elmer LS-5 spectrofluorimeter in cells with a square cm section, 1 cm in each dimension.

### **2.20 Statistical analysis -**

The non-parametric Mann-Whitney test was used for the analysis of results from *in vivo* experiments (N = 4 or 8) and results from *in vitro* experiments where N = 4. A difference at  $P < 0.05$  was considered to be statistically significant. For the rest of *in vitro* experiments, the number of animals from which samples were used was too small to be analysed. When an experiment was done on samples from two different animals from each treatment group, the results from each animal were given separately. When an experiment was done on more than two animal (three or four) per treatment group, the results were given as the mean  $\pm$  S.D. from all animals.

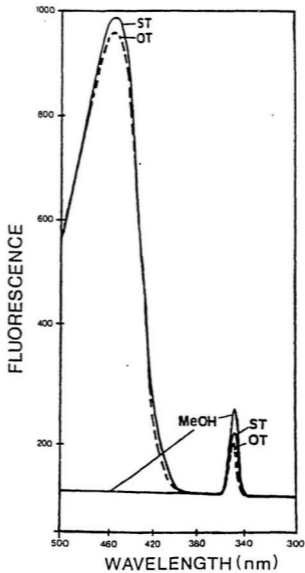
**Figure 5      The UV spectrum of OTA.**

The continuous line represents standard OTA from Sigma Chemical Co. (ST,—), while the broken line represents my OTA preparation (OT, --). Both samples were at a concentration of 25  $\mu$ M OTA in methanol. The instrument (Perkin Elmer Lambda 3B spectrophotometer) settings were: wavelength scan 400 - 200 nm, chart speed 60 mm/min and scan speed 60 nm/min.



**Figure 6      The fluorescence spectrum of OTA.**

The continuous line represents standard OTA from Sigma Chemical Co. (ST, —), while the broken line represents my OTA preparation (OT, --). Both samples were at a concentration of 50  $\mu$ M OTA in methanol. The instrument (Perkin Elmer LS-5 spectrofluorimeter) settings were as follows: Excitation wavelength 340 nm, while emission was scanned between 300 and 500 nm. The excitation/emission slits were set at 5/3 nm, chart speed 60 mm/min and scan speed 120 nm/min.



**Table 6. The  $R_f$  values of ochratoxin A.**

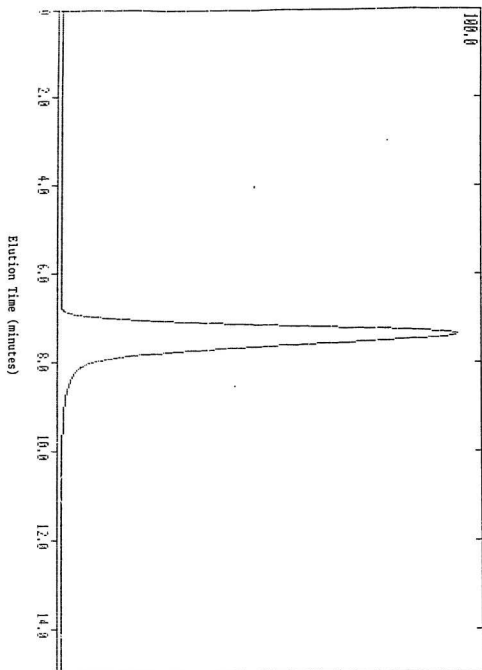
<b>Solvent system</b>	<b>Sigma OTA*</b>	<b>Prepared OTA*</b>
Benzene : acetic acid (4 : 1, v/v)	0.68	0.68
Benzene : acetic acid (8 : 1, v/v)	0.41	0.41
Benzene : acetic acid : Methanol (95 : 5 : 5, v/v/v)	0.45	0.45

\* 3  $\mu$ L of 25 mM were spotted on TLC plates with fluorescent indicator.

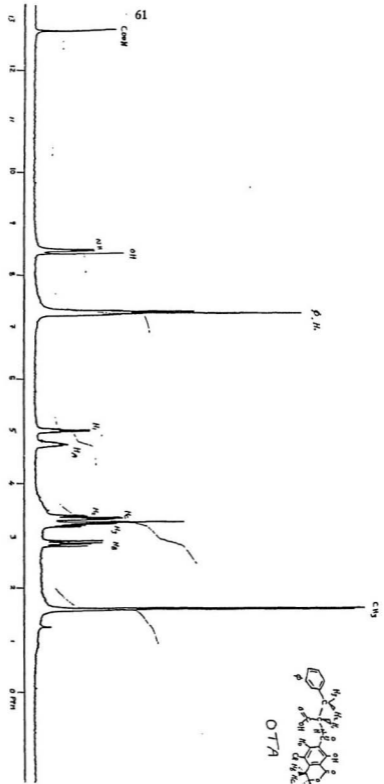
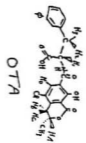
**Figure 7      The HPLC profile of our OTA preparation.**

Sample (20  $\mu$ L of 25  $\mu$ M solution in methanol) was injected into a Perkin Elmer-Series 4 liquid chromatograph. The column used was Partisil 10 ODS-2. The solvent system consisted of a mixture of a) acetonitrile:methanol (500:500, v/v) 65% and b) 5 mM sodium acetate:acetic acid (500:14, v/v) 35%. The flow rate was 1.5 mL/min and the fluorimetric detection was performed at excitation wavelength of 340 nm, emission wavelength of 465 nm and excitation/emission slits of 5/10 nm.

Detector Response (mvolts)



**Figure 8**      **Proton NMR spectrum of my OTA preparation.**



**Table 7. List of various treatments and cytochromes P-450 induced.**

Treatment	Major P-450 Enz. Induced	Route of Administration, Dose and Vehicle	Reference
Control	-	No treatment.	-
PB	IIB1	0.1% in drinking water for 5 days.	Graves, 1987
3-MC	IA1	20 mg/Kg in corn oil i.p. once each day for 3 successive days.	Guengerich, 1982
Clofibrate	IVA1	200 mg/Kg in 1 mL sucrose syrup containing 1% arabic gum given by gastric intubation once each day for 5 days.	Fournel, 1987
Isosafrole	IA1/IA2	120 mg/Kg in corn oil i.p. once each day for 3 successive days.	Fischer, 1981
PCN	IIIA1/IIIA2	100 mg/Kg in 1 mL 1% Tween 80 given by gastric intubation once each day for 4 days.	Graves, 1987
Isoniazid	IIE1	0.1% in drinking water for 10 days (pH 7.4).	Ryan, 1985

N = 4/treatment group.

## CHAPTER 3

### RESULTS

#### **3.1 Role of EDTA and cytochrome P-450 in the stimulation of OTA-induced lipid peroxidation in reconstituted systems.**

In a reconstituted microsomal lipid peroxidation system consisting of microsomal phospholipid, Fp,  $\text{Fe}^{3+}$ , OTA and NADPH (see Methods section 2.14.1), the concentrations of MDA obtained were 1.65 / 2.20 and 2.46 / 4.60 nmol/mL at the end of 20 and 40 min respectively (values from two different experiments). In this experiment, purified cytochrome P-450 and Fp (from 6-8 rats each) not microsomes from individual rats were used. Hence, experiments 1 and 2 were performed on two different days (each in duplicate) but using the same purified enzymes (as these were isolated only once). Addition of EDTA (25 nmol/mL) to the reconstituted system led to a three- to four-fold increase in the concentration of MDA formed yielding 6.62 / 7.45 and 10.24 / 13.60 nmol MDA/mL at 20 and 40 min respectively (Table 8). Replacement of EDTA by purified cytochrome P-450 IIB1 also led to a stimulation of lipid peroxidation. Figure 9 shows the extent of this stimulation in the presence of increasing concentrations of cytochrome P-450 IIB1. Concentrations up to 0.3 nmol cytochrome P-450/mL had a stimulatory effect on MDA production, which amounted to 6.5 / 8.5 and 10 / 13 nmol MDA at the end of 20 and 40 min respectively. Intact cytochrome P-450 seems to be essential since the heat-denatured enzyme, hematin, or a variety of hemoproteins were all ineffective in stimulating lipid peroxidation (Table 9), but carbon monoxide (CO) did not inhibit the stimulatory effect of cytochrome P-450. It is clear that the addition of only cytochrome P-450, but not other heme or hemoproteins stimulated lipid peroxidation (in this experiment, purified cytochrome P-450 from 6-8 rats not microsomes from individual rats was used).

### **3.2 Effect of microsomes from Co-heme pretreated rats on OTA-stimulated lipid peroxidation.**

This was studied as described in Methods sections 2.5.2, 2.12 and 2.14.2. The data in Figure 10 suggest that cytochrome P-450 may also be involved in OTA-dependent microsomal lipid peroxidation. The rate of MDA formation was highest in microsomes isolated from untreated rats. Thus, at the end of a 10-min incubation period, microsomes from untreated rats produced 7.6 / 9.4 nmol of MDA (values from two different experiments). In contrast, microsomes isolated from Co-heme-pretreated rats showed a much lower rate of lipid peroxidation which, at 10 min, amounted to only 2.2 / 2.5 nmol of MDA. Treatment of rats with Co-heme has been shown to lower hepatic cytochrome P-450 levels (Drummond and Kappas, 1982; Spaethe and Jollow, 1989), and also to reduce Fp activity (Spaethe and Jollow, 1989). Microsomes from Co-heme-pretreated rats contained 0.6 nmol of cytochrome P-450 and 22 units of Fp per mg protein. These values are 20% and 25% of the respective values in control microsomes (Table 10). To establish that the greatly reduced rate of MDA formation by microsomes from Co-heme-pretreated rats was not due primarily to lower Fp levels, microsomes from Co-heme-pretreated rats fortified by Fp were examined for their ability to carry out OTA-dependent lipid peroxidation. Fp fortified microsomes contained 78% of Fp of control microsomes. The results show that Fp-fortification of microsomes from Co-heme-pretreated rats only marginally increased MDA formation to 3.4 / 4.1 nmol at 10 min.

### **3.3 Effect of various cytochrome P-450 inducers on OTA metabolism by liver microsomes.**

The structures of OTA and its metabolites 4(R)- and 4(S)-4-OH-OTA are shown in Figure 1. Figure 11 shows the HPLC profile of OTA and its metabolites. Table 7 lists the various methods of inducing cytochrome P-450 (Methods section 2.5.1), together with the

major isoforms of cytochrome P-450 induced (the unified nomenclature by Nebert et al., 1987 is used). The effect of these inducers on the hepatic cytochrome P-450 concentration in liver microsomes is shown in Table 11. Some of the cytochrome P-450 inducers increased the total cytochrome P-450 concentration (e.g. PB, 2.5 fold increase over control; 3MC, 2 fold; CLF and PCN, about 1.7 fold and ISF, about 1.2 fold) while other only induced specific cytochrome P-450 isozymes without increasing the total content (e.g. INH). The extent of OTA metabolism by microsomes from rats treated with the cytochrome P-450 inducers is shown in Figure 12. After the induction of cytochrome P-450 in liver with 3MC, ISF, PB and PCN, the microsomal metabolism of OTA *in vitro* (Methods section 2.15.1) gave 4(R)-4-OH-OTA as the major metabolite. After induction with CLF both 4(R) and 4(S) isomers were produced in increased but equal amounts, and after induction with INH the 4(S) isomer predominated. Results from a completely different batch (Batch II) are shown in Figure 13. Figure 14 shows the effect of vehicle controls of the different inducers of cytochrome P-450 on the metabolism of OTA.

#### 3.4 Substrate specificity of cytochromes P-450 IIB1 and IA1/IA2.

The substrates 7-pentoxoresorufin (specific for PB inducible cytochrome P-450 IIB1, Lubet et al., 1985) and 7-ethoxoresorufin (specific for 3MC-inducible cytochrome P-450 IA1/IA2, Burke and Mayer, 1983) showed selective or preferential reactions with the appropriate cytochrome P-450 isoforms. Thus, liver microsomes from PB-treated rats dealkylated 7-pentoxoresorufin about 30 times more rapidly than those from control rats, and liver microsomes from 3MC-treated rats dealkylated it at a rate of only about 4.5 times that of microsomes from control animals. In contrast, microsomes from 3MC treated rats dealkylated 7-ethoxoresorufin about 82 / 88 times as rapidly as those from controls, and microsomes from PB treated rats increased its rate of metabolism only about 10.5 / 20 fold over controls (Table 12).

### 3.5 Effect of specific inhibitors of cytochromes P-450 1A1/1A2 and 1B1 on the metabolism of OTA *in vitro*.

Figure 15 shows the effect of  $\alpha$ -naphthoflavone (a known inhibitor of cytochrome P-450 1A1/2, Wiebel et al., 1971) and metyrapone (a known inhibitor of cytochrome P-450 1B1, Jonen et al., 1974) on the metabolism of OTA to 4(R)-4-OH-OTA by microsomes from PB, 3MC, and ISF treatments.  $\alpha$ -Naphthoflavone selectively inhibited the formation of 4(R) isomer by microsomes from rats pretreated with 3MC and ISF (~ 89 / 95% inhibition in both cases; results from two different experiments). The inhibition was ~ 18 / 41% using microsomes from rats treated with PB. On the other hand, metyrapone inhibited the formation of 4(R) isomer by microsomes from rats treated with PB (~ 94 / 96% inhibition), and also inhibited (to a lesser extent) its formation (~ 40 / 11% and ~ 40 / 16% inhibition) by microsomes from 3MC and ISF treated rats respectively (Figure 15). The effect of the two inhibitors on 4(S) isomer formation was less selective (Figure 16).

### 3.6 Effect of monoclonal antibodies (MAbs) against cytochromes P-450 on the metabolism of OTA by liver microsomes *in vitro*.

Two main monoclonal antibodies were used: clone 1-7-1 against cytochrome P-450 1A1/1A2 and clone 2-66-3 against cytochrome P-450 1B1/1B2. The preincubation of liver microsomes from rats pretreated with 3MC, with monoclonal antibody 1-7-1 reduced the subsequent formation of 4(R)-4-OH-OTA from OTA *in vitro* (Figure 17a). At a MAb to microsomal protein ratio of 5, the formation of the 4(S)-isomer was also partially inhibited (Figure 17a). Rabbit IgG was used to determine the non specific inhibition of OTA metabolism *in vitro*. The preincubation of liver microsomes from rats pretreated with 3MC, with rabbit IgG slightly inhibited the formation of the 4(R)-isomer only at IgG to microsomal protein ratio of 5 (Figure 17b). Preincubating liver microsomes from rats treated with PB, with MAb 2-66-3 reduced the subsequent formation of 4(R)-4-OH-OTA

from OTA *in vitro* (Figure 18a). At MAb to microsomal protein ratios of 2, 3 and 5, the formation of the 4(S)- isomer was also reduced (Figure 18a). The non specific inhibition of OTA metabolism resulting from preincubating rabbit IgG with liver microsomes from PB treated rats is shown in Figure 18b. Table 13 shows the cross reactivity of each of the two monoclonal antibodies with microsomes from rats treated with PB and 3MC. With respect to inhibition of the 4(R)-isomer formation, MAb 2-66-3 showed greater specificity towards microsomes from PB-treated rats whereas MAb 1-7-1 showed greater specificity towards microsomes from 3MC treated rats (Table 13). However, both antibodies especially 2-66-3 showed substantial cross-reactivity. MAbs against cytochrome P-450 enzymes are very expensive; if they were to be purchased, the cost would have been several thousand dollars. the fact that we obtained them as a gift and in small quantity, we were not able to repeat these experiments again.

