2,5,2',5'- TETRACHLOROBIPHENYL AND 3,4,3',4'- TETRACHLOROBIPHENYL AS INDUCERS OF HEPATIC MICROSOMAL ENZYMES IN RHESUS MONKEYS

Kathryn Anne Nielsen-Smith

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The thesis " 2,5,2',5'- Tetrachlorobiphenyl and 3,4,3',4'-Tetrachlorobiphenyl as Inducers of Hepatic Microsomal Enzymes in Rhesus Monkeys " has been examined and approved by the following examination committee:

G. Doyle Daves, Jr., Thesis Advisor
Professor

Michael H. Gold
Associate Professor

James F. Pankow
Assistant Professor

Wilbur P. McNulty
Senior Scientist,
Oregon Regional Primate Research Center

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To Tim

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ABSTRACT

2,5,2',5'-Tetrachlorobiphenyl and
3,4,3',4'-Tetrachlorobiphenyl as
Inducers of Hepatic Microsomal
Enzymes in Rhesus Monkeys

Kathryn Anne Nielsen-Smith, M.S.
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Supervising Professor: G. Doyle Daves, Jr.

Single doses of tetrachlorobiphenyls, shown to be free of any chlorinated dibenzodioxins or dibenzofurans to a level of 1 ppm, were given to rhesus monkeys (*Macaca mulatta*) and the hepatic microsomal enzyme response measured periodically for 32 days. Liver biopsies, obtained by a closed needle technique, provided the tissue samples. Aryl hydrocarbon hydroxylase (measured as benzo[a]pyrene hydroxylase), aminopyrine - N - demethylase and cytochrome P-450 were measured on each sample. Animals treated with 25 mg/kg body weight of 2,5,2',5'-tetrachlorobiphenyl showed a marked increase in the demethylase activity, which peaked at 1 to 2 days after treatment, and had no effect on the

hydroxylase activity or the position of the absorption maximum of the CO difference spectrum of cytochrome P-450. This is phenobarbital like inductive behavior. The animals treated with 1 mg/kg body weight of 3,4,3',4'-tetrachlorobiphenyl showed a 3-methylcholanthrene type inductive profile. The hydroxylase activity was significantly increased and peaked at 2 days after treatment. The demethylase activity was not changed. The position of the cytochrome P-450 difference spectrum absorption maximum was shifted to 448 nm. The control animals received just the acetone / corn oil vehicle and showed no changes in any of the biochemical activities measured in the course of this experiment. This data supports the structure - activity relationships, established in rats, for chlorobiphenyls as inducers of hepatic microsomal enzyme activity in rhesus monkeys.

INTRODUCTION

The polychlorinated biphenyls (PCBs)* are a class of chlorinated aromatic compounds which have found widespread applications because of their chemical stability and inertness as well as their dielectric properties.

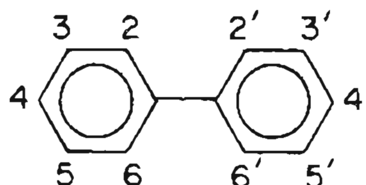

Figure 1. Structure and numbering of the biphenyl ring system.

Two hundred and nine different chlorobiphenyls theoretically exist. [1] However it is theoretically and mechanistically unlikely for them all to be formed in the technical chlorination process. These commercial mixtures were sold under the trade name Aroclor [R] in the United States and Great Britian, Clophen [R] in West Germany, Phenoclor [R] in France and Kanechlor [R] in Japan. All Arochlor [R] products are characterized by a four digit number. The first two digits denote the

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*In this thesis, the abbreviation PCB will refer to technical chlorobiphenyl mixtures or samples (extracts from environmental sources) having similar GC characteristics. Individual compounds will be referred to by their proper names (or abbreviations thereof) or generally as chlorobiphenyls.

type of molecule; 12 = chlorinated biphenyl. The last two digits give the weight percent of chlorine in the mixture. The exception is a more recent formulation, Aroclor 1016 ^{R}, which is 41% chlorine by weight.

PCBs are no longer being manufactured. However many electrical capacitors and transformers containing PCBs are still in use and only slowly being replaced. Formerly PCBs were included in hydraulic fluids, adhesives, dedusting agents, carbonless reproducing paper and microscope immersion oils. PCBs were also used as plasticizers in rubber manufacturing and as pesticide extenders. [2,3]

HISTORY

PCBs first found their use in industry in the early 1930's, and have been used extensively until recently. Except for early reports of skin disturbances in occupationally exposed workers, [4] they were not generally regarded as chemicals of high toxicity or as potentially important environmental contaminants.

The chemical properties of PCBs which make them industrially useful also cause them to persist in the environment. These include thermal stability, resistance to chemical oxidation and hydrolysis, water insolubility and solubility in organic solvents. [5] Essentially

the same pattern of persistence in the environment is becoming apparent for PCBs as has been found for the persistent organochlorine pesticides.[6]

In the early 1960's, PCBs were first identified as environmental pollutants when they were found in large concentrations in fish throughout Sweden.[7] Since then, PCBs have been detected in the environment from the Arctic to the Antarctic and subsequently in marine organisms, fish, birds and mammals.[8,9,10,11] Since PCBs are no longer in use, the levels of PCB detected in the environment and in animals appear to be gradually declining.[11]

Chemical evidence of human contamination has been found in adipose tissue[12,13,14] and in human milk.[15] The most severe episode of human contamination occurred in Japan in 1968.[16] The poisoning, called Yusho (rice oil disease) which affected more than 1600 persons, was caused by the ingestion of rice oil contaminated at levels of 2000 - 3000 ppm with Kanechlor - 400 [R], used as a heat exchange fluid during the manufacture of the rice oil.

PATHOLOGICAL EFFECTS

PCBs have extensive and varied pathological effects on species ranging from bacteria[17], plants[18], insects[19], crustaceans[20], fish[21], birds[22,23] and small mammals[24,25,26,27] to nonhuman primates[28,29,30] and humans.[16,31,32] The physical manifestations

of these effects range as widely as the species affected. In birds, the most important are fluid in the pericardial sac, kidney damage and reduced spleen. [33] Reproductive defects have also been noted. [22] In small mammals such as mice, rats and rabbits, pathologic changes are primarily limited to the liver and include adenofibrosis and hepatomas, [34] carcinomas, [35] and alterations in the hepatic endoplasmic reticulum. [36] In addition, the dermatologic signs seen in man and nonhuman primates (chloracne) are imitated on the inner surface of a rabbits ear lobe [37] and in certain hairless mice. [38]

As might be expected, nonhuman primates most closely resemble man in their biological response to PCBs. [39] Rhesus monkeys (Macaca mulatta) develop acne, subcutaneous edema, hair loss, conjunctivitis and extreme gastric mucosal hypertrophy and hyperplasia (a lesion characteristic of PCB intoxication) when treated with PCBs. [40] Many of these signs were observed in Yusho patients as well. [41]

The exact relationship between the pathological effects of chlorinated biphenyls and their metabolism is not at all understood. [42] The study of the metabolism or toxic effects is complicated by several factors. First, commercial PCBs are mixtures of many individual chlorobiphenyls. Each isomer* may have very different metabolic and toxic properties. [43-48] Secondly, industrial preparations of chlorobiphenyls

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*The term isomer applies to chlorobiphenyls with identical molecular composition.

have been found to be contaminated with trace amounts of polychlorinated naphthalenes or dibenzofurans.[49] Each of these is of considerable toxicological significance.[50-57]

BIOCHEMICAL EFFECTS

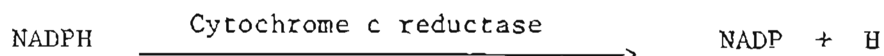
The relationship between the toxicity of chlorobiphenyls and a biochemical effect, induction of certain hepatic microsomal drug metabolizing enzymes, has been more clearly demonstrated.[45,48] The microsomal enzymes in the liver metabolize not only drugs and exogeneous chemicals, but also a variety of normal body constituents: steroid hormones, fatty acids, tyramine and other sympathomimetic amines, thyroxin, methylated purines and various indoles such as tryptamine and indoleacetic acid.[58] These enzymes are present in many tissues, but are particularly abundant in the liver cells. It is the smooth surfaced endoplasmic reticulum that contains most of them.[59] When liver cells are disrupted by homogenization, the endoplasmic reticulum is fragmented, and the fragments can be isolated by ultracentrifugation. These fragments are commonly called microsomes.[59]

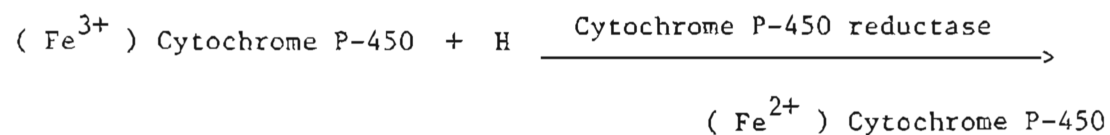
The microsomal enzymes generally act only on lipid soluble materials. In fact they generally convert lipid soluble compounds to more polar substances that can more easily be excreted from the

kidney and biliary tract.

The microsomal material contains a membrane bound oxidase system. It consists of a system enabling electron transport between compounds through the action of a variety of reductases plus a group of heme proteins that possess oxidase properties. The oxidase system (often called mixed function or mono-oxygenase) is capable of attacking molecular oxygen by reducing one atom of oxygen with the formation of water and incorporating the other atom of oxygen into a substrate.

The system requires NADPH and molecular oxygen. The NADPH reduces a component of the microsomes which reacts with molecular oxygen to form an " active oxygen " intermediate which oxidizes the substrate.[59,60] The reactions are summarized below:





The induction* of liver microsomal drug metabolizing enzymes is important pharmacologically, for it leads to accelerated biotransformation of drugs *in vivo* and so alters the duration and intensity of their action. More than 200 drugs, insecticides, carcinogens and other chemicals are known to stimulate the activity of drug metabolizing enzymes in liver microsomes. The uses and biological actions of these compounds are extremely diverse, e.g. phenobarbital (hypnotic), butylated hydroxytoluene (food antioxidant), DDT (pesticide), and 3-methylcholanthrene (carcinogen). The only features they all seem to have in common are that they are lipid soluble and so become localized in the hepatic endoplasmic reticulum and that they are substrates of, or become bound to the microsomal drug metabolizing enzymes,[58,61] or its cytosolic receptor site.[54-56]

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* In this thesis, induction simply refers to a relative increase in enzyme activity as determined by an increased rate of product formation. This may represent an increase in the rate of *de novo* synthesis of an enzyme, in the rate of activation of an enzyme from pre-existing components, or both when compared to the rate of degradation. The rate of degradation of the enzyme may also be decreased. No attempt was made in this investigation to distinguish these possibilities.

ENZYME INDUCTION

Inducers of hepatic microsomal enzyme activity are of at least two types, exemplified by phenobarbital and 3-methylcholanthrene.[58] Many compounds are like phenobarbital in stimulating varied pathways of metabolism by liver microsomes, including oxidation and reduction reactions, glucuronide formation and de-esterification. More specifically the activities of cytochrome P-450 and NADPH-cytochrome c reductase are increased. In contrast, polycyclic aromatic hydrocarbons, typified by 3-methylcholanthrene and benzo[a]pyrene stimulate a much more limited group of reactions, principally aromatic hydroxylation. In this case, a modified form of cytochrome P-450, cytochrome P-448 is induced, but the activity of NADPH-cytochrome c reductase is not affected.[61] Table one lists examples of the enzyme inductive effects of phenobarbital and polycyclic aromatic hydrocarbons.[58,62,63]

Table One

Effects of polycyclic aromatic hydrocarbons and phenobarbital on microsomal pathways of drug metabolism

Pathway Studied	Substrate	Enhanced enzyme activity after treatment with	
		Polycyclic Aromatic Hydrocarbons	Phenobarbital
Aromatic Hydroxyl-ation	Benzo[a]pyrene	++	±
	Acetanilid	+	
	Aniline	+	0
	Zoxazolamine	+	+
Aliphatic Hydroxyl-ation	Hexobarbital	0	+
	Pentobarbital	0	+
	Testosterone and other steroids	±	+
N-Dealkyl-ation	Aminopyrine	0	+
	Ethylmorphine	0	+
	p-Nitroanisole	0	+
	Meperidine	0	+
Sulfoxi-dation	Chlorpromazine	0	+
Glucuron-idation	Bilirubin	+	+
	Salicylamide		+

Aryl hydrocarbon hydroxylase (measured as benzo[a]pyrene hydroxylase) was chosen to represent 3-methylcholanthrene type inductive activity in this investigation since its activity is induced by 3-methylcholanthrene and not significantly affected by phenobarbital. [55] When measured as benzo[a]pyrene hydroxylase, the assay is sensitive and easy to perform on a small scale. The aryl hydrocarbon hydroxylase (AHH) system has been extensively studied because of its relationship to the field of oncology. [64] Polycyclic aromatic hydrocarbons have been shown to be potent inducers of AHH in both experimental animals and man. [65,66] It is believed that a metabolic activation of some chemical carcinogens is required to form a chemically reactive, electrophillic, ultimate carcinogen. It has been established that arene oxides (epoxides) are the activated metabolites of certain polycyclic hydrocarbons. [67] The formation of epoxides is usually carried out by the cytochrome P-448 associated mixed function oxidase system. There has been a report of an arene oxide being formed as a metabolic intermediate of 2,5,2',5'-tetrachlorobiphenyl. [68,69] PCBs possessing the enzyme inductive properties of certain polycyclic aromatic hydrocarbons may be of considerable importance in increasing the cancer risk of the environment. [70]

Phenobarbital type inductive activity can be assessed by measuring the aminopyrine - N - demethylase activity. It appears to be a good selective response for phenobarbital like activity since it is induced by phenobarbital, but not significantly affected by 3-methylcholanthrene. [55] This enzyme was shown to be more sensitive to the

inductive effects of PCBs than other O- or N- demethylases in rats.[71]

