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thesis entitled

CHEMISTRY AND BIOCHEMICAL PHARMACOLOGY

OF POLYBROMINATED BIPHENYL CONGENERS

presented by

Robert William Moore

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Biochemistry

Major professor

Date 20 February 1978

O-7639

CHEMISTRY AND BIOCHEMICAL PHARMACOLOGY OF POLYBROMINATED BIPHENYL CONGENERS

By

Robert William Moore

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1978

ABSTRACT

CHEMISTRY AND BIOCHEMICAL PHARMACOLOGY
OF POLYBROMINATED BIPHENYL CONGENERS

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The objectives of this research were to identify the chemical structures of the components of polybrominated biphenyl mixtures (PBBs), to determine if PBB congeners can be bioactivated into metabolites capable of binding covalently to protein and DNA, and to determine the effects of pure PBB congeners on rat liver microsomal drug metabolizing enzymes.

PBBs are complex mixtures which can contain thirty or more components, however, only the structure of the major Firemaster component, 2,2',4,4',5,5'-hexabromobiphenyl, had been identified. Two PBB mixtures have been fractionated by alumina chromatography and recrystallization, and the resultant purified congeners were analyzed by their ^{1}H-NMR, ^{13}C-NMR, and infrared spectra, and by gas chromatography-mass spectrometry. The major components of Firemaster, in order of their elution by gas chromatography, are 2,2',4,5,5'-penta-, 2,3',4,4',5-penta, hexa-, 2,2',4,4',5,5'-hexa-, 2,2',3,4,4',5'-hexa-, 2,3',4,4',5,5'-hexa-, hepta- and hexa-, 2,2',3,4,4',5,5'-hepta-, hepta-, hepta-, octa-, and 2,2',3,3',4,4',5,5'-octabromobiphenyl. Similarly, the major components of a higher molecular weight PBB mixture are 2,2',4,4',5,5'-hexa-, 2,2',3,4,4',5,5'-hepta-, hepta-, octa-, 2,2',3,3',4,4',5,5'-octa-, 2,2',3,3',4,4',5,5',6-nona-, and 2,2',3,3',4,4',5,5',6,6'-decabromobiphenyl.

The possibility that PBB components can covalently bind to cellular

macromolecules was investigated by aerobically incubating ^{14}C-PBBs with rat liver microsomes and NADPH. The substrate consisted almost exclusively of 2,2',4,4',5,5'-hexabromobiphenyl (HBB_6) and 2,2',3,4,4',5,5'-heptabromobiphenyl (HBB_7). Following exhaustive organic extractions, less than 0.05% of the radioactivity was found in the microsomes, regardless of whether control microsomes, or the microsomes from 3-methylcholanthrene-, phenobarbital-, or PBB-pretreated rats were used. When DNA was included in these incubation mixtures, no binding of PBBs or their metabolites could be detected. It thus appears that the two major Firemaster components are not metabolically activated into reactive species. Parallel incubations demonstrated the covalent binding of ^{3}H-benzo[a]pyrene metabolites to DNA, and that the microsomes isolated from PBB-pretreated rats enhanced this binding six-fold.

HBB_6 and HBB_7 comprise 56 and 27%, respectively, of the Firemaster mixture of PBBs. The effects of HBB_6, HBB_7, and of the suspected trace component 2,2'-dibromobiphenyl (DBB) on liver microsomal drug metabolizing enzymes were examined. Rats were injected i.p. with 90 mg/kg of these compounds, and sacrificed at intervals up to twenty-two days later. HBB_6 and HBB_7 increased liver weights, and strongly induced microsomal protein, NADPH-cytochrome P450 reductase, cytochrome P450, aminopyrine demethylation, and epoxide hydratase. Both caused only small inductions in benzo[a]pyrene hydroxylation and p-nitrophenol-UDP-glucuronyltransferase, and neither shifted the cytochrome P450 spectral maximum from 450 nm. These results, and the results of SDS-polyacrylamide gel electrophoresis, demonstrate that HBB_6 and HBB_7 affect microsomes in a manner very similar to phenobarbital, and that their

inductions are distinct from those caused by either 3-methylcholanthrene or PBBs. DBB had little if any effect on any parameter examined, a result which demonstrates that not all brominated biphenyls are microsomal inducers.

While HBB_6 and HBB_7 are both strictly phenobarbital-type inducers of liver microsomal drug metabolizing enzymes, PBBs cause a mixed-type induction of these enzymes. Seventeen percent by weight of Firemaster remains uncharacterized; one or more of these components must be responsible for the 3-methylcholanthrene-like aspects of the induction caused by the PBB mixture.

ACKNOWLEDGMENTS

I wish to thank my thesis advisor, Steve Aust, for his guidance and suggestions. It is also a pleasure to acknowledge other members of the lab for their help, advice, and friendship, including Bruce Svingen, Fred O'Neal, John Buege, Tom Pederson, and especially Ann Welton. I have collaborated on PBB-related research (not presented in this Thesis) with Cathy Troisi and Ghazi Dannan, to what I trust has been our mutual benefit. I have been extremely fortunate to have been assisted at various times by Jane Albright, Fred "Results" Askari, Debi Metcalf, and Poonsin Olson. I am grateful to all four, whose help was uniformly excellent and valuable, but especially so to Poonsin, because she helped the longest, and through the most difficult parts.

A number of individuals are deserving of special thanks. John Dent and Kevin McCormack provided preprints of their papers, Larry Besaw provided part of the heptabromobiphenyl and assisted with some of the animal work, Joelle André performed the GC-MS analysis, the MSU Chemistry Department (Frank Bennis) determined the ^{1}H-NMR spectra, John O'Conner and John Pierce determined the ^{13}C-NMR spectra, and Paul Kindel lent us his GC thereby making this whole project possible. A very special thanks goes to Stuart Sleight of the Department of Pathology, who performed all the pathological analyses of the experimental animals. His work has added greatly to the value of the research we have performed.

I thank NIH for a predoctoral traineeship, and a variety of people in and out of the Department for their friendship, and for advice and assistance on matters large and small. Lastly, Bob Bernstein, Ed Faleski, Rosemary Schraer, and the members of The Intergalactic Society provided crucial support, moral and otherwise, when my chances for a career as a scientist were quite precarious and when support was needed the most. In particular, without Bob's encouragement, this Thesis would not have been possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

butylated hydroxytoluene	2,6-ditert-butyl-*p*-cresol
C	control
Ci	Curie
cpm	counts per minute
DBB	2,2'-dibromobiphenyl
DCB	2,2'-dichlorobiphenyl
dimethyl-POPOP	1,4-bis[2-(4-methyl-5-phenyloxazolyl]-benzene
DNA	deoxyribonucleic acid
HBB_6	2,2',4,4',5,5'-hexabromobiphenyl
HBB_7	2,2',3,4,4',5,5'-heptabromobiphenyl
HCB_6	2,2',4,4',5,5'-hexachlorobiphenyl
HCCH	1,2,3,4,5,6-hexachlorocyclohexane
Hz	Hertz (cycles per second)
i.p.	intra peritoneal
MC	3-methylcholanthrene
NADH	β-nicotinamide adenine dinucleotide, reduced form
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
NMR	nuclear magnetic resonance

OBB	octabromobiphenyl PBB mixture
Pb	phenobarbital
PBBs	polybrominated biphenyls
PCBs	polychlorinated biphenyls
PCN	pregnenolone-16α-carbonitrile
PEG	polyethylene glycol
ppm	parts per million
PPO	2,5-diphenyloxazole
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SE	standard error of the mean
TCA	trichloroacetic acid
TLC	thin layer chromatography
Tris	tris (hydroxymethyl)aminomethane
UDP	uridine 5'-diphosphate

ORGANIZATION

Research has been performed on the chemistry of PBB mixtures, the binding of PBB metabolites to macromolecules, and the effects of PBBs and purified PBB components on microsomal drug metabolizing enzymes. Each of these topics is described in a separate Chapter, and is presented in a format similar to that used in most scientific journals. These Chapters are preceded by a Literature Review in which microsomal drug metabolizing enzymes, and the effects of PBBs on these enzymes, are discussed. All references cited in these four sections are presented together at the end of the Thesis. A brief history of PBB contamination incidents, with a separate list of general references, is included in the Introduction. The Introduction does not deal strictly with science, and unlike the other sections of this thesis, it is not fully referenced.

Unless stated otherwise, the term PBBs refers to industrial mixtures of polybrominated biphenyls, including whatever non-polybrominated biphenyl contaminants may be present. In Chapters 2 and 3, the terms PBBs and Firemaster are used interchangeably. The terms cytochrome P450 or cytochrome P450 hemoproteins, unless specifically stated otherwise, refer to the sum total of all such microsomal hemoproteins, whether or not λ_{max} is at 450 nm. The term MC (or HBB_6, etc.)-induced microsomes is frequently used. This refers to the microsomes isolated from organs of animals which had been pretreated with MC (or other agents) before sacrifice.

INTRODUCTION

Polybrominated biphenyls (PBBs) were manufactured in response to a growing need for flame retardant textiles, plastics, electronic parts, and other components. They have, unfortunately, made their way into the environment, with serious consequences. A major contamination began in 1973, when PBBs were mistaken for a mineral supplement (magnesium oxide) and added to cattle feed in Michigan. Because the error was undetected for a number of months, most residents of the state consumed contaminated milk, beef, and other products, and built up small amounts of PBBs in their blood, fat, and milk as a result. It appears that 500 to 1000 or more pounds of PBBs, averaging 75% bromine by weight, entered the food chain.

The consequences of the contamination in Michigan have been enormous. Thirty-thousand cattle, 6000 hogs, 1500 sheep, and 1,500,000 chickens have died as a result, although nearly all of these were deliberately sacrificed because they were too heavily contaminated to be sold. In addition, 850 tons of feed, and several dozen tons of dairy products have been destroyed. At least $30,000,000 in damages have been paid, with numerous cases still in court or awaiting trial. PBBs have been a cause of widespread concern among the general Michigan population, particularly among women contemplating breast feeding their babies, and among the most heavily exposed farm population. The fact that it was a dairy farmer, Rick Halbert, who was the motivating force

behind the eventual identification of PBBs as an environmental contaminant does not speak well for a number of institutions, governmental or otherwise. To put it mildly, most people in positions of authority have not distinguished themselves by their responses to the PBB problem. Fortunately, the preponderance of scientific evidence now indicates that PBBs are not highly toxic, and in that sense the residents of Michigan have been very lucky, because there is little reason to believe that a more toxic environmental contaminant would have been removed from the food supply any more quickly.

In 1977, PBBs were found in human hair, fish, plants, soil, and water in the vicinity of PBB manufacturing plants in New York and New Jersey. More recently, PBBs have been reported in fish in the Ohio River. The Environmental Protection Agency was also reported to be "urgently" examining fish, human hair, and breast milk around plants in Illinois, California, Mississippi, Ohio, Tennessee, and Pennsylvania where PBBs are used in large amounts. Since PBBs are also manufactured in Europe, it does not seem unreasonable to expect environmental contamination to be discovered there as well. Because thousands of tons of PBBs have been manufactured, and because of their stability and persistence, it is likely that the effects of those PBBs already in the environment will be felt for a number of years, and that future incidents of contamination will occur as a consequence of the disposal of materials containing PBBs.

PBB research in Dr. Aust's lab began in the spring of 1975. The initial experiments were designed to determine the effects of PBBs on rat liver microsomal drug metabolizing enzymes. Work done mainly by Cathy Troisi, with my collaboration, demonstrated that PBBs are excellent

inducers of these enzymes (see Catherine Troisi, Masters Thesis, Michigan State University, 1975). At about the same time, I began to more fully investigate the properties of PBBs, both chemically and biologically. Ghazi Dannan and I initiated a collaborative project designed to determine the effects of PBBs on drug metabolizing enzymes in lactating and nursing rats. The results demonstrated that PBBs could exert their full range of effects by being transmitted through rats' milk, and that nursing pups were more sensitive to the effects of PBBs than were the dams.

The research described in this Thesis was then initiated. PBBs were found to be a mixture of many different chemicals, and while numerous useful studies can be and have been done with mixtures of chemicals, such studies only rarely elucidate the mechanisms by which biological effects are manifested. The example provided by vitamin D research serves as a perfect case in point. For several decades, little progress in this area was made, until DeLuca, Norman, and others fractionated vitamin D preparations, identified the components, and began to investigate the biological effects of these purified components. It is now known that most of the effects once attributed to the major compounds are actually caused by trace quantities of very potent derivatives, and the mechanisms by which vitamin D acts are consequently much more thoroughly understood.

Nearly every research project on PBBs has been conducted knowing only the general chemical formula for PBBs and the structure of the major component of the mixture. However, as demonstrated in Chapter 3, and by the metabolic investigations by Ghazi Dannan, not all brominated biphenyls have the same biological effects or metabolic fates, nor

should they be expected to. Fractionation and characterization of PBB components, and research on the purified components, is vital to any attempt to understand the pharmacokinetics and pharmacodynamics of PBBs, or to doing something as fundamental as assaying the proper component(s) when evaluating the extent of environmental contamination.

Because such knowledge is central to all biological studies, the chemical structures of PBB components had to be identified. The first objective of this research was therefore to find out what PBBs consist of. In several cases, structures were elucidated by analyzing nearly homogeneous preparations. Other congeners, while enriched 10-30-fold, were still not homogeneous, but structural identification was still achieved. The second objective was to determine some of the biological properties of purified PBB components. This work primarily concerns the effects of PBB congeners on liver microsomal drug metabolizing enzymes. The relative levels of these enzymes is of more than just academic interest, because changes in these levels affect the pharmacology, toxicology, and carcinogenicity of a variety of other exogenous compounds. Enzyme induction also alters steroid metabolism, which can result in a variety of secondary responses. Even the immune system may be affected by induction; polychlorinated biphenyls are immunosuppressive, perhaps by inducing adrenal drug metabolizing enzymes, thereby increasing the serum levels of corticosteroids and causing atrophy of the thymus. Although with one exception the secondary effects of microsomal enzyme induction by either PBBs or their purified constituents were not evaluated, it is hoped that such research will be conducted in the future.

To this date, the only chemical structure reported in the literature for PBB components is that of the major congener. Chapter 1 presents the structures of eight additional congeners. Several other congeners have been partially characterized.

Chapter 2 describes the results of a brief set of experiments designed to determine whether or not several of the PBB congeners can be enzymatically activated into metabolites capable of binding to cellular macromolecules including DNA.

Chapter 3 represents the beginning of biological experiments with pure PBB components. The two major congeners in Firemaster, which together comprise greater than 80% of the mixture of PBBs which contaminated the Michigan food chain, were isolated from crude PBB mixtures, and their effects on liver microsomal drug metabolizing enzymes were examined. The effects of a third, rapidly metabolized congener were also determined. The results serve to underscore the point that PBBs consist of a variety of chemicals whose biological effects can differ widely. Additional purified congeners will be tested in the near future in Dr. Aust's lab for their effects on microsomal drug metabolizing enzymes.

References on the history of PBB contamination incidents are given below.

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LITERATURE REVIEW

Introduction

This Thesis is concerned with the chemistry and biochemical pharmacology of polybrominated biphenyl congeners. Although a massive literature exists concerning the closely related polychlorinated biphenyls (PCBs), I will not attempt to review it here, except for certain relevant aspects. The book by Hutzinger et al. (1974) is an excellent presentation of the chemical aspects of PCBs, and reviews of the toxicity of PCBs and related compounds by Kimbrough (1974) and by Fishbein (1974) are also extremely useful. An additional source of information concerning PCBs, their contaminants, and related molecules may be found in Volumes 1 and 5 of Environmental Health Perspectives (1972, 1973), a journal published by the National Institute of Environmental Health Sciences which presents the results of various symposia on environmental chemicals.

Four years ago, virtually nothing was known about polybrominated biphenyls (PBBs). The contamination incident in Michigan has provided the impetus for a large amount of research on PBBs, and the literature is now expanding rapidly. Again, no effort will be made to review all papers dealing with PBBs, although a considerable number will be discussed. On October 24 and 25, 1977, a workshop on the Scientific Aspects of Polybrominated Biphenyls-PBB was held at Michigan State University, and virtually every research group to have investigated PBBs

presented their results. The proceedings of this conference are scheduled to be published in the April 1978 issue of Environmental Health Perspectives, and the interested reader is referred to this volume for information concerning the entire range of research on PBBs.

The literature on the chemistry of PCBs is relatively straightforward, while that concerning PBBs is essentially nonexistent. Both will be presented as needed in Chapter 1, which deals strictly with chemistry. In order to fully understand the results presented in Chapters 2 and especially 3, which deal with the biochemical aspects of the research, some knowledge of microsomal drug metabolizing enzymes is required. This Literature Review is therefore concerned with the nature of these microsomal enzymes, their inducibility by PBBs and other chemicals, and the biological consequences of induction of microsomal drug metabolizing enzymes.

Microsomes

Microsomal drug metabolizing enzymes have been found in many mammalian tissues, however, the vast majority of research on these enzymes has been with liver. This discussion will therefore deal primarily with hepatic microsomes.

Microsomes are formed during tissue homogenization as the endoplasmic reticulum breaks up and reseals as small spheres. This process appears to not alter the enzymatic properties of the constituents. Following homogenization, the microsomes are isolated by differential centrifugation (Claude, 1969). Microsomes are often rehomogenized in a buffer containing a chelating agent in order to detach ribosomes and adsorbed proteins, then repelleted by ultracentrifugation to obtain a

more pure membrane preparation, designated washed microsomes (Welton and Aust, 1974a; and many others).

Microsomal Drug Metabolizing Enzymes

Microsomes contain an NADPH-dependent electron transport chain which catalyzes the hydroxylation of a wide variety of substrates, both endogenous and exogenous. The first component is NADPH-cytochrome P450 reductase, a flavoprotein which transfers reducing equivalents from NADPH to the terminal oxidases. Because of its ability to reduce exogenous cytochrome c, it is often referred to as NADPH-cytochrome c reductase (Gillette et al., 1972). There appear to be at least eight different terminal oxidases in rat liver microsomes (Guengerich, 1977); these are the cytochrome P450 hemoproteins. The combination of NADPH-cytochrome P450 reductase with the cytochrome P450 hemoproteins is often referred to as the microsomal mixed-function oxidase system (Conney, 1967; Gillette et al., 1972).