### **3.7 Time course of OTA metabolism by liver microsomes from rats treated with INH and PCN.**

OTA metabolism by liver microsomes from untreated (control) rats was very poor giving rise to 4(R)-4-OH-OTA and 4(S)-4-OH-OTA levels that averaged 0.015 and 0.014 nmol/mg protein respectively over a 30 min incubation period (Table 14). Microsomes from INH-treated rats were chosen to further study the 4(S)-isomer formation since this pretreatment induces 4(S)-isomer formation (about 7 fold) without concomitantly increasing 4(R)-isomer formation. Microsomes from PCN-treated rats were selected to study 4(R)-isomer formation since this pretreatment increased 4(R)-4-OH-OTA formation 55-fold over control microsomes. PCN pretreatment also increased 4(S)-isomer formation by 10-fold over control microsomes (Table 14).

Figure 19 shows the time course of OTA metabolism and lipid peroxidation using microsomes from INH-pretreated rats. There was very little 4(R)-4-OH-OTA formation

which reached a maximum of 0.017 nmol/mg protein at 40 min. In contrast, 4(S)-4-OH-OTA formation continued to increase steadily reaching a maximum of 0.080 nmol/mg protein at 40 min, the final time point tested (Figure 19a). In parallel measurements, MDA formation also increased steadily reaching 16 nmol/mg protein by 40 min (Figure 19b). The time course of OTA metabolism and lipid peroxidation by microsomes from PCN-pretreated rats is shown in Figure 20. In contrast to microsomes from INH-treated rats, microsomes from PCN-treated rats efficiently catalyzed the formation of 4(R)-4-OH-OTA which steadily increased to about 1 nmol/mg protein at 40 min, the final time point tested. 4(S)-4-OH-OTA formation also increased steadily with time to reach 0.17 nmol/mg protein by 40 min (Figure 20a). As in the case of microsomes from INH-treated rats, MDA formation also increased with time reaching 20.5 nmol/mg protein at 40 min (Figure 20b).

### 3.8 Effect of pH on OTA metabolism and lipid peroxidation.

The pH optima for the formation of 4(S)-4-OH-OTA (Figure 21a) and lipid peroxidation (Figure 21b) by microsomes from INH-treated rats were 7.0 and 6.5 - 7.5 respectively. 4(R) did not show a very distinct pH optimum, possibly because of the very low levels produced. The pH optima for the formation of 4(R)-4-OH-OTA (Figure 22a) and lipid peroxidation (Figure 22b) by microsomes from PCN-treated rats were 6.5 and 7.0 - 7.5 respectively. 4(S) did not show a distinct pH optimum, possibly because of low levels of production.

### 3.9 Effect of active oxygen scavengers, antioxidants and iron chelators on OTA metabolism and lipid peroxidation.

Tables 15 and 16 shows the effect of superoxide dismutase (SOD), catalase, mannitol, butylated hydroxyanisole (BHA), N,N-diphenyl-1,4-phenylenediamine (DPPD),

bathophenanthrolinedisulfonic acid (BPS) and Desferal addition on OTA metabolism and lipid peroxidation by microsomes from INH- and PCN-pretreated rats. With microsomes from INH-pretreated rats, 4(S)-4-OH-OTA formation was inhibited 100% by BHA, over 90% by DPPD, Desferal and BPS, about 30% by mannitol and about 15% by SOD and catalase. In contrast, none of these agents inhibited 4(R)-4-OH-OTA formation with the exception of DPPD which exerted about 40% inhibitory effect. In fact, both Desferal and BPS increased the yield of 4(R)-4-OH-OTA by 60-170% with microsomes from both sources (Table 16). Parallel determinations of lipid peroxidation indicated that BHA, DPPD, Desferal and BPS all inhibited MDA formation by >90%, mannitol was slightly inhibitory (15%) while catalase and SOD had no effect.

When microsomes from PCN treated rats were used, both antioxidants (BHA and DPPD) inhibited 4(S)-4-OH-OTA formation by ~ 50% while BPS exerted a 35% inhibitory effect, but SOD, catalase and Desferal increased 4(S)-4-OH-OTA levels by 35%, 50% and 30% respectively; mannitol was without effect. 4(R)-4-OH-OTA formation was not affected except by catalase, Desferal and BPS which increased it by about 30%, 100% and 170% respectively. As expected, MDA formation was strongly inhibited by BHA, DPPD, Desferal and BPS (>90%) while both catalase and mannitol were mildly inhibitory (15 - 20%). Figure 23 shows that varying concentrations of BHA inhibited to roughly the same extent both 4(S)-4-OH-OTA formation and lipid peroxidation by liver microsomes from INH treated rats. Thus, 2.5  $\mu$ M and 5  $\mu$ M BHA were required to completely inhibit 4(S)-4-OH-OTA formation at 10 min and 30 min respectively (Figure 23a), and 5  $\mu$ M BHA was required to inhibit MDA formation (Figure 23b) at 10 min and 30 min. Lower concentrations of BHA were correspondingly less inhibitory. In contrast, 4(R)-4-OH-OTA formation was not inhibited (data not shown; see also Tables 16 and 17).

Incubation of liver microsomes from INH- or PCN-treated rats with NADPH and OTA for 30 min resulted in the destruction of cytochrome P-450 by 40% and 75%

respectively. Inclusion of Desferal provided complete protection against cytochrome P-450 loss in the case of microsomes from INH-treated animals, and reduced the cytochrome P-450 loss from 75% to 37% in the case of microsomes from PCN-treated animals (Table 16).

Replacement of NADPH by ascorbate/ $\text{Fe}^{2+}$  almost virtually eliminated 4(R)-4-OH-OTA formation by microsomes from PCN-treated rats, but did not affect 4(S)-4-OH-OTA formation by microsomes from either PCN- or INH-treated rats (Table 17). As expected, the inclusion of BHA strongly inhibited 4(S)-4-OH-OTA formation as well as MDA formation (~90%) by microsomes from both sources. Addition of mannitol did not appreciably change the formation of 4(S)-4-OH-OTA, 4(R)-4-OH-OTA or MDA.

### **3.10 Effect of various hemoproteins and hydroperoxides on OTA metabolism.**

Cumene hydroperoxide (CHP, 0.5 mM) could effectively replace NADPH in catalyzing the formation of 4(R)-4-OH-OTA and 4(S)-4-OH-OTA by liver microsomes from PCN-treated rats (2 mg microsomal protein/mL) giving 0.53 and 0.03 nmol metabolite/nmol heme respectively (Table 18). By comparison,  $\text{H}_2\text{O}_2$  (5 mM) was much less effective giving only 0.015 and 0.013 nmol of the 4(R)- and 4(S)-isomers respectively, while linoleic acid hydroperoxide (LAHP, 65  $\mu\text{M}$ ) was totally ineffective. When microsomes were replaced with other hemoproteins e.g. hemoglobin or HRP (20 nmol/mL), or by hematin (5 nmol/mL) no OTA metabolism was observed in the presence of a variety of hydroperoxides (CHP, 0.5 mM;  $\text{H}_2\text{O}_2$ , 5 mM or LAHP, 65  $\mu\text{M}$ ) (Table 18).

### 3.11 Effect of pH on OTA metabolism and lipid peroxidation by liver microsomes, and on cytochrome P-450 content of these microsomes.

The pH optima for the formation of 4(R)-4-OH-OTA and 4(S)-4-OH-OTA, and for lipid peroxidation in liver microsomes from rats given different treatments are given in Figures 22, 24 and 25. The pH optimum for the formation of 4(R)-isomer was 6.0 using microsomes from rats treated with 3MC (Figure 25) and ISF (data not shown), and 6.5 using microsomes from rats treated with PB (Figure 24a) and PCN (Figure 22). The pH optimum for the formation of 4(S)-isomer was 7.0 (Figures 21a, 24a and 25), which coincided with that for lipid peroxidation (Figures 21b, 22b and 24b). Table 19 shows a comparison between the effect of pH 6.0 and 7.4 on OTA metabolism using microsomes from rats treated with 3MC, ISF and PB. The formation of 4(R)-isomer increased at pH 6.0 (compared to pH 7.4) by 7.7- and 5.0-fold using liver microsomes from 3MC and ISF treated rats respectively. At pH 7.4 (compared to pH 6.0), the formation of 4(S)-isomer increased 3.4- and 2.7-fold using liver microsomes from ISF and PB treated rats. Table 20 shows the MDA formed and cytochrome P-450 contents at the end of 30 min incubation using liver microsomes from rats treated with 3MC, ISF and PB at pHs 6.0 and 7.4. More MDA and lower cytochrome P-450 contents were observed at pH 7.4 as compared to pH 6.0. Thus, MDA formed at pH 7.4 was 26.8, 17.1 and 20.2 nmol/mg protein using liver microsomes from rats treated with 3MC, ISF and PB as compared to 18.9, 8.0 and 11.8 nmol/mg protein respectively at pH 6.0 for microsomes from the same sources. At pH 7.4, the percentage loss of cytochrome P-450 contents was 57, 43 and 59 using liver microsomes from rats treated with 3MC, ISF and PB as compared to 32, 15 and 51 respectively at pH 6.0 for microsomes from the same sources (Table 20). It seems that the loss in cytochrome P-450 at pH 6.0 for microsomes from 3MC and ISF treated rats is not as much as that for microsomes from PB treated rats. This may, partly, explain the 5.0- and 7.7-fold increase in the 4(R) isomer formation (at pH 6.0) using microsomes from ISF

and 3MC treated rats, and the 1.8-fold increase using microsomes from PB treated rats (Table 19). It also seems that the loss in cytochrome P-450 is greater when there is more lipid peroxidation (i.e. at pH 7.4 compared to pH 6.0, Table 20).

### **3.12 Effect of pH on the Km and Vmax of OTA hydroxylase in liver microsomes from rats treated with 3MC and ISF.**

The Km for OTA hydroxylation (measured at pH 7.4) was calculated to be 74  $\mu$ M for microsomes from rats treated with 3MC (Figure 26a), whereas the Vmax was 38 pmol/min/mg protein. At pH 6.0, Km dropped to 31  $\mu$ M and Vmax increased to 195 pmol/min/mg protein (Figure 26b). The Km and Vmax for OTA hydroxylation (measured at pH 7.4) by microsomes from rats treated with ISF were 92  $\mu$ M and 13 pmol/min/mg protein (Figure 27a). The corresponding values at pH 6.0 were 150  $\mu$ M and 279 pmol/min/mg protein (Figure 27b).

### **3.13 In vivo experiments.**

#### **3.13.1 Effect of PB treatment on OTA-induced release of enzymes in the urine.**

Figure 28 shows urine alkaline phosphatase levels both in PB-treated and control rat groups on day 0 (before OTA treatment) and days 1-5 of OTA treatment. Enzyme level was elevated significantly (compared to base line, day 0) on the fourth day of OTA treatment in the control group (four fold increase), whereas there was no increase in enzyme level for the PB-treated group. In case of  $\gamma$ -glutamyl transferase, enzyme levels in the urine increased on days 1, 2, 3 and 4 of OTA treatment in the control group by 2.5-, 2.0-, 1.7- and 3.5-fold (compared to base line, day 0). For the PB group, enzyme levels increased at days 2 and 3 by 2.7- and 2.6-fold (Figure 29).

Transport of the anion p-amino hippurate (PAH) in kidney cortex slices obtained from PB-pretreated rats treated with OTA (PB + OTA) and rats treated with OTA (OTA) was significantly inhibited to the same extent (55% - 60%) as compared to control animals (C) (Figure 30). However, there was no significant difference between the two groups (OTA and PB + OTA) in terms of their inhibition of PAH transport (Figure 30).

### 3.13.2 Effect of PB treatment on the distribution of OTA.

Figure 31 shows the percentage of administered [ $^3\text{H}$ ]OTA found in serum and urine of PB-treated and control rats 6 hr after OTA administration. Even though it was not significantly different, there was a tendency of increased OTA concentration in the serum of control (12.4% of administered dose) as compared to PB-treated rats (8.4%), whereas there was an increase in OTA concentration in the urine of PB treated rats (5.2%) as compared to control rats (2.0%). OTA levels in the liver (1.5% for control group and 1.24% for the PB-treated group) and the kidney (0.15% for control group and 0.13% for PB group) did not show a significant difference between the two groups (Figure 32). The percentage of the toxin accumulated in the whole organ is more in the liver (1.2% - 1.5%) as compared to the kidney (0.13% - 0.15%), but on a per gram basis, more toxin accumulated in the kidney than in the liver. This is in agreement with the finding of Galtier et al. (1979). The percentage of administered [ $^3\text{H}$ ]OTA in the gut tissues (stomach, the four portions of small intestine and cecum) are shown in Figure 33. The levels of OTA in the gut contents are shown in Figure 34. Higher OTA levels were found in the small intestinal tissue (2 - 6 fold increase) and gut contents (4 - 8 fold increase) of control rats than of PB rats.

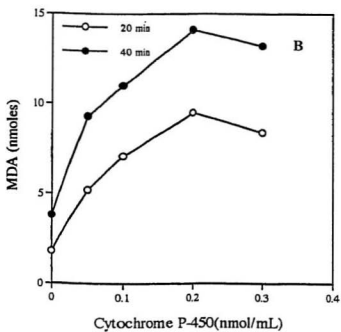
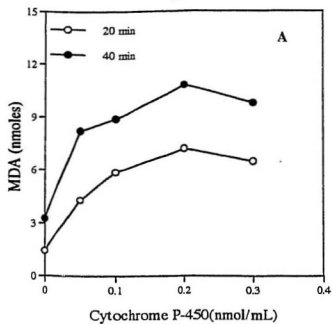
**Table 8. Involvement of EDTA or cytochrome P-450 in the stimulation of lipid peroxidation by OTA in a reconstituted system.**

Addition to system	MDA formed (nmol/mL)			
	20 min		40 min	
	Exp.1	Exp.2	Exp.1	Exp.2
None	1.65	2.20	2.46	4.60
EDTA (25 nmol/mL)	6.62	7.45	10.24	13.6
EDTA (50 nmol/mL)	5.14	6.30	7.96	11.20
Cyt. P-450 (0.3 nmol/mL)	4.30	6.20	7.11	10.03
Cyt. P-450 (0.5 nmol/mL)	5.35	4.40	8.45	9.78

Incubations were carried out as described in the Methods section 2.14.1. Each of the two experiments were carried out in duplicate. MDA values are means of the two separate incubations.