Phenobarbital and 3-methylcholanthrene type inductive activities can be further differentiated by their effects on cytochrome P-450. This cytochrome is a hemeprotein which is the terminal oxidase for many microsomal enzyme systems, binding lipid soluble substrates and molecular oxygen.[72] Many mono-oxygenase systems have been reported to contain cytochrome P-450 as the oxygen activating component. Its occurrence is not limited to mammalian tissues; fish, birds, insects, yeasts, fungi, plants and bacteria also contain cytochrome P-450 dependent mono-oxygenases.[73] In mammals, these P-450 dependent enzyme systems can be found in the adrenal, kidney, intestine, brain and lung, although in much smaller quantities than in the liver.[73]

Recent studies show that many slightly different forms of cytochrome P-450 can be electrophoretically separated from the livers of animals treated with various enzyme inducers.[74-77] The cytochrome P-450 which is present in untreated animals is similar to that which is induced by phenobarbital. The molecular weight of the purified preparation is 48,000 - 52,000, and the absorption maximum of its carbon monoxide difference spectrum is at 450 nm in both cases.[75] 3-Methylcholanthrene induces a hemeprotein that differs in spectral, immunological and catalytic properties from the cytochrome P-450 present in untreated or phenobarbital treated animals. This protein, designated cytochrome P-448 or P_1-450, has a molecular weight of about 56,000 and an absorption maximum of its carbon monoxide difference

spectrum at 448 nm. [75] The fact that different chemicals induce different forms of cytochrome P-450 may partially explain their induction of different types of enzyme activity. [73,78]

Enzyme induction can have a profound effect on metabolism and metabolism a profound effect on toxicity. [58] When an enzyme that acts on a drug is induced, the drug is metabolized more rapidly and the metabolite formed more quickly. The consequences for the organism depend on the relative activities of the drug and its metabolite. When the metabolite has little activity of its own, enzyme induction speeds the termination of the action of the drug by accelerating its inactivation. Examples are diphenylhydantion [79] and several barbiturates. [80]

When the metabolite has the same effect as the parent compound and is of comparable or greater potency, enzyme induction may intensify the effects by accelerating the production of the metabolite. Codeine [81] and polycyclic hydrocarbons [82] are examples. When a drug is not appreciably metabolized by the induced enzymes, no change in the duration or intensity of its action would be expected. This is the case with barbital in the rat. [83]

HEPATIC MICROSOMAL ENZYME INDUCTION BY PCB S

The ability of PCBs to induce hepatic microsomal drug

metabolizing enzymes is well documented.[40,43-45,47,48,50,64,71,84-107] Although the interpretation of these data is very complex, it is interesting to note the wide variety of biochemical effects attributed to treatment with PCBs. Table two summarizes the recent research on hepatic enzyme inductive effects of commercial PCB mixtures.

Table Two

Effects of commercial PCB mixtures on hepatic enzyme activities in mammals

Species	a) Compounds b) Route c) Dosage	Effects in liver	Ref.
Rat	a) Aroclor 1016 and Aroclor 1254 b) intraperitoneal (ip) c) 25 mg/kg/day x 6 day	Marked increase in microsomal hemeprotein and cytochrome P-450 (P-450). Induction of ethylmorphine-N-demethylase (EM-N-demethylase) and aniline hydroxylase (AH). 1254 was more potent inducer than 1016.	84
Rat	a) Aroclors 1242, 1248, 1254 and 1260 b) fed in diet c) 0.5, 5, 50 or 500ppm for 4 weeks	Liver to body weight ratio increased for all Aroclors at 500 ppm. Induction of P-450, aminopyrine (AP), codeine and EM -N-demethylases and *p*-nitroreductase by all Aroclors at 50 ppm and by 1254 and 1260 at 5 ppm. No linear relationship between effectiveness as inducer and chlorine content.	85
Mouse	a) Aroclor 1254 b) fed in diet c) 1500 ppm for 7 days	Induction of P-450 and mixed function oxidase activities	86
Rat	a) "PCBs" b) skin application c) 25 mg/kg/day x 6 day	Induction of P-450, EM -N-demethylase, AH, and aryl hydrocarbon hydroxylase (AHH)	87
Rhesus Monkey	a) Aroclor 1248 b) fed in diet c) 300 ppm for 90 days	Enlargement of the liver associated with cell hypertrophy and proliferation of the endoplasmic reticulum. AP-N-demethylase activity was increased, while AH, esterase and nitroreductase activities were decreased	29

Species	a) Compound b) Route c) Dosage	Effects in liver	Ref.
Rat	a) Aroclor 1242 b) ip injection c) 100 mg/kg	Increased liver weight, acetanilid hydroxylase, AP -N-demethylase, P-450 and cytochrome b_5 activities 24 hours after the dose. Enzyme levels remained elevated 10 days after the dose.	27
Rat	a) Aroclor 1221, 1254 and 1260 b) fed in diet c) 1, 5, 10, 25 and 50 ppm for 1-13 weeks	AP-N-demethylase and phosphorothioate detoxification (EPN substrate) activities were increased in proportion to the chlorine content of the Aroclors.	71
Infant Rhesus Monkey	a) Aroclor 1248 b) oral c) 35 mg/kg/day x 30 days	No significant increases in AH, nitroreductase, N-demethylase, glucose-6-phosphatase or p-nitrophenyl-acetate hydroxylase (esterase) activities.	88
Rat	a) Aroclor 1254 b) ip injection c) 25 mg/kg/day x 6 day	Induced 10 fold increase in AHH and significant increase in EM-N-demethylase. P-450 activity increased 3 fold with a shift in the absorption maximum to 448 nm.	89
Rat	a) Aroclor 1254 b) fed in diet c) 0.1, 1 or 10 ppm for 12 weeks	Significant increases in p-nitroanisole-O-demethylase, AH and P-450 only in group fed at 10 ppm.	90
Rat	a) Aroclor 1254 b) fed in diet c) 100 ppm for up to 13 months	P-450 and microsomal heme were increased at 1 week. Induction of δ-aminolevulinic acid (ALA) synthetase and hepatic porphyrin occurred after 2-7 months exposure.	91

Species	a) Compound b) Route c) Dosage	Effects in liver	Ref.
Rat	a) Aroclor 1254 b) fed in diet c) 50 ppm	Significant increases in P-450, EM-N-demethylase, nitro-reductase and pentobarbital hydroxylase observed in 7 days.	92
Rat	a) Clophen A50 (50% chlorinated) b) ip injection c) 15 mg/kg/day x 6 day	When measured 24 hr after the last dose, AHH was increased 7.5 fold, PNA-O-demethylase 16 fold and P-450, 3 fold. The absorption maximum of P-450 was shifted to 448 nm.	64
Rat	a) Biphenyl, Aroclors 1016, 1221, 1232, 1242, 1248, 1254 and 1260 b) ip injection c) 50 mg/kg/day x 3 day	When measured 4 days after the last dose, biphenyl and all Aroclors induced cytoplasmic BSP-GSH conjugating activity. All Aroclors decreased DNA, increased RNA and protein content, PNA-O-demethylase, AH and carboxylesterase activities. The greatest inductive effects were exerted by the more highly chlorinated mixtures.	93
Rat	a) Aroclors 1016 and 1242 b) fed in diet c) 100 or 500 ppm for 6 months	At 1 week, 1242 markedly increased liver weight, P-450, AP-N-demethylase, AH, nitroreductase and glucuronyl transferase activities while 1016 had minimal effects. At 6 months, both had significant effects, although 1016 was much less potent than 1242 as an inducer.	94
Rat	a) Aroclors 1248, 1254 or 1260 b) fed in diet c) 100 ppm for 52 week	Significant increases in total serum lipids and cholesterol. Distinct morphological changes in the liver.	95
Rat	a) Aroclor 1254 b) fed in diet c) 1,5 or 25 ppm for 140 days	No significant effects of 1 ppm diet. The 5 and 25 ppm diets produced dose related increases in pentobarbital hydroxylase, AP-N-demethylase and acetanilid hydroxylase activities after 35, 70 and 140 days.	96

Species	a) Compound b) Route c) Dosage	Effects in liver	Ref.
Rat	a) Aroclor 1254 b) ip injection c) 25 mg/kg/day x 6 day	P-450, AP-N-demethylase and AHH were induced in the livers of newborns whose mothers were given doses 2 days after giving birth. Increased enzyme activity was found in the livers of fetuses of rats treated during pregnancy.	97
Rabbit and Rat	a) Clophen 50A b) oral c) 50 mg/kg/day x 5 day	In rabbits, no induction of AP-N-demethylase, PNA-O-demethylase, AH or 4-chlorobiphenyl hydroxylase was found, although P-450 was increased. In rats, these enzymes were increased 2 - 5 fold.	98
Rat	a) " PCBs " b) oral c) various doses	AP-N-demethylase and AHH were significantly increased even at 1.0×10^{-3} mg/kg , when measured 24 hours after the last dose.	99
Rat	a) Phenoclor DP6 b) fed in diet c) 100 ppm for 8 days	Female rats were less affected than the male rats, as measured by phenobarbital sleeping time.	100

Several of these studies are of particular interest. Alvares *et al*.[89] classified PCBs as a new type of enzyme inducer, intermediate in action between phenobarbital and 3-methylcholanthrene. They measured hepatic enzymes 24 hours after treating rats with Aroclor 1254 ^{R}, phenobarbital or 3-methylcholanthrene. Benzo[a]pyrene hydroxylase activity was increased approximately the same amount by the Aroclor and the 3-methylcholanthrene. The carbon monoxide binding spectrum of cytochrome P-450 showed a shift of the absorption maximum to 448 nm in these groups also. Ethylmorphine-N-demethylase activity was significantly increased only in the Aroclor 1254 ^{R} and phenobarbital treated groups.

The results suggested two possibilities. Since Aroclor 1254 ^{R} induced properties characteristic of both the phenobarbital and 3-methylcholanthrene classes of inducers, it seemed reasonable that a mixture of polychlorinated biphenyls would contain compounds each having the inductive properties of one or the other class. However a second possibility could not be ruled out, that a single component of the mixture could possess the combined properties of the two classes of inducers, so that the characteristic inducing action of Aroclor 1254 ^{R} they described is due to chlorobiphenyls that individually are mixed type inducers.

The ability of the liver to recover from PCB treatment was investigated by Litterst and Van Loon.[92] Rats were maximally induced with PCBs and then allowed to recover for ten days. Levels

of induced enzymes appeared to return to pretreatment levels more rapidly when induced by phenobarbital than when induced by PCBs.

Goldstein _et al._ [94] compared two Aroclors of similar chlorine content, as enzyme inducers. Aroclor 1242 ^{R} is 42% chlorine by weight and contains 9% biphenyls with five or more chlorines. A more recent formulation, Aroclor 1016 ^{R} is 41% chlorine by weight, but contains only 1% biphenyls with five or more chlorines. Aroclor 1242 ^{R} was found to be much more effective than 1016 at inducing hepatic drug metabolizing activity in rats. This greater potency was ascribed to its greater content of more highly chlorinated biphenyls. Ecobichon and Comeau [93] also demonstrated that the more highly chlorinated PCB mixtures were more potent as inducers of hepatic microsomal enzymes.

Clearly the interpretation of the results of these enzyme induction studies is complicated by the multiplicity of the commercial preparations with their uncertain and varied composition and unknown contamination with toxicologically important agents. Variations in the mode of administration, duration of treatment and species of test animal used further confound interpretation.

Many questions were raised. Do all chlorobiphenyls of the same degree of chlorination produce comparable toxicological responses? Are the pathological responses observed due to the difference in number or position of the chlorines around the biphenyl nucleus, or both? Is the duration of their effect related to their persistence in the body? Can an individual chlorinated biphenyl really be a new

type of enzyme inducer, intermediate in effect between phenobarbital and 3-methylcholanthrene? How important is the presence of contaminants in the commercial mixtures for their biological effects? These questions encouraged many groups to begin working with individual chlorobiphenyls. The results of these investigations are summarized in Table three.

Table Three

Effects of individual chlorobiphenyl isomers on hepatic enzyme activities in birds and mammals

Species	a) Compound b) Purity c) Route d) Dosage	Effects in liver	Ref.
Rabbit	a) 2,4,5,2',4',5'-hexaCB and Aroclor 1260 b) furans unlikely in hexaCB, none detected in Aroclor c) skin application d) 120 mg/day x 28 day	Acne like lesions developed earlier and were more severe in the Aroclor treated group. Enhanced liver weights were found in both groups, but liver injury (lesions and serum transaminase) was more severe in the hexaCB treated group. The mean liver content of the chemicals was about the same, 239 ppm hexaCB and 236 ppm Aroclor.	101
Rat	a) 2,3,4,5- and 3,5,3',5'-tetraCB b) greater than 95% by GC c) oral d) 5, 20 or 40 mg/kg/day x 5 days	When assayed 2 days after the last dose, 2,3,4,5-TCB slightly induced EM-N-demethylase and AH, even at the lowest dose level. 3,5,3',5'-TCB induced these enzymes in male rats, but decreased their activity in female rats at higher doses. UDP-glucuronyl transferase and ALA synthetase activities were not affected by either TCB.	43
Rat	a) Biphenyl, isomerically pure chlorobiphenyls b) homogeneous by TLC and GC c) ip injection d) 50 mg/kg/day x 3 day	No induction by biphenyl or 4-monoCB. Increases in O- and N- demethylases, AH, and nitroreductase activities were correlated with the degree and position of chlorination, with the more highly chlorinated isomers being more active.	44

Species	a) Compound b) Purity c) Route d) Dosage	Effects in liver	Ref.
Rat	a) Mixtures of di-, tetra- or hexaCBs b) isomeric composition and purity not stated c) oral d) single dose of various amounts	P-450, NADPH-cytochrome c reductase, PNA-O-demethylase and AH were induced in a dose dependent manner, with the hexa-CBs being the most active.	102
Rat	a) Isomerically pure chlorobiphenyls b) used as purchased from Analabs c) ip injection d) 50 mg/kg/day x 3 day	When measured 4 days after the last dose and compared to biphenyl, those isomers chlorinated in the 4- and 4'-positions induced enzymes associated with the endoplasmic reticulum (mixed function oxidases). Cytoplasmic enzymes were induced by biphenyl, although higher chlorinated isomers were more active.	45
Rat	a) 2,5,2',5'-TCB b) purity not stated c) oral d) 1.25 g/kg, single dose	Animals pretreated with phenobarbital were able to survive a dose which killed half the control group and all of the group pretreated with a hepatic microsomal enzyme inhibitor.	40
Rhesus Monkey	d) 18 mg/kg, single dose	Slight proliferation of the smooth endoplasmic reticulum. No significant changes in glucose-6-phosphatase, esterase, AHH, N-demethylase or nitroreductase, although P-450 was significantly increased.	40
Chicken	a) hexaCBs b) greater than 99% pure c) fed in diet d) 400 ppm for 3 weeks	The most active inducer of durg metabolism were 2,3,4,2',3',4'- and 2,4,6,2',4',6'-hexaCB, while 2,4,5,2',4',5'-hexaCB produced intermediate effects and 2,3,6,2',3',6'-hexaCB was a poor inducer.	103