More than two hundred substrates with diverse structures can be metabolized by the cytochrome P450 hemoproteins (Conney, 1967), in a variety of overall reactions, all of which incorporate one atom of oxygen from molecular oxygen into the substrate as a hydroxyl group or epoxide. The other atom of oxygen is incorporated into water (Gillette et al., 1972; Gunsalus et al., 1975). The cytochrome P450 substrates include endogenous compounds such as fatty acids and steroids, as well as numerous exogenous foreign compounds (xenobiotics) such as drugs and environmental pollutants (Conney, 1967; Kuntzman, 1969; Gillette et al., 1972; Conney and Burns, 1972). In many cases, the hydroxylated product is unstable, and so the net reaction appears to be something

different, such as an N-demethylation (Brodie et al., 1958); Gillette et al., 1972). The eight or more cytochrome P450 hemoproteins differ in their substrate specificities (Guengerich, 1977), which in part accounts for the capacity of the system to metabolize so many diverse substrates.

Many of the cytochrome P450 metabolic products can in turn serve as substrates for several additional enzymes. Epoxides can either rearrange nonenzymatically to form phenols, or they can be converted into far less reactive dihydrodiols by the action of epoxide hydratase. Both the formation (by cytochrome P450 hemoproteins) and cleavage (by epoxide hydratase) of epoxides are of considerable interest to persons investigating chemical carcinogenesis, because many epoxides are unstable electrophilic compounds which can covalently bind to protein, RNA, and DNA. Epoxides can also be conjugated with reduced glutathione, both nonenzymatically and as catalyzed by a family of glutathione-S-epoxide transferases (Sims and Grover, 1974; Jerina and Daly, 1974). Some hydroxylated cytochrome P450 metabolites are conjugated with glucuronic acid by the action of a family of enzymes called UDP-glucuronyltransferases (Dutton, 1975; Parke, 1975). Conjugations to different sugars, such as galacturonic acid, have also been reported (Vessey and Zakim, 1973), although these appear to be minor pathways. In general, both the mixed-function oxidases and the above-mentioned enzymes convert hydrophobic molecules into more hydrophilic derivatives, which can then be more easily excreted in the bile or by the kidneys.

Induction of Microsomal Drug Metabolizing Enzymes

One interesting property of the microsomal drug metabolizing enzymes is the ability of compounds (often substrates) to induce the enzymes which catalyze drug metabolism. The two prototype inducers are the barbiturate phenobarbital (Pb) and the carcinogen 3-methylcholanthrene (MC), each of which alone causes a distinct and characteristic pattern of induction. Within the past several years, evidence has been presented for the existence of an additional distinct inducer, pregnenolone-16α-carbonitrile (PCN). Because of the differing biological implications of induction by these prototype compounds, much research effort has gone into characterizing the type of microsomal induction caused by various agents.

Pb causes liver enlargement, and a large increase in the quantity of microsomal protein. NADPH-cytochrome P450 reductase is strongly induced, and the total amount of cytochrome P450 is greatly increased. The spectral maximum in the reduced carbon monoxide difference spectrum is at 450 nm, and there is no change in the ratio of the 455 nm/430 nm peaks of the reduced ethyl isocyanide difference spectrum. Pb induces the cytochrome P450-catalyzed N-demethylation of benzphetamine, aminopyrine, and ethylmorphine, and the aliphatic hydroxylation of hexobarbital, pentobarbital, and the 16 α position of testosterone. These activities are at most only slightly induced by MC (Conney, 1967; Parke, 1975). Pb also strongly induces epoxide hydratase (Bresnick et al., 1977), the UDP-glucuronyltransferase-catalyzed glucuronidation of chloramphenicol (Bock et al., 1973), and one or more 45,000 dalton microsomal hemoproteins (Welton and Aust, 1974b). Induction by MC causes none of these effects.

MC has little effect on liver weights, and causes a smaller increase in microsomal protein than does Pb. It has no effect on NADPH-cytochrome P450 reductase, and while it induces the total amount of cytochrome P450, the magnitude of the induction by MC is smaller than that caused by Pb. MC shifts λ_{max} in the reduced carbon monoxide difference spectrum to 448 nm, and increases the ratio of the 455 nm/430 nm peaks in the reduced ethyl isocyanide difference spectrum. Unlike the strong inductions caused by Pb, MC has little effect on the cytochrome P450-catalyzed metabolism of benzphetamine, aminopyrine, ethylmorphine, hexobarbital, pentobarbital, or the 16 α position of testosterone (Conney, 1967; Parke, 1975). Unlike Pb, MC strongly induces the aromatic hydroxylation and epoxidation of benzo[α]pyrene, the hydroxylation of biphenyl at the 2 position (Parke, 1975), and the O-deethylation of ethoxyresorufin (Burke et al., 1977) and ethoxycoumarin (Thomas et al., 1976). MC also has no effect on epoxide hydratase (Bresnick, 1977), it induces the conjugation of *p*-nitrophenol but not chloramphenicol by UDP-glucuronyltransferases (Bock et al., 1973), and it induces one or more 53,000 dalton microsomal hemoproteins (Welton and Aust, 1974b).

Both MC and Pb also increase the metabolism of a variety of other cytochrome P450 substrates, such as *p*-nitroanisole (Conney, 1967), but assays of these activities provide no information as to the nature of the microsomal induction.

The effects of PCN on microsomal drug metabolizing enzymes have not been well characterized. It induces NADPH-cytochrome P450 reductase, and appears to cause partial inductions of several cytochrome P450-catalyzed activities, but these are all also induced by either MC

or Pb. PCN causes a slight shift in the reduced carbon monoxide difference spectrum towards 449 nm (Lu et al., 1972; Birnbaum et al., 1976). Its only unique feature is the induction of the 50,000 dalton hemoprotein, which is the hemoprotein which predominates in control microsomes (Birnbaum et al., 1976). For the remainder of this thesis, the PCN-type induction of microsomal enzymes will be ignored, and microsomes will be considered to be control, MC, Pb, or mixed-type (see below). It is hoped, however, that future research on drug metabolism will include the development of assays specific for each different cytochrome P450 hemoprotein, so that more refined analyses of the nature of inductions caused by various compounds, such as halogenated biphenyls, can be made.

One term which should be defined is "mixed-type inducer." This refers to an inducing agent which causes the responses in microsomes seen following the administration of both MC and Pb. Hexachlorobenzene, 2,2',3,3',4,4'-hexachlorobiphenyl, and 2,2',3,4,4',5'-hexachlorobiphenyl are the only single chemicals reported to cause a mixed-type induction (Stonard and Nenov, 1974; Stonard, 1975; Stonard and Greig, 1976), whereas mixtures of chemicals can easily cause such an induction provided that both MC- and Pb-type inducers are present.

Consequences of Induction of Microsomal Drug Metabolizing Enzymes

The induction of microsomal drug metabolizing enzymes can have pharmacological, toxicological, physiological, and carcinogenic consequences.

Microsomal enzymes can both activate and inactivate drugs, and changes in the levels of these enzymes can alter the pharmacological

effects of numerous drugs. Phenobarbital treatment decreases the plasma concentrations of the oral anticoagulant bishydroxycoumarin, hydrocortisone (cortisol) administration enhances the elimination of antipyrine, and cigarette smoking decreases the plasma concentration of phenacetin; these and numerous other interactions are attributed in large part to the induction of microsomal enzymes. Many drugs also stimulate their own rates of metabolism through induction, which in part explains certain cases of drug tolerance (Breckenridge, 1975).

The toxicity of certain compounds can be altered by enzyme induction. Bromobenzene is capable of causing hepatic necrosis and death, and pretreatment of rats with Pb enhances bromobenzene toxicity, while MC decreases the toxicity. In Pb-pretreated rats, the major metabolite is 3,4-bromobenzene epoxide, which is capable of depleting reduced glutathione levels and covalently binding to cellular macromolecules. When rats are pretreated with MC, bromobenzene metabolism is not markedly accelerated, the less reactive 2,3-bromobenzene epoxide is the major product, and toxicity is reduced (Gillette et al., 1974; Parke, 1975). The hepatotoxicity of carbon tetrachloride is likewise influenced by the nature of microsomal enzymes. Rats pretreated with Pb are far more susceptible to CCl_4 hepatotoxicity than are control rats, while MC induction of microsomes has a protective effect. When rats are given a sublethal dose of carbon tetrachloride, then challenged with what would normally be a lethal dose, no rats die, because the first dose destroyed much of the cytochrome P450 responsible for bioactivating the molecule into its reactive intermediates (Recknagel and Glende, 1973).

Enzyme induction can have a variety of physiological consequences, particularly on the metabolism of steroids. Pb induces both the biosynthesis and breakdown of cholesterol, the hydroxylation of cholic acids, the 6 β-hydroxylation of cortisol, and the hydroxylation of testosterone, estrogens, and progesterone. A variety of secondary effects can result from these changes (Parke, 1975).

Of perhaps the greatest interest and importance are the effects of microsomal induction on chemical carcinogenesis. Polynuclear aromatic hydrocarbons are not themselves carcinogenic. They are, however, bioactivated by cytochrome P450 hemoproteins into reactive intermediates, such as epoxides and diol epoxides, which can be highly mutagenic and carcinogenic (Sims and Grover, 1974; Jerina and Daly, 1974). The dependence of mutagenicity on activation for many compounds can clearly be demonstrated, for example, by the Ames test for mutagenicity. Bacteria incubated with most carcinogens are not mutated unless both NADPH and microsomes, preferably induced microsomes, are included in these incubations (Ames et al., 1973).

Studies of the effects of induction on chemical carcinogenesis have yielded mixed results. Depending on the type of induction and the chemical being tested, either protection against or increased susceptibility to a secondary chemical carcinogen can be observed (Jerina and Daly, 1974; Parke, 1975). While PBBs have not been tested in this regard, PCBs have been shown to protect against some carcinogens and enhance the effects of others. These results will be discussed below.

Polyhalogenated Biphenyls and Chemical Carcinogenesis

Both PCBs and PBBs are complex mixtures consisting primarily of halogenated biphenyls, but other halogenated compounds including naphthalenes and dibenzofurans can often be present in small amounts (Fishbein, 1974; Hutzinger et al., 1974; Kay, 1977). Because no research has been performed on the relationship between purified or partially purified halogenated biphenyls and chemical carcinogenesis, this discussion concerns the effects of these mixtures as a whole, including their impurities.

Multiple adenomatous nodules were observed in the livers of rats fed approximately one gram of PCBs (Kanechlor-400) over a four hundred day period. These nodules, which were found only in the female rats, were considered to be benign neoplastic lesions (Kimura and Baba, 1973).

A wide variety of morphological changes were seen in the livers of male and female rats fed up to 1000 ppm PCBs (Arochlors 1254 and 1260) for eight months, however, no cancerous or precancerous changes were observed (Kimbrough et al., 1972). In a similar investigation, clusters of pancreatic-type cells were observed in the livers of a number of rats fed 500 ppm PCBs (Arochlor 1254) for six months, but again, no cancerous or precancerous changes were seen (Kimbrough, 1973). However, when nearly two hundred female rats were fed 100 ppm PCBs (Arochlor 1260) for twenty-one months, different results were obtained. Hepatocellular carcinomas were found in 14% of the PCB-fed rats, but in only 0.6% of the control animals. Neoplastic nodules were found in the livers of 79% of the treated rats, but in none of the controls. While not proof of carcinogenicity, neoplastic nodules are a part of the

spectrum of responses to hepatocarcinogens. Ninety-nine percent of the PCB-treated rats had areas of hepatocellular alteration, compared with an incidence of 16% in the controls. No such PCB-dependent effects were seen in other organs. This study showed PCBs to have a hepatocarcinogenic effect in rats (Kimbrough et al., 1975). Neoplastic nodules have also been observed in the livers of both male and female rats given a single one gram dose of PBBs (Firemaster FF-1) by gavage (Kimbrough et al., 1977).

In both cases in which the effects of PCBs on mice were examined, evidence of carcinogenicity was obtained. Ito et al. (1973) found nodular hyperplasia and well-differentiated hepatocellular carcinomas in the livers of male mice fed 500 ppm PCBs for thirty-two weeks. These neoplasms were only observed using a PCB mixture averaging five chlorines per biphenyl (Kanechlor 500); when mixtures averaging three or four chlorines per molecule were used (Kanechlors 300 and 400, respectively), no neoplasms were seen. Kimbrough and Linder (1974) found hepatomas in nine of twenty-two male mice feed 300 ppm PCBs (Arochlor 1254) for eleven months, while none were found in mice fed a control diet.

In addition to these investigations with laboratory animals, there is tentative epidemiological evidence for an increased incidence of malignant malanoma among human workers heavily exposed to PCBs (Bahn et al., 1976; Lawrence, 1977; Bahn et al., 1977).

It is not known which components of these PCB mixtures are carcinogenic, nor whether these components require metabolic activation in order to initiate carcinogenesis.

No studies have yet appeared on the effects of PBBs on the carcinogenicity of secondary agents. Several such studies have been reported with PCBs, the results of which are presumed to reflect altered levels of microsomal drug metabolizing enzymes.

Uchiyama et al. (1974) surgically implanted MC-impregnated threads into the uteri of mice. Dietary PCBs (Kanechlor 400) had no effect on the MC-induced changes in the cervical epithelium, although dietary DDT (a microsomal inducer) increased the severity and frequency of precancerous and cancerous tissue changes.

Ito et al. (1973) fed male mice 250 ppm of a PCB mixture averaging five chlorines per molecule (Kanechlor 500) for twenty-four weeks. Although twice this dose for thirty-six weeks caused nodular hyperplasias and well-differentiated hepatocellular carcinomas, neither of these neoplasms were observed with this regimen. The β isomer of 1,2,3,4,5,6-hexachlorocyclohexane (HCCH) was fed simultaneously with PCBs at levels up to 250 ppm, and while β-HCCH caused no effects by itself at any dose tested, mice fed both PCBs and this compound had a significant incidence of both nodular hyperplasia and hepatocellular carcinoma. The α isomer alone was carcinogenic at high dietary levels, and simultaneous feeding of PCBs with α-HCCH at this level increased the incidence of carcinomas. Nodules were not seen at lower doses of α-HCCH unless PCBs were fed simultaneously.

PCBs have also been shown to decrease the carcinogenic effects of compounds. Makiura et al. (1974) investigated the effects of dietary PCBs (Kanechlor 500) on the incidence of carcinogenesis caused by the hepatic carcinogens 3'-methyl-4-dimethylaminoazobenzene, N-2-fluorenylacetamide, and diethylnitrosamine. While hepatocellular carcinomas

developed in a majority of rats given these three compounds, either alone or in pairs, rats fed PCBs in addition to these carcinogens developed only a few tumors. PCB treatment alone (500 ppm for twenty weeks) caused no liver tumors. PCBs also protected against the development of nodular hyperplasias, oval cell infiltration, and bile duct proliferation.

Induction of Microsomal Drug Metabolizing Enzymes by PBBs

With one very recent exception (Poland and Glover, 1977), all previous research concerning the effects of brominated biphenyls on microsomal drug metabolizing enzymes has been conducted using mixtures of PBBs, which are known to contain brominated naphthalenes (Kay, 1977) and possibly also brominated dibenzofurans, in addition to a large number of brominated biphenyl congeners. While this research has provided much useful information concerning microsomal induction, it provides no clues as to which of the components induce microsomal enzymes, or of the nature of the inductions caused by the individual components. Research on the effects of pure brominated biphenyl congeners will be presented in Chapter 3.

It appears that Farber and Baker (1974) were the first to demonstrate microsomal induction by what they called "hexabromobiphenyl." Since this research has only appeared in abstract form, it cannot be determined exactly what they studied, but it was probably the Firemaster mixture of PBBs. Rats were fed 0, 0.5, 5, 50, or 500 ppm "hexabromobiphenyl" for thirty days. Liver weights, microsomal protein, NADPH-cytochrome P450 reductase, cytochrome P450, and two of the Pb-inducible cytochrome P450-catalyzed drug metabolism activities were induced. By

comparison with a PCB mixture averaging five chlorines per molecule, it was concluded that "hexabromobiphenyl" was at least five times as potent as PCBs as a microsomal inducer.

Cecil et al. (1975) gave Japanese quail a single oral dose of PBBs, at 100 mg/kg body weight, and determined pentobarbital sleeping times at intervals thereafter. Pentobarbital is inactivated by hydroxylation, a reaction catalyzed by one or more Pb-inducible enzymes, so what they were studying was the extent of the Pb-type induction caused by PBBs. Pentobarbital sleeping times were found to be markedly reduced.

Babish et al. (1975) fed Japanese quail 0, 10, 20, or 100 ppm PBBs for nine weeks. Cytochrome P450, aminopyrine demethylation (Pb-inducible), and three activities inducible by both Pb and MC were all elevated, some by as little as 10 ppm PBBs.

Babish et al. (1976) and Farber et al. (1976) have published abstracts showing that PBBs can cause at least partial microsomal inductions in rats and dogs, respectively. Corbett et al. (1975) noted that PBBs induced cytochrome P450 and a 55,000 dalton hemoprotein in mouse liver, and Sleight and Sanger (1976) found that as little as 1 ppm PBBs in the diet of rats could induce microsomal drug metabolizing enzymes.

The first demonstration that PBBs are a mixed-type inducer was carried out in Dr. Aust's laboratory in 1975. Both a single 90 mg/kg i.p. injection and a diet of 10 ppm PBBs caused major increases in liver weight, microsomal protein, NADPH-cytochrome P450 reductase, cytochrome P450, aminopyrine demethylation, and benzo[α]pyrene hydroxylation, all within a week. PBBs caused greater increases in liver weights, microsomal protein, and cytochrome P450 than did either MC or Pb when either was given alone at a maximally effective dose. Induction

by PBBs was distinct from that caused by either MC or Pb alone, but was quite similar to simultaneous injections of both prototype compounds, thereby demonstrating that PBBs cause a mixed-type induction. A single injection of 90 mg PBBs/kg was as effective as five daily injections of this magnitude, and inductions were still pronounced ten days after a single injection (the effects of MC or Pb were more transient) (Troisi, 1975). Subsequent analyses of these microsomes (unpublished) demonstrated that *p*-nitrophenol-UDP-glucuronyltransferase was induced (an index of MC-type induction), and that hemoproteins at 53,000 daltons (MC-type) and especially 45,000 daltons (Pb-type) were induced by PBBs. Complement fixation assays, using an antibody prepared against the 45,000 dalton hemoprotein, showed that this hemoprotein was induced to comparable extents by both Pb and PBBs.