**Figure 9      Effect of cytochrome P-450 concentration on OTA stimulated lipid peroxidation.**

Incubations were carried out in duplicate at 37°C for 20 and 40 min in 0.25 M Tris-HCl buffer, pH 6.8/0.25 M NaCl and contained per mL: phospholipid vesicles (1  $\mu$ mol P), 177 ng Fp (3.2 units), 500 nmol OTA, varying amounts of cytochrome P-450 (0 - 0.3 nmol), 110 nmol  $\text{Fe}^{3+}$  and 200 nmol NADPH. The reaction volume was 1 mL. Results of two separate experiments (A and B) are shown.



**Table 9. Requirement for cytochrome P-450 in NADPH-dependent lipid peroxidation.**

<b>Hemoprotein/Treatment</b>	<b>MDA formed (nmol/mL)</b>
None	1.98
Cyt. P-450	7.56
Cyt. P-450 (heat denatured) <sup>1</sup>	2.02
Cyt. P-450 + carbon monoxide <sup>2</sup>	7.33
Hematin	2.12
Hemoglobin	2.67
Cyt. <i>c</i>	2.43
Horseradish peroxidase	2.38

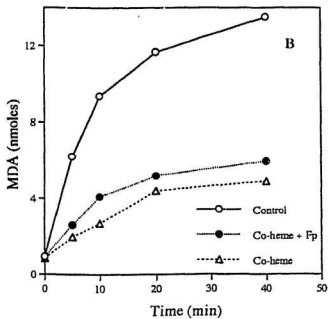
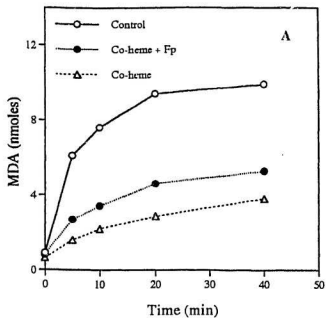
Incubations were carried out for 40 min as described in the Methods section 2.14.1. Hematin and all hemoproteins were added in an amount corresponding to 0.3 nmol heme/mL. MDA values are means from quadruplicate incubations that did not vary from each other by more than 10%.

<sup>1</sup> 100°C for 2 min.

<sup>2</sup> Carbon monoxide was bubbled through the incubation mixture for 30 sec prior to addition of OTA.

**Figure 10**      **Effect of depleting cytochrome P-450 on OTA stimulated lipid peroxidation.**

Incubations were carried out in duplicate at 37°C for 0, 5, 20 and 40 min in 0.1 M phosphate buffer, pH 7.4 and contained per mL: 2 mg microsomal protein (from control or Co-heme treated rats), and 125 nmol OTA. The reaction was initiated with 1 mM NADPH (final concentration). The reaction volume was 1 mL. Microsomes from rats treated with Co-heme (before use in lipid peroxidation) were first fortified for Fp by incubating them with the purified enzyme preparation (as described in the Methods section 2.12. Results of two separate experiments (A and B) from two different animals are shown.



**Table 10.** Effect of Co-heme pretreatment on liver microsomal NADPH-cytochrome P-450 reductase (Fp) and cytochrome P-450 levels.

Treatment	P-450 Reductase <sup>1</sup>	Cytochrome P-450 <sup>2</sup>
None	88.2 ± 7.3	2.92 ± 0.31
Co-heme	22.3 ± 2.7*	0.60 ± 0.05*
Co-heme + Fp	70.1 ± 6.8*	0.57 ± 0.05*

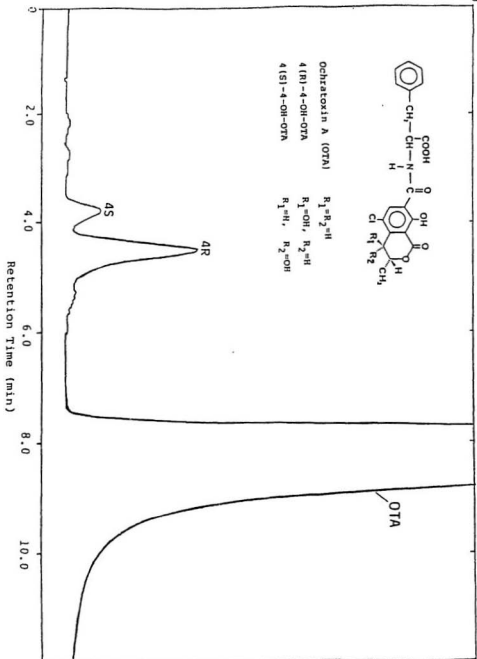
Animals received either Co-heme (50 µmol/kg body weight; sub-cutaneously) or saline 9 days and 2 days prior to being sacrificed. NADPH-cytochrome P-450 reductase and cytochrome P-450 levels were determined as described in the Methods sections 2.11 and 2.9. Results are means ± S.D of values from four individual rats/treatment group. \*, means significantly different from no treatment ( $p < 0.05$ , Mann Whitney test).

<sup>1</sup> nmol cytochrome *c* reduced/min/mg protein

<sup>2</sup> nmol/mg protein

**Figure 11**     **HPLC profile of OTA and its metabolites 4(R)- and 4(S)-4-OH-OTA.**

Typical HPLC profile of OTA metabolites formed during incubation of OTA with liver microsomes from PCN treated rats. OTA metabolites were analysed as described in the Methods section 2.15.3. Extracted sample (50  $\mu$ L of 0.5 mL) was injected into a Perkin Elmer-Series 4 liquid chromatograph. The column used was Partisil 10 ODS-2. The solvent system consisted of a mixture of a) acetonitrile: methanol (500:500, v/v) 65% and b) 5 mM sodium acetate:acetic acid (500:14, v/v) 35%. The flow rate was 1.5 mL/min and the fluorometric detection was performed at excitation wavelength of 340 nm, emission wavelength of 465 nm and excitation/emission slits of 5/10 nm.



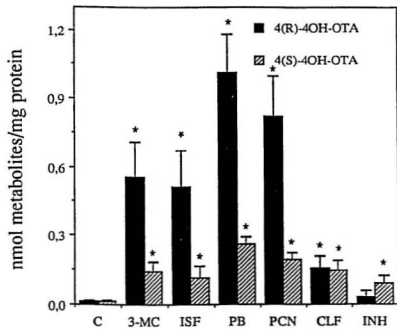
**Table 11. Total cytochromes P-450 content of liver microsomes isolated from rats following various treatments.**

<b>Treatment*</b>	<b>Cyt. P-450 content (nmol/mg protein)</b>
Control	0.85 $\pm$ 0.07
PB	2.15 $\pm$ 0.14*
3-MC	1.70 $\pm$ 0.18*
Clofibrate	1.40 $\pm$ 0.20*
Isosafrole	1.00 $\pm$ 0.02*
PCN	1.45 $\pm$ 0.17*
Isoniazid	0.80 $\pm$ 0.03

\* The various treatments are given in Table 7. Values shown are means  $\pm$  S.D. of duplicate determinations from four individual rats per treatment group. \*, means significantly different from control ( $p < 0.05$ , Mann Whitney test).

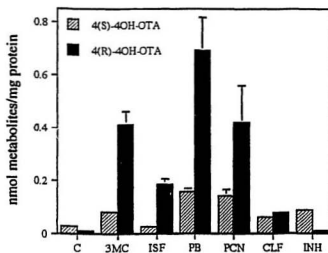
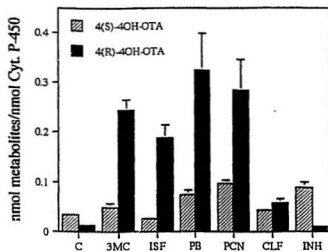
**Figure 12**     **The formation of 4(R)- and 4(S)-4-OH-OTA *in vitro* using liver microsomes from rats treated with different inducers of cytochrome P-450.**

Incubations were carried out in triplicate at 37°C for 30 min in 0.1 M potassium phosphate buffer (pH 7.4) and contained per mL: 2 mg microsomal protein, 125 nmol OTA and a NADPH-regenerating system (consisting of 0.4  $\mu$ mol NADP<sup>+</sup>, 5  $\mu$ mol MgCl<sub>2</sub>, 5  $\mu$ mol DL-isocitrate and 0.65 units of isocitric dehydrogenase). Details are described in the Methods section 2.15.3 for analysis of metabolites. Results are means  $\pm$  S.D. of four individual rats per treatment group. These results are from Batch III. Results from Batch II (a completely different batch of all treatments) are shown in figure 13. Batch I (not shown) was a trial run to determine the optimum doses of the various cytochrome P-450 inducers and their effects on OTA metabolism. \*, means significantly different from control ( $p < 0.05$ , Mann-Whitney test).



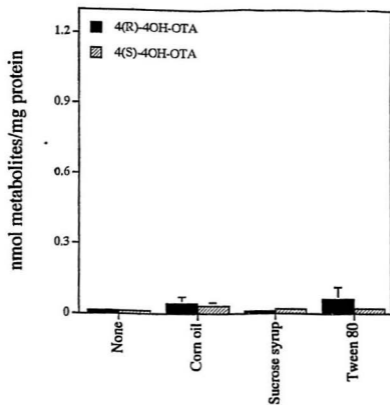
**Figure 13**    **The formation of 4(R)- and 4(S)-4-OH-OTA *in vitro* using liver microsomes from rats treated with different inducers of cytochrome P-450 (Batch II).**

Incubations were carried out in triplicate at 37°C for 30 min in 0.1 M potassium phosphate buffer (pH 7.4) and contained per mL: 2 mg microsomal protein, 125 nmol OTA and a NADPH-regenerating system (consisting of 0.4  $\mu$ mol NADP<sup>+</sup>, 5  $\mu$ mol MgCl<sub>2</sub>, 5  $\mu$ mol DL-isocitrate and 0.65 units of isocitric dehydrogenase). Details are described in the Methods section 2.15.3 for analysis of metabolites. Results are means  $\pm$  S.D. of three individual rats per treatment group.



**Figure 14**    **The formation of 4(R)- and 4(S)-4-OH-OTA *in vitro* using liver microsomes from control rats treated with the vehicles of the different inducers of cytochrome P-450.**

Incubations were carried out in triplicate at 37°C for 30 min in 0.1 M potassium phosphate buffer (pH 7.4) and contained per mL: 2 mg microsomal protein, 125 nmol OTA and a NADPH-regenerating system (consisting of 0.4  $\mu$ mol NADP<sup>+</sup>, 5  $\mu$ mol MgCl<sub>2</sub>, 5  $\mu$ mol DL-isocitrate and 0.65 units of isocitric dehydrogenase). Details are described in the Methods section 2.15.3 for analysis of metabolites. Results are means  $\pm$  S.D. of three individual rats per treatment group. Results are plotted to the same scale as the figure of the actual treatments (Figure 12 in the Results Section) for comparison.



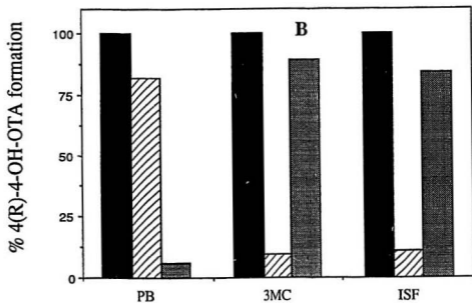
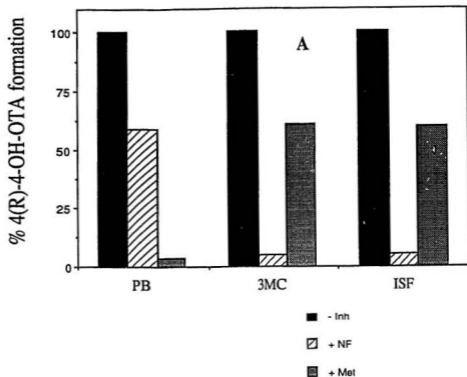
**Table 12. Ethoxy- and pentoxyresorufin O-dealkylation by liver microsomes from control, 3MC and PB treated rats.**

Sample	PROD (pmol resorufin released/min/mg protein)		EROD	
	Exp.1	Exp.2	Exp.1	Exp.2
Control	17.8	19.4	22.4	19.0
3MC	82.2	83.3	1840.0	1678.2
PB	543.6	594.7	234.1	379.7

Details are described in the Methods section 2.16. Results are means of triplicate incubations from two separate experiments.

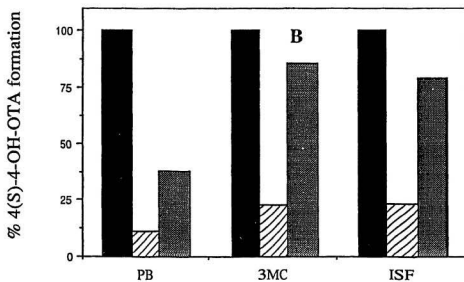
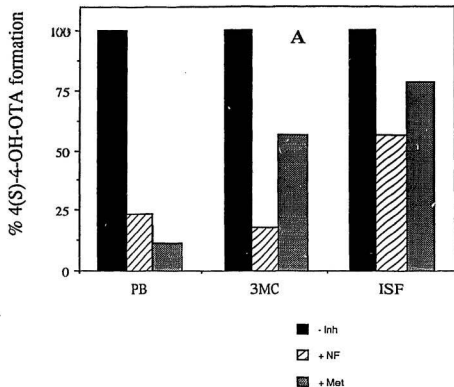
**Figure 15** Effect of the cytochrome P-450 inhibitors  $\alpha$ -naphthoflavone (50  $\mu$ M) and metyrapone (100  $\mu$ M) on the metabolism of OTA to 4(R)-4-OH-OTA by liver microsomes from PB, 3MC and ISF treated rats.

Incubations were carried out in duplicate at 37°C for 20 min in 0.1 M potassium phosphate buffer (pH 7.4) and contained per mL: 2 mg microsomal protein, 125 nmol OTA and a NADPH-regenerating system (consisting of 0.4  $\mu$ mol NADP<sup>+</sup>, 5  $\mu$ mol MgCl<sub>2</sub>, 5  $\mu$ mol DL-isocitrate and 0.65 units of isocitric dehydrogenase). Details are described in the Methods section 2.15.3 for analysis of metabolites. Results are means of two separate experiments (A and B) from two different animals. The uninhibited activity was normalized to 100%. The uninhibited values were 0.58/0.53 for PB, 0.38/0.87 for 3MC and 0.51/1.19 for ISF nmol 4(R)-4-OH-OTA/mg protein.