Species	a) Compound b) Purity c) Route d) Dosage	Effects in liver	Ref.
Rat	a) Tetra- and hexaCBs b) greater than 99% pure by GC with FID c) ip injection d) various doses	3,4,3',4'-TCB and 3,4,5,3',4',5'-hexaCB were the most toxic and increased P-448, AHH and glucuronyl transferase but decreased AP-N-demethylase. 2,4,2',4'-TCB and 2,4,5,2',4',5'-, 2,3,4,2',3',4'- and 2,4,6,2',4',6'-hexaCBs increased P-450 and AP-N-demethylase activities. Isomers chlorinated in only one ring, or in both but not in the 4- or 4'- positions had little activity as inducers.	48
Rat	a) HexaCBs b) purity determined by melting point, nmr c) ip injection d) 25 mg/kg/day x 4 days	When assayed 4 days after the last injection, 2,4,5,2',4',5'- and 2,3,4,2',4',5'-hexaCB caused increases in P-450 and AP-N-demethylase activities. 2,3,4,2',3',4'-hexaCB caused very small increases in these enzymes. All isomers caused significant increases in AHH, but small when compared to treatment with Aroclor 1260. Only 2,3,4,2',4',5'-hexaCB caused a shift from P-450 to P-448.	46
Rat	a) Isomerically pure chlorobiphenyls b) used as purchased from Analabs c) ip injection d) 0.2 mmole/kg single dose	2,5,2',5'-TCB was rapidly eliminated from the liver, causing minimal changes in morphology or enzyme activity. Residues of 2,4,2',4'-TCB and 2,4,5,2',4',5'-hexaCB were more persistent and caused marked induction of microsomal enzymes and changes in liver morphology within 3 days of the dose.	47
Chicken Embryo	a) Many chloro- and bromobiphenyls b) greater than 99% pure by mass spectrometry c) injection into air sac d) various doses	3,4,3',4'-TCB, 3,4,5,3',4',5'-hexaCB and 3,4,5,3',4',5'-hexabromobiphenyl were active inducers of AHH and did not induce AP-N-demethylase.	104

Species	a) Compound b) Purity c) Route d) Dosage	Effects in liver	Ref.
Rat	a) 4,4'-dihalobiphenyls b) purified repeatedly by TLC c) ip injection d) 0.2 mmole/kg x 3 days	4,4'-Difluorobiphenyl did not persist and caused no biochemical changes in the liver. 4,4'-Dichloro- and 4,4'-dibromobiphenyls induced PNA-O-demethylase and AH, with the dibromo compound being silghtly more persistent. 4,4'-Di-iodobiphenyl did not induce enzyme activity although it was very persistent.	105
Rat	a) 2,4,5,2',4',5'-hexaCB b) used as purchased from Analabs c) ip injection d) various doses daily for 3 days	As purchased, 2,4,5,2',4',5'-hexaCB was a mixed type inducer but when rigorously purified, it was a pure phenobarbital type inducer. TCDF, a pure 3-methylcholanthrene type inducer was identified as the major contaminant at a level of 44ppm. It is potent enough to account for the mixed type induction seen with the commercial hexaCB.	51
Rat	a) HexaCBs b) dibenzofurans at less than 5ppm c) oral d) 50 mg/kg, single dose	3,4,5,3',4',5'- , 2,4,5,2',4',5'- and 2,3,4,2',3',4'-hexaCB were shown to induce only P-450 when measured 72 hr after the dose.	106
Rat	a) Several chlorobiphenyl isomers b) " exhaustively purified " c) ip injection d) various single doses	N-demethylation was increased by 2,5,2',5'- and 2,4,2',4'-TCBs at 100 mg/kg and by 2,3,4,3',4'-pentaCB at 10 mg/kg, but decreased by 3,4,5,3',4'-pentaCB and 3,4,5,3',4',5'-hexaCb at 10 mg/kg. AHH was markedly increased by 3,4,5,3',4'-pentaCB and 3,4,5,3',4',5'-hexaCB and slightly increased by 2,4,3',4'- and 3,4,3',4'-TCB and 2,3,4,3',4'-pentaCB.	107

Johnstone *et al.*[44] were among the first to conduct an extensive investigation of the hepatic effects of various chlorobiphenyl isomers. Biphenyl and 4-chlorobiphenyl did not induce microsomal enzymes although they and all the other agents tested did induce the cytoplasmic bromosulphophthalein - glutathione conjugating system. Microsomal mono-oxygenases were induced by hexa- and octachlorobiphenyls and by those di- and tetrachlorobiphenyls with chlorines in the 3- and 4- positions. In all cases the more highly chlorinated biphenyls were more potent.

Ecobichon and Comeau[45] carried out a very complete study of the influence of degree and position of chlorination of isomerically pure chlorobiphenyls on hepatic function in the rat. They observed induction of activity of enzymes closely associated with the endoplasmic reticulum with agents chlorinated in the 4- and 4'- positions, irrespective of chlorination at other positions. Cytoplasmic activity was stimulated by biphenyl itself, although enhanced cytoplasmic enzyme activity was observed with the higher chlorinated isomers.

Hansell *et al.*[47] correlated persistence in the liver with the ability to affect microsomal enzyme activity and liver morphology. Those isomers which accumulated in the liver were able to induce mixed function oxidase activity and changes in cell morphology. Those which did not persist caused only minimal changes.

The structure - activity relationships for chlorobiphenyls as hepatic enzyme inducers were outlined by Goldstein *et al.*[48] and

Poland and Glover.[104] Their work clearly demonstrated that symmetrically chlorinated biphenyls were either phenobarbital or 3-methylcholanthrene type inducers, or inactive, but that no single compound possessed both types of activity. Those isomers chlorinated in both the meta- and para- positions, but not in the ortho- positions were the most toxic (as measured by the rats weight loss) and induced aryl hydrocarbon hydroxylase, glucuronyl transferase and cytochrome P-448, all 3-methylcholanthrene type activities. Biphenyls chlorinated in the ortho- and para- positions induced aminopyrine - N - demethylase and cytochrome P-450, both phenobarbital type activities. Goldstein further stated that compounds chlorinated in just one ring, or in both rings, but not in the para- positions, had very little activity as inducers of liver enzyme activity in rats.

Poland and Glover extended their study of the structure - activity relationships of chlorinated hydrocarbons as inducers of hepatic mixed function oxidases to include chlorinated dibenzodioxins, dibenzofurans and biphenylenes.[52-56,104]

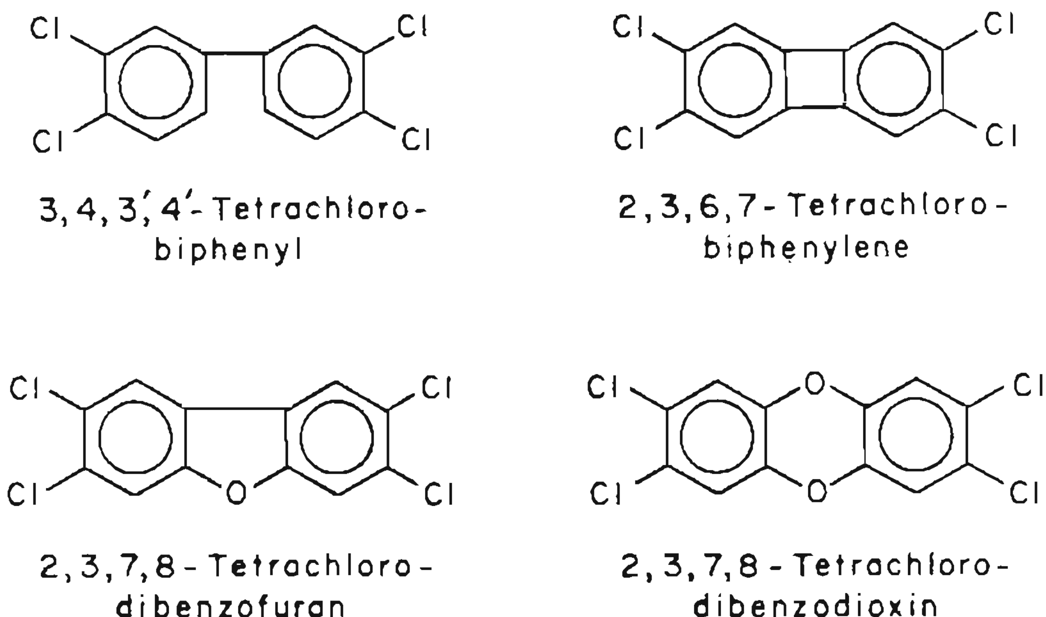

Figure 2. Comparison of chemical structures of approximately isosteric molecules.

They found that 2,3,7,8-tetrachlorodibenzodioxin (TCDD) produced similar effects on microsomal drug metabolizing enzymes in rat liver as 3-methylcholanthrene. However, on a molar basis, TCDD was 30,000 times more potent than 3-methylcholanthrene as an inducer of aryl hydrocarbon hydroxylase. [55] They found a cytosolic induction receptor site to which TCDD binds. The drug - receptor interaction initiates a series of events that ultimately result in the induction of aryl hydrocarbon hydroxylase activity. Both TCDD and 3-methylcholanthrene induced enzyme activity to the same maximum level. Administration of both chemicals at maximally effective doses evoked

no further increase in enzyme activity, and they produced parallel dose - response curves. Polycyclic hydrocarbons did compete with [^{3}H]-TCDD for specific cytosol binding. These observations suggested that TCDD and 3-methylcholanthrene share the same receptor.[54-56]

Using a large number of variously substituted dibenzo - dioxins, their data led to the formation of rules for the induction of aryl hydrocarbon hydroxylase activity in the chick and rat: (a) positions 2,3,7 and 8 must contain at least three halogen substituents; (b) substituents at these lateral positions have the order of activity Br > Cl > F > NO_2 ; (c) at least one hydrogen atom must remain on the dibenzodioxin nucleus.[53]

When the chlorinated biphenyls were compared to TCDD as inducers of aryl hydrocarbon hydroxylase, only those molecules having adjacent halogen atoms in at least two of the lateral positions of each ring (3,4,3',4' or 3,4,5,3',4',5') and having no halogen substitution adjacent to the biphenyl bridge (2,2' or 6,6') were found to be active.[104]

These observations were related to the overall shape of the molecule when 3,4,3',4'-tetrachlorobiphenyl was shown to be an approximate isostereomer of TCDD, since its substitution pattern can allow it to assume a planar or nearly planar conformation. If a halogen atom is substituted adjacent to the biphenyl bridge, free rotation about the bond is sterically hindered and the molecule cannot become planar. 2,3,7,8-Tetrachlorodibenzofuran (TCDF),

3,4,3',4'- tetrachloroazoxybenzene and 2,3,6,7-tetrachlorobiphenylene were all shown to be approximate isostereomers of TCDD, and to be potent inducers of aryl hydrocarbon hydroxylase. Their molecular structures can be thought of as fitting into a rectangle approximately 3 x 10 angstroms, with halogens at all four corners. The chlorinated biphenyls that did induce aryl hydrocarbon hydroxylase activity and cytochrome P-448 were capable of assuming a planar conformation, and were shown to interact with the cytosolic receptor site for TCDD. The differences in inductive potency between the biphenyls and the corresponding biphenylenes and dibenzodioxins might be due to the biphenyl's ability to rotate about their biphenyl bridge, where the more potent biphenylenes and dibenzodioxins are tightly held in a planar conformation. [104] Poland and Glover also demonstrated that binding to the hepatic cytosol TCDD receptor was necessary for induction of aryl hydrocarbon hydroxylase activity and showed a good correlation between that ability and the capacity to produce toxic responses (thymic atrophy and edema) in chickens. [104]

In the first study to show that a 99% pure chlorobiphenyl can contain sufficient quantities of dibenzofuran contaminants to appreciably alter its biological effects, Goldstein _et al._ [51] demonstrated that rigorously purified 2,4,5,2',4',5'-hexachloro-biphenyl resembled the phenobarbital class of enzyme inducers while the commercial 2,4,5,2',4',5'-hexachlorobiphenyl it was prepared from possessed the inducing properties of both the phenobarbital and

3-methylcholanthrene classes of inducers. The major contaminant, TCDF, was a pure 3-methylcholanthrene type inducer, and potent enough to account for the differences in inductive effects between the commercial and rigorously purified hexachlorobiphenyls. To confirm this, 50 ppm of TCDF was added to the purified hexachlorobiphenyl and tested for inductive activity. The result was indistinguishable from that obtained using the commercially available material.

This work points out a serious practical problem. A purity of 99% may not be sufficient for biological studies when the impurities include very potent enzyme inducers such as chlorinated dibenzodioxins or dibenzofurans. This fact further complicates interpretation of toxicological data obtained even in studies using "pure" chlorobiphenyl isomers. In most cases the material was not checked for the presence of chlorinated dibenzodioxins or dibenzofurans, or the identities of the contaminants were not reported.

Keeping these drawbacks in mind, some general conclusions can be drawn from the available data. First, enzyme inductive activity can be roughly correlated with degree of chlorination [43] and with persistence of the chlorobiphenyl in the body. [102,103] The more highly chlorinated biphenyls were more active as inducers and were preferentially retained in the body, over the less chlorinated biphenyls. The biphenyl nucleus itself was shown to be insufficient for induction of microsomal enzyme activity. [44,45] Second, if an

individual, symmetrically chlorinated biphenyl is rigorously purified, the enzyme induction seen is either phenobarbital or 3-methylcholanthrene type. The structure - activity relationships asserted by Goldstein *et al.* [48] and Poland and Glover [104] appear to be valid for the studies reviewed.