Dent, Gibson, and co-workers have characterized many aspects of the microsomal induction caused by PBBs, in liver as well as in kidney and mammary gland, and a review of this work and research in other laboratories is in press (Dent, 1978).

The initial experiment (Dent et al., 1976a) was a dietary dose-response investigation, in which PBBs were shown to be potent inducers of hepatic microsomal drug metabolizing enzymes. The pattern of induction included the characteristics of microsomes induced both by Pb and by MC. Enzymes assayed included the Pb-responsive NADPH-cytochrome P450 reductase, epoxide hydratase, and ethylmorphine demethylation, as well as the MC-responsive ethoxycoumarin-O-deethylation and benzo[α]-pyrene hydroxylation, all of which were strongly induced. Cytochrome P450 was also induced, and λ_{max} in its reduced carbon monoxide difference spectrum was lowered by 1.4 nm.

Dent et al. (1976b) also investigated the effects of a single i.p. injection of PBBs, at either 25 or 150 mg/kg body weight. The rats were killed at various times thereafter, and their microsomes were compared with the liver microsomes isolated from rats pretreated with MC, Pb, or MC plus Pb. The assays listed above were performed, with essentially the same results. PBB-pretreated and MC plus Pb-pretreated rats both had mixed-type inductions of their hepatic microsomal drug metabolizing enzymes.

McCormack et al. (1977) fed 100 ppm PBBs to rats for three months, then analyzed hepatic and renal drug metabolizing enzymes and renal function. Enzyme activities responsive to both MC and Pb (biphenyl 4-hydroxylation) and to MC alone (biphenyl 2-hydroxylation and benzo-[α]pyrene hydroxylation) were induced in both liver and kidney. One interesting result was that while hepatic epoxide hydratase, a Pb-responsive enzyme, was induced, the renal activity was decreased to 14% of the control values. Since epoxide hydratase is normally considered beneficial, in that it converts reactive epoxides formed by the cytochrome P450s into more stable dihydrodiols, it was postulated that this combination of enzyme activities may increase the susceptibility of the kidney to toxicological damage caused by a second agent. Renal function was not affected by the PBB treatment, however.

The composition of microsomal drug metabolizing enzymes varies with age, and neonates typically have much lower levels of these enzymes than do adults (Conney, 1967; Parke, 1975). McCormack et al. (1978) therefore investigated the effects of a 150 mg/kg i.p. injection of PBBs on the hepatic and renal drug metabolizing enzymes in neonatal rats. The rats were examined at intervals up to sixty-three days

later, at which time they were seventy days old. In liver, peak activities of ethoxyresorufin-O-deethylation, biphenyl-2-hydroxylation, and benzo[α]pyrene hydroxylation were reached before the peak activities of hexobarbital hydroxylation, glutathione S-epoxide transferase, and epoxide hydratase were attained, indicating that the MC-like aspects of the mixed-type induction were fully expressed somewhat more rapidly than were the Pb-like aspects. Pb-responsive renal activities were either non-detectable (hexobarbital and biphenyl hydroxylation) or not induced (epoxide hydratase), while both benzo[α]pyrene hydroxylation and ethoxyresorufin O-deethylation were strongly induced.

Dent et al. (1977a) examined the effects of PBBs on drug metabolism in liver and mammary gland in lactating rats. The animals were fed 50 ppm PBBs from day 8 of gestation through day 14 postpartum, and PBBs increased liver weights, microsomal proteins, benzo[α]pyrene hydroxylation, and epoxide hydratase. Mammary benzo[α]pyrene hydroxylation was increased, while epoxide hydratase was reduced by 50%. This combination of activities has also been observed in the kidney (McCormack et al., 1977), and could play a role in the toxicity of xenobiotics being secreted into milk, since the enzymes forming epoxides are elevated and the enzyme to cleave them is depressed. The pups from this study were cross fostered in order to examine the effects of prepartum and postpartum exposure to PBBs (Dent et al., 1977b). Benzo[α]pyrene hydroxylation and epoxide hydratase were induced in pups exposed in utero only, as well as in pups exposed only during lactation. Pups exposed both transplacentally and by nursing were more strongly induced than were pups exposed by either of these routes alone.

Another investigation of drug metabolizing enzymes in PBB-fed lactating rats and their nursing pups has been carried out by Moore et al., 1976, 1978). Lactating rats were fed 0, 0.1, 1.0, or 10. ppm PBBs for the eighteen days following delivery, at which time mothers and pups were sacrificed. Pups nursing from mothers fed 10 ppm PBBs showed significant increases in liver weights and microsomal protein, and both mothers and pups had increased cytochrome P450, aminopyrine demethylation, benzo[α]pyrene hydroxylation, and *p*-nitrophenol-UDP-glucuronyltransferase. Pups nursing from mothers fed 1.0 ppm had increases in microsomal protein, cytochrome P450, aminopyrine demethylation, and benzo[α]pyrene hydroxylation, while their mothers were unaffected. PBBs caused a mixed-type induction in both the lactating rats and their nursing pups, therefore, components of the PBB mixture responsible for both the MC- and Pb-like aspects of the induction must be transmitted through milk. The neonates were approximately ten-fold more sensitive to the effects of PBBs in their mothers' diets as were the dams. The approximate no-effect level for microsomal induction in nursing rat pups was 0.1 ppm PBBs in the diet of the adult.

Dent et al. (1977c) have demonstrated that PBBs cause a mixed-type induction of liver microsomal drug metabolizing enzymes in mice as well as rats. Ethylmorphine demethylation and epoxide hydratase peaked forty-eight hours after a 150 mg/kg i.p. injection of PBBs, while ethoxycoumarin O-deethylation and benzo[α]pyrene hydroxylation peaked ninety-six hours after injection. These activities, plus the ethyl isocyanide difference spectra, showed the Pb-like induction to occur more rapidly in mouse hepatic microsomes than did the maximal MC-like induction. This shift was also verified by following the toxicity of

bromobenzene at various time points after PBB administration (Roes et al., 1977). In mice, Pb decreases the median time to death following an injection of bromobenzene moreso than does MC, and MC also alters the slope of the time-lethality curve. When bromobenzene was injected at various times after PBBs were given, the responses to it went first from the Pb-type reaction to the MC-type response.

In order to better characterize the nature of the PBB-inducible cytochrome P450 hemoproteins, Dent et al. (1977d) examined the ethyl isocyanide spectral properties, reaction kinetics, inhibitor effects, and SDS-polyacrylamide gel electrophoretic profiles of liver microsomes induced by treating female rats with 150 mg PBBs/kg. Evidence was obtained for both a shift from Pb- to MC-, and from MC- to Pb-type inductions. Also, a 58,000 dalton hemoprotein unique to MC-induced microsomes was observed. This hemoprotein was found in the microsomes of rats pretreated with MC plus Pb, but not in PBB-induced microsomes, and so it was concluded that PBBs have some but not all of the properties of a mixed-type inducing agent. This hemoprotein appears to be absent from male rats, however, regardless of whether or not the animals were pretreated with microsomal inducing agents (Welton and Aust, 1974b).

Conclusions

The papers cited above all agree, or are consistent with the idea, that PBBs cause a mixed-type induction of rat (and mouse) liver microsomal drug metabolizing enzymes. PBBs, however, are a mixture of at least thirty different chemicals, and these papers provide no information as to which PBB components are responsible for or are capable of causing the induction. Indeed, with one very recent exception to be

discussed in Chapter 3, no biological effects of any sort have been shown to result from any individual brominated biphenyl.

In order to begin to understand the biological effects of brominated biphenyls, and to better understand the effects of PBB mixtures, brominated biphenyls were purified from two crude PBB mixtures, and the structures of a number of the congeners were determined. These results are presented in Chapter 1. The results of experiments designed to examine the covalent binding of the two major PBB components to macromolecules are presented in Chapter 2. Chapter 3 represents the beginning of experiments designed to elucidate the biological effects of pure brominated biphenyls. The effects of three pure congeners on rat liver microsomal drug metabolizing enzymes were determined.

CHAPTER 1

PURIFICATION AND CHARACTERIZATION OF POLYBROMINATED BIPHENYL CONGENERS

ABSTRACT

Polybrominated biphenyls (PBBs) are complex mixtures which can contain thirty or more components. Sundström et al. (1976a) and Jacobs et al. (1976) have shown the major congener of the Firemaster mixture of PBBs to be 2,2',4,4',5,5'-hexabromobiphenyl, however, no other structures have been published. The molecular weights of the more prominent components of two different PBB mixtures have been determined by gas chromatography-mass spectrometry. A number of congeners have been purified from these mixtures by chromatography and recrystallization, and characterized by 'H-NMR spectroscopy. When possible, the ^{13}C-NMR spectra, infrared spectra, and melting points of these congeners were also determined. By these analyses, the structures of eight additional polybrominated biphenyl congeners have been determined, these are: 2,2',4,5,5'-penta-, 2,3',4,4',5-penta-, 2,2',3,4,4',5'-hexa-, 2,3',4,4',5,5'-hexa-, 2,2',3,4,4',5,5'-hepta, 2,2',3,3',4,4',5,5'-octa-, 2,2',3,3',4,4',5,5',6-nona-, and 2,2',3,3',4,4',5,5',6,6'-decabromobiphenyl.

INTRODUCTION

Polybrominated biphenyls (PBBs) were industrially synthesized for use as flame retardants. Although the exact procedures used in the manufacturing process have not been published, the reaction probably

involves the addition of molecular bromine to biphenyl in the presence of a catalyst such as $FeCl_3$, heat, and/or pressure. Depending upon the choice of reaction conditions, different compositions can be obtained in these mixtures, and two such mixtures have been utilized in these investigations. One was the product Firemaster, which contaminated much of the Michigan food chain beginning in 1973 (Carter, 1976), and which averages six bromines per molecule. The other mixture is of an unknown origin, although it has a gas chromatographic profile very similar to that reported by Zitko (1977) for "octabromobiphenyl" (OBB), manufactured by Dow Chemical.

More than thirty components can be seen when Firemaster is fractionated by column chromatography and assayed by gas chromatography. OBB is much simpler, although at least eleven components may likewise be visualized. Different biological consequences may be expected to result from exposure to the components of these and similar mixtures, and while much useful information can be gained by studying the effects of PBB mixtures on biological systems, an understanding of these effects at the molecular level requires studies with the individual purified compounds. Differences in the biological fates of PBB components may also be expected to occur, as indeed has been shown by Dannan (1978).

PBBs have not been well characterized chemically. The molecular weights of the more prominent congeners have been published (Fries and Marrow, 1975; Jacobs et al., 1976; Zitko, 1977). The structure of the major component of Firemaster has been identified as 2,2',4,4',5,5'-hexabromobiphenyl (Sundström et al., 1976a; Jacobs et al., 1976), and brominated naphthalenes and a methyl brominated furan have also been found in Firemaster (Kay, 1977). Sundström et al. (1976b) have also

compiled chemical information about a large number of brominated biphenyls having four or fewer bromines.

Because of the importance of determining the biological effects of pure PBB components, and of elucidating the structure-function relationships for both the pharmacokinetics and pharmacodynamics of these components, it was decided to attempt to purify and structurally identify as many of them as possible. The results of these chemical characterizations are presented in this Chapter.

MATERIALS AND METHODS

Materials

Glass-distilled chloroform, ethyl acetate, acetonitrile, and nonspectro hexane, all suitable for pesticide analysis, were purchased from Burdick and Jackson Laboratories, Muskegon, Michigan. Hexane was purified before use by passing it over a column of basic alumina. Neutral and basic alumina were obtained from Sigma Chemical Co., St. Louis, Missouri. Deuterochloroform, 99.8 atom % D, tetramethylsilane, and OBB were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. 2,2'-Dibromobiphenyl was obtained from K and K Rare and Fine Chemicals, Plainview, New York. Deuterobenzene, 99.5 atom % D, was from ICN, Irvine, California. The Firemaster PBB mixture was manufactured by the Michigan Chemical Corp., St. Louis, Michigan. The sample was obtained from a feed mixing plant shortly after the accidental contamination of the Michigan food chain was discovered, and is most probably Firemaster FF-1, lot 7042. For the purification of several brominated biphenyls, Firemaster BP-6, lot 6244A, was used as the starting material. The

column packing for gas chromatography, 3% OV-1 on Gas Chrom Q, was purchased from Supelco, Bellefonte, Pennsylvania. The carrier and purge gas, 95% argon, 5% methane, was from Matheson Gas Products, Joliet, Illinois.

Gas Chromatography

PBBs were assayed with a Hewlett Packard Model 402 gas chromatograph equipped with a pulsed ^{63}Ni electron capture detector. The column, 3% OV-1 on Gas Chrom Q, 100-120 mesh, was maintained at 270°, using 95% argon-5% methane for both carrier and purge gas. The normal settings used were range = 10 and attenuation = 8.

Gas Chromatography-Mass Spectrometry

An LKB Type 9000 gas chromatograph-mass spectrometer was used to determine the molecular weights of the PBB components. Source temperature was 290°, a 3500 volt accelerating voltage was used, and the column was 3% OV-1. I thank Joelle André of the Michigan State University Mass Spectrometry Facility for these analyses.

Determination of Spectra

180 MHz ^1H-NMR spectra were obtained on a Bruker WP 180 spectrometer at ambient temperature. Dilute solutions (less than 3% w/v) were prepared in $CDCl_3$, and chemical shifts are reported in ppm relative to the internal standard tetramethylsilane. I thank the Michigan State University Chemistry Department and Frank J. Bennis for these spectra.

15.08 MHz ^{13}C-NMR spectra were determined at ambient temperature on a Bruker WP 60 spectrometer, equipped with quadrature detection. Both $CDCl_3$ (for peaks 6, 8, and 12) and deuterobenzene (for peak 12)

were used as solvents; all solutions were saturated. (See Figure 1 for the numbering system.) Chemical shifts are reported in ppm relative to internal tetramethylsilane. I am grateful to John O'Conner and John Pierce for determining these spectra.

Infrared spectra were taken as KBr pellets on a Perkin-Elmer 167 grating infrared spectrophotometer.

Other Determinations

Melting points were determined with a Hoover Unimelt capillary melting point apparatus.

Estimates of the percent purity of congeners were made assuming that the electron capture detector on the gas chromatograph responded with equal intensity to each congener. Values obtained by this method were in good agreement with those estimated by the 'H-NMR spectra.

The percentage compositions of peaks 4 and 8 in Firemaster and OBB were determined by gas chromatographic analyses of standard (w/v) solutions.

Fractionation of Firemaster and OBB Mixtures - General Procedures

Chromatography on alumina in the presence of hexane was the only chromatographic method found to fractionate the PBB congeners. A large number of other column packings and solvents were tried, without success. These included: silicic acid with hexane, Florisil with hexane, silanized silica gel with acetone, hexane, acetonitrile and 95% ethanol, Sephadex LH-20 with ethyl acetate:methanol (4:1), acetonitrile, and hexane, activity grade IV (hydrated) alumina with hexane, $AgNO_3$ impregnated alumina with hexane, paraffin oil-saturated alumina with paraffin oil-saturated methanol, Bio-Beads S-X2 (BioRad, neutral

porous styrene-divinylbenzene copolymer beads) with hexane, acetonitrile, and methanol, Celite with hexane, and phenyl derivatized silica gel with methanol. In all cases, all congeners eluted with or very close to the void volume, with no evidence of separation of congeners. Vacuum distillation met with only limited success as a fractionation procedure. Recrystallization of partially purified congeners was at times a very selective purification technique.

Exact procedures for preparing the individual bromobiphenyls will be given below, following a general discussion of the protocol used. Most separations were performed in a 2.2x35 cm glass column fitted with a fritted glass bottom and a Teflon stopcock. The column was filled with hexane, then typically 110-115 g of alumina powder was added while the column was drained to prevent overflowing and to minimize sticking of the alumina to the top of the column. Excess hexane was drained, then the sample was applied in a minimum volume of hexane (about 40 ml/g of Firemaster, and 400 ml/g of OBB). The samples were washed into the column with several small volumes of hexane before the reservoir was filled. Basic, neutral, and acidic alumina all gave comparable results, the only difference being in the retention volumes, which decreased with decreasing pH. Neutral alumina was used for all purifications described in this Chapter. Hydrated alumina did not retain PBBs.

The order of elution of Firemaster congeners was as follows (see Figure 1 for nomenclature): trace quantities of relatively volatile components including (probably) 2,2'-dibromobiphenyl (see Chapter 3), nearly all the peak 4, then peaks 1, 6, 7, and 8, 6a immediately followed by 2, then 5 and 3, then 10, 11, and 12. Peak 9 and a number of

additional unidentified components, plus substantial quantities of peaks 10, 11 and 12, could then be eluted with a more polar solvent, such as ethyl acetate, chloroform, or acetonitrile. Mixtures of compounds eluted from alumina columns with these solvents are referred to as the polar fractions. When OBB was chromatographed on alumina, the order of elution was peaks 4, 8, 6a, 7, 11, 12, 10, 13, and 14. Components of these mixtures having identical gas chromatographic retention times also had indistinguishable column chromatographic properties and were therefore assumed to be identical.

All peaks visible by gas chromatographic analyses of Firemaster or OBB behaved as single components during column chromatography. A large number of trace components were revealed in the course of various purifications which had retention times similar but not identical to those of the major congeners.

One property encountered during column chromatography was that retention volumes were dependent upon the quantity of sample applied. For example, a small number of fractions containing only peaks 1 and 4 can be obtained once about 90% of the peak 4 has eluted from alumina, although the peak 1 is only about 20% pure. But when the partially purified peak 1 preparation is rechromatographed, the peak 4 displays a much longer elution volume, and only a small portion of it elutes before peak 1. This change in relative elution volumes was encountered a number of times with other partially purified fractions, and it severely limited the possible purifications of a number of the congeners.

Column fractions were assayed by gas chromatography. Except when very small amounts of bromobiphenyls were being chromatographed, or when more than about 500 fractions (about 20 ml each) were collected,

one µl injections were usually sufficient to monitor the progress of the column chromatographic separations.

Fractions were pooled and concentrated using a rotary evaporator. While the solvents used did not bump, bromobiphenyls could usually be found in the trap, indicating that a small amount of co-distillation could take place. Because a dibromobiphenyl could be found in the distilled solvent, solvents were not reused.