**Figure 16** Effect of the cytochrome P-450 inhibitors  $\alpha$ -naphthoflavone (50  $\mu$ M) and metyrapone (100  $\mu$ M) on the metabolism of OTA to 4(S)-4-OH-OTA by liver microsomes from PB, 3MC and ISF treated rats.

Incubations were carried out in duplicate at 37°C for 20 min in 0.1 M potassium phosphate buffer (pH 7.4) and contained per mL: 2 mg microsomal protein, 125 nmol OTA and a NADPH-regenerating system (consisting of 0.4  $\mu$ mol NADP<sup>+</sup>, 5  $\mu$ mol MgCl<sub>2</sub>, 5  $\mu$ mol DL-isocitrate and 0.65 units of isocitric dehydrogenase). Details are described in the Methods section 2.15.3 for analysis of metabolites. Results are means of two separate experiments (A and B) from two different animals. The uninhibited activity was normalized to 100%. The uninhibited values were 0.18/0.20 for PB, 0.13/0.14 for 3MC and 0.09/0.13 for ISF nmol 4(R)-4-OH-OTA/mg protein.

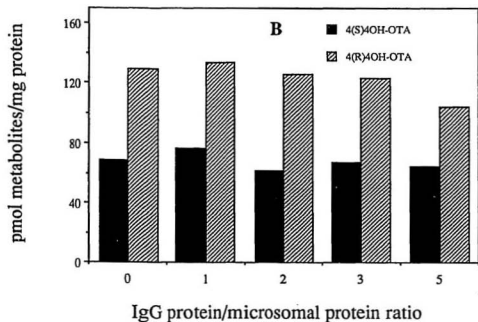
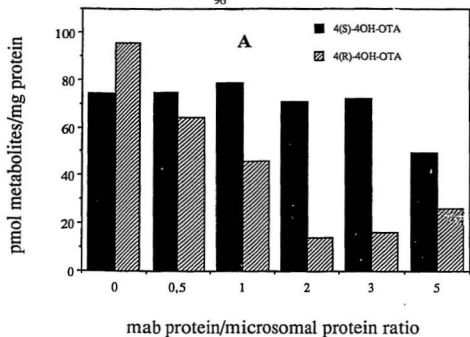


**Figure 17a** **Effect of monoclonal antibody (MAb) 1-7-1 on the metabolism of OTA by microsomes from rats treated with 3MC.**

MAb was preincubated with microsomes in 0.1 M potassium phosphate buffer (pH 7.4) for 30 min at room temperature. Microsomal protein concentration was 2 mg/mL and the total incubation volume was 100  $\mu$ L. Reaction was started by adding substrate (OTA, 125  $\mu$ M) and NADPH-regenerating system and was carried out in duplicate at 37°C for 30 min.

**Figure 17b** **Effect of rabbit IgG on the metabolism of OTA by microsomes from rats treated with 3MC.**

IgG was preincubated with microsomes in 0.1 M potassium phosphate buffer (pH 7.4) for 30 min at room temperature. Microsomal protein concentration was 2 mg/mL and the total incubation volume was 100  $\mu$ L. Reaction was started by adding substrate (OTA, 125  $\mu$ M) and NADPH-regenerating system and was carried out in duplicate at 37°C for 30 min.

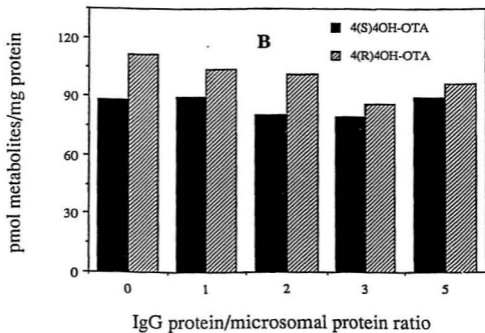
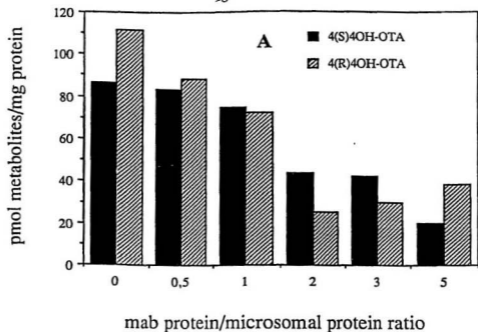


**Figure 18a**    **Effect of monoclonal antibody (MAb) 2-66-3 on the metabolism of OTA by microsomes from rats treated with PB.**

MAb was preincubated with microsomes in 0.1 M potassium phosphate buffer (pH 7.4) for 30 min at room temperature. Microsomal protein concentration was 2 mg/mL and the total incubation volume was 100  $\mu$ L. Reaction was started by adding substrate (OTA, 125  $\mu$ M) and NADPH-regenerating system and was carried out in duplicate at 37°C for 30 min.

**Figure 18b**    **Effect of rabbit IgG on the metabolism of OTA by microsomes from PB treated rats.**

IgG was preincubated with microsomes in 0.1 M potassium phosphate buffer (pH 7.4) for 30 min at room temperature. Microsomal protein concentration was 2 mg/mL and the total incubation volume was 100  $\mu$ L. Reaction was started by adding substrate (OTA, 125  $\mu$ M) and NADPH-regenerating system and was carried out at 37°C for 30 min.



**Table 13.** Cross reactivity of MAb 1-7-1 and 2-66-3 with cytochromes P-450 in liver microsomes isolated from rats treated with PB or 3MC.

Treatment	4(R)-4-OH-OTA Inhibition (%)	
	1-7-1*	2-66-3*
PB	23.2	58.3
3MC	63.8	39.0

Treatment	4(S)-4-OH-OTA Inhibition (%)	
	1-7-1*	2-66-3*
PB	19.2	38.0
3MC	0.0	15.0

Conditions are as described in the legend of Figure 17a.

\* MAb protein/microsomal protein 2 : 1.

**Table 14. Effect of INH- and PCN-pretreatments on hepatic cytochrome P-450 levels and OTA metabolism.**

Pretreatment	Cyt. P-450	4(R)-4-OH-OTA	4(S)-4-OH-OTA
		<i>nmol/mg protein</i>	
None	0.85 ± 0.07	0.015 ± 0.004	0.014 ± 0.006
INH	0.80 ± 0.03	0.032 ± 0.030 (2)	0.093 ± 0.031 (7)*
PCN	1.45 ± 0.17*	0.816 ± 0.150 (55)*	0.194 ± 0.040 (14)*

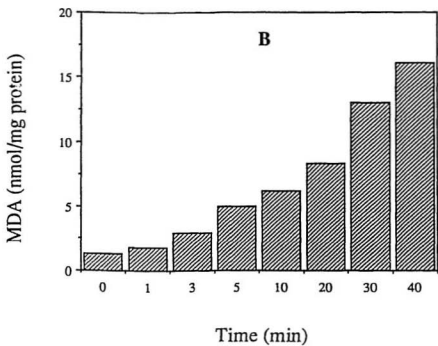
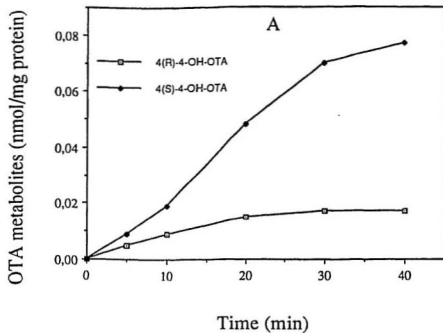
Pretreatment of rats and measurement of cytochrome P-450 levels and OTA metabolites was carried out as described in the Methods (sections 2.5.1, 2.9 and 2.15.3). Values shown are means ± S.D. from four individual rats. The numbers in parenthesis are fold increase in OTA metabolites compared to no treatment.

\*, means significantly different from no treatment ( $p < 0.05$ , Mann Whitney test).

**Figure 19     A. Time course of OTA metabolism by liver microsomes from INH-treated rats.**

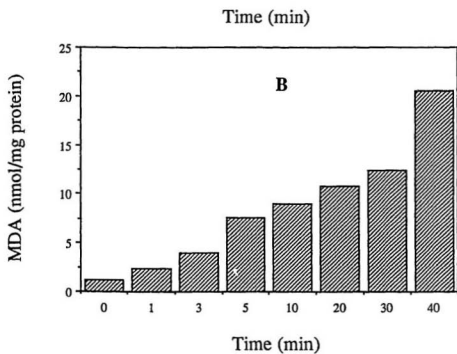
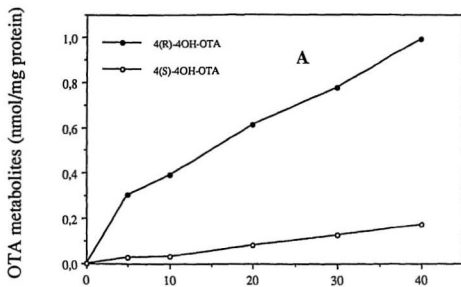
**B. Time course of lipid peroxidation in liver microsomes from INH-treated rats.**

Incubations were carried out at 37°C in 0.1 M potassium phosphate buffer (pH 7.4) and contained per mL: microsomal protein (2 mg), 125 nmol OTA and a NADPH-regenerating system (see Methods section 2.15.1). At timed intervals, two 0.5 mL samples were withdrawn, one for HPLC analysis of OTA metabolites, and the other for measurement of MDA levels. Details are described in the Methods sections 2.15.3 and 2.14.2. Results are means of duplicate determinations from each of two separate experiments. The difference between the two means at all time points was no more than 23% for metabolism and 16% for lipid peroxidation. The relatively high degree of variation is likely due to the cytochrome P-450 content of liver microsomes from every individual animal, also due to the fact that lipid peroxidation is a chain reaction and the rates of propagation are known to vary greatly from an experiment to another.



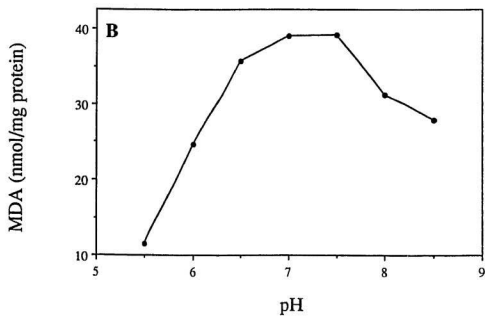
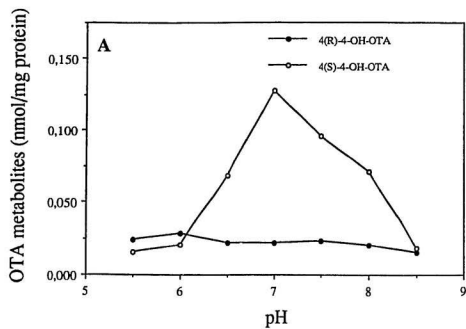
**Figure 20**    **A. Time course of OTA metabolism by liver microsomes from PCN-treated rats.**  
**B. Time course of lipid peroxidation in liver microsomes from PCN-treated rats.**

Incubations were carried out at 37°C in 0.1 M potassium phosphate buffer (pH 7.4) and contained per mL: microsomal protein (2 mg), 125 nmol OTA and a NADPH-regenerating system (see Methods section 2.15.1). At timed intervals, two 0.5 mL samples were withdrawn, one for HPLC analysis of OTA metabolites, and the other for measurement of MDA levels. Details are described in the Methods sections 2.15.3 and 2.14.2. Results are means of duplicate determinations from each of two separate experiments. The difference between the two means at all time points was no more than 18% for metabolism and 21% for lipid peroxidation. The relatively high degree of variation is likely due to the cytochrome P-450 content of liver microsomes from every individual animal, also due to the fact that lipid peroxidation is a chain reaction and the rates of propagation are known to vary greatly from an experiment to another.



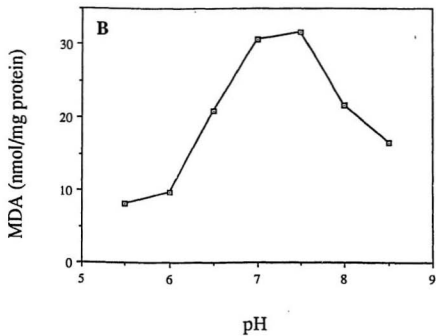
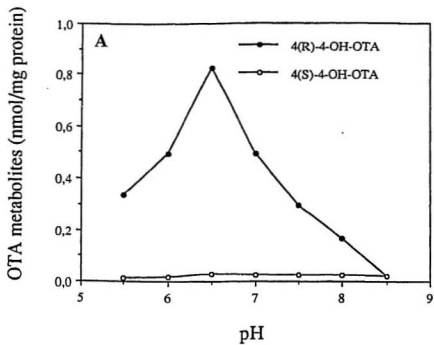
**Figure 21**      **Effect of pH on OTA metabolism (A) and lipid peroxidation (B) by liver microsomes from INH-treated rats.**

Incubations were carried out at 37°C in 0.1 M potassium phosphate (pH 5.5 - 8.5) and contained in a total volume of 1 mL: microsomal protein (2 mg), 125 nmol OTA and a NADPH-regenerating system (see Methods section 2.15.1). At the end of 30 min, 0.5 mL from each incubation was withdrawn for HPLC analysis of OTA metabolites, and the remaining 0.5 mL was used for measurement of MDA levels. Details are described in the Methods sections 2.15.3 and 2.14.2. Results are means of duplicate determinations from each of two separate experiments. The difference between the two means at all time points was no more than 20% for metabolism and 10% for lipid peroxidation. The relatively high degree of variation is likely due to the cytochrome P-450 content of liver microsomes from every individual animal.



**Figure 22**      **Effect of pH on OTA metabolism (A) and lipid peroxidation (B) by liver microsomes from PCN-treated rats.**

Incubations were carried out at 37°C in 0.1 M potassium phosphate (pH 5.5 - 8.5) and contained in a total volume of 1 mL: microsomal protein (2 mg), 125 nmol OTA and a NADPH-regenerating system (see Methods section 2.15.1). At the end of 30 min, 0.5 mL from each incubation was withdrawn for HPLC analysis of OTA metabolites and the remaining 0.5 mL was used for measurement of MDA levels. Details are described in the Methods sections 2.15.3 and 2.14.2. Results are means of duplicate determinations from each of two separate experiments. The difference between the two means at all time points was no more than 40% for metabolism and 16% for lipid peroxidation. The high degree of variation is likely due to the cytochrome P-450 content of liver microsomes from every individual animal, also due to the fact that lipid peroxidation is a chain reaction and the rates of propagation are known to vary greatly from an experiment to another.