Unsymmetrically chlorinated biphenyls present additional difficulties in determining enzyme inductive properties. Recently, Parkinson *et al.* demonstrated a mixed type of induction profile using 2,3,4,5,3',4',5'-heptachlorobiphenyl, which was carefully purified to remove any dibenzofuran contaminants, although this purity was not fully documented. [108] This isomer was chosen for study since it contains the structural requirements for aryl hydrocarbon hydroxylase inductive activity (3,4,5 substitution) in one ring while the other ring contains that and a chlorine in the 2 position, which should prevent planarity and aryl hyrdocarbon hydroxylase activity.

A Japanese group investigated the toxicological and enzyme inductive properties in rats of several unsymmetrically chlorinated biphenyls that were shown to be retained in the bodies of Yusho patients for several years after their exposure to the PCBs. [32] Their evidence strongly suggested that 2,3,4,5,2',4',5'- and 2,3,4,5,2',3',4'-heptachlorobiphenyls were phenobarbital type inducers while 2,3,4,5,3',4'-hexachlorobiphenyl was a 3-methylcholanthrene type inducer of moderate strength. 2,4,5,3',4'-Pentachlorobiphenyl was found to be a mixed type inducer. They explained their findings

by stating that further chlorination at the _ortho-_ position of a potent 3-methylcholanthrene type chlorobiphenyl reduces the intensity, but it still retains the ability to induce 3-methylcholanthrene type activity. For example, 3,4,5,3',4'-pentachlorobiphenyl, a potent 3-methylcholanthrene type inducer, chlorinated in an _ortho-_ position, becomes 2,3,4,5,3',4'-hexachlorobiphenyl, a less potent 3-methylcholanthrene type enzyme inducer. However, chlorination of a less potent 3-methylcholanthrene type inducer, such as 3,4,3',4'-tetrachlorobiphenyl, results in conversion to the mixed type, exemplified by 2,3,4,3',4'-pentachlorobiphenyl. If the 3-methylcholanthrene type chlorobiphenyls are chlorinated at both _ortho-_ positions, they are converted to the phenobarbital type, exemplified by 2,3,4,5,2',4',5'- and 2,3,4,5,2',3',4'-heptachlorobiphenyls. The issue of the possibility of a single, pure chlorobiphenyl compound being a new, mixed type of hepatic enzyme inducer is far from being settled and bears further investigation.

Virtually all the toxicological data on PCBs have been obtained using rats as the experimental animal. Since large variations in responses to PCB treatment are seen between test species,[98] it seems logical to use a test animal, the rhesus monkey, _Macaca mulatta_, which more closely resembles man in its biological response to PCBs.[39] This should facilitate the extrapolation of the data obtained to humans. However there are a few drawbacks. Special facilities are needed to handle primates and because of the greater expense, fewer

animals can be used.

ONGOING RESEARCH

This thesis research is part of a larger, ongoing study of the metabolism and toxicology of polychlorinated biphenyls in rhesus monkeys being conducted jointly by the Oregon Regional Primate Research Center and the Oregon Graduate Center. Previous work by this group has shown that rhesus monkeys are very susceptible to the toxic effects of Aroclors and certain chlorobiphenyls.[28,30,109,110] Groups of three young, male monkeys were fed diets containing no PCBs, 3 ppm 3,4,3',4'-tetrachlorobiphenyl (3,4-TCB) or 3 ppm 2,5,2',5'-tetrachlorobiphenyl (2,5-TCB). The 3,4-TCB and 2,5-TCB were purchased from Analabs,Inc. Selected ion monitoring by gas chromatography - mass spectrometry showed them both to contain several contaminants with masses assignable to tetra- and pentachlorodibenzodioxins and dibenzofurans. However all contaminants were present at levels of less than 1 ppm, and the TCBs were used without further purification. The control diet was also checked, and found to be negative for PCB contamination.

During 219 days of exposure, the animals receiving the control or 2,5-TCB diet gained weight and showed no clinical signs of

illness. The animals receiving the 3,4-TCB diet quickly showed signs of toxicity. Swelling and redding of the eyelids was noted in two monkeys on day 14 and in the third on day 21. Two lost weight while the other continued to gain slowly. Starting on day 23, the 3,4-TCB diet was given only twice a week, a reduction of exposure to an average of 1 ppm. In spite of this, the clinical condition of all three animals continued to deteriorate with severe swelling of the eyelids, loss of hair, dry scaly skin and weight loss. One animal died during a radiographic procedure on day 29.

On day 50 the exposure was further reduced by providing a diet containing 0.3 ppm of 3,4-TCB on a daily basis. A second animal was killed when moribund on day 62. The third monkey improved slightly. The dietary level of 3,4-TCB was then raised to 1 ppm on day 104. Signs of toxicity returned and the animal died on day 215, emaciated and almost hairless.

In a similar experiment, 3,4-TCB, 2,5-TCB and Aroclor 1242 [R] were added at 1 ppm to the diet of three groups of five young, male rhesus monkeys. Four animals served as controls, consuming an uncontaminated diet. The animals eating the control, 2,5-TCB and Aroclor 1242 [R] diets remained well throughout the 133 day exposure period. However four of the five monkeys on the 3,4-TCB diet showed swelling of the eyelids and elevation of the fingernails during the fourth week. One of these developed a bacterial diarrhea resistant to antimicrobial therapy and died on day 33.

Signs of toxicity grew more pronounced in the three remaining 3,4-TCB animals and the control diet was restored on day 38. The fifth animal in the 3,4-TCB treated group had not yet shown any signs of toxicity and was maintained on the 3,4-TCB diet. Swelling of the eyelids and elevation of the fingernails were finally seen on day 106, and the animal switched to the control diet on day 133. These experiments clearly show that within one level of chlorination there are very significant differences in chlorobiphenyl toxicity for monkeys. There also appears to be substantial variation in the response to PCB treatment between individual animals.

PROJECT RATIONALE

There has been no systematic comparison of the whole animal toxicity of pure chlorobiphenyl isomers with their hepatic enzyme inductive characteristics in primates. It is very tempting to speculate that 3-methylcholanthrene type induction is meaningfully related to overall toxicity, possibly through the ability of the compounds involved to assume a planar conformation, but this remains to be proved.

The purpose of the experiment performed for this thesis was to test the hypothesis that, within one level of chlorination,

the positions of the chlorines around the biphenyl ring system would determine the persistence in the body and the type and duration of enzyme induction produced in rhesus monkeys.

It was expected that the most toxic isomer used, 3,4-TCB, would produce signs of toxicity and 3-methylcholanthrene type hepatic microsomal enzyme activity, and the less toxic isomer, 2,5-TCB, would produce no toxic signs at the dose used, and that if any enzyme induction was observed, it would be the phenobarbital type.

To more effectively use the animals, data on the kinetics of storage and excretion of the TCBs were obtained in the course of this experiment. Those results will be reported elsewhere.

MATERIALS AND METHODS

CHEMICALS

3,4,3',4'- Tetrachlorobiphenyl and 2,5,2',5'-tetrachlorobiphenyl were purchased from Analabs, Inc. Selected ion monitoring mass spectrometry showed them both to contain several contaminants with masses assignable to tetra- and pentachlorodibenzodioxins and dibenzofurans. All were present at less than 1 ppm and they were used without further purification.[109] All reagents used in the enzyme assays were analytical reagent grade or the purest grade available from normal commercial sources. The standard for the aryl hydrocarbon hydroxylase assay, 3-hydroxybenzo[a]pyrene, was provided by the National Cancer Institute Chemical Repository.

ANIMALS

Nine individually caged, immature, captive born male rhesus monkeys weighing 2 to 3 kg were used in this experiment. After baseline samples were obtained, two groups of three animals

were dosed with either 1 $^{mg}/kg$ body weight of 3,4-TCB or 25 $^{mg}/kg$ body weight of 2,5-TCB in 10% acetone/ corn oil by nasogastric tube. The control group received an equivalent volume of the acetone/ corn oil vehicle. One animal in the 3,4-TCB treated group vomited a few hours after being dosed and was eliminated from the experimental protocol.

SAMPLING METHODS

Blood, urine and feces samples and liver and fat biopsies were taken before and at 1/3, 1, 2, 4, 8, 11, 16, 22 and 32 days after treatment. The blood, urine, feces and fat samples will be used in the portion of the study concerned with the kinetics of TCB storage and excretion.

The liver biopsies were obtained using a closed needle technique.[111] The animals were fasted overnight before a biopsy. Ketamine anesthesia was shown not to induce any of the enzymes measured in this study when repeatedly administered, and was used in this experiment.[112,113] The animals were restrained in a supine position. The point of entry into the peritoneum was between the seventh and ninth thoracic ribs, along or slightly anterior to, the mid axillary line. The skin was perforated with a scalpel blade.

The Menghini needle (2.0 mm o.d., 4 cm length, attached to a 10 ml Luer lock disposable syringe containing 2 - 3 ml of sterile normal saline) was guided through the intercostal space without contacting a rib. Using the index fingers to guard against a sudden deep penetration, the needle was rotated while being pushed through the peritoneal membrane until its entry into the peritoneal cavity. About 0.5 ml of saline was expelled to clear the needle of muscle or connective tissue. The liver was located by gently probing the peritoneal cavity with the needle, until a soft resistance was felt. At times, applying pressure with the palm from the opposite side of the animals' body helped. When the liver was located, moderate negative pressure was applied to the syringe and a short positive thrust directed toward the liver. The negative pressure was maintained until the needle was withdrawn. Multiple punches could be made, if needed to obtain more tissue. Light pressure was usually sufficient to control superficial bleeding and an aerosol dressing applied.

The contents of the syringe were gently emptied into a sterile petri dish and rinsed with normal saline. Any obvious bits of adipose or fibrous tissue or blood clots were removed before the liver sample was placed in a small aluminium foil envelope, labelled and quickly placed in liquid nitrogen.

The success rate for obtaining liver biopsy samples was 100%. Familiarity with the animals as individuals aided the process.

The average sample ranged from 40 - 70 mg. Over the course of the experiment, over 80 liver and fat biopsies were performed and few side effects of the surgical procedures observed. Some animals picked at their fat biopsy sutures resulting in local irritation or infection. A few animals vomited during or just after the liver biopsy procedure.

The liver biopsy samples were stored in a liquid nitrogen freezer at -70° C until the enzyme assays could be performed. The freezing and storage was shown to have no significant effect on the activities of the enzymes measured. Samples of fresh liver tissue were obtained from a monkey at autopsy. The enzyme assays were performed on the fresh tissue and on samples after freezing and storage for periods up to 33 days. Both enzymes were shown to vary less than 15% under these conditions.[114]

The enzyme activities of liver homogenates measured in this experiment were aryl hydrocarbon hydroxylase (measured as benzo[a]-pyrene hydroxylase) as a prototype of 3-methylcholanthrene type enzyme activity and aminopyrine - N - demethylase as a measure of phenobarbital type activity. Microsomal cytochrome P-450 or P-448 was estimated when sample size allowed.

ENZYME ASSAY METHODS

To begin the assay procedures, the liver sample was homogenized in 0.050 M Tris-HCl buffer, pH 7.5, using a Potter - Elvejhem glass - teflon homogenizer. All operations were carried out at 0 - 4° C. The volume was adjusted to obtain a homogenate with a protein concentration of about 1.5 to 2.5 mg/ml, by adding buffer to a final volume of 3 to 4 ml. The activity of both enzymes was linear within this concentration range (Figure 7).

The aryl hydrocarbon hydroxylase (AHH) assay is based on the enzymatic conversion of benzo[a]pyrene to hydroxylated metabolites and subsequent fluorometric measurement of these metabolites.

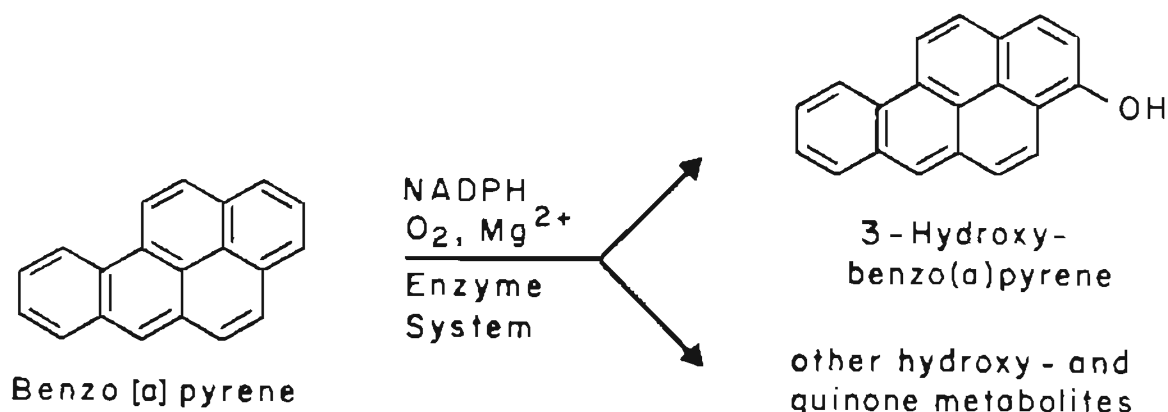

Figure 3. Enzymatic conversion of benzo[a]pyrene to hydroxylated metabolites.

Although 3-hydroxybenzo[a]pyrene accounts for less than half of the total amount of metabolism of benzo[a]pyrene, (the rest going to other phenolic, quinone, arene oxide and dihydrodiol metabolites) the fluorescence of this isomer is so intense it accounts for virtually all the alkali extractable fluorescence. [115,116]

The activity of AHH in liver homogenates was determined by the method of Nebert and Gelboin. [117] The assay was carried out in an incubation mixture of 1 ml total volume.

0.660 ml 0.050 M Tris-HCl buffer, pH 7.5

0.100 ml 0.0036 M NADPH

0.100 ml 0.003 M $MgCl_2$

0.100 ml liver homogenate (1.5 - 2.5 mg protein/ml)

0.040 ml 0.002 M benzo[a]pyrene in methanol

The reaction was started by addition of the substrate, and the mixture incubated aerobically in a 37° C water bath, with shaking, for 30 minutes (Figure 8). The reaction was stopped by addition of 1.0 ml of cold acetone. After the acetone, 3.25 ml of hexane was added and the mixture again warmed to 37° C, to facilitate the extraction. After vortex mixing, a 1.0 ml aliquot of the organic phase was transferred to a fresh tube containing 3.0 ml of 1 M NaOH. The layers were vortexed carefully to avoid emulsion formation, and as soon as the layers had separated completely, a portion of the

basic layer was transferred to a clean 10 x 75 mm tube which served as a cuvette for the fluorescence measurements. The overall efficiency of the extraction procedure for 3-hydroxybenzo[a]pyrene was 66%.