Samples for recrystallization were dissolved in hexane with continuous swirling in round bottom flasks, using an electric heating mantle as the source of heat. Samples were always filtered through a Pasteur pipet tightly plugged with glass wool before beginning recrystallizations in order to remove particulate matter. Unless the volume of solvent was very small, recrystallizations were allowed to proceed overnight at room temperature. Flasks were then placed in an ice bath for several hours, followed by fifteen minutes in a dry ice-acetone bath. Mother liquors were then removed with a Pasteur pipet.

Purification of Individual Bromobiphenyl Congeners

Peak 1 was purified 20-30 fold, by two different routes. One preparation was the first mother liquor from the peak 6 purification (see below), a mixture which contained approximately 35-40% peak 1, as shown in the insert in Figure 2. This is designated peaks 1 plus 6, while the second is designated as peaks 1 plus 4. The second preparation contained approximately 60% peak 1, with peak 4 as the predominant contaminant, as shown in the insert in Figure 3, and was prepared as follows. One gram of Firemaster was applied to each of two alumina columns. Fractions containing peaks 1, 4, and 6 only were pooled and

reapplied to a third column of alumina. The final preparation consisted of the peak 1-containing fractions which eluted before peak 6 began to emerge from the column.

Peak 2 was purified approximately ten-fold. The starting material was one gram of Firemaster from which most of peaks 4, 1, and 6 had been removed by alumina chromatography. This was further purified by three additional columns until a final purity of 35-40% was achieved (see Figure 4, insert).

Peak 4 could easily be isolated in large quantities. One gram of Firemaster was applied to an alumina column. Trace quantities of material with short gas chromatographic retention times eluted, followed by most of the peak 4. The peak 4 fractions were pooled and recrystallized twice from hexane. More than 450 mg of 99.9% pure crystals could be obtained from one gram of Firemaster.

Peak 5 was obtained in approximately 80% purity. Fractions containing the highest concentrations of peak 5 (about 10% by weight of the starting material) were pooled from the first two columns used to purify the peak 1 plus 4 preparation, then rechromatographed on alumina. While this third column had relatively little effect on the purity, it did remove all the contaminating peak 3, and increased the purity of peak 5 by about 10%. This peak 5 preparation was the only one which behaved as an oil, and attempts to recrystallize it from several solvents were unsuccessful.

Peak 6 was prepared from Firemaster to about 95% purity. After the fractions from the last column used for the final peak 1 plus 4 preparation eluted, the column was washed with chloroform and acetonitrile. The remaining fractions, which contained all the peak 6, were

then applied to another alumina column. Peaks 1 and 4 eluted first, then the column was washed with chloroform and acetonitrile and all fractions containing peak 6 were combined. This preparation was then recrystallized twice, to remove peak 4 and most of the peak 1 (the first mother liquor was the peak 1 plus 6 preparation).

Peak 8 and all subsequent peaks were purified from OBB rather than from Firemaster. One gram of OBB was dissolved in hexane and applied to an alumina column. The most concentrated fractions of peak 8, which were also contaminated with peaks 4, 11, and 12, were pooled and reapplied to a second column. Peak 8-containing fractions which eluted before peaks 11 and 12 did were pooled and recrystallized from hexane. About 150 mg of 98% pure crystals were obtained per gram of OBB.

The first column used to purify peak 8 was also used to purify peak 11. Fractions containing the greatest concentration of peak 11, but with only small amounts of peak 13, were pooled and applied to another alumina column. Once most of the peak 8 eluted, all fractions before peak 10 began to emerge were pooled and recrystallized. The first mother liquor was enriched in peak 11, and was in turn recrystallized. The second mother liquor contained peak 11 in about 80% purity.

Peak 12 was purified by a series of columns and recrystallizations. The starting material was two grams of OBB from which most of the peak 8 (and 4) had been removed by chromatography on two alumina columns. This was applied to a third column, and fractions containing peaks 10, 11, and 12 (and some 8) were pooled and recrystallized. The mother liquor contained most of the peaks 10 and 11, while the crystals were mostly peaks 8 and 12. The crystals were dissolved in hexane, then applied to a fourth column. Peak 8 eluted first, then chloroform was

applied, and all fractions containing peak 12 were pooled. A fifth column identical to the fourth was then run, resulting in peak 12 at a purity of about 90%. Peaks 10 and 11 were easily removed by recrystallization, but peak 8 tended to co-crystallize with peak 12, and three recrystallizations were required to raise the purity of peak 12 to 98%.

The preparation of peak 13 began with the third column used to purify peak 12. After most of the peaks 8, 10, 11, and 12 had eluted from this column, chloroform was applied to elute all remaining congeners. The chloroform fractions contained much of the original peak 13 (about 60% pure), and all of the peak 14, plus other contaminants. They were reapplied to a fourth column, and all fractions containing peak 13 but not 14 were pooled and recrystallized four times. A final purity of 95% was achieved.

Peak 14 was obtained from the final column used to purify peak 13. After peak 14 began to elute, a number of fractions containing most of the remaining peaks 10, 11, 12, and 13 were eluted. Peak 14 was then obtained at 30% purity by washing the column with chloroform.

Nomenclature

Rules for naming halogenated biphenyls are as presented by Hutzinger *et al.* (1974).

1. An unprimed locant is of lower order (i.e., preferred) than its corresponding primed locant. For example, 2 is lower than 2', but 2' is lower than 3.

2. Locants as low as possible should be given to all substituents, ignoring primes at this stage.

3. As few primed locants as possible should be used.

4. When the number of halogen substituents in each ring is the same, the ring with the lower numbered substituents receives the unprimed numbers. If everything else is equal, the locant cited first is unprimed.

RESULTS

The gas chromatographic profiles of Firemaster and OBB are shown in Figure 1. The isomeric compositions of these mixtures had previously been partially determined by gas chromatography-mass spectrometry in three laboratories. Fries and Marrow (1975) identified Firemaster peak 4 as a hexabromobiphenyl, and stated that peak 8 was a mixture of hepta- and octabromobiphenyl. Jacobs et al. (1976) identified Firemaster peaks 1 and 2 as pentabromobiphenyls, peaks 3, 4, 5, and 6 as hexabromobiphenyls, and peaks 7 and 8 as heptabromobiphenyls. Zitko (1977) identified OBB peak 8 as a heptabromobiphenyl, the 10 and 11 region and peak 12 as octabromobiphenyls, and peak 13 as a nonabromobiphenyl.

The results of gas chromatographic-mass spectrometric analyses of Firemaster, partially purified Firemaster fractions, and OBB are presented in Table 1. Resolution improved considerably with each run and was excellent for the last several samples, but the initial poor resolution limited the number of positive assignments which could be made with the Firemaster and OBB mixtures. Partially purified samples allowed the unambiguous assignment of molecular weights to several

Figure 1. GAS CHROMATOGRAPHIC PROFILES AND NUMBERING SYSTEM FOR THE COMPONENTS OF POLYBROMINATED BIPHENYL MIXTURES

The sample of Firemaster contained 400 ng PBBs, while the "octabromobiphenyl" sample contained 800 ng.

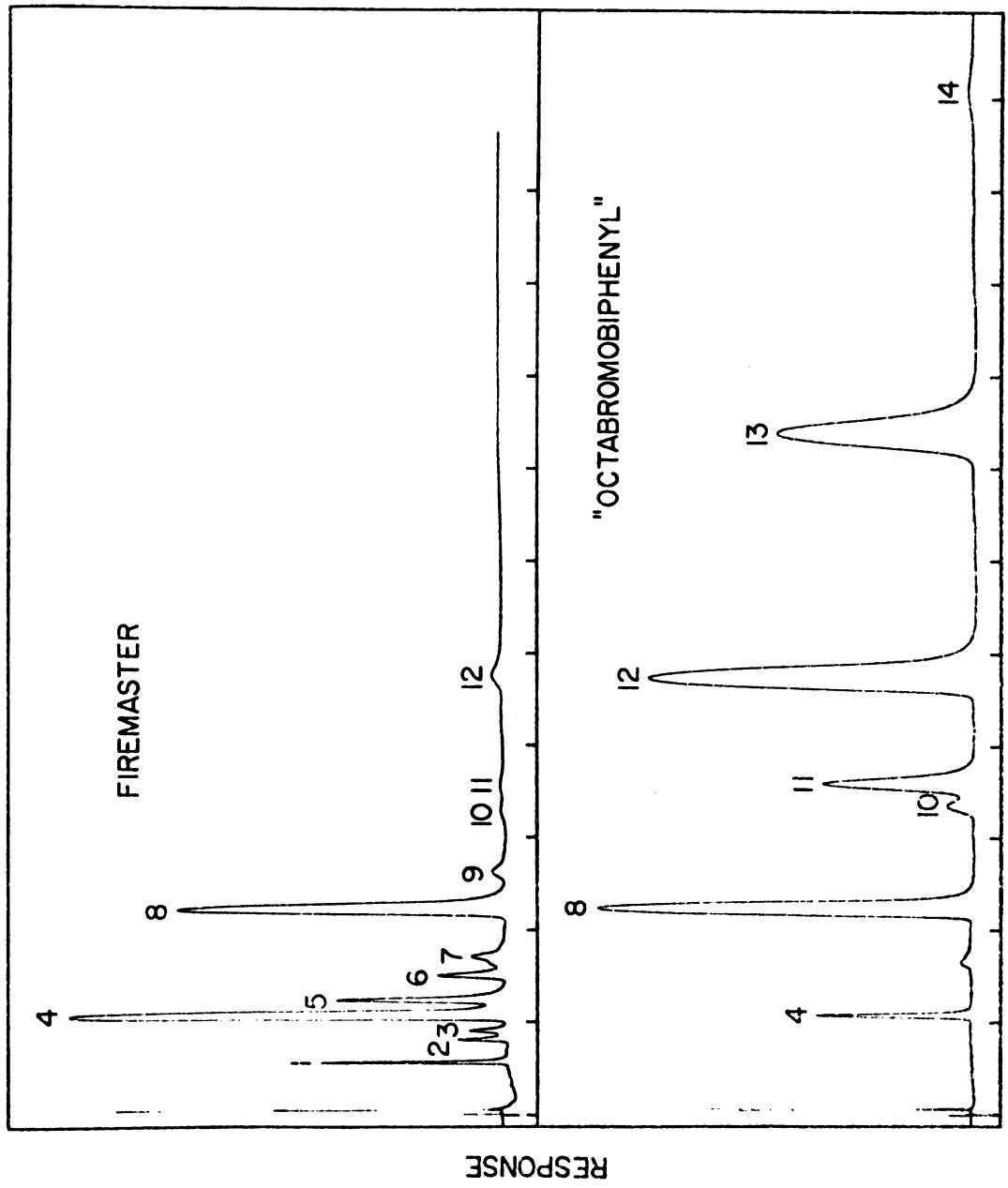

FIREMASTER
"OCTABROMOBIPHENYL"
RESPONSE
RETENTION TIME (min.)
0
6
12
18
24
30
36
42
48
54
60
66
2
3
4
5
6
7
8
9
10
11
12
13
14

Figure 1

Table 1

DETERMINATION OF THE NUMBER OF BROMINES PRESENT IN POLYBROMINATED BIPHENYL CONGENERS

	Peak Number													
Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Firemaster	5	5		6		6		7				8		
OBB				6				7				8	9	
Partially purified peak 14													9	10
Partially purified peak 3*		5	6		6			7						
Firemaster polar fraction									7	7	8	8		

*This preparation contained no peak 4 and therefore allowed the molecular weights of peaks 3 and 5 to be unambiguously determined.

congeners with very similar gas chromatographic retention times or which were present in only small concentrations in the mixtures.

The only disagreement with the literature was the finding that peak 8 contained no octabromobiphenyl. Peak 6 was shown to contain six bromines by taking the molecular weight scan at the leading edge of the peak. The molecular weight of peak 7, and of the shoulder between peaks 6 and 7, could not be unambiguously determined. Heptabromobiphenyl was clearly present in one or both of these peaks, but a hexabromobiphenyl may also be present.

These analyses have resulted in four new molecular weight assignments. While Zitko (1977) had previously shown the peak 10 and 11 region to contain an octabromobiphenyl, it was shown here that peak 10 is a heptabromobiphenyl and peak 11 is an octabromobiphenyl. Peak 9 was shown to be a heptabromobiphenyl, and peak 14 was found to contain ten bromines. Since the maximum number of bromines a biphenyl can contain is ten, these analyses identify the structure of peak 14 to be 2,2',3,3',4,4',5,5',6,6'-decabromobiphenyl. Other structural analyses cannot be made based on the fragmentation patterns of halogenated biphenyls because of rearrangement during fragmentation (Sundström et al., 1976a).

Characterization of Peak 1

Peak 1 is a pentabromobiphenyl, of which forty-six possible isomers can exist. Although it could not be completely purified, peak 1 was obtained in highly enriched form in two different mixtures, whose gas chromatographic profiles are shown in the inserts in Figures 2 and 3. Fortunately, since the NMR spectra of the two major contaminants,

peaks 6 and 4 respectively, are known, it was possible to clearly assign NMR signals to peak 1, and in this way the structure of the molecule was determined. As shown in Figure 2, a complicated 'H-NMR spectrum was obtained from the peak 1 plus 6 mixture. Peak 6 gives 'H-NMR signals at δ = 7.93 (1H), 7.59 (2H) and 7.54 (1H) ppm, however, in this spectrum, the integrals for these areas are nearly 2:2:1 rather than 1:2:1, so there must be one proton at δ = 7.93 ppm from peak 1. The other signals must all arise from peak 1. In addition to the signal at δ = 7.93 ppm, there is another unsplit signal at 7.49 ppm of equal magnitude. While the remaining signals were not electronically integrated, the 1:1 ratio of peak areas appears to hold.

Unsplit signals on a halogenated biphenyl can only result from two types of structures. The proton can be the lone proton on one ring (at any position), or two protons can exist <u>para</u> to each other on one ring (necessarily in the positions <u>ortho</u> and <u>meta</u> to the bridge carbon). Since there are five protons in this molecule it is impossible for these signals to arise from lone protons on each ring, therefore, one ring must contain two protons <u>para</u> to each other. This ring must be brominated at the 2, 4, and 5 positions. The bromination of benzene deshields the <u>ortho</u> protons by 0.19 ppm (Williams and Fleming, 1973), and since the proton at the 3 position is adjacent to two bromines, it must give rise to the singlet at 7.93 ppm. The proton at the 6 position is less deshielded, being adjacent to only one bromine, and causes the singlet at 7.49 ppm. The quartet centered at δ = 7.53 ppm shows <u>ortho</u> and <u>para</u> splitting, the quartet at 7.41 is split <u>ortho</u> and <u>meta</u>, and the quartet centered at 7.36 ppm is split <u>meta</u> and <u>para</u>. Based on these splitting patterns, only three structures for the second

Figure 2. ^{1}H-NMR SPECTRUM AND STRUCTURE OF 2,2',4,5,5'-PENTABROMOBIPHENYL (PEAK 1)

The gas chromatographic profile of this partially purified preparation is shown in the insert. Only the values of the signals due solely or partially to peak 1 are noted.

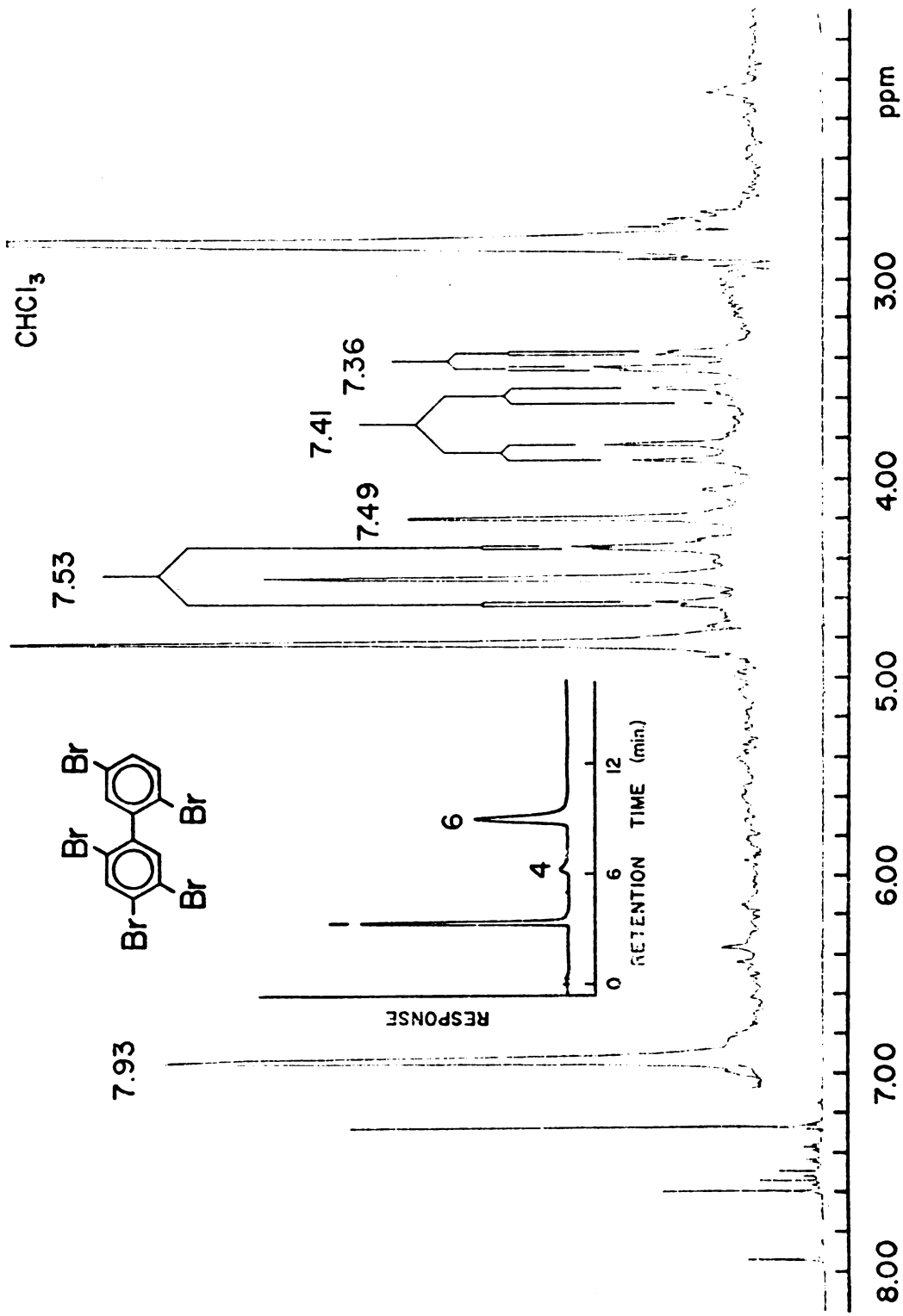

CHCl3
7.53
7.49
7.41
7.36
7.93
8.00
7.00
6.00
5.00
4.00
3.00
ppm
Br
Br
Br
Br
Br
Br
RESPONSE
1
4
6
0
6
12
RETENTION TIME (min)

Figure 2

ring are possible. If the bromines are at the 2 and 4 positions, the signal split para and ortho would have to occupy the 6 position, but δ = 7.53 ppm is far too downfield for an ortho proton adjacent to another unsubstituted carbon. If the bromines are at the 3 and 4 positions, the signal split ortho and meta would have to occupy the 6 position and be adjacent to an unsubstituted carbon. The shift of 7.41 ppm is again too far downfield for such a proton between a bridge carbon and an unsubstituted carbon. As will be shown, this is actually the structure of peak 2. The third posssibility is for bromines to occupy the 2 and 5 positions, which results in very reasonable chemical shift values for the three protons on this ring. The protons at the 3, 4, and 6 positions have 'H-NMR signals centered at δ = 7.53, 7.41, and 7.36 ppm, respectively. The ortho, meta, and para coupling constants are 8.4, 2.4, and 0.4 Hz, respectively, in agreement with the coupling constants of a wide variety of halogenated aromatic compounds.