**Table 15. Effect of active oxygen scavengers and antioxidant on NADPH-dependent OTA metabolism and lipid peroxidation.**

Addition	4(R)-4-OH-OTA	4(S)-4-OH-OTA	MDA
	<i>nmol/mg protein</i>		
	Exp.1/ Exp.2	Exp.1/ Exp.2	Exp.1/ Exp.2
<b>INH microsomes</b>			
None	0.012 / 0.016	0.094 / 0.106	26.4 / 26.9
SOD (35 units)	0.012 / 0.018	0.078 / 0.092	26.2 / 27.5
Catalase (800 units)	0.012 / 0.018	0.079 / 0.093	24.3 / 26.5
Mannitol (11 mM)	0.012 / 0.016	0.060 / 0.082	22.7 / 22.9
<b>PCN microsomes</b>			
None	0.63 / 0.71	0.19 / 0.21	23.1 / 24.7
SOD (35 units)	0.66 / 0.80	0.24 / 0.30	19.0 / 23.4
Catalase (800 units)	0.78 / 0.94	0.28 / 0.34	20.5 / 20.8
Mannitol (11 mM)	0.69 / 0.84	0.20 / 0.28	19.1 / 19.8

Incubations were carried out for 30 min as described in the Methods section 2.15.1. Results are means of duplicate determinations from each of two separate experiments.

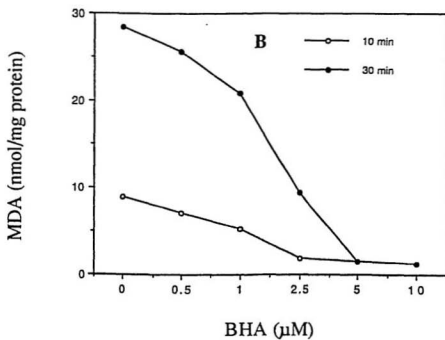
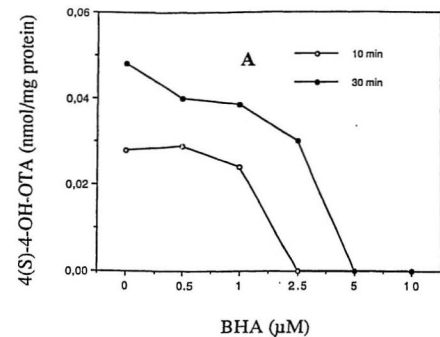
**Table 16. Effect of antioxidants and iron chelators on OTA metabolism, lipid peroxidation and cytochrome P-450 content.**

Addition	4(R)-4-OH-OTA	4(S)-4-OH-OTA	MDA	CytochromeP-450*
	<i>nmol/mg protein</i>			
	Exp.1/ Exp.2	Exp.1 / Exp.2	Exp.1 / Exp.2	
<b>INH microsomes</b>				
None	0.012 / 0.016	0.094 / 0.106	24.5 / 25.1	0.49 (40 % Loss)
Desferal (50 μM)	0.025 / 0.031	0.005 / 0.008	1.15 / 1.31	0.83 ( 0 % Loss)
BPS (100 μM)	0.019 / 0.025	0.007 / 0.009	0.81 / 0.89	
BHA (10 μM)	0.011 / 0.015	< 0.001	1.93 / 2.05	
DPPD (10 μM)	0.005 / 0.011	0.010 / 0.012	0.68 / 0.72	
<b>PCN microsomes</b>				
None	0.63 / 0.71	0.19 / 0.21	21.9 / 23.3	0.33 (75 % Loss)
Desferal (50 μM)	1.32 / 1.54	0.24 / 0.28	1.20 / 1.64	0.82 (37 % Loss)
BPS (100 μM)	1.74 / 1.86	0.12 / 0.14	1.00 / 1.10	
BHA (10 μM)	0.65 / 0.69	0.09 / 0.11	1.90 / 2.4	
DPPD (10 μM)	0.74 / 0.78	0.09 / 0.11	0.81 / 0.89	

Incubations were carried out for 30 min as described in the Methods section 2.15.1. Results are means of duplicate determinations from two separate experiments. \* The original cytochrome P-450 contents were 0.83 and 1.32 nmol/mg protein for microsomes isolated from rats treated with INH and PCN respectively.

**Figure 23**      **Effect of varying BHA concentrations on (A) OTA metabolism and (B) lipid peroxidation by liver microsomes from INH-treated rats.**

Incubations were carried out at 37°C in 0.1 M potassium phosphate (pH 7.4) for 10 and 30 min in the presence of 0 - 10 µM BHA, and contained in a total volume of 1 mL: microsomal protein (2 mg), 125 nmol OTA and a NADPH-regenerating system (see Methods section 2.15.1). At the end of 30 min, 0.5 mL from each incubation was withdrawn for HPLC analysis of OTA metabolites and the remaining 0.5 mL was used for measurement of MDA levels. Details are described in the Methods sections 2.15.3 and 2.14.2. Results are means of duplicate determinations from each of two separate experiments. The difference between the two means at all time points was no more than 33% for metabolism and 30% for lipid peroxidation. The relatively high degree of variation is likely due to the cytochrome P-450 content of liver microsomes from every individual animal, also due to the fact that lipid peroxidation is a chain reaction and the rates of propagation are known to vary greatly from an experiment to another.



**Table 17. Effect of BHA and mannitol on ascorbate-dependent OTA metabolism and lipid peroxidation.**

Addition	4(R)-4-OH-OTA	4(S)-4-OH-OTA	MDA
	<i>nmol/mg protein</i>		
	Exp.1 / Exp.2	Exp.1 / Exp.2	Exp.1 / Exp.2
<b>INH microsomes</b>			
None	0.018 / 0.028	0.125 / 0.155	33.3 / 35.7
Mannitol (11 mM)	0.014 / 0.020	0.150 / 0.184	29.5 / 34.1
BHA (10 $\mu$ M)	0.014 / 0.021	0.012 / 0.028	2.50 / 2.96
<b>PCN microsomes</b>			
None	0.010 / 0.050	0.129 / 0.137	28.2 / 33.4
Mannitol (11 mM)	0.021 / 0.037	0.122 / 0.138	26.1 / 29.0
BHA (10 $\mu$ M)	0.020 / 0.026	0.008 / 0.016	0.77 / 0.81

Incubations were carried out for 30 min as described in the Methods section 2.15.1 with the exception that ascorbate/ $\text{Fe}^{2+}$  (1 mM/5  $\mu$ M, final concentrations) were used instead of NADPH-regenerating system. Results are mean of duplicate determinations from two separate experiments.

**Table 18.** Ability of various hemoproteins/hydroperoxides to metabolize OTA.

System	4(R)-4-OH-OTA	4(S)-4-OH-OTA
	<i>nmol / nmol heme</i>	
PCN microsomes/CHP	0.530 / 0.536	0.017 / 0.039
PCN microsomes/H <sub>2</sub> O <sub>2</sub> <sup>a</sup>	0.014 / 0.016	0.012 / 0.015
PCN microsomes/LAHP	< 0.001 <sup>b</sup>	< 0.001
Hb/CHP	< 0.001	< 0.001
Hb/H <sub>2</sub> O <sub>2</sub>	< 0.001	< 0.001
Hb/LAHP	< 0.001	< 0.001
Hematin/CHP	0.005 / 0.008	0.007 / 0.015
Hematin/H <sub>2</sub> O <sub>2</sub>	0.006 / 0.009	0.002 / 0.004
Hematin/LAHP	< 0.001	< 0.001

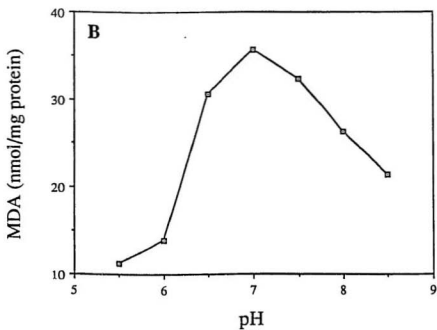
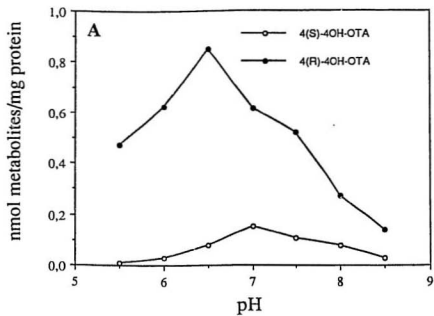
Incubations were carried out for 30 min as described in the Methods section 2.15.1. For the different reagents, the following concentrations were used (in 0.5 mL reaction volume): PCN microsomes (1 mg protein), Hb (10 nmol), Hematin (2.5 nmol), CHP (0.5 mM), H<sub>2</sub>O<sub>2</sub> (5 mM), LAHP (65  $\mu$ M). Results are means of duplicate incubations from each of two separate experiments.

<sup>a</sup> 150  $\mu$ M hydroxylamine included to inhibit contaminating catalase.

<sup>b</sup> detection limit.

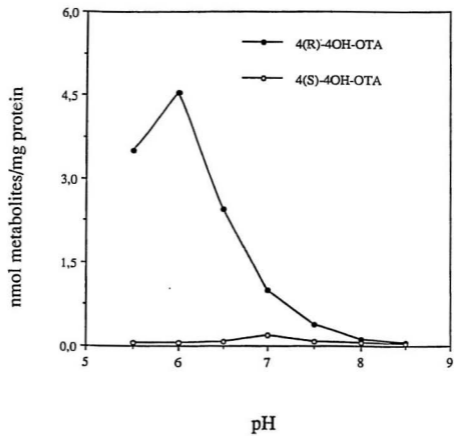
**Figure 24**      **Effect of pH on OTA metabolism (A), and lipid peroxidation (B) by liver microsomes from PB treated rats.**

Incubations were carried out in duplicate at 37°C for 30 min in 0.1 M potassium phosphate buffer (pH 5.5 - 8.5), and contained in a total volume of 1 mL: microsomal protein (2 mg), 125 nmol OTA and a NADPH-regenerating system (see Methods section 2.15.1). At the end of 30 min, 0.5 mL from each incubation was withdrawn for HPLC analysis of OTA metabolites, and the remaining 0.5 mL was used for measurement of MDA levels. Details for analysis of metabolites are described in the Methods section 2.15.3. Results are means of duplicate determinations from each of two separate experiments. The difference between the two means at all time points was no more than 17% for metabolism and 16% for lipid peroxidation. The relatively high degree of variation is likely due to the cytochrome P-450 content of liver microsomes from every individual animal, also due to the fact that lipid peroxidation is a chain reaction and the rates of propagation are known to vary greatly from an experiment to another.



**Figure 25**      **Effect of pH on OTA metabolism by liver microsomes from 3MC treated rats.**

Incubations (0.5 mL volume) were carried out in duplicate at 37°C for 30 min in 0.1 M potassium phosphate buffer (pH 5.5 - 8.5), and contained per mL: microsomal protein (2 mg), 125 nmol OTA and a NADPH-regenerating system (see Methods section 2.15.1). Details for analysis of metabolites are described in the Methods section 2.15.3. Results are means of duplicate determinations from each of two separate experiments. The difference between the two means at all time points was no more than 37%. The high degree of variation is likely due to the cytochrome P-450 content of liver microsomes from every individual animal.



**Table 19.** Comparison of the ability of microsomes isolated from rats treated with 3MC, ISF and PB to metabolize OTA at pH 6.0 and 7.4.

Group	pH	4(S)-4-OH-OTA (nmol/mg protein)	4(R)-4-OH-OTA
3MC	6.0	0.057 ± 0.030	7.98 ± 3.10
3MC	7.4	0.109 ± 0.060	1.03 ± 0.45
ISF	6.0	0.033 ± 0.010	5.58 ± 4.02
ISF	7.4	0.111 ± 0.045	1.12 ± 0.66
PB	6.0	0.042 ± 0.033	0.63 ± 0.21
PB	7.4	0.112 ± 0.053	0.34 ± 0.23

Details for conditions are described in the Methods section 2.15.1. Incubations were carried out in duplicate. Results are means ± S.D. from three different animals.

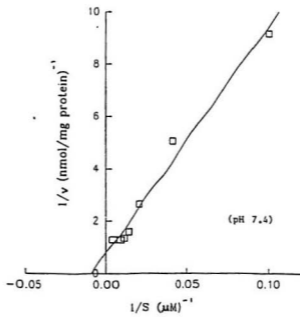
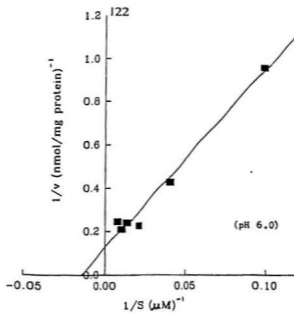
**Table 20.** The effect of pH 6.0 and 7.4 on the ability of microsomes isolated from rats treated with 3MC, ISF and PB to undergo lipid peroxidation and cytochrome P-450 destruction.

Treatment	pH	MDA (nmol/mg protein)	Cyt. P-450 Loss (%)
3MC	6.0	18.9 ± 8.3	31.6 ± 7.0
3MC	7.4	26.8 ± 7.2	57.3 ± 3.5
ISF	6.0	8.0 ± 6.9	14.7 ± 20.5
ISF	7.4	17.1 ± 5.6	42.6 ± 20.8
PB	6.0	11.8 ± 8.8	51.1 ± 23.9
PB	7.4	20.2 ± 10.2	59.2 ± 25.4

Incubations were carried out at 37°C for 30 min in 0.1 M potassium phosphate buffer (pH as indicated in the Table), and contained per mL: 2 mg microsomal protein, 125 nmol OTA and a NADPH-regenerating system (see Methods section 2.15.1). The reaction volume was 3 mL. At the end of 30 min, 0.5 mL was withdrawn for measurement of lipid peroxidation and the remaining volume was diluted 1:1 using the same incubation buffer (pH 7.4) to give 1 mg microsomal protein/mL for measurement of cytochrome P-450 (see Methods section 2.9). Results are mean ± S.D. from three different animals.

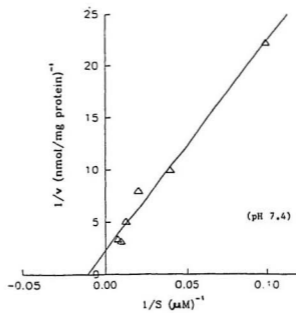
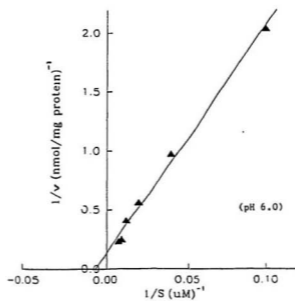
**Figure 26**    **Effect of pH on the  $K_m$  and  $V_{max}$  of OTA hydroxylation by liver microsomes from 3MC treated rats.**

Incubations were carried out in duplicate at 37°C for 30 min in 0.1 M potassium phosphate buffer (pH 6.0 or pH 7.4), and contained per mL: microsomal protein (2 mg), OTA (10, 25, 50, 75, 125 and 250 nmol) and a NADPH regenerating system (see Methods section 2.15.1). The amounts of hydroxylated OTA in nmol/mg protein (the initial velocities) were measured at 30 min (the reaction was linear up to 60 min). Only the major metabolite 4(R)-4-OH-OTA was considered in this case. Details for analysis of metabolites are described in the Methods section 2.15.3. Results are means of duplicate determinations from each of two separate experiments. The difference between the two means at all time points was no more than 30%.