The concentration of the extracted hydroxylated benzo[a]-pyrene metabolites in the basic phase was determined fluorometrically with activation at 396 nm and emission at 522 nm in a Farrand ratio filter fluorometer. The measurements were made as rapidly as possible after the extraction into the NaOH, since the hydroxylated metabolites have been reported to be unstable to base. [118] The fluorescent metabolite obtained from the actual incubation mixture cochromatographed with authentic 3-hydroxybenzo[a]pyrene on HPLC.*

Enzyme activities were determined in triplicate and compared to a blank to which acetone had been added before incubation. The standard curve was prepared by subjecting buffered solutions of 3-hydroxybenzo[a]pyrene to the same incubation and extraction steps as in the actual assay. The activity was reported as μg metabolite/hr/g protein.

In an earlier experiment, rats were treated with TCDD to maximally induce AHH activity. Portions of liver from this experiment,

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*Instrument: Waters Associates HPLC
Column: Chromsorb 18 - RP, 10 μm, 250 x 4.6 mm
Solvent: 100% Methanol at 1 ml/min.

frozen and stored in liquid nitrogen, were used as positive controls during the AHH assays.

Aminopyrine - N - demethylase (AP-N-demethylase) activity was determined essentially by the method of Schoene et al. [119] with modification as suggested by Matsubara et al. [120] The assay is based on the colorimetric measurement of formaldehyde formed from the enzymatic oxidative demethylation of aminopyrine.

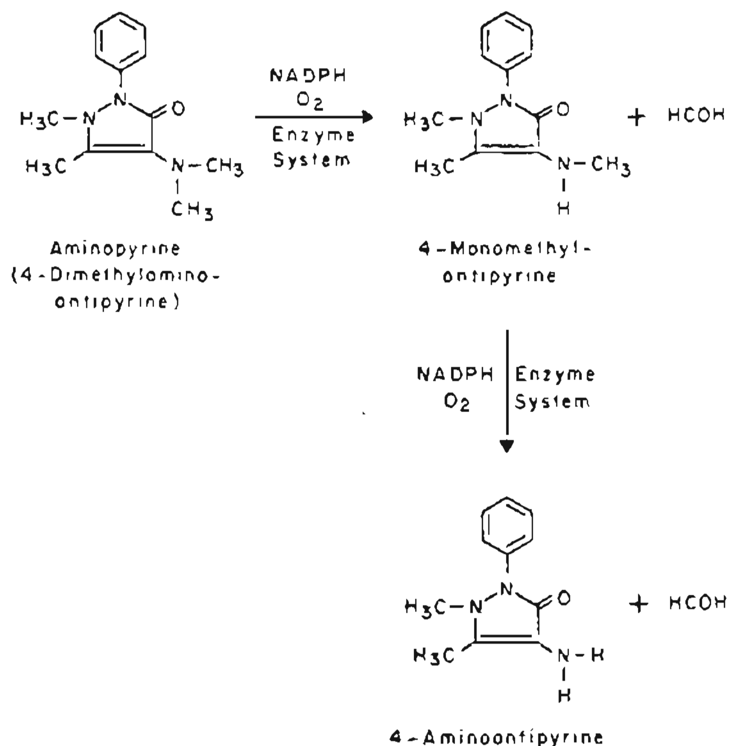

Figure 4. Enzymatic oxidative N-demethylation of aminopyrine.

The following incubation mixture was used:

0.200 ml 0.020 M $MgCl_2$

0.100 ml 0.008 M NADP

0.100 ml 0.070 M isocitric acid

0.005 ml isocitric dehydrogenase, Sigma Type IV

0.200 ml liver homogenate (1.5 - 2.5 mg protein/ ml)

0.050 ml 0.100 M aminopyrine, in 50% aqueous methanol

After five minutes preincubation at 37° C, the reaction was started by the addition of the aminopyrine, and carried out aerobically at 37° C for six minutes with moderate shaking. Within the range of protein concentrations used, formaldehyde production was linear with time in the range of four to ten minutes (Figures 7, 9). The reaction was stopped by the addition of 0.330 ml of 10% (w/v) trichloroacetic acid. After centrifugation, the protein free supernatant was quantitatively transferred to a clean tube and treated with 0.490 ml of the Nash reagent (acetylacetone, ammonium acetate and distilled water).[121,122] The absorbance of the resulting yellow solution was measured at 412 nm using a Beckman Model DU spectrophotometer.

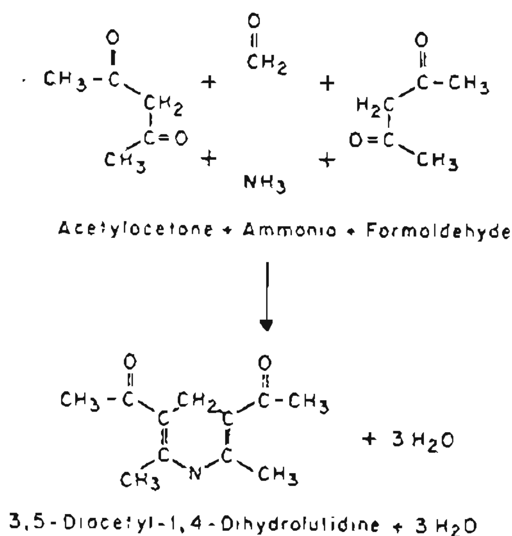

Figure 5. Reaction of formaldehyde with the Nash reagent.

The reaction was run in duplicate and compared to a blank which consisted of the reaction mixture to which trichloroacetic acid was added before the substrate. NADP and a NADPH generating system were used since they are much less expensive than NADPH itself. No greater enzyme activity resulted when NADPH was added to the incubation mixture. Liver from a monkey which had been given a large dose of 2,5-TCB for a metabolic experiment and was shown to have a high level of AP-N-demethylase activity served as a positive control for this assay. A known amount of formaldehyde carried through the incubation and assay procedure served as the standard. Enzyme activity was reported as nmole formaldehyde / min / mg protein.

Cytochrome P-450 was estimated by the method of Omura and Sato [123] using microsomes prepared by the calcium ion sedimentation method of Cinti *et al.*, [124] whenever sufficient sample remained after the determination of AHH and AP-N-demethylase activities.

The microsomes were prepared by centrifuging the liver homogenate at 12,000 x g for 10 minutes to bring down the cell debris, nuclei and mitochondria. The pellet was discarded and the supernatant transferred to a clean tube. One volume of 0.024 M $CaCl_2$ was added to each two volumes of supernatant to give a final concentration of 0.008 M $CaCl_2$. This was well mixed and centrifuged at 27,000 x g for 15 minutes. The supernatant was discarded and the pellet washed free of hemoglobin by resuspending in 1.15% (isotonic) KCl, and recentrifuging at 27,000 x g for 15 minutes. The supernatant was discarded and the pellet covered with 0.5 ml of 0.01 M phosphate buffer, pH 7.6 in isotonic KCl. This was kept at 0° C overnight. All centrifugation was accomplished in a Sorvall RC2 - B refrigerated centrifuge.

The cytochrome P-450 content of the microsomes was estimated the day after they were prepared. The buffer was discarded, and the microsomal pellet resuspended in at least 0.6 ml of the same buffer in a Potter - Elvejhem glass - teflon homogenizer. In most cases, the minimum amount of buffer needed to fill the cuvettes (0.5 ml) was used, since the sample was limited.

Difference spectra of the microsomal preparations were

obtained with a Beckman 25 spectrophotometer with microcuvettes of 1 cm optical path. Their capacity was 0.250 ml each. The microsomal preparations, usually containing 0.15 to 0.30 mg protein/ ml, was placed in both the sample and reference cells. The cells were previously flushed with nitrogen. After the baseline was recorded from 490 to 440 nm, the difference was induced by both cells being reduced with sodium dithionite, and the sample cuvette saturated with carbon monoxide gas by carefully bubbling it through the solution for about 20 seconds. The spectrum was recorded again over the same interval. All spectrophotometric measurements were made at room temperature, 20 - 25° C.

A wavelength calibration with a holmium oxide filter was run after each difference spectrum to allow precise location of the absorption maxima. With full scale deflection adjusted to equal 0.1 absorbance unit, the concentration of the cytochrome was calculated from the maximum peak height around 450 nm, the protein concentration of the microsomal suspension, the volume of the cuvette and an extinction coefficient of 91 $m\underline{M}^{-1}cm^{-1}$.[122]

$$\frac{\text{Peak height in abs. units} \times 1000}{91 \times \text{mg protein in cuvette}} = \text{nmole P-450/}_{\text{mg microsomal protein}}$$

Figure 6. Formula for calculation of P-450 concentration from difference spectra.

Because the sample size was limited, in no case was it possible to estimate the P-450 content more than once per sample. Occasional positive control samples were run using the same samples as for the AHH or AP-N-demethylase assays.

In all cases the concentration of protein in the liver homogenates or microsomal preparations were determined by the method of Lowry *et al.*[125] using crystalline bovine serum albumin as the standard. The unknown samples were run at two dilutions, to ensure that one was within the linear range of the assay.

STATISTICAL METHODS

Statistical significance of the variations of the enzymes measured in the treated animals compared to those in control animals were measured by the Mann - Whitney U test.[126]

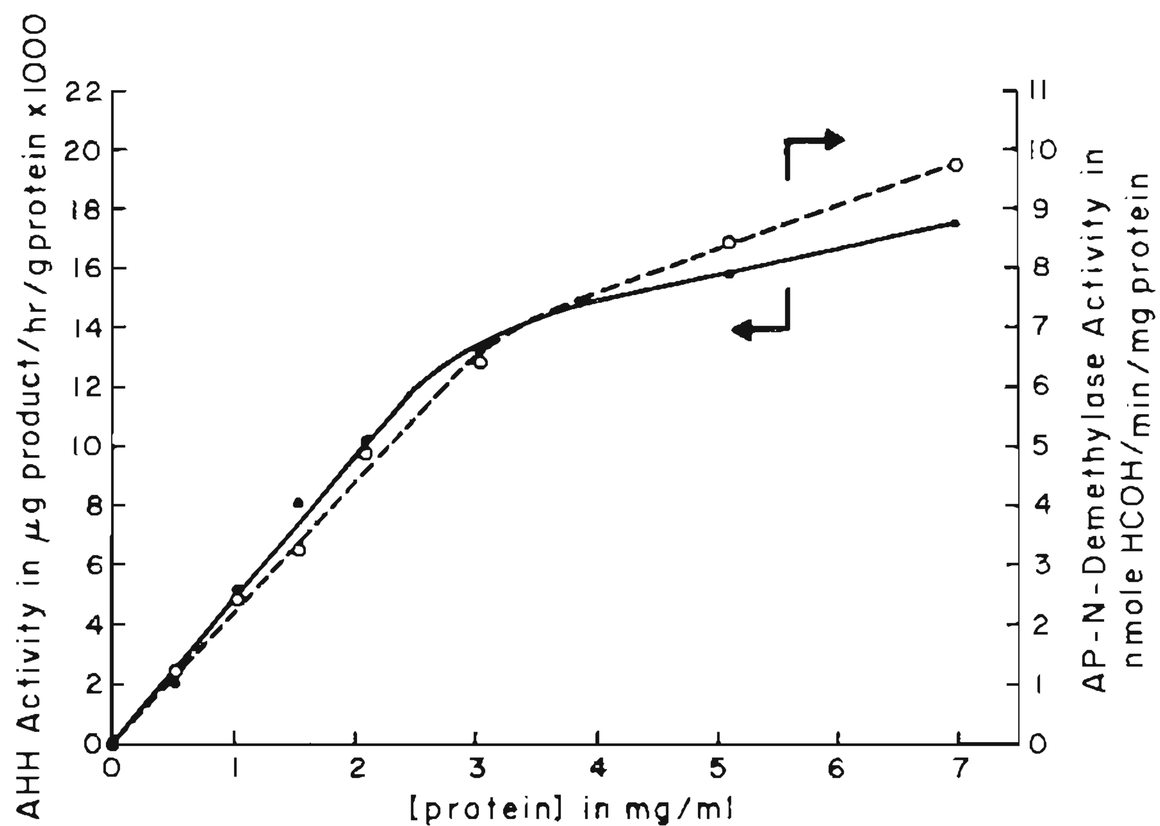

Figure 7. Linearity of the AHH and AP-N-demethylase assays with variation in protein concentration of the liver homogenate.

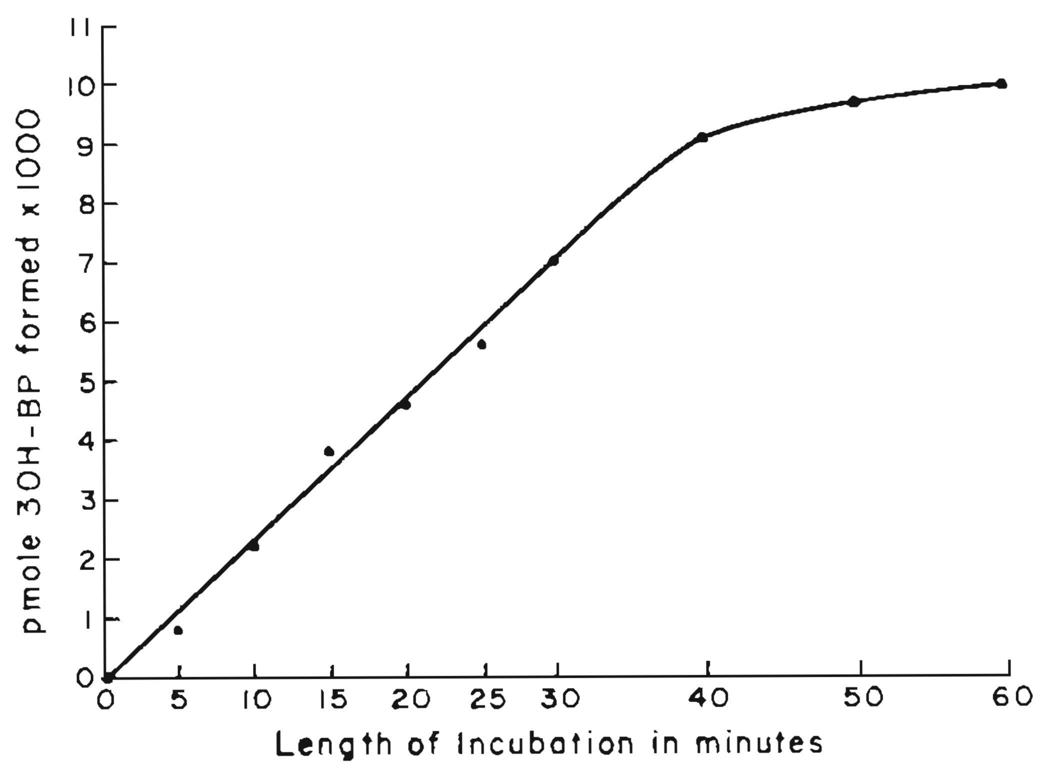

Figure 8. Linearity of product formation with length of incubation in the AHH assay.