Analysis of the 'H-NMR spectrum of the peak 1 plus 4 preparation (Figure 3) permits the same conclusions to be drawn. The signals at δ = 7.59 and 7.54 ppm are absent because peak 6 is absent. The peak 4 signals are at 7.47 and 7.93 ppm (Sundström et al., 1976a), and several other smaller signals which are absent from the peak 1 plus 6 NMR spectrum can be seen to arise from one or more compounds obscured by peak 4 in the gas chromatographic analysis. When the peak 4 contribution is subtracted from the integral of the signal at 7.93 ppm, an excellent ratio of unity is obtained for the integrated areas of the signals from all five of the peak 1 protons. From both the peak 1 plus 4 and 1 plus 6 preparations, the structure of peak 1 is determined to be 2,2',4,5,5'-pentabromobiphenyl, as shown in Figures 2 and 3.

Figure 3. ^{1}H-NMR SPECTRUM AND STRUCTURE OF 2,2',4,5,5'-PENTABROMOBIPHENYL (PEAK 1)

The gas chromatographic profile of this partially purified preparation is shown in the insert. Only the values of the signals due solely or partially to peak 1 are noted.

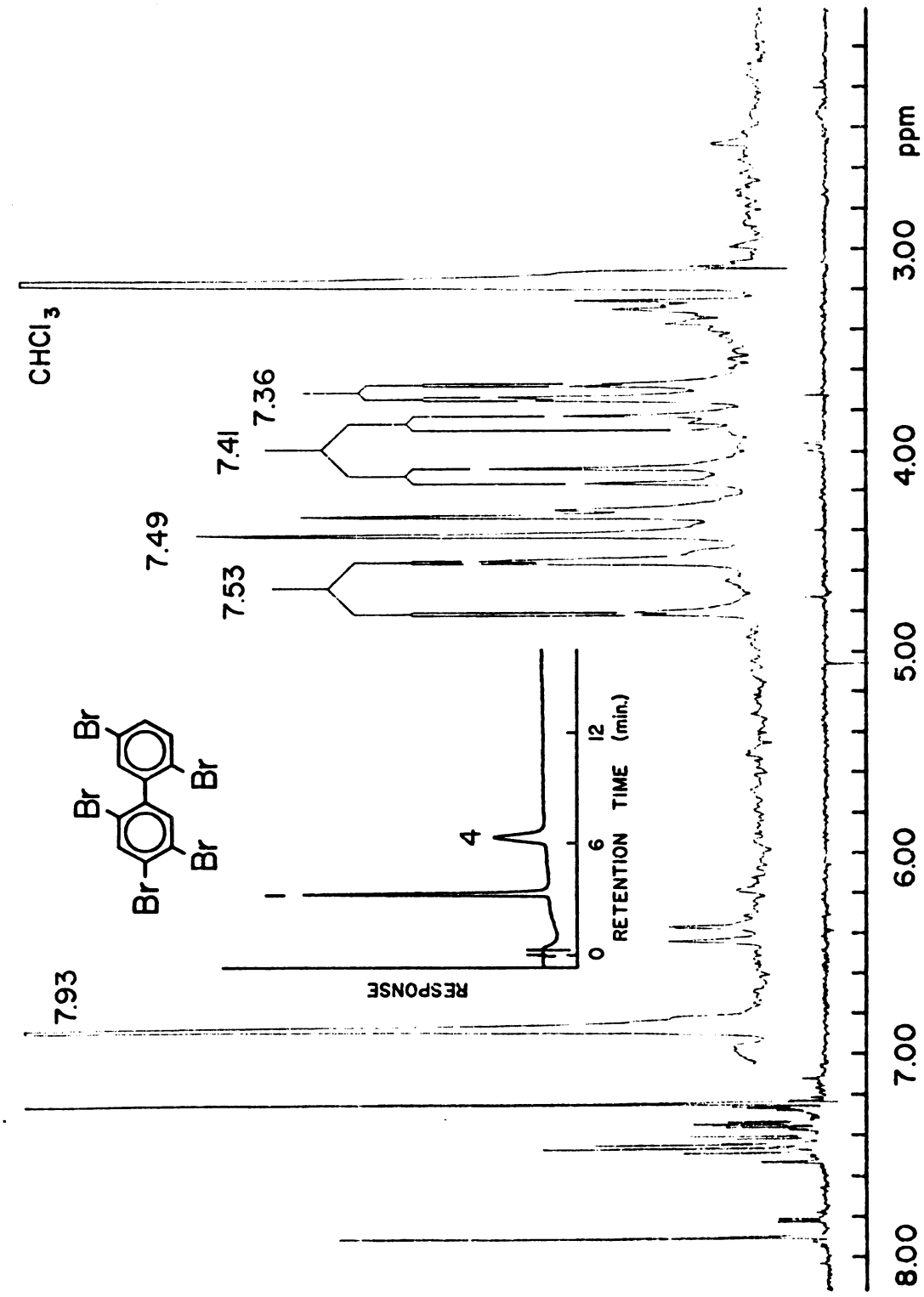

CHCl3
7.93
7.49
7.53
7.41
7.36
Br
Br
Br
Br
Br
RESPONSE
1
4
0
6
12
RETENTION TIME (min.)
8.00
7.00
6.00
5.00
4.00
3.00
ppm

Figure 3

Characterization of Peak 2

Like peak 1, peak 2 was not completely purified, as shown by the gas chromatographic analysis presented in the insert of Figure 4. Peak 6 gives rise to singlets at 7.93, 7.59 (2H), and 7.54, while the other major contaminant (peak 8) gives rise to singlets at 7.93, 7.47 and 7.45 ppm. When these signals are disregarded, and the splitting patterns shown in Figure 4 are assigned, the five protons from this pentabromobiphenyl are found to cause NMR signals of nearly equal intensity at the shift values shown in Figure 4.

Two singlets are observed, and by the same reasoning as was used during the assignment of the structure of peak 1, one ring must be brominated at the 2, 4, and 5 positions. The proton at the 3 position causes the signal at δ = 7.93 ppm, while the proton at the 6 position causes the 7.54 ppm signal.

Para splitting is not observed in the second ring. Based on the splitting pattern observed, only three possible structures can be drawn for the second ring. If the bromines are at the 2 and 5 positions, the proton at the 4 position, which is adjacent to one bromine and one proton, would cause the signal at 7.18 ppm, which is not nearly downfield enough. Indeed, this is the structure of peak 1. If the bromines are at the 2 and 4 positions, the proton at the 5 position would cause the signal at 7.18 ppm, which again isn't nearly far enough downfield for a proton adjacent to one bromine. However, if the bromines are at the 3 and 4 positions, the 2, 5, and 6 protons would cause the reasonable shifts of 7.63, 7.68, and 7.18 ppm, respectively. The structure of peak 2 is therefore 2,3',4,4',5-pentabromobiphenyl (Figure 4). Ortho, meta and para coupling constants are 8.4, 2.2, and <0.5 Hz, respectively.

Figure 4. ^{1}H-NMR SPECTRUM AND STRUCTURE OF 2,3',4,4',5-PENTABROMOBIPHENYL (PEAK 2)

The gas chromatographic profile of this partially purified preparation is shown in the insert. Only the values of the signals due solely or partially to peak 2 are noted.

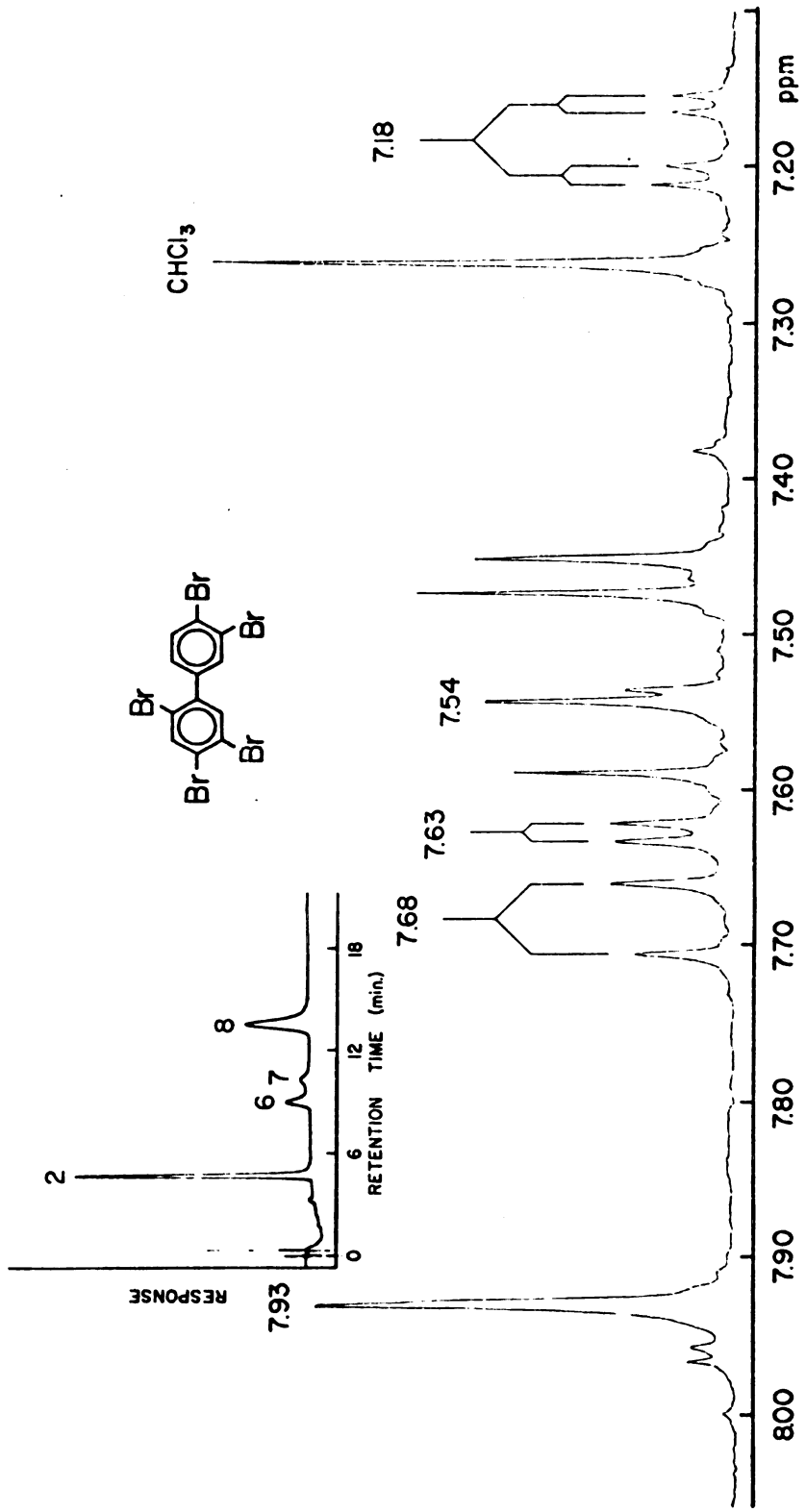

RESPONSE
2
6
7
8
0
6
12
18
RETENTION TIME (min.)
Br
Br
Br
Br
7.93
7.68
7.63
7.54
CHCl3
7.18
8.00
7.90
7.80
7.70
7.60
7.50
7.40
7.30
7.20
ppm

Figure 4

Characterization of Peak 4

Peak 4 is the only PBB congener for which the structure had been elucidated. Sundström et al. (1976a) concluded that the structure was 2,2',4,4',5,5'-hexabromobiphenyl, based on the 'H-NMR spectrum which showed two singlets ($J < 1$ Hz) of equal intensity at 7.47 and 7.93 ppm. Jacobs et al. (1976) similarly obtained the structure by ^{13}C-NMR spectroscopy. Only six signals were observed, indicating that the molecule is symmetrical. Structural assignments were as follows: bridge carbons, 140.3; C-2 (Br), 122.4; C-3 (H), 136.7; C-4 (Br), 125.9; C-5 (Br), 123.8; and C-6 (H), 134.8 ppm. Peak 4 was obtained in a virtually homogeneous form, and was found to have a melting point of 159-160°, in agreement with the value of 159-161° reported by Norstrom et al. (1976), and far higher than the values of 128-130° reported by Sundström et al. (1976a) for an impure preparation. The infrared spectrum has not yet been published, and is presented in Figure 14. By gas chromatographic analysis of standard solutions, peak 4 was found to comprise 56% by weight of the Firemaster lot (#7042) which contaminated much of the Michigan food chain. The structure of peak 4, which is 2,2',4,4',5,5'-hexabromobiphenyl, is shown in Figures 15 and 16.

Characterization of Peak 5

The sample of peak 5 was approximately 80% pure, being contaminated with peak 8 (δ = 7.93, 7.47, and 7.45 ppm) and a small amount of peak 2. When the contributions from the peak 8 signals were subtracted, an excellent ratio of unity was found between the signals arising from peak 5. Peak 5 is a hexabromobiphenyl (forty-two possible structures)

and contains four protons. The doublets centered at δ = 7.68 and 7.02 ppm (J = 7.8 Hz) arise from protons ortho to each other, and since these are not also meta split, they must be the only two protons on one ring. There are only two possible substitution patterns for such a ring. If bromines occupy the 2, 3, and 6 positions, each proton will be ortho to one proton and one bromine. However, the doublet centered at 7.02 ppm is far too upfield to be compatible with this structure. The second possibility is for the bromines to occupy the 2, 3, and 4 positions, which is what the spectrum shows to be the case. The signals centered at 7.68 ppm are from the proton at the 5 position, and the doublet centered at 7.02 ppm represents the 6 proton, which is adjacent to a bridge carbon and to an unsubstituted carbon.

The second ring must contain the remaining two protons. Since neither ortho nor meta splitting is observed, these protons must be para to each other, and so the bromines occupy the 2, 4, and 5 positions on this ring. The downfield signal (δ = 7.93 ppm) must be from the proton at the 3 position, while the upfield one at 7.46 ppm must be from the proton at the 6 position. The structure of peak 5 is therefore 2,2',3,4,4',5'-hexabromobiphenyl, as shown in Figure 5.

Characterization of Peak 6

Peak 6 is a hexabromobiphenyl, for which forty-two isomeric structures may exist. The ^{1}H-NMR spectrum (Figure 6) is very simple, with δ = 7.93 (1H), 7.59 (2H), and 7.54 (1H) ppm. Since no splitting is observed, and since three different protons are present, two of the protons must be para to each other on one ring, and the other two protons must occupy equivalent positions on the second ring. The first

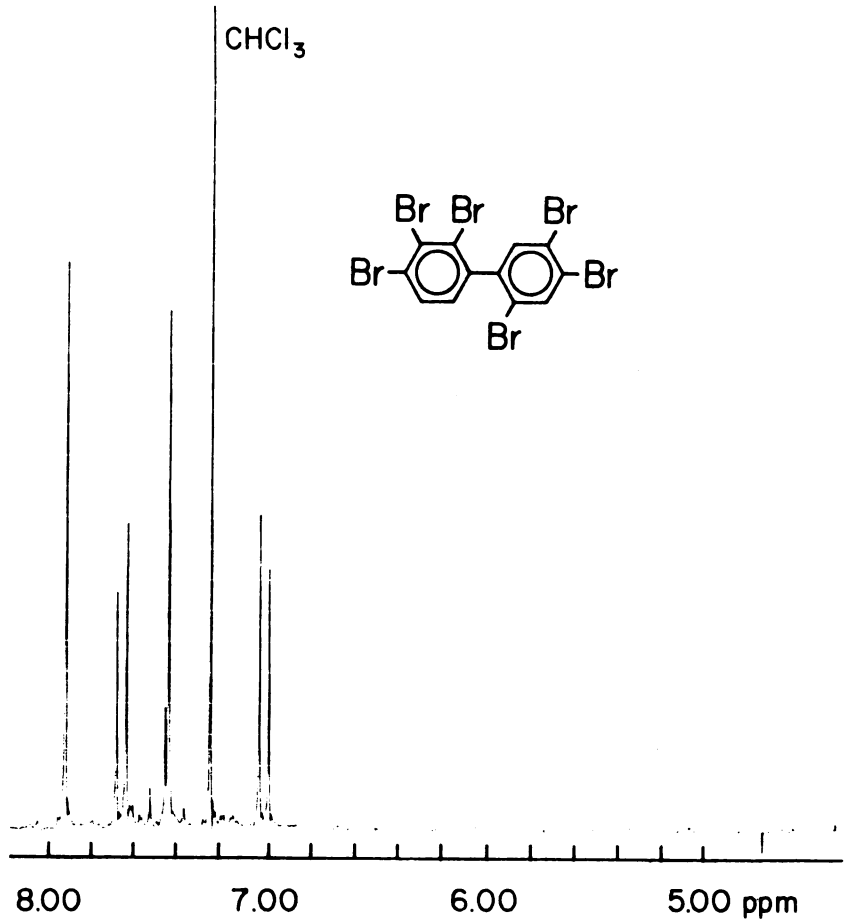


Figure 5. ^{1}H-NMR SPECTRUM AND STRUCTURE OF 2,2',3,4,4',5'-HEXA-BROMOBIPHENYL (PEAK 5).