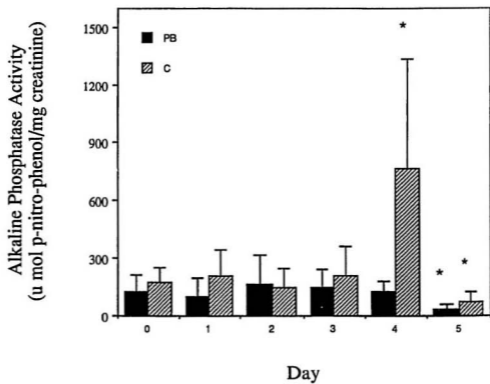
**Figure 27**      **Effect of pH on the  $K_m$  and  $V_{max}$  of OTA hydroxylation by liver microsomes from ISF treated rats.**

Incubations were carried out in duplicate at 37°C for 30 min in 0.1 M potassium phosphate buffer (pH 6.0 or pH 7.4), and contained per mL: microsomal protein (2 mg), OTA (10, 25, 50, 75, 125 and 250 nmol) and a NADPH-regenerating system (see Methods section 2.15.1). The amounts of hydroxylated OTA in nmol/mg protein (the initial velocities) were measured at 30 min (the reaction was linear up to 60 min). Only the major metabolite 4(R)-4-OH-OTA was considered in this case. Details for analysis of metabolites are described in the Methods section 2.15.3. Results are means of duplicate determinations from each of two separate experiments. The difference between the two means at all time points was no more than 30%.



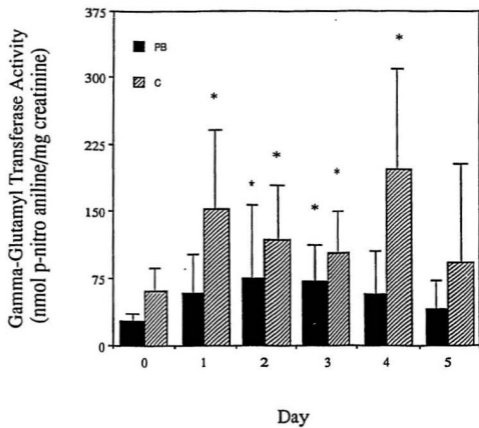
**Figure 28      Alkaline phosphatase activity in urines of control and PB-treated rats following OTA administration.**

Both groups of rats were given orally OTA (0.5 mg/kg in 50 mM NaHCO<sub>3</sub> daily for 5 days). Details of pretreatments, treatments and enzyme measurements are given in the Methods sections 2.5.1, 2.6.1 and 2.18.1. Results are means  $\pm$  S.D. (n = 8 animals per treatment group). \*, means significantly different from day 0 ( $p < 0.05$ , Mann-Whitney test).



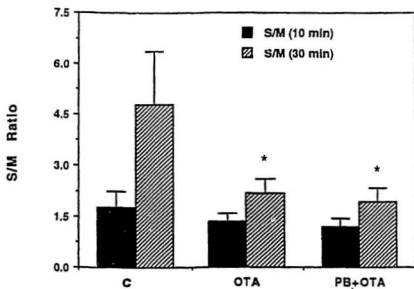
**Figure 29**      **$\gamma$ -Glutamyl transferase activity in urines of control and PB-treated rats following OTA administration.**

Both groups of rats were given orally OTA (0.5 mg/kg in 50 mM NaHCO<sub>3</sub> daily for 5 days). Details of pretreatments, treatments and enzyme measurements are given in the Methods sections 2.5.1, 2.6.1 and 2.18.1. Results are means  $\pm$  S.D. (n = 8 animals per treatment group). \*, means significantly different from day 0 (p < 0.05, Mann-Whitney test).



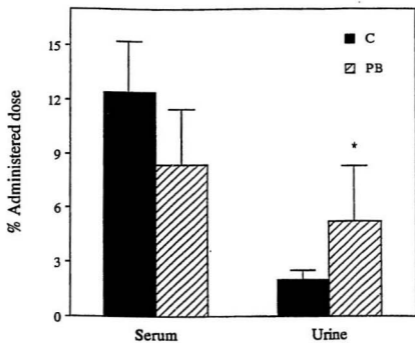
**Figure 30**     **[<sup>14</sup>C]PAH transport in renal cortex slices from control (C), OTA treated (OTA) and PB-pretreated treated with OTA (PB + OTA) rats.**

Rats were given orally OTA (0.5 mg/kg in 50 mM NaHCO<sub>3</sub> daily for 5 days. Details of pretreatments, treatments and PAH transport are given in the Methods sections 2.5.1, 2.6.1 and 2.17. Results are expressed as S/M (slice/medium) ratio at 10 and 30 min and represent means  $\pm$  S.D. (n = 4 animals per treatment group). \*, means significantly different from control (p < 0.05, Mann-Whitney test).



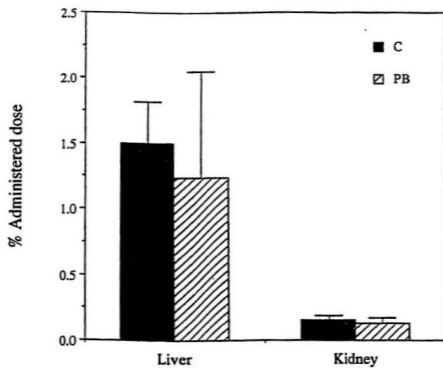
**Figure 31** [3H]OTA levels in serum and urine of control and PB-treated rats 6 hours after one oral treatment with [3H]OTA (288 µg/kg in 50 mM NaHCO<sub>3</sub>).

Details for treatments, sample processing and radioactivity counting are given in the Methods sections 2.6.2 and 2.18.2. Results are means  $\pm$  S.D. (n = 4 animals per treatment group). \*, means significantly different from control (p < 0.05, Mann-Whitney test).



**Figure 32**     **[<sup>3</sup>H]OTA levels in liver and kidney of control and PB-treated rats 6 hours after one oral treatment with [<sup>3</sup>H]OTA (288 µg/kg in 50 mM NaHCO<sub>3</sub>).**

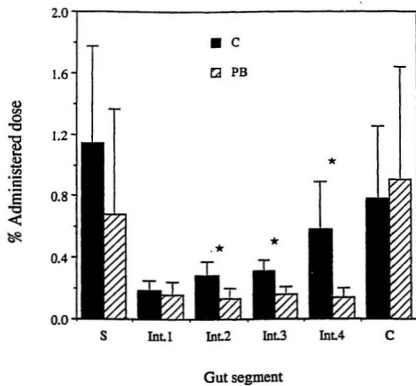
Details for treatments, sample processing and radioactivity counting are given in the Methods sections 2.6.2 and 2.18.2. Results are means  $\pm$  S.D. (n = 4 animals per treatment group).



**Figure 33**    **[<sup>3</sup>H]OTA levels in the gut of control and PB-treated rats 6 hours after one oral treatment with [<sup>3</sup>H]OTA (288 µg/kg in 50 mM NaHCO<sub>3</sub>).**

Details for treatments, sample precessing and radioactivity counting are given in the Methods sections 2.6.2 and 2.18.2. Results are means  $\pm$  S.D. (n = 4 animals per treatment group).

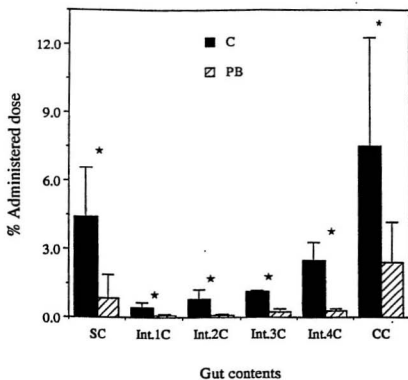
\*, means C and PB are significantly different from each other (p < 0.05, Mann-Whitney test).



**Figure 34**     **[<sup>3</sup>H]OTA levels in the gut contents of control and PB-treated rats 6 hours after one oral treatment with [<sup>3</sup>H]OTA (288 µg/kg in 50 mM NaHCO<sub>3</sub>).**

Details for treatments, sample precessing and radioactivity counting are given in the Methods sections 2.6.2 and 2.18.2. Results are means  $\pm$  S.D. (n = 4 animals per treatment group).

\*, means C and PB are significantly different from each other (p < 0.05, Mann-Whitney test).



## CHAPTER 4

### DISCUSSION

#### 4.1 Involvement of cytochrome P-450 in the stimulation of ochratoxin A-induced lipid peroxidation.

Recently, using a reconstituted microsomal lipid peroxidation system consisting of microsomal phospholipid, the flavoprotein NADPH cytochrome P-450 reductase (Fp), EDTA and  $\text{Fe}^{3+}$ , we demonstrated that OTA induced lipid peroxidation by chelating  $\text{Fe}^{3+}$  and that the resulting OTA- $\text{Fe}^{3+}$  chelate was readily reducible by the flavoprotein NADPH cytochrome P-450 reductase to the OTA- $\text{Fe}^{2+}$  complex. The latter, in the presence of oxygen, provided the active species that initiated lipid peroxidation (Omar et al., 1990). In our reconstituted system, the addition of 25  $\mu\text{M}$  EDTA gave a maximum stimulation (4.0-fold at 20 min) in lipid peroxidation. Pederson and Aust (1972) first developed a reconstituted lipid peroxidation system consisting of phospholipid vesicles, Fp, NADPH, and iron chelates. In their system, an EDTA- $\text{Fe}^{3+}$  chelate was required in addition to the standard ADP- $\text{Fe}^{3+}$  chelate. Microsomal lipid peroxidation was first described by Hochstein and Ernster (1963), who demonstrated the enzymatic nature of the process and a requirement for NADPH and an ADP- $\text{Fe}^{3+}$  chelate (Hochstein et al., 1964). The observation that only ADP- $\text{Fe}^{3+}$  is necessary to promote lipid peroxidation in microsomes, while both EDTA- $\text{Fe}^{3+}$  and ADP- $\text{Fe}^{3+}$  are needed to promote it in a reconstituted system, suggests that there may be microsomal component(s) that directly reduce ADP- $\text{Fe}^{3+}$  for which EDTA- $\text{Fe}^{3+}$  can substitute in the reconstituted system (Morehouse and Aust, 1988). Cytochrome P-450, being the last electron acceptor among the catalytic components of the NADPH-dependent electron transport chain, is a likely candidate. Ekstrom and Ingelman-Sundberg (1984) were the first to show that incorporation of one of several purified rabbit liver cytochrome P-450 isozymes into phospholipid vesicles containing Fp increased lipid

peroxidation. Later, Morehouse and Aust (1988) demonstrated that purified rat liver cytochrome P-450b could effectively replace EDTA-Fe<sup>3+</sup> in a reconstituted lipid peroxidation system consisting of phospholipid vesicles, EDTA-Fe<sup>3+</sup>, ADP-Fe<sup>3+</sup>, Fp, and NADPH. Our results show that cytochrome P-450 IIB1 is also able to enhance lipid peroxidation in a reconstituted system where OTA-Fe<sup>3+</sup> is the chelated form of Fe<sup>3+</sup>. That cytochrome P-450 plays a functional role, and is not merely a convenient source of heme-iron, is evident from the observation that heat-denatured cytochrome P-450, hematin, or a variety of hemoproteins were either ineffective or only marginally effective in stimulating lipid peroxidation (Table 9). Even cytochrome c, a hemoprotein readily reducible by NADPH-cytochrome P-450 reductase, only weakly stimulated lipid peroxidation. Carbon monoxide (CO) did not inhibit lipid peroxidation. We are unaware of any work in the literature showing the effects of CO on lipid peroxidation in a reconstituted mixed-function oxidase system. However, Ernster and Nordenbrand (1982) showed that CO did not inhibit the NADPH-dependent ADP/Fe<sup>3+</sup>-stimulated lipid peroxidation of liver microsomes. The reason for this lack of inhibition by CO is not clear.

The role of cytochrome P-450 in OTA-stimulated NADPH-dependent microsomal lipid peroxidation is also implicated from the data showing that microsomes isolated from Co-heme treated rats underwent less lipid peroxidation than those from control rats (Figure 10). Co-heme treatment has been shown to reduce both cytochrome P-450 and Fp levels, and thus the reduced ability of Co-heme microsomes to peroxidize might be due to decreased cytochrome P-450 and/or Fp levels. It is well known that the activity of Fp greatly exceeds the overall mixed-function oxidase activity of the various microsomal cytochrome P-450-dependent mixed-function oxidases (Spaethe and Jollow, 1989). Thus, it seems unlikely that the decrease in Fp activity observed (Table 10) would play a significant role in the depression of lipid peroxidation. In support of this assertion, we

have observed that fortification\* of Co-heme microsomes with Fp (to levels 78% of that in control microsomes; Table 10) only partially increased the extent of lipid peroxidation to 45% of that of control microsomes (Figure 10). This suggests that lower cytochrome P-450 levels rather than lower Fp levels were the main cause of decreased lipid peroxidation in Co-heme microsomes. However, it is possible that externally added Fp is not well integrated into the microsomes and thus may not be able to function as effectively as the Fp in control microsomes.

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\* Co-heme microsomes (2 mg protein) were preincubated with X4 Fp (640 nmol) which was subsequently removed by centrifugation, leaving only Fp actually incorporated into the membrane or adhering to it.

#### 4.2 Role of cytochrome P-450 in OTA metabolism.

Stormer and Pederson (1980) were the first to show that OTA was metabolized to a hydroxylated product (4-OH-OTA) by rat liver microsomes. Later, they (Stormer et al., 1981) showed the formation of both 4(R)- and 4(S)-4-OH-OTA by human liver microsomes as well as liver microsomes from control rats and pigs. A subsequent study by Stormer et al. (1983) examined the metabolism of OTA by liver microsomes from control and PB treated rabbits, and Ueno (1985b) also used 3MC and PB as specific inducers of cytochrome P-450 enzymes in a study of OTA metabolism. Recently, Oster et al. (1991) showed that isolated cytochrome P-450 fractions (not identified) were able to metabolize OTA. Hietanen et al. (1991) showed that a cytochrome P-450 isozyme similar to that induced by 3MC is responsible for OTA metabolism. None of the above studies were of sufficient detail to examine under the same experimental conditions the effect of several major cytochrome P-450 inducers on OTA metabolism. We, therefore, carried out a detailed study of OTA metabolism by liver microsomes from rats treated with inducers of the major cytochrome P-450 isoforms.