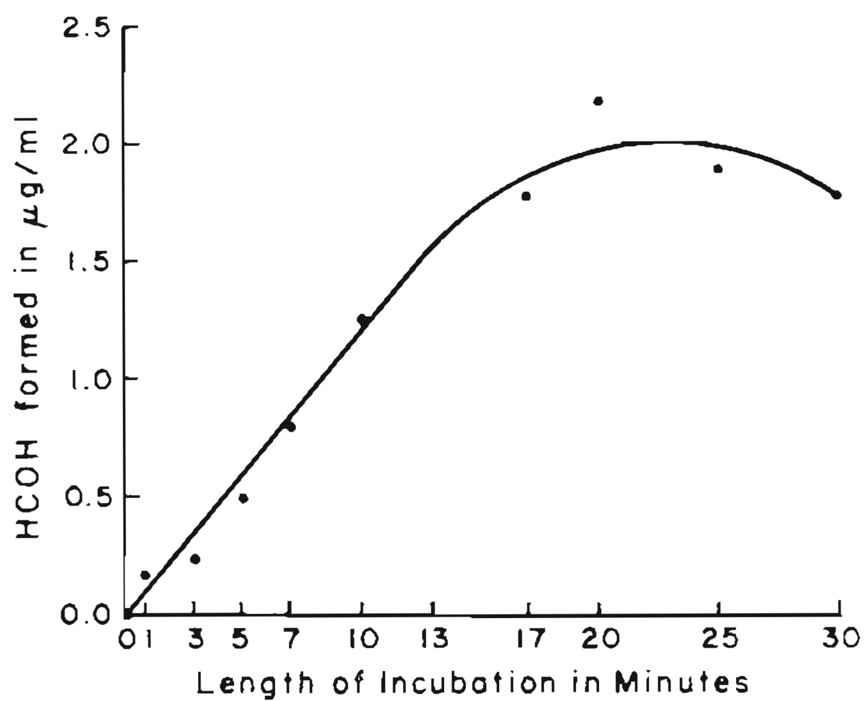

Figure 9. Linearity of product formation with length of incubation in the AP-N-demethylase assay.

RESULTS

Clinically, the animals dosed with the acetone/ corn oil vehicle only or with 2,5-TCB remained well. The 3,4-TCB treated animals developed mild periorbital edema, some loss of eyelashes and elevation of the nails. These signs of toxicity were first observed about 22 days after treatment. The animals did not become severely ill and eventually recovered.

Biochemically, the changes in hepatic microsomal AHH, AP-N-demethylase and cytochrome P-450 activities are presented in figures ten through seventeen. Each point is the average of triplicate (AHH) or duplicate (AP-N-demethylase) determinations. Each point on the cytochrome P-450 graphs represent a single determination.

The aryl hydrocarbon hydroxylase activity in the control and 2,5-TCB treated groups did not change over the course of this experiment (Figures 10,11). The 3,4-TCB treated animals exhibited a marked increase in AHH activity by eight hours after treatment, to 280% of baseline activity. The peak increase occurred two days after treatment (530% of baseline activity) with a steady decrease in activity until 32 days after treatment. At that point, the AHH activity had returned to a level only slightly greater than the pretreatment value (140% of baseline activity, Figure 12).

This figure includes data obtained from a pilot experiment in which a single animal was given 500 $^{\mu g}/kg$ body weight of 3,4-TCB, and the AHH response measured. (This is one half the dose used in the current experiment.)

The increase in AHH activity at two days after treatment in the 3,4-TCB treated group was significantly different from the control group by the Mann - Whitney U test, when $p = 0.05$. Under the same conditions, there was no significant difference between the 2,5-TCB treated and control groups. When the AHH assay was performed several times on many different occasions, using portions of a single frozen liver sample, the variation between replicates and from day to day was less than 10%.

The aminopyrine - N - demethylase activities of the control and 3,4-TCB treated groups were not appreciably changed by the experimental treatment (Figures 13,15). Those animals dosed with 2,5-TCB showed a marked increase in the activity of the enzyme. Peak activity (approximately 600% of baseline activity) was seen at one or two days after treatment, with decay to essentially pretreatment levels by 32 days (Figure 14).

The peak increase at two days after treatment with 2,5-TCB was significantly different from the control group by the Mann - Whitney U test, when $p = 0.05$. There was no significant difference between the 3,4-TCB and control groups under the same conditions. The AP-N-demethylase assay procedure was also shown to vary less than 10%

between replicates and from day to day.

The change in the amount of cytochrome P-450 in the liver microsomes of the experimental groups are illustrated in figure 16. There appears to be an increase in both the 2,5-TCB and 3,4-TCB treated groups over the control group. However, due to the small sample size, each point represents only a single determination. There is also a great deal of variation between individuals in the pretreatment levels, from 0.70 to 1.42 nmole P-450/ mg microsomal protein. The data would be much more conclusive if larger samples had been available. There was a definite shift in the position of the absorption maxima, from 450 nm to 448 nm in the 3,4-TCB treated group (Figure 17). The shift was apparent by one to two days after treatment, and had returned to 450 nm by 22 days after treatment. When the position of the absorption maxima were compared to the control group at two days after treatment, the shift of the absorption maxima in the 3,4-TCB treated group was significant by the Mann - Whitney U test when $p = 0.05$. There was no significant difference in the positions of the absorption maxima between the 2,5-TCB treated and the control groups under the same conditions.

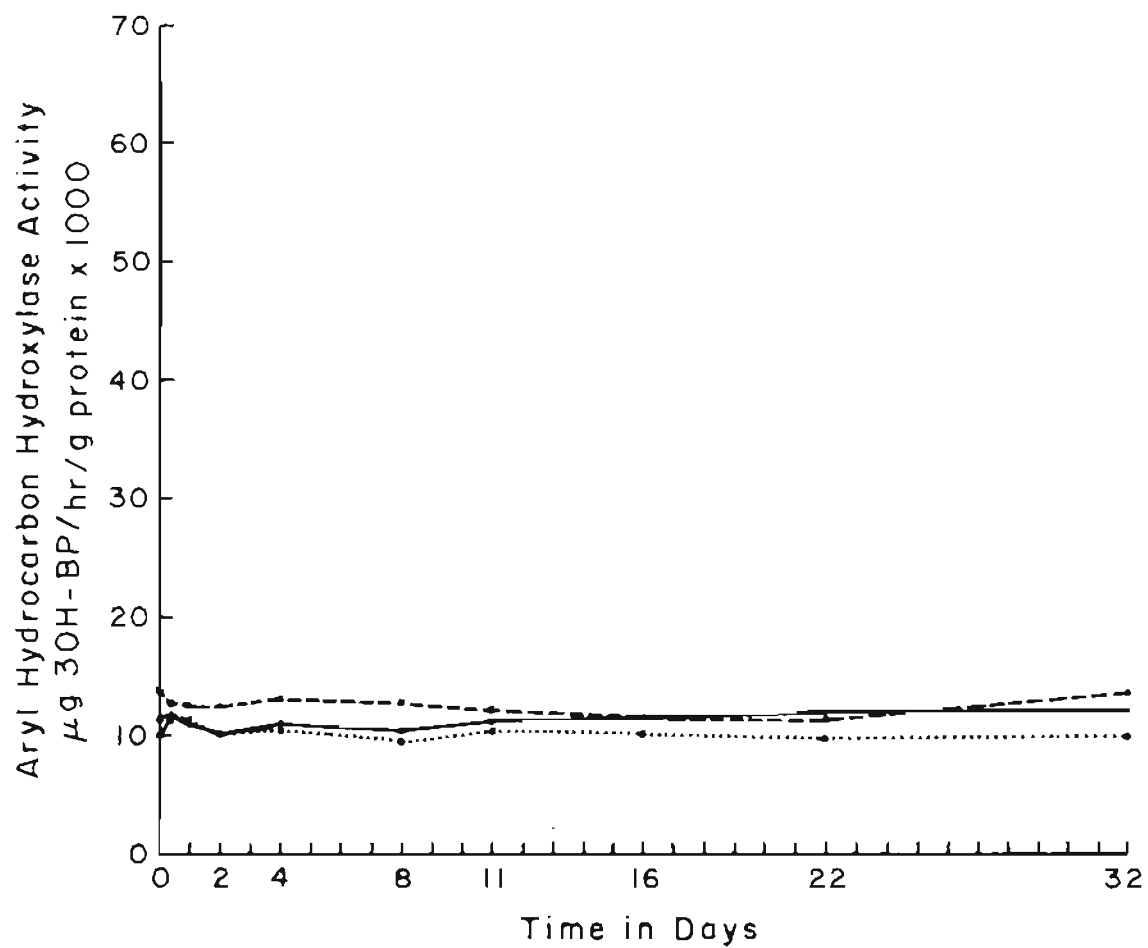

Figure 10. Time course of induction of hepatic AHH activity in control animals treated with acetone / corn oil vehicle.

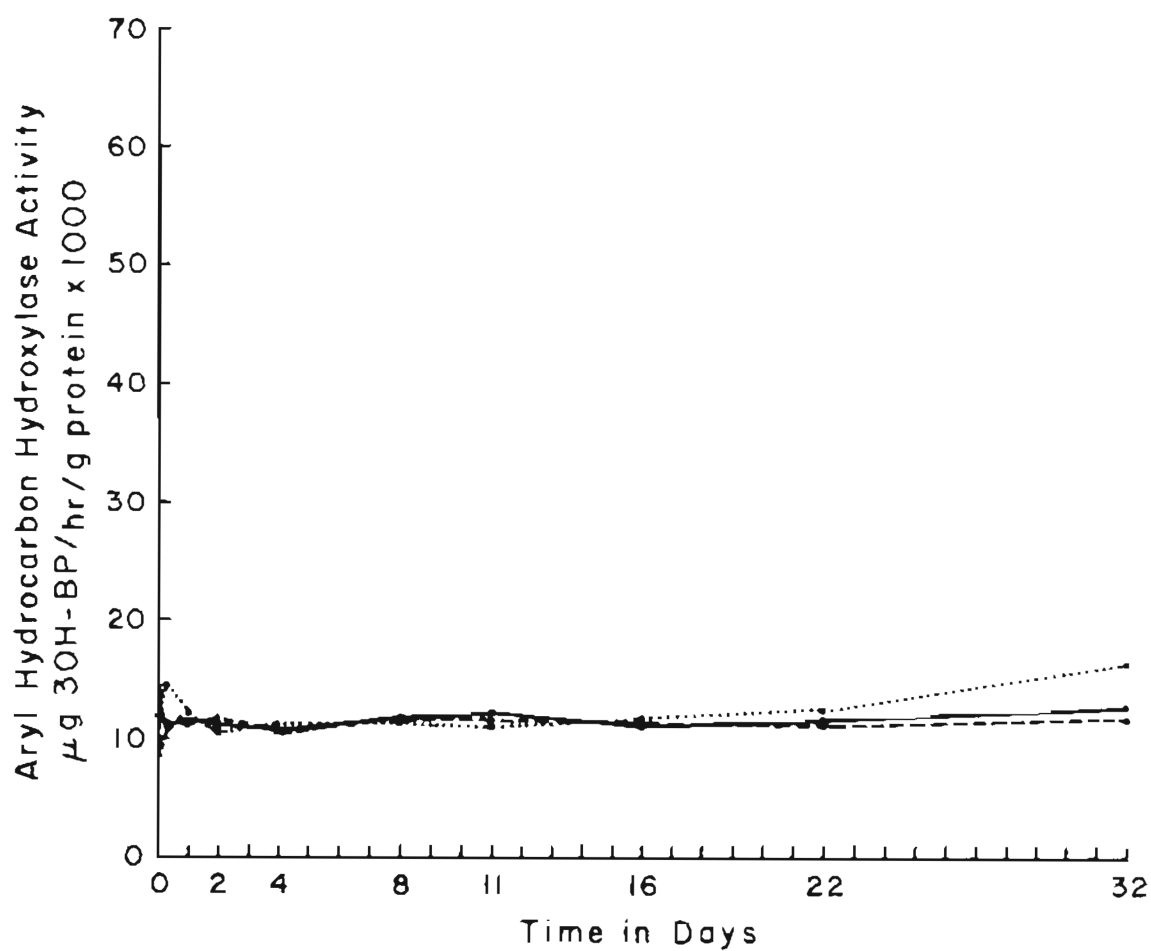

Figure 11. Time course of induction of hepatic AHH activity in animals treated with 25 mg/kg body weight of 2,5-TCB.