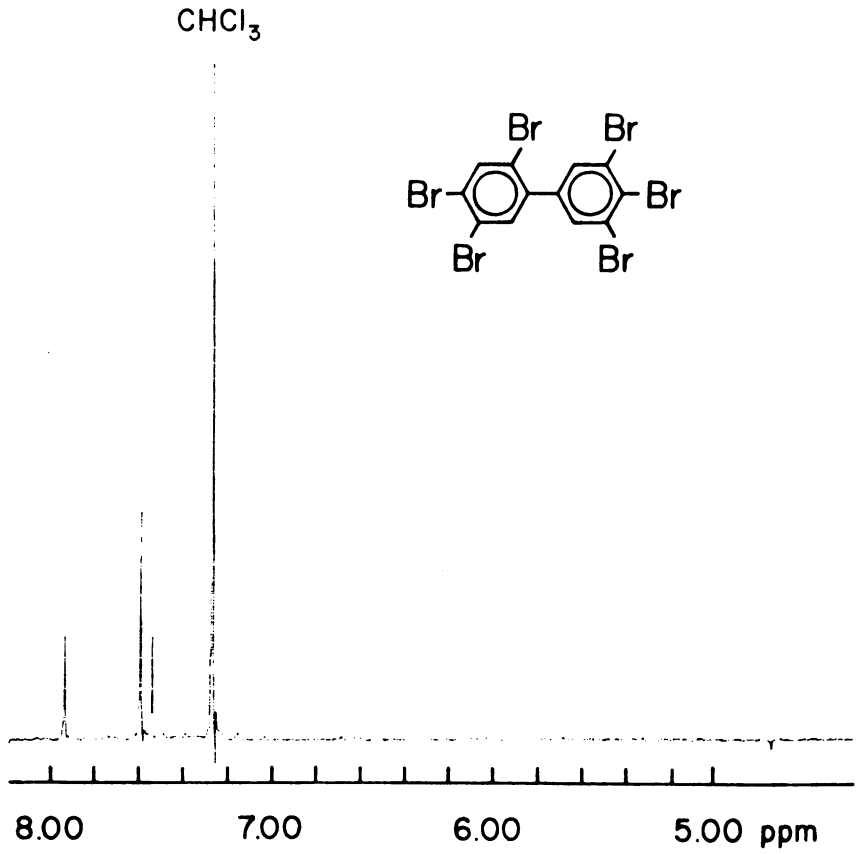


Figure 6. ^{1}H-NMR SPECTRUM AND STRUCTURE OF 2,3',4,4',5,5'-HEXA-BROMOBIPHENYL (PEAK 6)

ring must therefore be brominated at the 2, 4, and 5 positions. The signal at 7.93 ppm is assigned to the 3 proton, and the 7.54 ppm signal is assigned to the 6 proton.

Two possible structures can be postulated for the second ring. If the bromines occupy the 2, 4, and 6 positions, the protons would be expected to cause NMR signals at about 7.8 or 7.9 ppm, because each proton would be adjacent to two bromines. However, if the bromines occupy the 3, 4, and 5 positions, the two proton singlet at 7.59 ppm can be easily rationalized. As will become evident when the structures and ^{1}H-NMR spectra of several additional congeners are presented, the number of bromines in biphenyls ortho to the bridge carbons has a characteristic effect on the shifts of the protons ortho to the bridge carbons. All other things being equal, as bromines are added to the ortho positions, the signals of the ortho protons are shifted upfield by about 0.08 ppm. If the bromines on the second ring were at 2, 4, and 6 positions, the proton at the 6 position on the first ring would be expected to have a shift of about 7.37 ppm, not 7.54 ppm as was observed. However, with these bromines at the 3, 4, and 5 positions, only one bromine in the molecule occupies an ortho position, and the observed signals are where they would be expected to be. The structure of peak 6 is 2,3',4,4',5,5'-hexabromobiphenyl, as shown in Figure 6. The protons at the 2' and 6' positions are equivalent and are responsible for the singlet at δ = 7.59 ppm.

The quantity of peak 6 was not sufficient to determine the entire ^{13}C-NMR spectrum, however, the shifts of the protonated carbons were determined in chloroform solution (Figure 7). The signals at 137.9, 134.9, and 133.0 ppm are assigned to the 3, 4, and the equivalent 2'

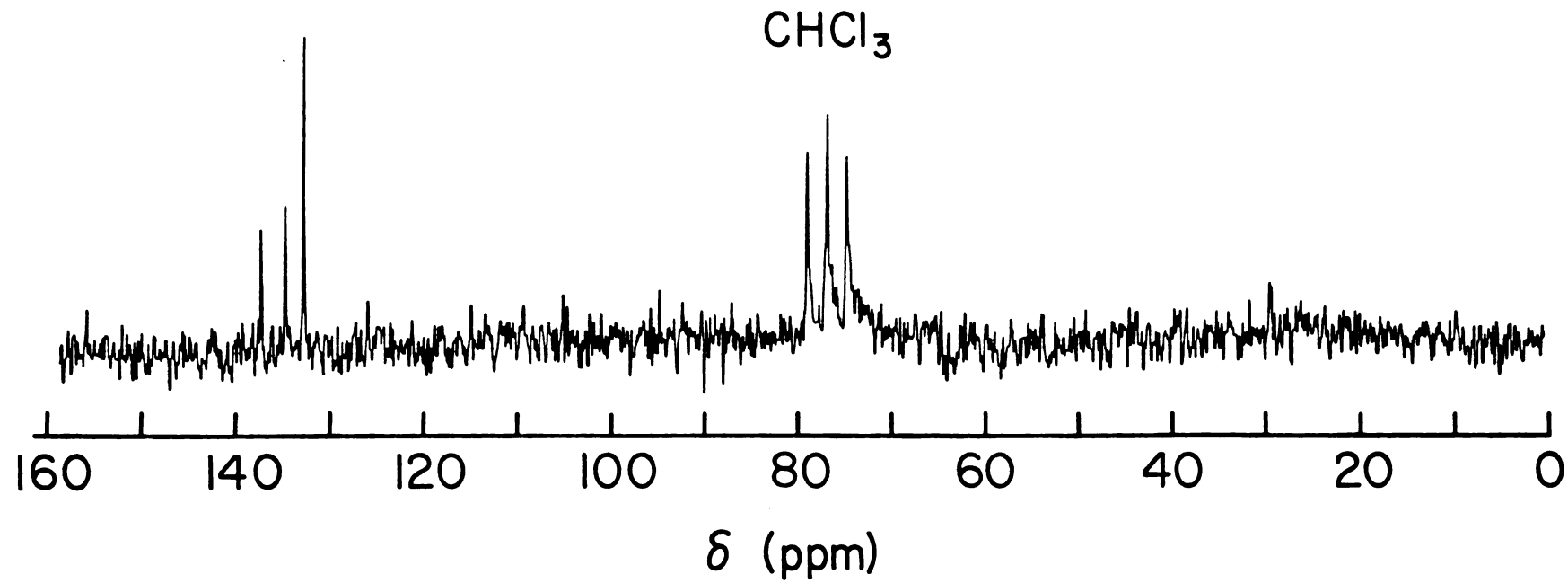


Figure 7. ^{13}C-NMR SPECTRUM OF 2,3',4,4',5,5'-HEXABROMOBIPHENYL (PEAK 6)

Experimental conditions were such that only the signals from protonated carbons could be visualized.

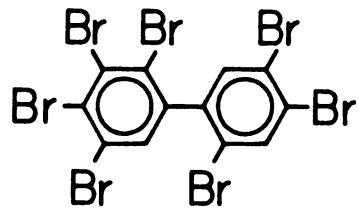
and 6' carbons, respectively. These assignments were made based on the ^{13}C-NMR shifts observed in biphenyl, benzene, and bromobenzene (Sadtler Research Laboratories, 1976), and on the shifts noted for the carbons in peak 4 (Jacobs et al., 1976). Although the 'H-NMR spectrum quickly allowed all but two possible structures for peak 6 to be ruled out, this partial ^{13}C-NMR spectrum was of no help in deciding between the two.

Peak 6 was found to melt at 165-166°, and its infrared spectrum is shown in Figure 14.

Characterization of Peak 8

There are twenty-four possible heptabromobiphenyls of which peak 8 is one. The 'H-NMR spectrum shows the presence of three distinct protons which do not observably split each other. This limits the possible structures to the three which have two protons para to each other on one ring (at the 3 and 6 positions) and a lone proton on the second ring. One of the upfield signals must be from the proton at the ortho position on the tribrominated ring, and since the other signal is very close to it, the lone proton must also be at an ortho position. The signal at δ = 7.93 ppm must be from the 3' proton, which is adjacent to two bromines. The signals at 7.47 and 7.45 ppm are assigned to the 6 and 6' protons, respectively, based on peak heights. The structure of peak 8 is therefore 2,2',3,4,4',5,5'-heptabromobiphenyl, as shown in Figure 8.

The ^{13}C-NMR spectrum of peak 8 was determined in $CDCl_3$ solution (Figure 9). The most useful information which could be derived from this spectrum is that there are ten distinct carbons in addition to



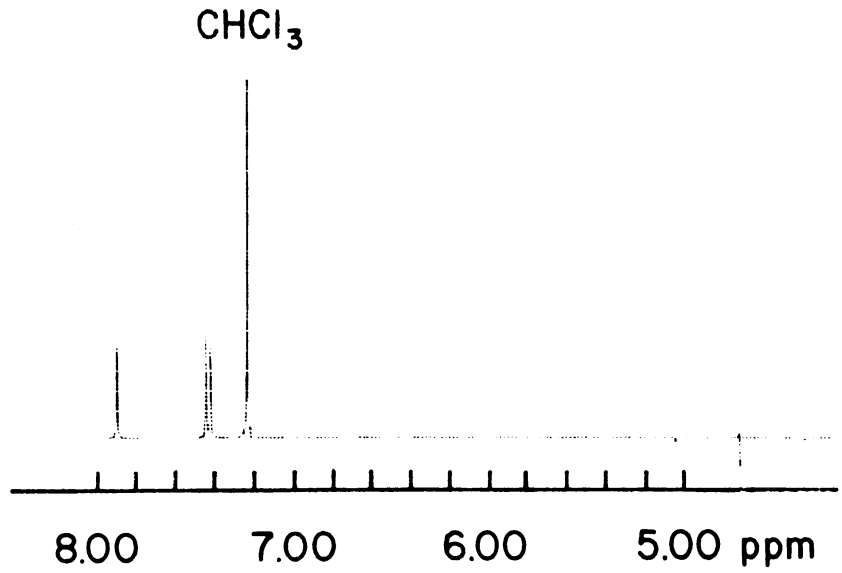


Figure 8. ^{1}H-NMR SPECTRUM AND STRUCTURE OF 2,2',3,4,4',5,5'-HEPTA-BROMOBIPHENYL (PEAK 8)

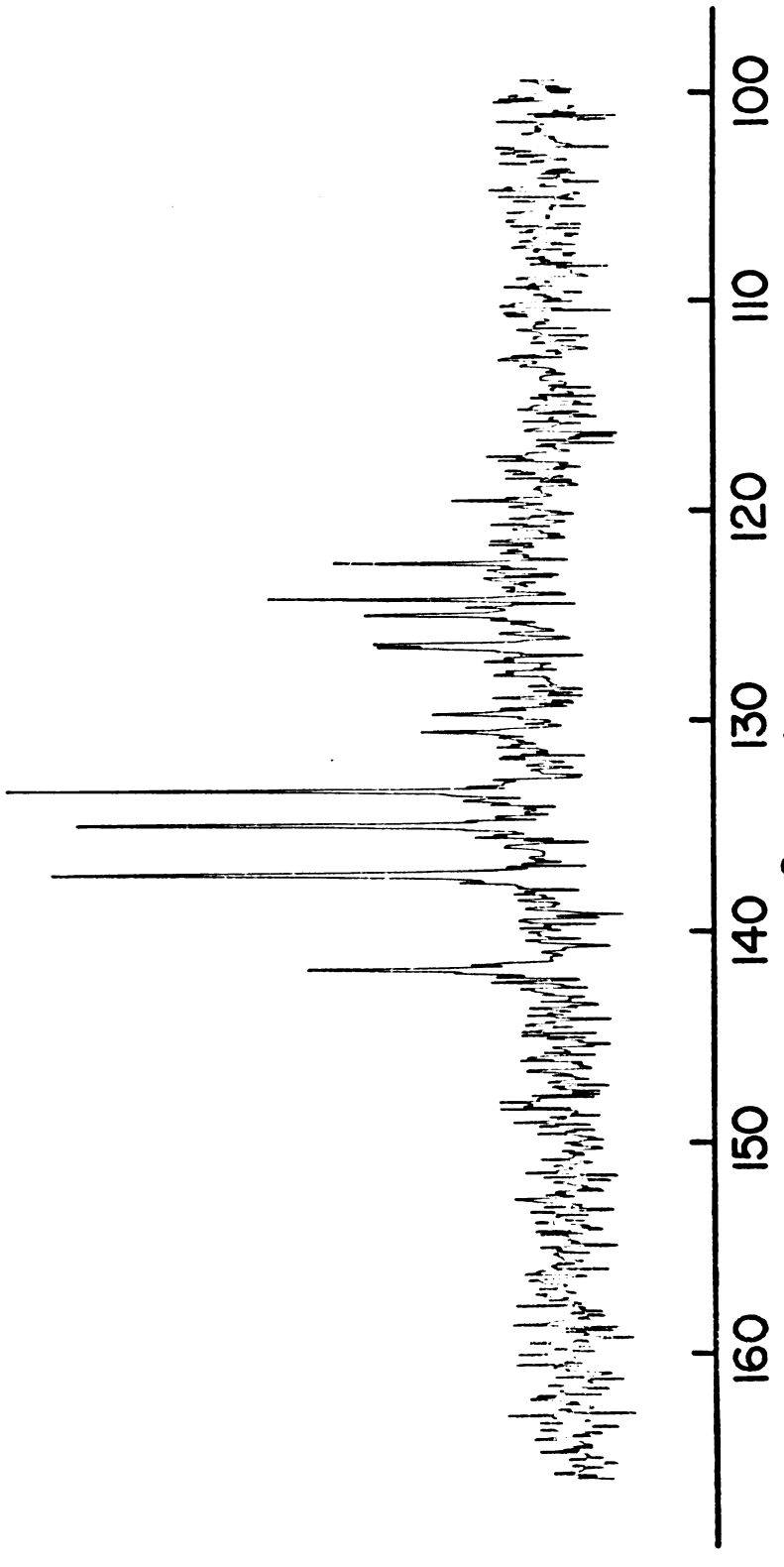


Figure 9. ^{13}C-NMR SPECTRUM OF 2,2',3,4,4',5,5'-HEPTABROMOBIPHENYL (PEAK 8)

the two which appear as a broadened peak at δ = 141.8 ppm. It has been shown, with chlorinated biphenyls, that chemically equivalent carbons on one ring do not give rise to ^{13}C-NMR peaks even when there is an asymmetric substitution pattern on the other ring (Hutzinger et al., 1974). Four of the possible structures have an axis of symmetry and would show a maximum of eight signals, while twelve are symmetrical on one ring and would show a maximum of ten peaks. Since eleven or twelve peaks are observed, these structures cannot be peak 8. Given the present scarcity of information about the ^{13}C-NMR properties of brominated biphenyls, it is not possible to rule out any of the final eight potential structures for peak 8 on the sole basis of its ^{13}C-NMR spectrum. However, since the structure was determined from its ^{1}H-NMR spectrum, and since the ^{13}C-NMR assignments have been made for the closely related molecule 2,2',4,4',5,5'-hexabromobiphenyl (peak 4) (Jacobs et al., 1976), it is possible to assign most of the signals to single carbons. The broadened peak at 141.8 ppm is assigned to the bridge carbons, while the large peaks at 137.2, 134.9, and 133.3 ppm are assigned to the protonated carbons at the 3', 6', and 6 positions, respectively. Other assignments are: C3 at 130.5; C4 at 129.7; and C2 at 122.5 ppm. The peaks at 126.5 and 126.3 ppm are extremely close; one is assigned to C4', while the other is from either C2 or C5. The peak at 125.0 ppm is from C2 or C5, or possibly C5', while the peak at 124.2 ppm is probably from C5', though possibly from C2 or C5. Wilson (1975) has shown that the substitution of chlorines onto biphenyl causes ^{13}C-NMR signals to shift in an approximately additive manner, and these assignments have been made based on this observation, the ^{13}C-NMR spectrum of peak 4 (Jacobs et al., 1976), and the shifts

observed upon the bromination of benzene (Sadtler Research Laboratories, 1976), and of biphenyl at the 3 position (Levy et al., 1973).

Peak 8 was found to melt at 165-166°, and to comprise 27% by weight of Firemaster lot #7042, and 25% of OBB. Its infrared spectrum is presented in Figure 14.