We examined the OTA metabolizing ability of liver microsomes from rats pretreated with the cytochrome P-450 inducers phenobarbital (PB), 3-methylcholanthrene (3MC), clofibrate (CLF), isosafrole (ISF), pregnenolone-16 $\alpha$ -carbonitrile (PCN) and isoniazid (INH). Proper controls for the vehicles in which inducers were given were done in addition to a no treatment control group. The microsomal cytochrome P-450 contents of livers from the different treatment groups were in good agreement with reported values (Guengerich et al., 1982b; Hietanen et al., 1986; Graves et al., 1987). For the formation of the major metabolite 4(R)-4-OH-OTA, liver microsomes from rats treated with PB, PCN, 3MC, ISF and CLF gave 67, 55, 39, 34 and 10 fold increase respectively as compared to control microsomes from untreated rats. For 4(S)-4-OH-OTA (minor metabolite) formation, liver microsomes from rats treated with PCN, PB, 3MC, CLF and

ISF gave a 14, 18, 10, 10 and 8 fold increase compared to control microsomes from untreated rats (Figure 12). Microsomes from INH-treated rats did not give any increase in the formation of the 4(R) isomer, but formation of the 4(S) isomer was increased about 7 fold as compared to microsomes from control rats (no treatment) (Figure 12). The large increases in OTA metabolism by microsomes from 3MC- and PCN-treated animals were not due to the vehicles (corn oil and Tween 80, respectively) in which they were administered. Both corn oil and Tween 80 did, however, increase the 4(R) and the 4(S) isomers formation over untreated controls by 2.9 / 2.4-fold and 4.3 / 1.6-fold respectively. Sucrose syrup, the vehicle in which clofibrate was administered, did not increase OTA metabolism (Figure 14).

OTA hydroxylation activity as well as its inducibility appears to vary with the strain of rat used. Hietanen et al. (1986) observed OTA 4-hydroxylation rates of about 2.5 and about 0.7 pmol/min/mg protein with liver microsomes from female Lewis and DA rats respectively. This activity was inducible, about 2.3-fold and about 1.3-fold in Lewis rats, and about 12-fold and about 2-fold in DA rats after 3MC and PB pretreatments, respectively. On the other hand, Stormer and Pedersen (1980) found a very high rate of OTA 4-hydroxylation (about 90 pmol/min/mg protein) in liver microsomes from male Wistar rats, and this activity was inducible 3.4-fold on PB pretreatment. In all the above studies, it was not indicated whether just 4(R)-4-OH-OTA (the major metabolite) or both epimers were measured. By comparison, we observed OTA 4-hydroxylation rate of about 1 pmol/min/mg protein [Table 14; combined 4(R) and 4(S) metabolites] which is more in line with the values observed by Hietanen et al. (1986). This activity was inducible about 3.8 fold on INH pretreatment but 33 fold on PCN pretreatment (Table 14, values given in text are for combined metabolites). However, the inability of INH pretreatment to induce 4(R)-4-OH-OTA formation suggests that cytochrome P-450 IIE1, the major isoform induced by INH (Ryan et al., 1985) is unable to catalyze its formation. The effect of INH

and PCN pretreatments on OTA metabolism has not previously been examined.

Cytochrome P-450 enzymes range from being very specific to non-specific towards substrates. A few substrates are known to react specifically with certain isoforms of cytochrome P-450 e.g. 7-pentoxoresorufin and 7-ethoxoresorufin are known to be specific substrates for cytochromes P-450 IIB1 (PB inducible) and IA1 (3MC inducible) respectively (Lubet et al., 1985; Burke and Mayer, 1983). We used these two substrates to confirm the induction of the desired cytochrome P-450 isoforms by the inducers. Thus, microsomes from PB treated rats showed a 30-fold increase in 7-pentoxoresorufin metabolism over control microsomes while microsomes from 3MC treated rats showed only a 4.5-fold increase. In contrast, microsomes from 3MC treated animals were 82 / 88-fold more effective than control microsomes in metabolizing 7-ethoxoresorufin while microsomes from PB treated animals were only 10.5 / 20-fold more efficient (Table 12). These results confirm that PB and 3MC treatments preferentially induced cytochromes P-450 IIB1 and IA1/IA2 respectively.

Cytochrome P-450 inhibitors are also useful tools in characterizing the involvement of specific cytochrome P-450 isoforms in metabolic processes. Some inhibitors react specifically with certain isozymes. For example,  $\kappa$ -naphthoflavone (Wiebel et al., 1971) and metyrapone (Jonen et al., 1974) are two inhibitors of cytochromes P-450 IA1/IA2 and IIB1 respectively. Inhibition by specific cytochrome P-450 inhibitors of a certain reaction that proceeds via a specific cytochrome P-450 isozyme would mean the direct involvement of that particular isozyme in the reaction.  $\kappa$ -Naphthoflavone and metyrapone were used in our studies.  $\kappa$ -Naphthoflavone selectively inhibited (89 / 95%) formation of 4(R)-4-OH-OTA by microsomes from 3MC and ISF treated rats, but it inhibited its formation by microsomes from PB treated rats by only 18 / 41% (Figure 15). On the other hand, metyrapone inhibited (94 / 96%) formation of the 4(R) isomer by microsomes from PB treated rats, but only inhibited its formation in microsomes from rats treated with 3MC and

ISF (40 / 11% and 40 / 16%) (Figure 15). The effect of the two inhibitors on 4(S)-4OH-OTA formation was less selective (Figure 16). Liver microsomes from rats pretreated with Cobalt protoporphyrin IX (total cytochrome P-450 depleted by >80%) failed to metabolize OTA indicating the necessity for cytochrome P-450 (data not shown).

Use of monoclonal antibodies (MAbs) against specific isoforms of cytochrome P-450 also provides additional evidence for the involvement of these isoforms in a reaction. Clones 1-7-1 (against cytochrome P-450 1A1/1A2) and 2-66-3 (against cytochrome P-450 IIB1) (Nakajima et al, 1990; Gelboin, personal communication) were used in these studies. The highest inhibition of OTA hydroxylation was given by only a two fold ratio of MAb protein/ microsomal protein using MAb from clone 1-7-1 with microsomes from 3MC treated rats (Figure 17a) and using MAb from clone 2-66-3 with microsomes from PB treated rats (Figure 18a). In both cases, a dose dependent inhibition was observed. Small non-specific reaction (inhibition of up to 22%) was observed with the two microsomes when rabbit IgG was used instead of the MAbs (Figures 17b and 18b). The specificity of MAbs differ with substrates. A given MAb may inhibit very specifically a reaction of a certain substrate, but show less or even no specificity towards another substrate; this can be seen from the results obtained by Gelboin et al. (1988). From my results, MAb from clone 2-66-3 inhibited more strongly (58% inhibition) OTA metabolism to 4(R)-4OH-OTA by microsomes from PB treated rats although it cross reacted substantially (39% inhibition) with microsomes from 3MC treated rats. On the other hand, MAb from clone 1-7-1 was more specific (64% inhibition) for microsomes from 3MC treated rats although it cross reacted weakly (23% inhibition) with microsomes from PB treated rats (Table 13).

#### 4.3 Possible mechanism of the formation of 4(S)-4-OH-OTA.

Microsomes from INH treated rats [which mainly form the 4(S) isomer] were chosen to investigate the mechanism of the formation of the 4(S) isomer. Microsomes from PCN treated rats [that give a high yield of the 4(R) isomer] were chosen to study its formation. PCN pretreatment increased the formation of 4(R)-4-OH-OTA more than 50-fold that in controls. In contrast, INH pretreatment did not substantially increase formation of the 4(R) isomer, but increased levels of the 4(S) isomer 7-fold over control (Table 14).

The formation of 4(R)-4-OH-OTA and 4(S)-4-OH-OTA showed clear differences with respect to pH optima, effect of iron chelator and antioxidants. The pH optima for the formation of the 4(R) isomer by microsomes from rats treated with INH and PCN were pH 6.0 and 6.5 respectively (Figures 21 and 22). At pH 7.5 there was a 60% decrease in the formation of the 4(R) isomer by microsomes from rats treated with PCN (Figure 22). A pH optimum of 6.5 for the formation of the 4(R) isomer was also observed for microsomes from rats treated with PB (Figure 24a), and of 6.0 for microsomes from rats treated with 3MC (Figure 25). Microsomes from ISF treated rats also showed a pH optimum of 6.0 with respect to the 4(R) isomer formation (data not shown). The pH optimum for cytochrome P-450-dependent oxidation of most substrates is around 7.5 so it is curious that the formation of the 4(R) isomer exhibits a low pH optimum. The phenolic hydroxyl of OTA has a pKa of 7.1 (Chu, 1971) so it is tempting to speculate that OTA with an unionized hydroxyl group binds preferentially to cytochrome P-450 thus possibly yielding more 4(R)-4-OH-OTA below this pH. The pH optimum of 6.5 for the formation of the 4(R) isomer differs from the value of 7.5 observed by Stormer and Pedersen (1980) with liver microsomes from phenobarbital treated Wistar rats, but agrees with the optimum pH obtained by Ueno (1985b) with liver microsomes from rats (strain not mentioned).

In contrast to 4(R)-4-OH-OTA, the pH optimum of 7.0 observed for the formation of 4(S)-4-OH-OTA with microsomes from rats treated with INH and PB also coincided with the pH optimum for MDA formation by these microsomes (Figures 21 and 24) suggesting that the two processes may be linked. Furthermore, an intact mixed-function oxidase system is required for the formation of 4(R)-4-OH-OTA, but not 4(S)-4-OH-OTA or lipid peroxidation. Replacement of NADPH by ascorbate results in the direct chemical reduction of the OTA-Fe<sup>3+</sup> complex giving rise to non-enzymatic lipid peroxidation. The ability of ascorbate to stimulate microsomal lipid peroxidation is well known (Ernster and Nordbrand, 1982). In the presence of ascorbate, only the 4(S) isomer was formed, suggesting the requirement for an intact mixed function oxidase system for the formation of the 4(R) isomer. The need for cytochrome P-450 in the formation of the 4(R) isomer is also substantiated by the observation that no OTA metabolites were detected when liver microsomes from Co-heme pre-treated rats were used. A correlation between lipid peroxidation and 4(S)-4-OH-OTA [but not 4(R)-4-OH-OTA] formation is further strengthened by the observation that both processes are remarkably sensitive to inhibition by the iron chelators Desferal and BPS and the antioxidants BHA and DPPD (Table 16). In fact, in the presence of varying concentrations of BHA (Figure 23), both lipid peroxidation and 4(S)-4-OH-OTA formation are inhibited in parallel suggesting that either lipid peroxides are responsible for 4(S)-4-OH-OTA formation or that a common species mediates the formation of both.

The inability of microsomes to produce 4(S)-4-OH-OTA in the presence of linoleic acid hydroperoxide (LAHP) suggests that lipid peroxides may not be involved in its formation (Table 18). In contrast, microsomes in the presence of CHP gave rise to substantial amounts of both 4(R)-4-OH-OTA and 4(S)-4-OH-OTA. Cytochrome P-450 is known to function as a peroxidase and the ability of CHP to catalyze the cytochrome P-450-dependent metabolism of xenobiotics has been well documented (Rahimtula and

O'Brien, 1974; Rahimtula and O'Brien, 1975). A variety of hemoproteins including HRP (Corbett and Corbett, 1987; O'Brien, 1988) and Hb (Catalano and Ortiz de Montellano, 1987) are also known to oxidize xenobiotics. However, these hemoproteins as well as hematin were unable to catalyze OTA oxidation in the presence of a variety of hydroperoxides suggesting that cytochrome P-450 may be essential (table 18).

The lack of substantial inhibition by SOD or catalase suggests that the superoxide anion or  $H_2O_2$  are not directly involved in the formation of 4(S)-4-OH-OTA or MDA by microsomes from rats treated with INH. Both SOD and catalase increased OTA metabolism by microsomes from rats treated with PCN, possibly by protecting cytochrome P-450 from radical-induced damage. Previously, we have shown (Hasinoff et al., 1990) that the presence of OTA stimulated superoxide and hydroxyl radical formation by liver microsomes. Mannitol, a known scavenger of hydroxyl radicals, did inhibit 4(S)-4-OH-OTA by about 45% with microsomes from rats treated with INH indicating that hydroxyl radicals may play a role. However, mannitol did not inhibit either the NADPH-dependent (by microsomes from PCN treated rats) or the ascorbate-dependent (by microsomes from INH and PCN treated rats) metabolism of OTA, or lipid peroxidation, indicating that hydroxyl radicals are probably not involved in either of these processes. Gutteridge (1982) showed that hydroxyl radicals do not play a significant role in NADPH-dependent microsomal lipid peroxidation.

The induction of lipid peroxidation (Omar et al., 1990) and active oxygen species (Hasinoff et al., 1990) by OTA can lead to the destruction of cytochrome P-450 which is responsible for 4(R)-4-OH-OTA formation. Desferal, by chelating iron, would prevent the formation of the active oxygen species responsible for lipid peroxidation and thus protect the cytochrome P-450 from destruction. This was indeed observed (Table 16). The fact that Desferal inhibited the formation of 4(S)-4-OH-OTA [but not that of 4(R)-4-OH-OTA] and MDA suggests that free iron is essential in their formation. This, together with

observations that cytochrome P-450 is essential and that free (unbound) reactive oxygen species are not involved, suggests that an active  $\text{Fe}^{2+}$ -oxygen complex may be responsible for both 4(S)-4-OH-OTA formation and lipid peroxidation (see Figure 35 at the end of this chapter). The precise mechanism by which such a hydroxylation occurs is presently not known. We have already demonstrated (Omar et al., 1990) that OTA induces lipid peroxidation by chelating  $\text{Fe}^{3+}$  and that the resulting OTA- $\text{Fe}^{3+}$  chelate is more readily reducible by the flavoprotein NADPH-cytochrome P-450 reductase to the OTA- $\text{Fe}^{2+}$  complex which, in the presence of oxygen, provides the active species that initiates lipid peroxidation. Cytochrome P-450 is required presumably to facilitate the reduction of the  $\text{Fe}^{3+}$  complex to the  $\text{Fe}^{2+}$  complex. The exact nature of the initiating species is presently not known and different investigators have implicated the perferryl ion (Tien et al., 1981; Ursini et al., 1989), the ferryl ion (Koppenol, 1985) and the  $\text{Fe}^{2+}\text{-O}_2\text{-Fe}^{3+}$  complex (Bucher et al., 1983).