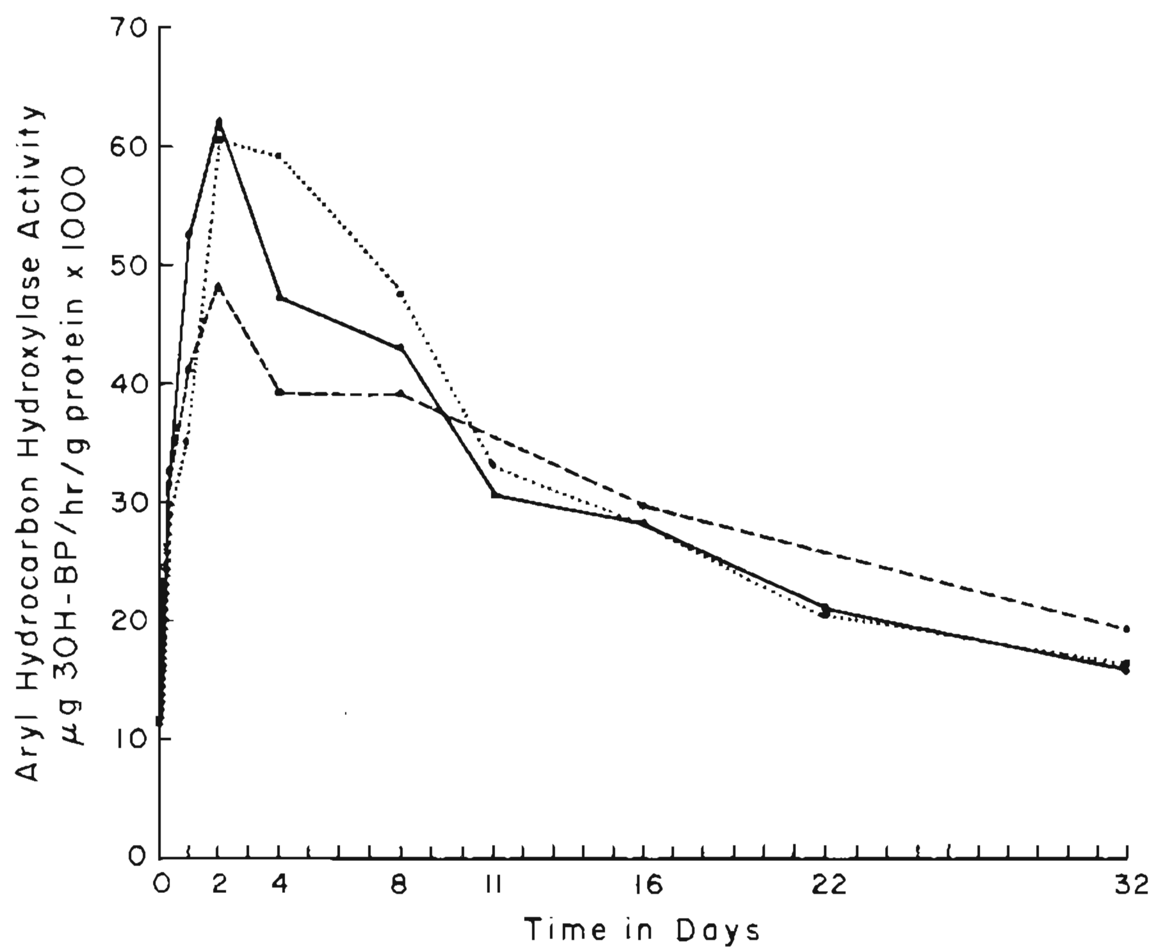

Figure 12. Time course of induction of hepatic AHH activity in animals treated with 1 mg/kg body weight of 3,4-TCB (solid and dotted lines) or 500 μg/kg body weight of 3,4-TCB (dashed line).

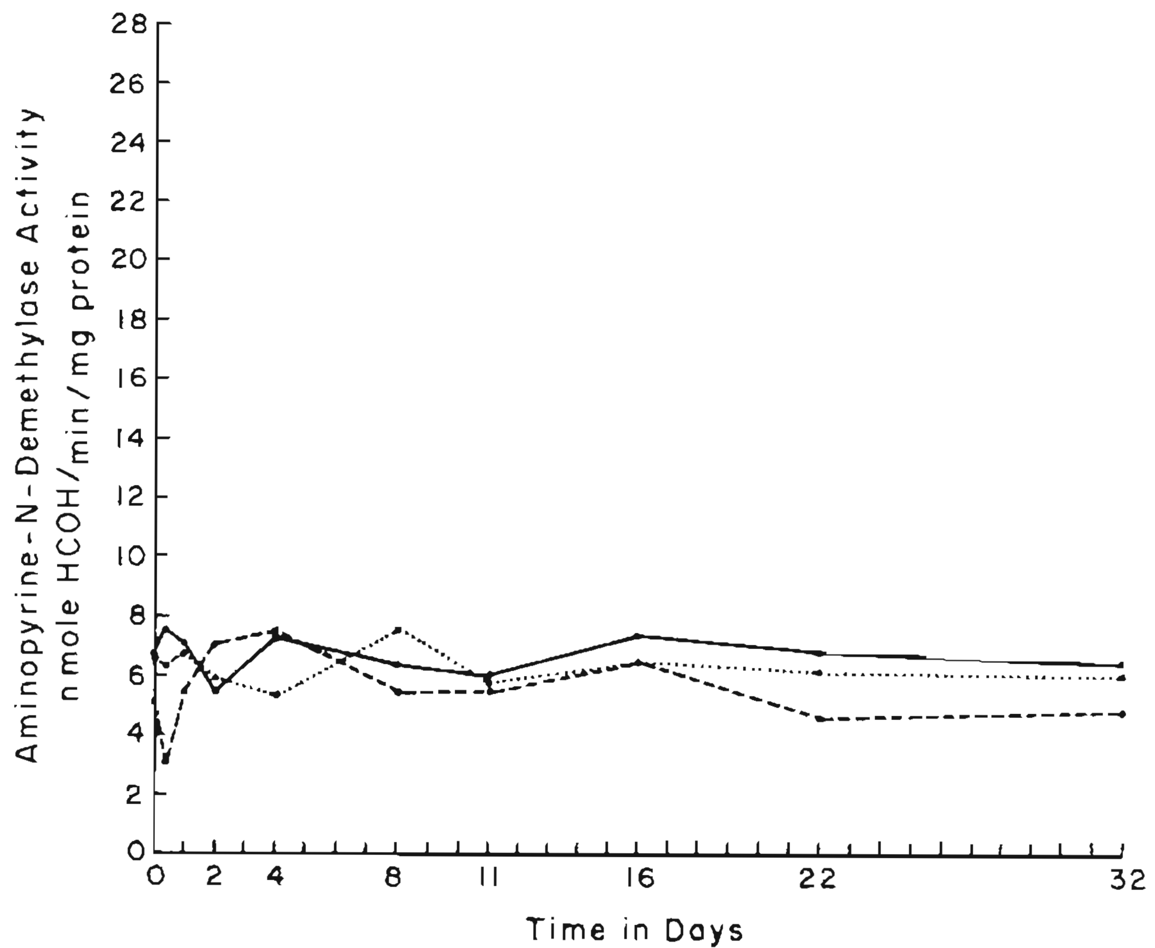

Figure 13. Time course of induction of hepatic AP-N-demethylase activity in control animals treated with acetone / corn oil vehicle.

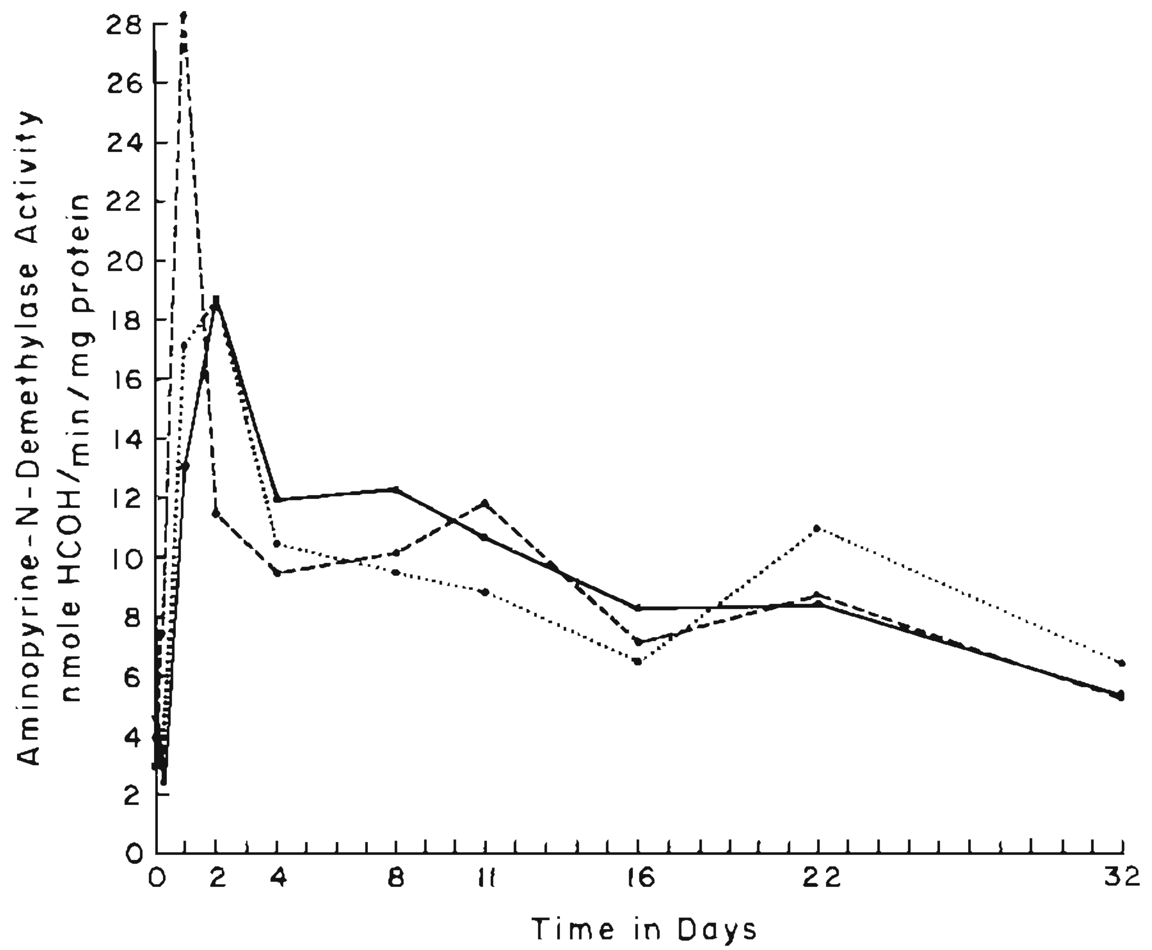

Figure 14. Time course of induction of hepatic AP-N-demethylase activity in animals treated with 25 mg/kg body weight of 2,5-TCB.

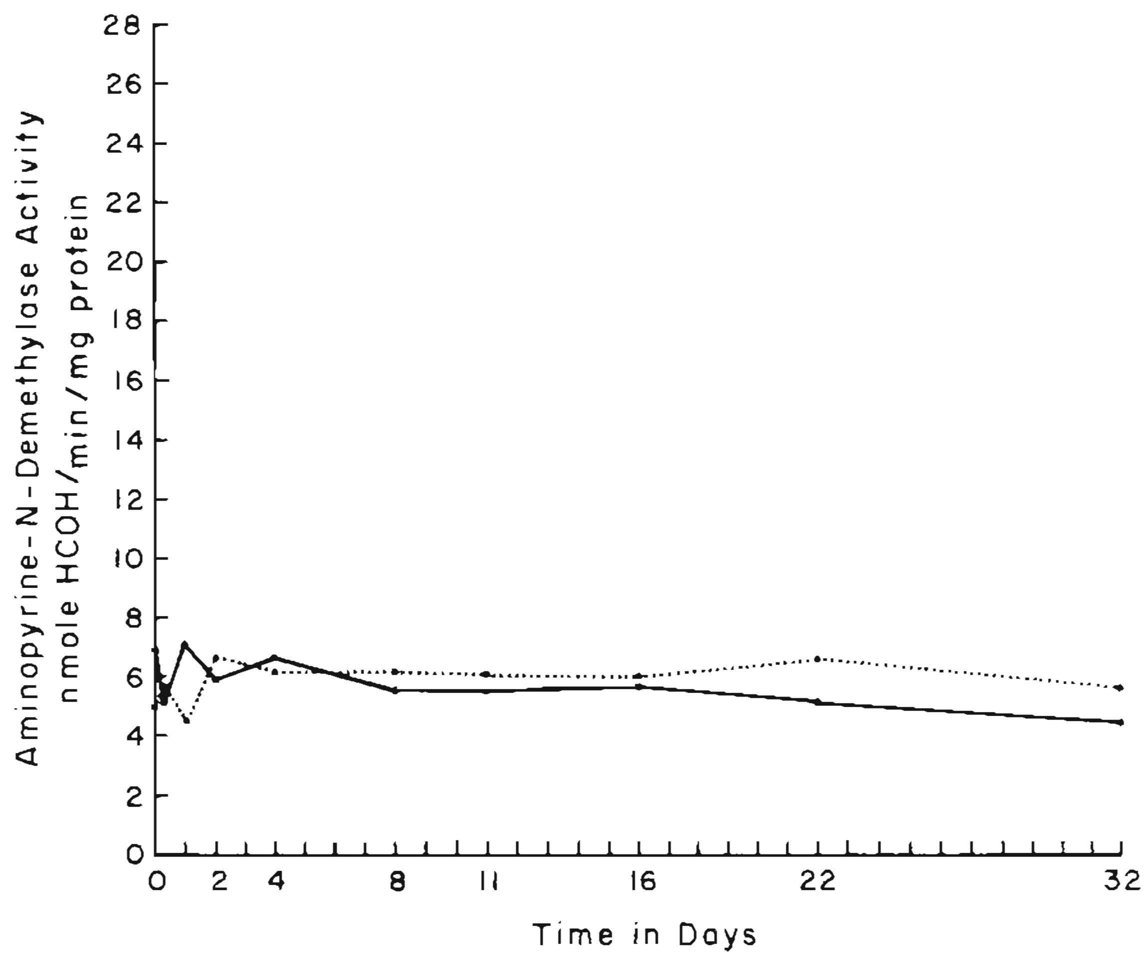

Figure 15. Time course of induction of hepatic AP-N-demethylase activity in animals treated with 1 mg/kg body weight of 3,4-TCB.