Characterization of Peak 11

Peak 11 was obtained at about 80% purity, being contaminated mainly with peak 12 (δ = 7.46 ppm). It has eight bromines and two protons, and these protons give rise to the major signals of equal intensity at 7.96 and 7.37 ppm (Figure 10). Based on the lack of observable splitting, the twelve possible structures are narrowed to four. The first one has one proton on each ring, one meta and one para to their respective bridge carbons. While the signal at 7.96 ppm could be attributed to either of these protons, the upfield signal is incompatible with either of these protons, each of which would be adjacent to two bromines. The second possible structure is fully brominated on one ring, and has a 2, 4, 5 substitution pattern on the other. The proton at the 3 position could cause the signal at 7.96 ppm, and the proton on the 6 carbon could give the 7.37 ppm signal. While such a shift for the 6 proton is very logical, the same is not true for the 3 proton. The 2, 4, 5 substitution pattern is found in peaks 1, 2, 4, 5, 6, and 8, and in every case, the signal from the 3 proton is at 7.93 ppm, not 7.96 ppm. While this structure cannot be definitively ruled out for peak 11, the 2, 4, 5 substitution pattern must be considered unlikely. The third and fourth possible structures are very similar, and it is not currently possible to determine which of them is correct. Both

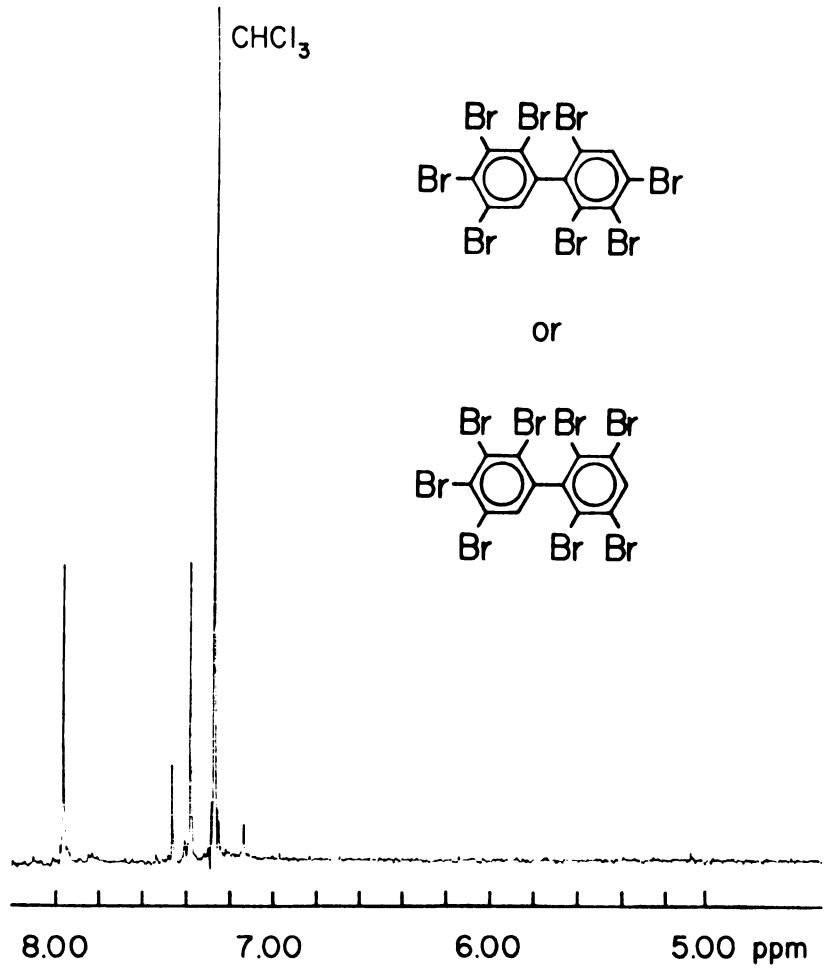


Figure 10. ^{1}H-NMR SPECTRUM OF PEAK 11

The two most likely structures are shown.

have single protons on each ring. In both structures, one proton occupies an ortho position, giving rise to the 7.37 ppm signal. The proton on the other ring would occupy either the meta or ortho position, and cause the 7.96 ppm signal. In either position, the proton on the second ring would be adjacent to two bromines and therefore greatly deshielded. These two possible structures for peak 11, 2,2',3,3',4,-5,5',6'-octabromobiphenyl or 2,2',3,3',4,4',5,6'-octabromobiphenyl, should be distinguishable by ^{13}C-NMR spectroscopy. Unfortunately, only a small quantity of peak 11 could be purified, and the amount was insufficient to attempt to determine such a spectrum.

Characterization of Peak 12

Peak 12, which contains eight bromines, was obtained in 98% purity from OBB, and displayed a lone ^{1}H-NMR signal at δ = 7.46 ppm (Figure 11). The two protons must be equivalent, which permits five possible structures to be considered. Three of these would have protons at the 3 and 3' positions, the 4 and 4' positions, or the 3' and 5' positions. In all these structures, though, each proton would be adjacent to two bromines, and shifts of 7.9 ppm or greater would be expected rather than the signal at 7.46 ppm which was observed. The fourth possible structure has both protons on the same ring, ortho to the bridge carbon, while the fifth has identical rings, each with one proton ortho to a bridge carbon. Neither structures can be definitively ruled out based on the ^{1}H-NMR spectrum of peak 12. Since these structures should give rise to eight and six ^{13}C-NMR signals, respectively, attempts were made to determine the ^{13}C-NMR spectrum of this compound. Unfortunately, peak 12 has only limited solubility in deuterochloroform, and the only

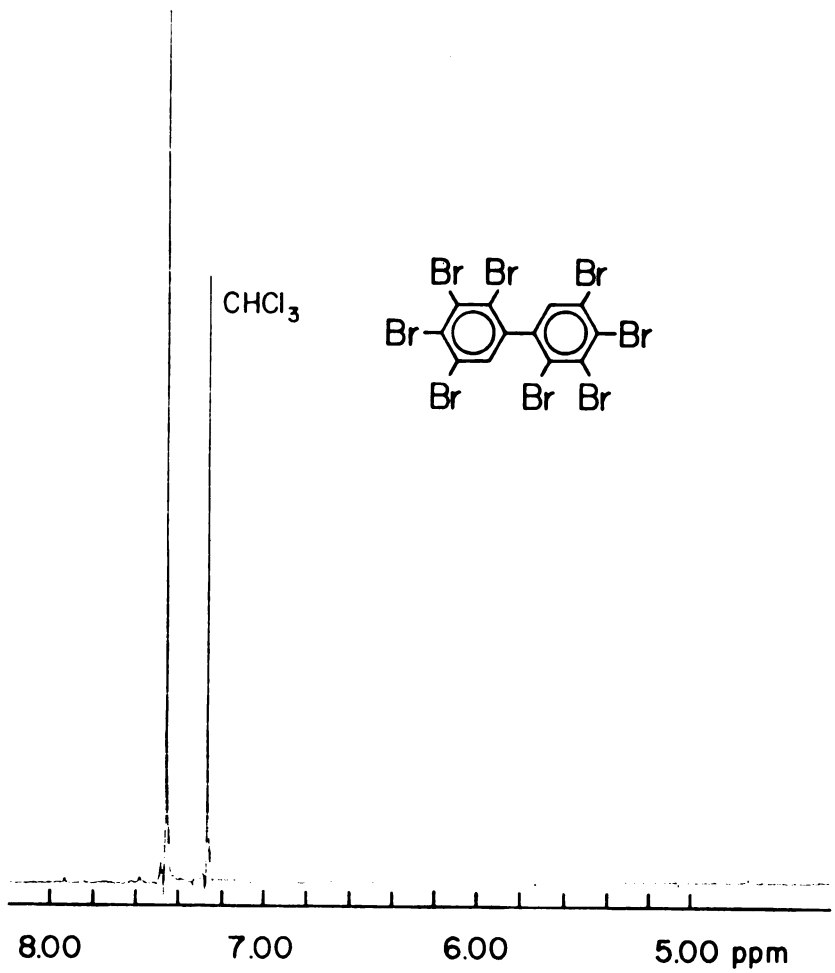


Figure 11. ^{1}H-NMR SPECTRUM AND STRUCTURE OF 2,2',3,3',4,4',5,5'-OCTABROMOBIPHENYL (PEAK 12)

signal which could be observed was that from the protonated carbons, at 132.8 ppm. The spectrum was then retaken in deuterobenzene, a much better solvent. While the solvent obscured the spectrum between 125 and 130 ppm, the region in which the bridge carbons would appear remained unaffected. The structure with both protons on one ring would have distinct bridge carbons, but only one signal was seen in the bridge carbon region, at δ = 142.7 ppm (Figure 12). Peak 12 was therefore identified by the combination of ^{1}H- and ^{13}C-NMR spectroscopy as 2,2',3,3',4,4',5,5'-octabromobiphenyl (Figure 11).

Peak 12 was found to melt at 232-233°, and its infrared spectrum is shown in Figure 14.

Characterization of Peak 13

Peak 13 was obtained in 95% purity. This nonabromobiphenyl gave a lone ^{1}H-NMR signal at δ = 7.38 ppm (Figure 13), which must arise from a proton ortho to a bridge carbon. The other two possible structures would have protons at the 3 or 4 positions, each of which would be adjacent to two bromines and would be expected to show a ^{1}H-NMR signal at 7.9 ppm or higher. The structure of peak 13 is therefore 2,2',3,3',4,4',5,5',6-nonabromobiphenyl (Figure 13). It decomposed at 263-264°, and its infrared spectrum is shown in Figure 14.

DISCUSSION

Results of structural analyses of polybrominated biphenyl components are presented in Figures 15 and 16. The structure of peak 4 (retention time six minutes) had previously been established, while

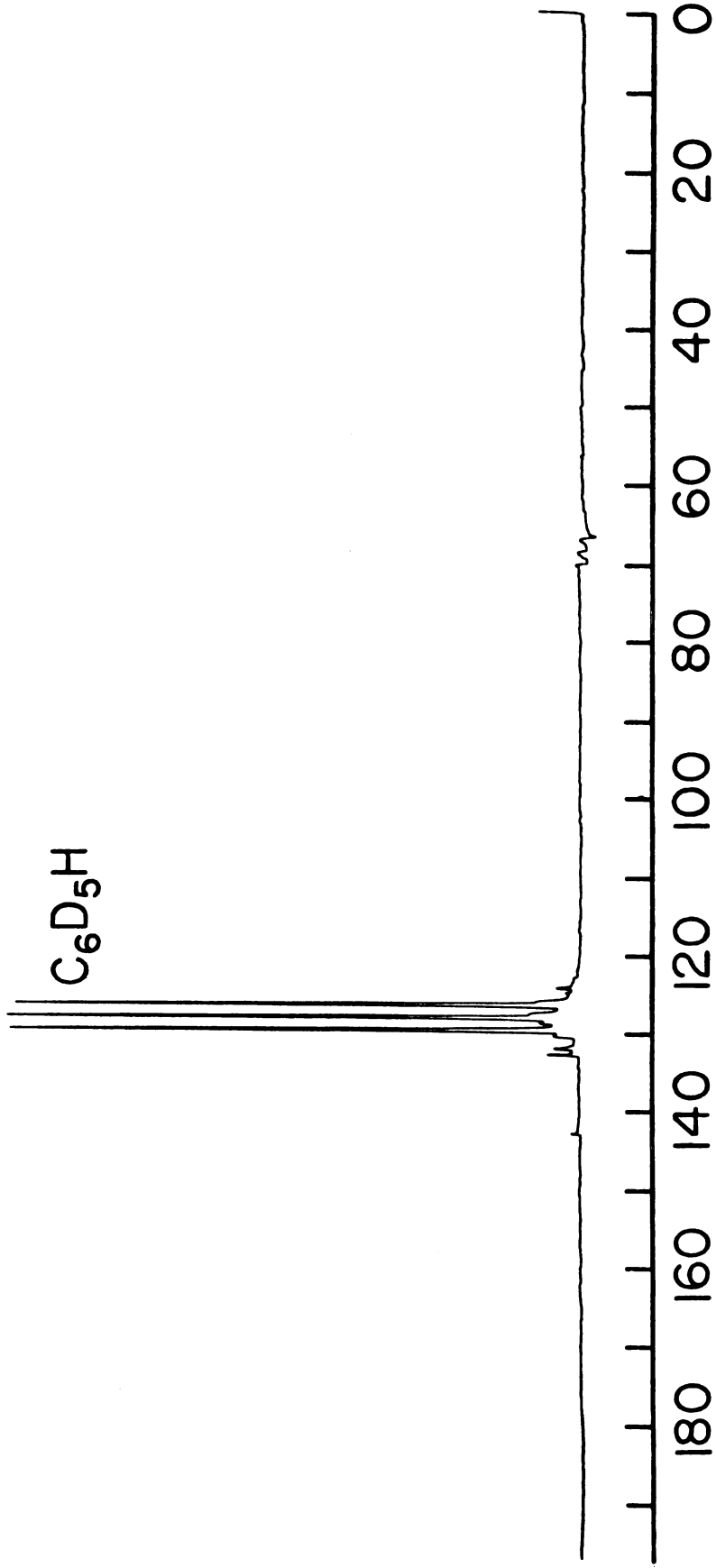


Figure 12. ^{13}C-NMR SPECTRUM OF 2,2',3,3',4,4',5,5'-OCTABROMOBIPHENYL (PEAK 12)

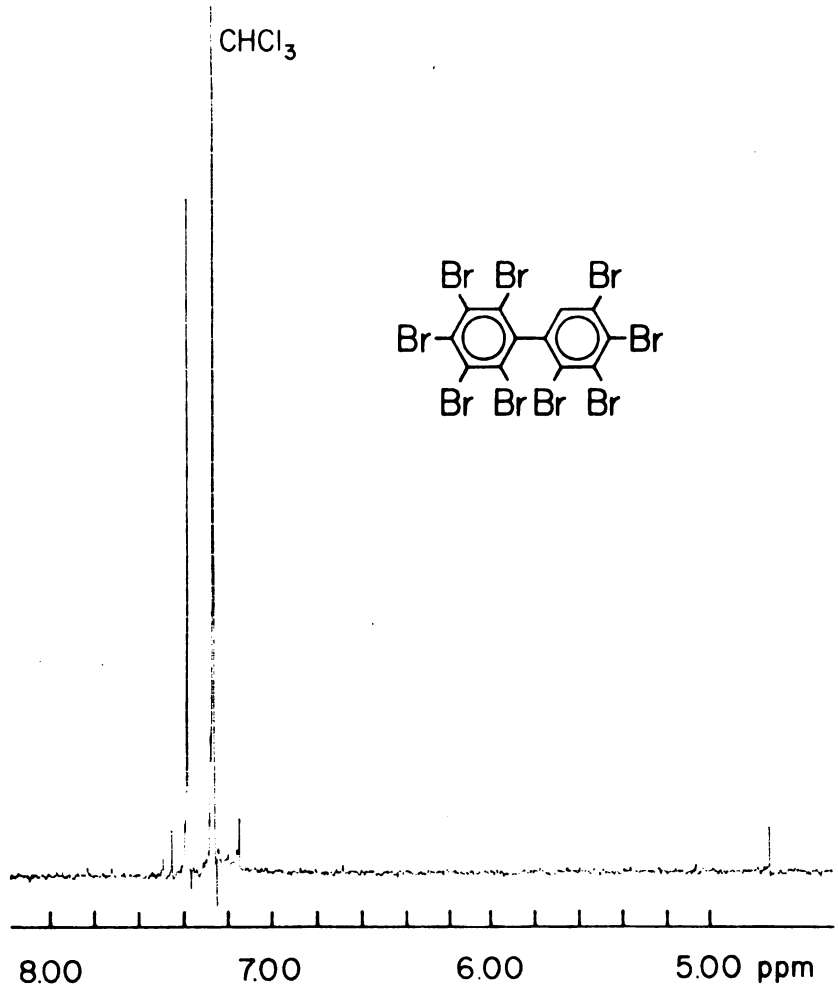


Figure 13. ^{1}H-NMR SPECTRUM AND STRUCTURE OF 2,2',3,3',4,4',5,5',6-NONABROMOBIPHENYL (PEAK 13)

Figure 14. INFRARED SPECTRA AND STRUCTURES OF FIVE PURE POLYBROMOBIPHENYL CONGENERS

From top to bottom, the spectra are of peaks 4, 6, 8, 12, and 13. All were at least 95% pure.

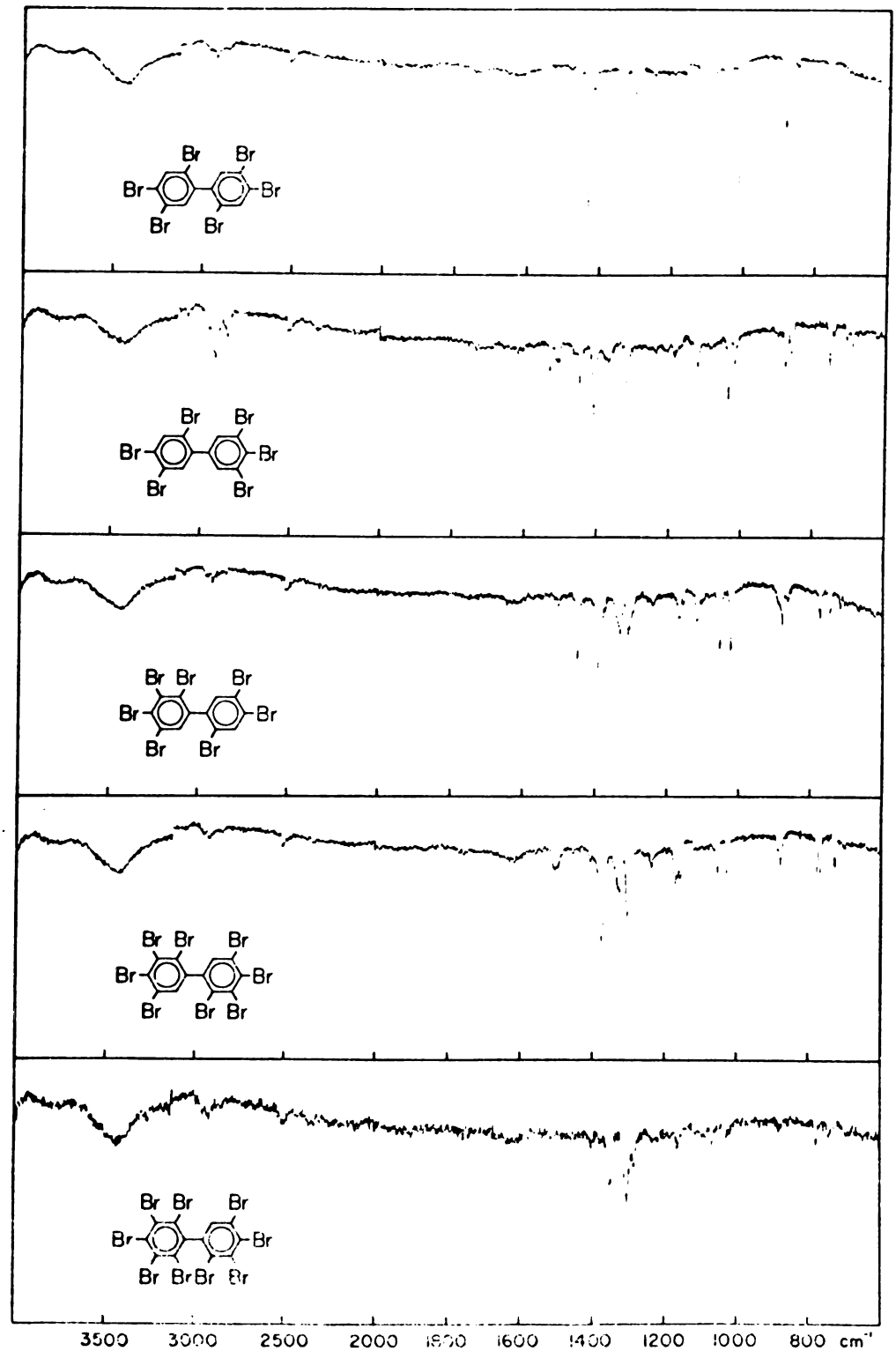


Figure 14

Figure 15. GAS CHROMATOGRAPHIC PROFILE OF POLYBROMINATED BIPHENYLS (FIREMASTER) AND STRUCTURES OF ITS COMPONENTS

A 400 ng sample was analyzed.