Formation of the 4(R) and 4(S) isomers showed clear difference with respect to pH optima. The pH optimum for the formation of the 4(R) isomer was 6.0 by microsomes from rats treated with 3MC (Figure 25) and ISF (data not shown), and 6.5 by microsomes from rats treated with PB (Figure 24), whereas that for the formation of the 4(S) isomer was 7.0 - 7.5; the latter coincided with the pH optimum for lipid peroxidation (Figures 21 b and 24 b). Because of this marked difference in the pH optima for the formation of the 4(R) and 4(S) isomers, OTA metabolism was measured at the two different pHs (pH 6.0, optimum for the formation of the 4(R) isomer by microsomes from rats treated with 3MC and ISF, and pH 7.5, optimum for the formation of the 4(S) isomer and lipid peroxidation, were chosen). At pH 6.0 (compared to pH 7.5), the formation of the 4(R) isomer increased by 7.7 and 5.0 fold using microsomes from rats treated with 3MC and ISF respectively. On the other hand, at pH 7.5, the formation of the 4(S) isomer increased by

3.4 and 2.7 fold using microsomes from rats treated with ISF and PB respectively as compared to pH 6.0 (Table 19). This increase in 4(R)-4-OH-OTA formation at pH 6.0 (versus pH 7.5) could be due to less ionization of OTA at pH 6.0 which would make it more lipophilic and possibly allow it to access cytochrome P-450 more readily, and/or it could be that less lipid peroxidation is occurring at pH 6.0 and thus cytochrome P-450 is more protected allowing for increased metabolism. Increased lipid peroxidation at pH 7.5 (versus pH 6.0) could also account for increased 4(S)-4-OH-OTA formation especially if the two processes are linked. Measurement of cytochrome P-450 at the end of an incubation at the two different pHs revealed that at pH 6.0, the percentages of cytochromes P-450 lost were 32, 15 and 51% using microsomes from rats treated with 3MC, ISF and PB, respectively (Table 20). The corresponding MDA amounts formed by microsomes from the same sources were 18.9, 8.0 and 11.8 nmol/mg protein. The percentages of cytochromes P-450 lost at pH 7.4 were 57, 43 and 59% using microsomes from rats treated with 3MC, ISF and PB, respectively (Table 20). The corresponding MDA amounts formed by microsomes from the same sources were 26.8, 17.1 and 20.2 nmol/mg protein. The greatest loss of cytochrome P-450 correlated with the highest MDA formed (Table 20). Thus, it seems that lipid peroxidation is an important factor that determines the percentage loss of cytochrome P-450 that in turn affects the metabolism of OTA.

The large increase in the rate of OTA hydroxylation at pH 6.0 as compared to pH 7.4 using microsomes from rats treated with 3MC or ISF can be only partially due to the protective effect on cytochrome P-450 at the lower pH. Similarly, an alteration in the  $K_m$  of the substrate also appears not to be the critically important factor because the  $K_m$  of OTA hydroxylation decreased at pH 6.0 using microsomes from rats treated with 3MC, but it increased using microsomes from rats treated with ISF. The most likely reason for the increase in OTA at pH 6.0 using microsomes from 3MC or ISF treated rats could be that at this pH the particular isozymes metabolizing OTA (cytochrome P-450 1A1/1A2) undergo a

conformational change in a manner that enhances OTA hydroxylation. The hydroxylating  $\text{Fe}^{3+}$ -Oxygen complex is probably brought into close proximity of the substrate at the site where hydroxylation occurs. Such a conformational change due to lowering of the pH may not favor the hydroxylation of other substrates which bind to the enzyme differentially. Also, other isozymes (for example, IIB1) having a different apoenzyme structure would undergo conformational changes that may not be conducive to OTA hydroxylation.

#### 4.4 Role of cytochrome P-450 in OTA-induced toxicity.

The hepatic microsomal cytochrome P-450 system (mainly cytochrome P-450 families I, II, III and IV) metabolizes a large number of xenobiotics (chemicals, drugs, and environmental contaminants). Most metabolism leads to detoxification of the xenobiotics and their rapid excretion, but some compounds are known to be activated to intermediates that are toxic and/or carcinogenic. For example, aflatoxin B<sub>1</sub> is activated by 3MC inducible cytochrome P-450-AFB (Fukuhara et al., 1989 and 1990). Activation of dimethylnitrosamine and tobacco-smoke-related nitrosamines to more genotoxic and carcinogenic products by different cytochromes P-450 (particularly IIE1 and IIA6) has also been shown (Yamazaki et al., 1992). Enalapril maleate, a drug used to treat hypertension, exerts greater hepatotoxicity upon induction of cytochrome P-450 IIIA by PCN (Jurima-Romet and Huang, 1992). Therefore, the cytochrome P-450 system is important in drug detoxification as well as in drug activation to mutagens, carcinogens and toxins, and genetically determined differences in the activity of these enzymes can influence individual susceptibility to adverse drug reactions, drug induced diseases and certain types of chemically induced cancers. Debrisoquine is used as a test drug to classify individuals into phenotypes based on their metabolic handling of it. There are two main phenotypes: the rapid acetylator/extensive metabolizer and slow acetylator/poor metabolizer (Eichelbaum et al., 1992).

Other important factors that alter the cytochrome P-450 isozyme profile include alcohol consumption, smoking and a variety of drugs and xenobiotics. Altering the cytochrome P-450 profile in the liver may result in serious consequences. Ethanol is known to induce cytochrome P-450 IIE1 (Ekstrom and Ingelman-Sundberg, 1989), therefore the fate of a certain drug/xenobiotic would differ in a person who consumes alcohol from that in a person who does not consume alcohol. One might be more prone to drug toxicity than the other. It was suggested that the bioactivity of N-nitroso-

dimethylamine (NDMA) by cytochrome P-450 IIE1 induced by chronic alcohol consumption plays a critical role in the hepatotoxicity of NDMA (Ma et al., 1991). Also, serious hepatotoxicity may develop in chronic alcoholics while they are taking therapeutic doses of acetaminophen. The mechanism of increased susceptibility involves induction of cytochrome P-450 by alcohol, and the depletion of hepatic glutathione reserves, both of which can result from chronic alcohol ingestion and both of which affect acetaminophen metabolism (Rex and Kumar, 1992). Anttila et al. (1991) suggested that smoking and peripheral type of cancer are related to high levels of pulmonary cytochrome P-450 IA1/2 (which is involved in metabolic activation of polycyclic aromatic hydrocarbons and aromatic amines) in lung cancer patients.

Ochratoxin A is primarily a nephrotoxin. We therefore reasoned that if induction of hepatic cytochrome P-450 leads to more rapid detoxification of OTA and its subsequent clearance, then the kidneys would be exposed to less toxin and for a shorter time. This should result in less damage to the kidneys as compared to the non-induced group. On the other hand, if hepatic cytochrome P-450 induction results in the activation of OTA to a more toxic metabolite then the kidneys would be exposed to a more toxic form of the toxin and would show more damage compared to the kidneys from a non-induced group. We therefore compared the effect of OTA administration to PB-treated rats (liver cytochrome P-450 induced) and to control rats. Excretion of renal enzymes in the urine is considered to be a sensitive, non-invasive indicator of renal toxicity and damage (Price, 1982; Ngaha and Plummer, 1977). Figures 28 and 29 show urine alkaline phosphatase and  $\gamma$ -glutamyl transferase levels both in PB treated and control rats on day 0 (before OTA treatment) and days 1 - 5 after OTA treatment. Levels of both enzymes were elevated on the fourth day after OTA treatment in control rats, but not in PB treated rats indicating that pretreatment with PB protected the kidneys. In the case of  $\gamma$ -glutamyl transferase, enzyme levels were also elevated on days 1, 2 and 3 for control group and on days 2 and 3 for PB group.

OTA being an anion is known to be transported into the kidneys through the anion transport channel (Sokol et al., 1988). It is also known that OTA inhibits the transport of the anion PAH in kidney cortex slices (Sokol et al., 1988). From our results, the anion transport system was not affected by the PB treatment (Figure 30) since PAH transport was inhibited by 55-60% in OTA treated rats compared to control rats, and there was no significant difference in the inhibition of PAH transport in OTA treated rats and in PB pretreated rats treated with OTA (Figure 30). Thus, the effect exerted by PB on OTA is more likely to be enhanced metabolism and faster elimination through induction of hepatic cytochrome P-450. This is more evident from the results obtained from the *in vivo* experiment using [ $^3\text{H}$ ]OTA. Quantitation of OTA in different tissues and body fluids is helpful because it can provide useful information about the amount absorbed, bound to serum albumin and excreted in the urine. Figure 31 shows OTA levels in serum and urine of PB treated rats and control rats 6 hr after OTA administration. Even though it was not significantly different, there was a tendency of increased OTA concentration in the serum of control rats compared to PB treated rats (OTA is known to bind tightly to serum albumin; Chu, 1971). Moreover, there was an increase in OTA concentration in the urine of the PB treated rats compared to control rats indicating that OTA was absorbed, metabolized and excreted faster in PB rats as compared to control rats. OTA levels in the tissues (liver, kidney, stomach, the four portions of small intestine and cecum), and in the gut contents are shown in Figures 32, 33 and 34. Higher OTA levels were found in the gut contents (contents of stomach, small intestine and cecum) of control rats compared to PB treated rats again indicating that OTA was absorbed, metabolized and excreted faster in the PB rats. More OTA was found in the four portions of the small intestinal tissues in the control rats; this may have been because there was more unabsorbed OTA in the intestinal contents of the control rats. The high OTA levels in cecum contents might represent, in part, excretion of OTA and metabolites in bile since OTA is known to undergo enterohepatic circulation

(Fuchs et al., 1988).

The above results suggest that the induction of cytochrome P-450 offers protection against OTA toxicity. In support of this view, Hutchison et al. (1971) showed that 4-OH-OTA was non-toxic to rats at doses up to 40 mg/kg. Further support came from Moroi et al. (1985) who showed that the acute toxicity of OTA was reduced in mice by pretreatment with PB for 1 week, and the LD<sub>50</sub> increased to 1.5-2.0 times control. They also observed increased OTA concentration (2-fold) in the bile extract of PB-pretreated mice (in which also they observed unknown metabolite) compared to control. In contrast, OTA concentration in the urine extract was decreased to one-third of control. Moroi et al. (1985) suggested that a change in the metabolism of OTA could cause the decrease in the toxicity of OTA in PB-pretreated mice. In other words, OTA hydroxylation is more likely to be a detoxification pathway. Thus, it appears that OTA itself is toxic rather than a hydroxylated metabolite. The kidney compared with the liver contains much lower activities of cytochromes P-450 enzyme system, thus preventing it from effectively detoxifying OTA. In the absence of rapid OTA metabolism in the liver, the kidney would be exposed to higher levels of OTA and thus suffer greater toxicity. In addition, OTA is transported into the kidney via the anion transport channel (Sokol et al., 1988) thus increasing its concentration in that organ. These reasons may account for OTA being more nephrotoxic than hepatotoxic.

#### **Relevance to human toxicity.**

OTA has been shown to be nephrotoxic and hepatotoxic to a variety of animals and is strongly suspected of being the main etiologic agent responsible for Balkan endemic nephropathy and associated urinary tract tumors in humans. The different cytochrome P-450 isozymes are mainly responsible for oxidizing the various structurally diverse chemicals to which an organism is exposed to readily excretable hydrophilic metabolites.

Many of these isozymes are highly inducible by a variety of xenobiotics and their induction leads to enhanced metabolism of their substrates. Toxicity to a particular tissue/organ is most often the end result of that tissue/organ being exposed for an appropriate length of time to a sufficient concentration of a toxic chemical or its reactive metabolite.

My results suggest that OTA is itself toxic since its enhanced metabolism in PB treated (cytochrome P-450 induced) rats protected against nephrotoxicity. If increased cytochrome P-450 levels protect against OTA nephrotoxicity in humans, then diets rich in cruciferous vegetables (cabbage, brussels, sprouts), broiled foods or w-3 fatty acids which induce cytochrome P-450 should protect against OTA nephrotoxicity. Discounting their side effects, cigarette smoke and a variety of cytochrome P-450 inducing drugs should also exert a protective effect. It is clear from my studies that several cytochrome P-450 isozymes are able to metabolize OTA.

High dietary fat is known to reduce absorption of chemicals and other nutrients from the gut. Studies carried out in our laboratory with OTA dissolved in corn oil versus bicarbonate have confirmed this. A high fat diet would thus be expected to increase the half-life of OTA in the body thus increasing the risk of tissue damage. In humans, therefore, a low fat diet might protect against OTA nephrotoxicity (reduce OTA toxicity, but not fully protect against it) by enhancing its absorption through the gut and subsequent elimination.

In conclusion, avoiding or at least minimizing exposure to OTA would offer the best protection against nephrotoxicity. However, a diet low in fat and rich in cytochrome P-450 inducers should protect against OTA nephrotoxicity.

This study should be important to humans and of relevance because humans have similarities in a number of their cytochromes P-450 to those of rats. Mostly, they have more than 70% homology between them on the level of amino acid sequence and DNA (see Table 21 at the end of this chapter).

## CONCLUSIONS

My data provide evidence for the stimulatory effect of cytochrome P-450 on OTA-induced lipid peroxidation in a reconstituted system, and strongly implicate cytochrome P-450 role in OTA-induced lipid peroxidation in microsomes.

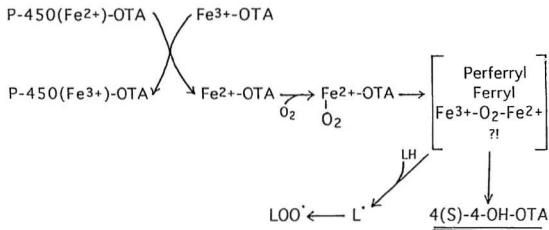
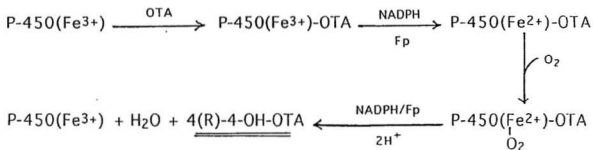
The results also show that OTA is metabolized by the main cytochrome P-450 isoforms IA1/IA2, IIB1 and IIIA1/IIIA2. This conclusion is based on studies using 1) specific cytochrome P-450 inducers, 2) specific substrates, 3) specific inhibitors and 4) monoclonal antibodies against specific cytochrome P-450 isoforms.

The results from *in vivo* experiments suggest a protective effect of PB against OTA toxicity, probably through enhanced metabolism and detoxification due to cytochrome P-450 induction.

**Key words for indexing:**

Ochratoxin, lipid peroxidation, cytochrome P-450, inducers, inhibitors, monoclonal antibodies, rat, platelets, aggregation, secretion.

**Figure 35** Scheme representing the possible mechanism of 4(R)- and 4(S)-4-OH-OTA formation and of OTA-stimulated lipid peroxidation.



**Table 21. Sequential homology between rat and human P-450 forms**

(Adapted from Soucek and Gut. , 1992).

Rat	Cyt. P-450 form		Sequential homology (%)	
		Human orthologue	DNA	amino acid
CYP1A1		CYP1A1	80	78
CYP1A2		CYP1A2	75	70
CYP1B1		CYP1B6	78	74
CYP1E1		CYP1E1	75	78
CYP1A1		CYP1A3	— a	78
CYP1A2		CYP1A5	— a	71
CYP1A1		CYP1A9	— a	— a

a Data not available

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