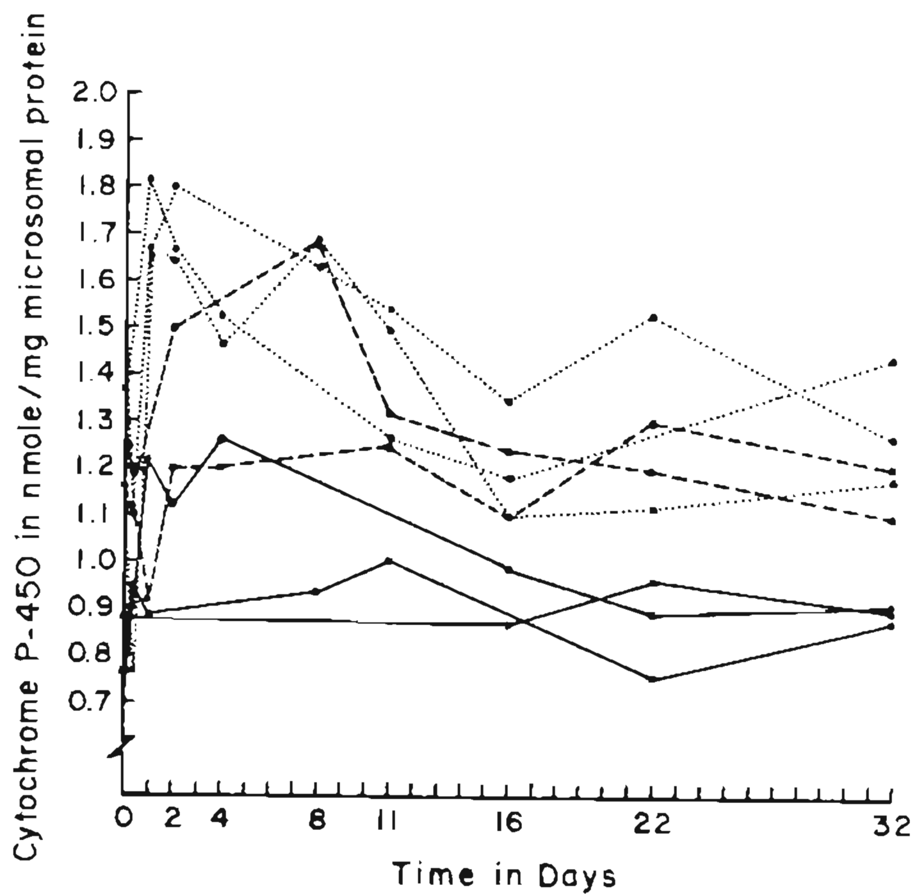

Figure 16. Time course of induction of hepatic microsomal cytochrome P-450 (P-448) in animals treated with acetone / corn oil vehicle (solid lines), 25 mg/kg body weight of 2,5-TCB (dotted lines), or 1 mg/kg body weight of 3,4-TCB (dashed lines).

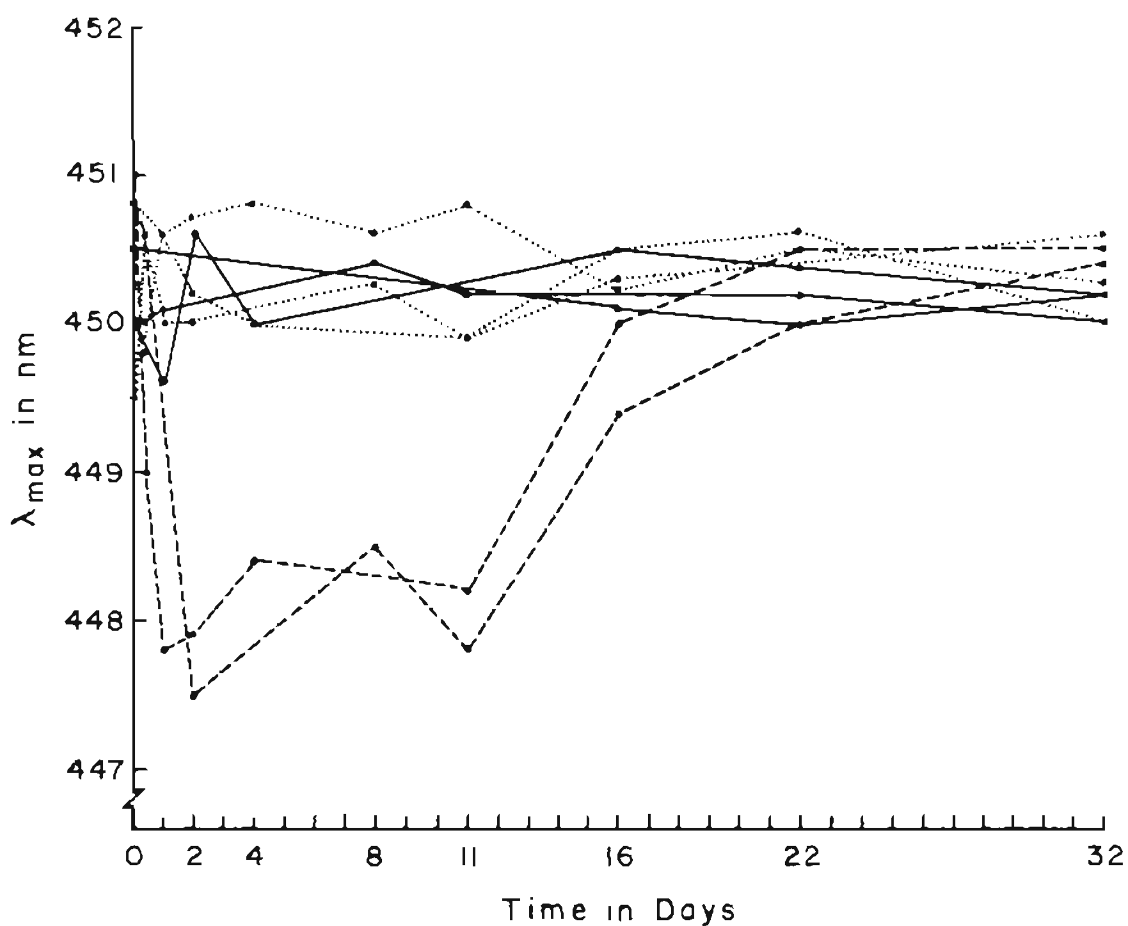

Figure 17. Time course of shift in position of absorption maxima in CO difference spectra of cytochrome P-450 (P-448) of liver microsomes obtained from animals treated with acetone / corn oil vehicle (solid lines), 25 mg/kg body weight of 2,5-TCB (dotted lines), or 1 mg/kg body weight of 3,4-TCB (dashed lines).

PILOT EXPERIMENT WITH HEXACHLOROBIPHENYLS

It is generally acknowledged that the more highly chlorinated biphenyls are more active and potent inducers of hepatic microsomal enzymes than the less chlorinated compounds.[44,45,102] Chronic feeding experiments with several hexachlorobiphenyls are being conducted by this research group. Preliminary results from a pilot study indicate that 3,4,5,3',4',5'-hexachlorobiphenyl is toxic at 1 or 0.1 ppm in the diet, although the signs take longer to develop at the lower dose. This isomer induced aryl hydrocarbon hydroxylase to approximately the same level with either dose, and had no, or a slightly depressant effect on aminopyrine - N - demethylase at two months into the experiment. When measured at two months, the absorption maxima of the carbon monoxide difference spectra of cytochrome P-450 was shifted to about 448 nm. The other isomers tested in the pilot experiment, 2,4,5,2',4',5'- and 2,4,6,2',4',6'-hexachlorobiphenyls were both fed at 65 ppm in the diet for two months. No clinical signs of toxicity appeared during this time period. At one and two months exposure, both these isomers showed phenobarbital type hepatic enzyme inductive behavior by stimulating aminopyrine - N - demethylase activity while not significantly affecting aryl hydrocarbon hydroxylase activity. The absorption maxima of the carbon monoxide difference spectra of microsomal

cytochrome P-450 remained at 450 nm for both of these treatment groups.

DISCUSSION

These results confirm in primates the structure activity relationships for tetrachlorobiphenyls as inducers of hepatic drug metabolizing enzymes established in chicken embryos and rats by Poland and Glover [104] and Yoshimura *et al.* [107] 3,4-TCB induced aryl hydrocarbon hydroxylase activity, caused a shift in the position of the absorption maxima of the carbon monoxide difference spectra of cytochrome P-450 to 448 nm and did not induce aminopyrine - N - demethylase. This is pure 3-methylcholanthrene type inductive behavior. In contrast to the observed inactivity of 2,5-TCB as an inducer of hepatic microsomal enzyme activity in rats, [48] it was found to possess pure phenobarbital type activity in the rhesus monkey, inducing aminopyrine - N - demethylase activity and cytochrome P-450, while having no effect on aryl hydrocarbon hydroxylase activity. These data agree with those of Kohli *et al.*,[106] that chlorination at the *para*- position is not necessarily required for enzyme inductive activity.

Although the pilot experiment with hexachlorobiphenyls is not conclusive, the structure - activity relationships demonstrated for tetrachlorobiphenyls appear to apply. [128] The only isomer tested without *ortho*- substitution, 3,4,5,3',4',5'-hexachlorobiphenyl, appears to be a pure 3-methylcholanthrene type inducer, and quite

toxic. Those isomers tested with _ortho_- substitution, 2,4,5,2',4',5'- and 2,4,6,2',4',6'-hexachlorobiphenyls, were phenobarbital type inducers, and appear to be nontoxic at this dose level. In the full scale experiment, these isomers and 2,3,6,2',3',6'-hexachlorobiphenyl will be more extensively tested.

This is the first comparison of the structure - activity relationships of chlorobiphenyls as inducers of hepatic microsomal drug metabolizing enzymes in primates. It is also one of the few toxicological studies conducted using chlorobiphenyl isomers that have been shown not to contain potent enzyme inducing contaminants at levels that could obscure any effect the chlorobiphenyl itself might have had.

If there was 1 ppm of TCDF in the chlorobiphenyls used in this experiment, the dose of TCDF would have been 0.001 μg/kg body weight in the 3,4-TCB treated group and 0.025 μg/kg body weight in the 2,5-TCB treated group. Very little quantitative data on the toxicity of TCDF for rhesus monkeys is available. McNulty _et al._[110] fed rhesus monkeys 5 ppb of TCDF in the diet for several months. Animals receiving a total dose of 45 or 90 μg/kg body weight showed only mild toxic signs. No information on enzyme induction was available. They also reported TCDF was about one tenth as potent a toxic agent as TCDD. Kitchin and Woods [129] reported that the lowest effective dose of TCDD as an AHH inducer in rats was 0.002 μg/kg. If the rats' sensitivity to these compounds is the same as the rhesus

monkeys', the lowest effective dose of TCDF as an AHH inducer would be 0.02 μg/kg body weight. Since no induction of AHH was seen in the 2,5-TCB treated animals in this experiment, it appears that a dose of 0.025 μg/kg body weight of TCDF is below the no effect level as an inducer of AHH in the rhesus monkey.

When data on the kinetics of storage and excretion of the chlorobiphenyls used in this experiment is available, correlations between persistence in the body and duration of the inductive effects can be made.

Striking differences in the whole animal toxicity between the treatment groups were observed. Animals receiving 1 mg/kg body weight of 3,4-TCB developed signs of toxicity while those dosed with 25 mg/kg body weight of 2,5-TCB did not clinically appear any different from the control group. No quantitative comparison of the potency of these two TCB isomers is possible from the data obtained in this experiment. It is not economically feasible to use many animals at different dosages to determine the dose - response characteristics of each isomer in primates.

For the tetrachlorobiphenyl isomers tested, the correlation between induction of aryl hydrocarbon hydroxylase activity and eventual whole animal toxicity was absolute. It is not generally believed that the induction of aryl hydrocarbon hydroxylase activity itself results in toxicity, (since the induction of the same activity by 3-methylcholanthrene does not produce the same toxic signs as the

toxic chlorobiphenyl isomers [107]) but that it is necessary, but not in and of itself sufficient to produce toxicity. [104] Poland and Glover recently proposed that the stereospecific binding of chlorinated aromatic hydrocarbons (TCDD, TCDF and planar chlorobiphenyls) by the induction receptor and the sustained expression (or repression) of one or more genes controlled by the receptor leads to the toxic responses characteristic of these compounds. [130]

Another aim of these metabolism and toxicity studies was to attempt to identify certain characteristic morphological or biochemical changes resulting from PCB treatment of primates that could be used as a screening test for occupational or environmental exposure of humans to PCBs. Biopsies of facial skin have so far not been helpful, since the characteristic microscopic signs of chloracne do not appear until the animal looks sick. Certain changes in hepatic microsomal enzymes appear to be strictly correlated with eventual toxicity, but liver biopsies are hardly a convenient screening procedure. Blood serum enzyme levels would be the most suitable and convenient large scale screening tool, if an appropriate, early indicator of toxicity could be found. Standard serum chemistry and liver function tests have proved not to be significantly affected until the animal is clinically sick. Preliminary results indicate that sorbitol dehydrogenase is significantly elevated quickly in animals being treated with those hexachlorobiphenyl isomers which eventually prove to be toxic. [127]

It is apparent from the large body of literature reviewed in this thesis that the toxicology of chlorinated biphenyls is a vast and complex field. This thesis reports a beginning of the investigation of the structure - activity relationships of chlorobiphenyls as inducers of hepatic microsomal enzymes in rhesus monkeys. There has been very little work of this kind in primates. The widespread contamination of the environment and exposure of people to polychlorinated biphenyls demands that the primate toxicology of these compounds be further explored.

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BIOGRAPHICAL NOTE

The author was born 11 November, 1956, in Eugene, Oregon. She attended public schools there and was graduated from Winston Churchill High School in 1974.

In 1978 she was graduated magna cum laude from Pacific University in Forest Grove, Oregon, with a Bachelor of Science degree in chemistry. During her stay at Pacific, she was employed as a laboratory teaching assistant and as a research assistant with Dr. James Currie. In 1977, she was awarded the Harold Zeh scholarship from the Portland Section of the ACS. She spent the summer of 1977 as a participant in the NSF - Undergraduate Research Participation program, working with Dr. R. C. Ronald at Washington State University.

The author began study at the Oregon Graduate Center in 1978, where she completed the requirements for the degree Master of Science in June, 1980.