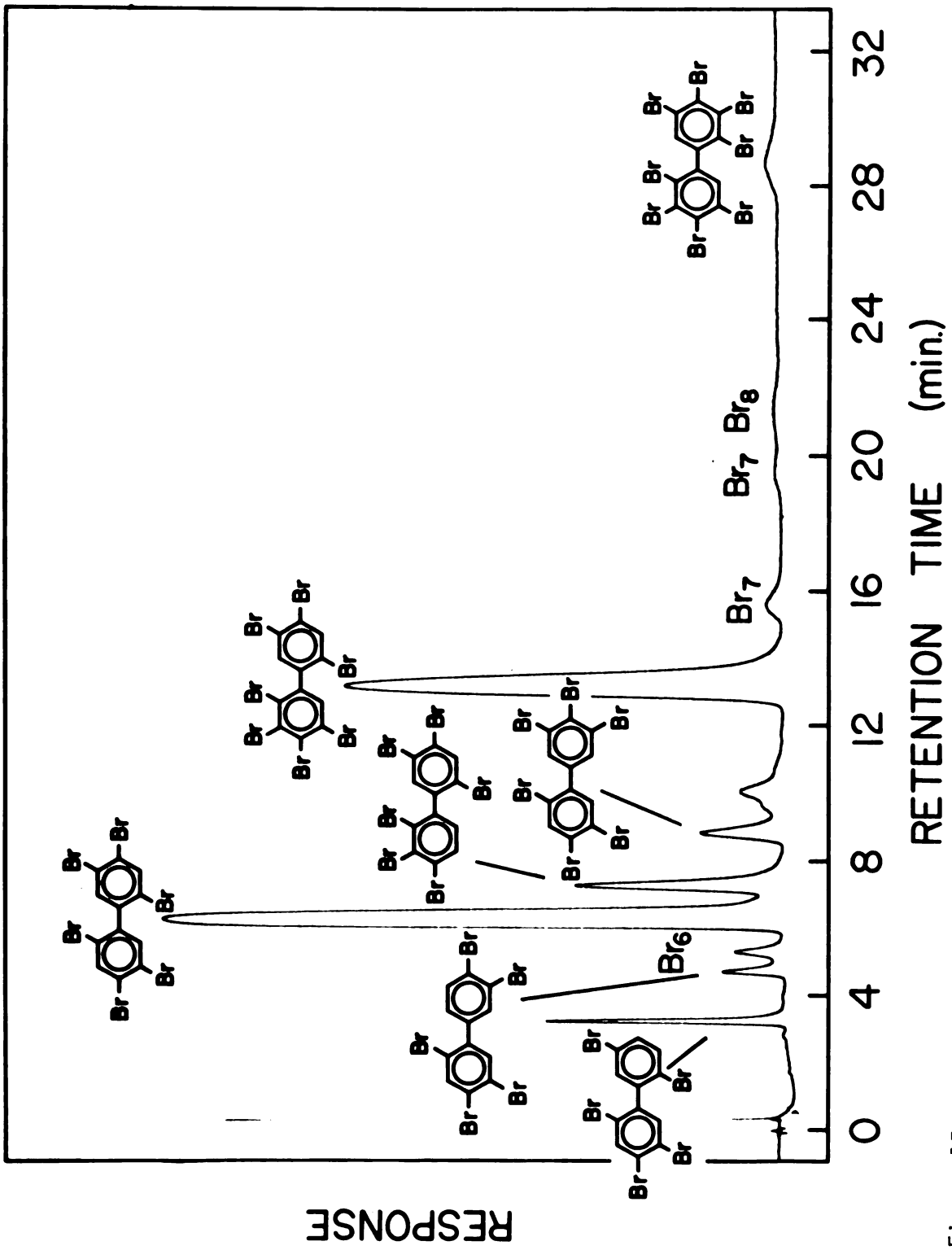

RESPONSE
0
4
8
12
16
20
24
28
32
RETENTION TIME (min.)
Br6
Br7
Br7 Br8

Figure 15

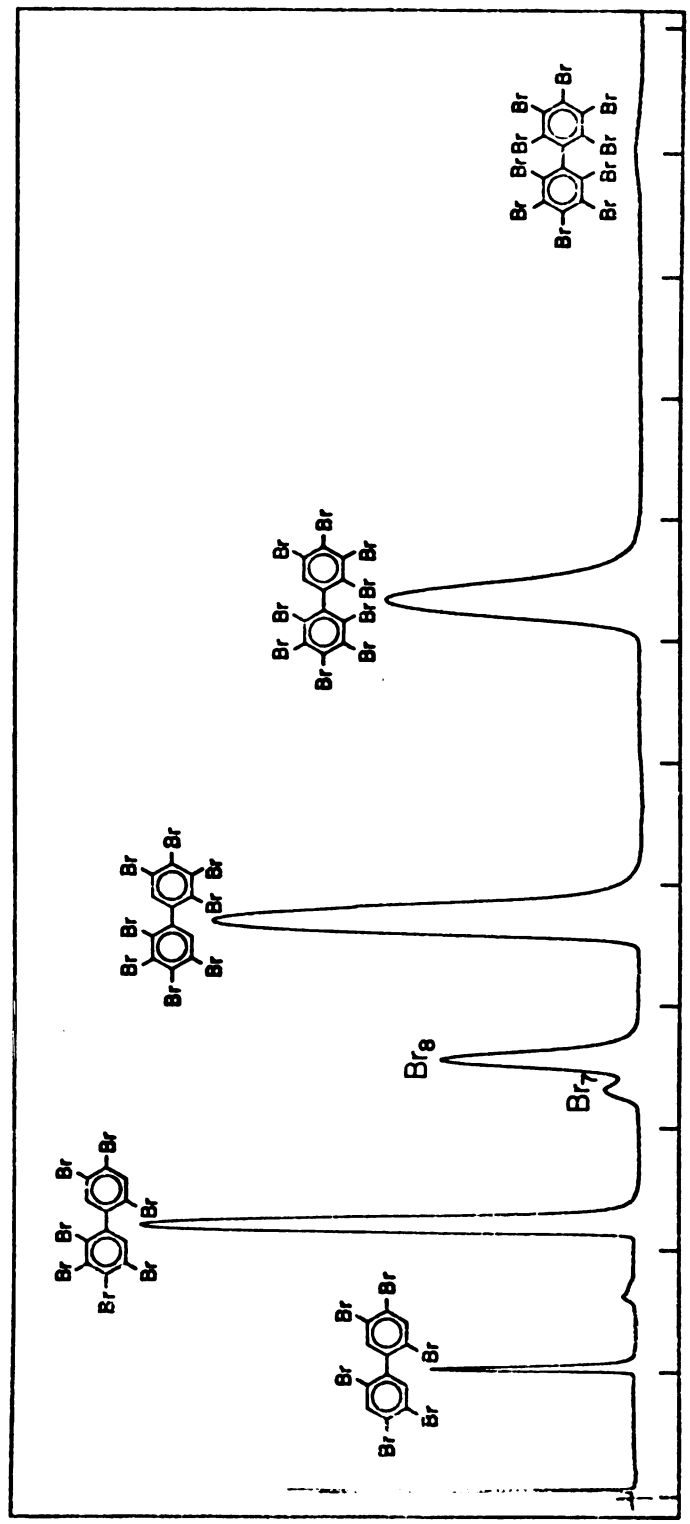


Figure 16. GAS CHROMATOGRAPHIC PROFILE OF POLYBROMINATED BIPHENYLS (OCTABROMOBIPHENYL) AND STRUCTURES OF ITS COMPONENTS

An 800 ng sample was analyzed.

all the other structures are the result of research described in this Chapter. Peak 11 has also been partially characterized, and it is one of the two structures pictured in Figure 10. The molecular weights of peaks 9, 10, 11 and 14 have also been determined, as shown.

It can be seen that the 2, 4, 5 substitution pattern is very common, occurring in peaks 1, 2, 4, 5, 6 and 8, while the 2, 3, 4, 5 substitution pattern may be seen in peaks 8, 11, 12, and 13. Other substitution patterns include: 2,5-dibromo, 3,4-dibromo, 2,3,4-tribromo, 3,4,5-tribromo, and 2,3,4,5,6-pentabromo. The third and fourth ortho positions appear to be the most difficult to brominate. In all cases for which the structures are known, at least one meta position on each ring is brominated, and with the exception of peak 1, all para positions are brominated.

The chemical shifts for the protons in the nine known structures, and for the one partially characterized structure, are presented in Table 2. It can be seen that the shifts for protons in these structures which are adjacent to two bromines all fall in the range of 7.96 to 7.93 ppm. Bromine is known to deshield the protons ortho to it; in bromobenzene, these proton signals are shifted downfield by 0.19 ppm (Williams and Fleming, 1973).

The chemical shifts for protons adjacent to both a bromine and a bridge carbon fall into three categories, depending upon the total number of ortho bromines. Peaks 2 and 6 each have one ortho bromine, and the shifts for the ortho protons are 7.54, 7.63, 7.54, 7.59, and 7.59 ppm. Peaks 1, 4, 5, 8 and 12 each have two ortho bromines, and the shifts of protons adjacent to bromines all fall in the range of 7.49 to 7.45 ppm, with one exception. The ortho proton on one peak 1 ring

Table 2

PROTON CHEMICAL SHIFTS IN POLYBROMINATED BIPHENYL CONGENERS

Peak Number	Structure	Proton Number									
		2	3	4	5	6	2'	3'	4'	5'	6'
1	2,2',4,5,5'-Br_5		7.932			7.486		7.533	7.409		7.357
2	2,3',4,4',5-Br_5		7.932			7.543	7.627			7.684	7.183
4	2,2',4,4',5,5'-Br_6		7.931			7.468		7.931			7.468
5	2,2',3,4,4',5'-Br_6				7.675	7.020		7.932			7.458
6	2,3',4,4',5,5'-Br_6		7.934			7.536	7.590				7.590
8	2,2',3,4,4',5,5'-Br_7					7.473		7.934			7.451
11	2,2',3,3',4,4',5,6'- or 2,2',3,3',4,5,5',6'-Br_8					7.370			(7.959)		
12	2,2',3,3',4,4',5,5'-Br_8					7.456					7.456
13	2,2',3,3',4,4',5,5',6-Br_9										7.379
14	2,2',3,3',4,4',5,5',6,6'-Br_{10}										

is at 7.36 ppm, but this might be because it is on the only ring among the known structures to have a proton at the para position. Peaks 11 and 13 both have three bromines in the ortho positions, and the proton signals are at 7.37 and 7.38 ppm, respectively. Only peaks 2 and 5 have ortho protons adjacent to another unsubstituted carbon, but again the trend towards decreasing chemical shifts with increasing ortho bromination is observed. These trends have been noted by Welti and Sissons (1972) in a comprehensive ^{1}H-NMR analysis of chlorinated biphenyls.

Although the absolute values differ somewhat, the patterns of chemical shifts and coupling constants are quite similar for both brominated and chlorinated biphenyls. The ^{1}H-NMR spectra for a number of chlorinated biphenyls, including the analogs of several of the structures presented in this Chapter, have been published by Tas and Vos (1971), Bartle (1972), Tas and Kleipool (1972), Welti and Sissons (1972), and Wilson (1975).

Sissons and Welti (1971) have noted that when chlorinated biphenyls are subjected to either gas chromatography or column chromatography (alumina with hexane), the congeners eluted in a characteristic order. Assuming the structure of one ring to be constant, those structures with 2,5-, 2,4-, 2,3-, 2,3,6-, 3,4-, 2,4,5-, 2,3,4-, and 2,3,4,5-substitution patterns in the second ring eluted from the gas chromatograph in the order given above. When chromatographed on alumina, the elution order was 2,4,5-, 2,5-, 2,3,4,5-, 2,4-, 2,3,6-, 3,4-, 2,3-, and 2,3,4-, again assuming a constant structure in the other ring. Results with polybrominated biphenyls are completely consistent with these observations.

At times, recrystallization was an extremely useful tool to purify the brominated biphenyl congeners. Peaks 6a and 7 were completely removed when peak 8 was recrystallized, while peak 4 co-crystallized with 8. Peak 8 was only removed from peak 12 by repeated recrystallizations, while peaks 10 and 11 were almost completely removed the first time. Peak 4 was completely removed in the first recrystallization of peak 6. In most other cases, only partial separations could be achieved by this route. In general, brominated biphenyls with the same number of ortho bromines tend to recrystallize together, probably because increasing ortho bromination would be expected to increase the twisting of the molecules.

When it was possible to do so, the melting points and infrared spectra of the purified compounds were determined. Although the infrared spectra were not helpful in elucidating structures, it can be seen that each compound does have a completely distinct spectrum (Figure 14).

The results presented in this Chapter should be of use in a variety of biological investigations. Dannan (1978) found that when rats were treated with PBBs, the relative concentrations of congeners in milk and liver microsomes were different from those of the starting material. He has also demonstrated that only two of the Firemaster components could be detectably metabolized in vitro by liver microsomes. Results presented in Chapter 3 demonstrate that of three brominated biphenyls tested as inducers of microsomal drug metabolizing enzymes, two were strictly phenobarbital-type inducers, and one was not an inducer. A fourth bromobiphenyl has been shown to be only a 3-methylcholanthrene-type inducer of these drug metabolizing enzymes (Poland

and Glover, 1977). The different congeners are expected to vary, sometimes greatly, in their biological effects and metabolic fates. A knowledge of the structures of components found in mixtures of PBBs should be useful in efforts to elucidate the nature of the biological effects of such mixtures. Such chemical information is obviously required to elucidate the structure-function relationships observed when these compounds interact with biological systems.

CHAPTER 2

STUDIES ON THE COVALENT BINDING OF POLYBROMINATED BIPHENYLS TO PROTEIN AND DNA

ABSTRACT

It has been postulated that polybrominated biphenyls may exert their toxic effects by being metabolized to epoxides capable of binding covalently to cellular macromolecules. This hypothesis has been investigated by aerobically incubating ^{14}C-bromobiphenyls with rat liver microsomes and NADPH. The radioactive preparation consisted almost exclusively of 2,2',4,4',5,5'-hexabromobiphenyl and 2,2',3,4,4',5,5'-heptabromobiphenyl, unlike the Firemaster mixture of polybrominated biphenyls which contains 17% by weight of other constituents. Following exhaustive post-incubation extractions, less than 0.05% of the radioactivity was associated with microsomal protein. No metabolites could be detected by thin layer chromatography of the organic extracts. There was no binding to exogenous DNA included in similar incubation mixtures, regardless of whether control microsomes, or microsomes induced by 3-methylcholanthrene, phenobarbital, or polybrominated biphenyls were used. The limit of sensitivity for DNA binding was approximately one metabolite bound per 10^8 DNA bases. Under the same conditions, ^{3}H-benzo[α]pyrene was shown to bind to DNA, and this binding was greatly enhanced in microsomes induced by 3-methylcholanthrene and polybrominated biphenyls, but not phenobarbital. It is concluded that the two major components of polybrominated biphenyls may be activated into protein-binding metabolites at an extremely slow rate, although impurities in the ^{14}C-material could also account for this binding. These congeners are not metabolically activated

into DNA-binding metabolites.

INTRODUCTION

The mechanisms by which the carcinogenic potentials of polycyclic aromatic hydrocarbons are expressed are under intensive investigation in a large number of laboratories. It appears that many of these compounds, which are relatively inert chemically, are not themselves carcinogenic. Rather, they are bioactivated by the microsomal mixed-function oxidase drug metabolizing enzymes into electrophilic derivatives (usually epoxides) capable of covalently binding to cellular macromolecules including DNA. Mutagenicity and carcinogenicity can be consequences of this binding (Jerina and Daly, 1974; Sims and Grover, 1974).

Not all epoxides that are formed covalently bind to macromolecules. Epoxides can nonenzymatically isomerize into phenols, and in the process, migration and retention of deuterium, tritium, halogens, and alkyl groups can occur, a process known as the "NIH shift". Any derivative of a parent molecule in which a substituent has migrated is presumed to have undergone an epoxidation. The enzymatic hydration of an epoxide to a dihydrodiol represents a third possible fate of an epoxide. The reaction is catalyzed by epoxide hydratase, a microsomal enzyme, and the formation of a dihydrodiol is considered as evidence for epoxide formation. Lastly, epoxides can be conjugated with reduced glutathione, both enzymatically and nonenzymatically. The presence of glutathione conjugates or their degradation products, the so-called "mercapturic acids", is also taken as evidence for the prior existence of an epoxide (Jerina and Daly, 1974; Sims and Grover, 1974).

Safe et al. (1976) and Kohli and Safe (1976) have provided evidence that 4,4'-dibromobiphenyl can undergo an NIH shift, and is therefore presumably metabolized through an epoxide intermediate. They fed 4,4'-dibromobiphenyl to a rabbit and a pig and isolated metabolites from urine and feces. In both cases, a metabolite with a molecular weight and ^{1}H-NMR spectrum consistent with 3,4'-dibromobiphenyl-4-ol was obtained. Such a compound would have to be a rearrangement product from an epoxide. NIH shifts of bromine in 1,4-dibromobenzene and in 1,4-dibromonaphthalene have also been observed, by Ruzo et al. (1976a,b).

Wyndham et al. (1976) showed that 4-chlorobiphenyl was metabolized in vitro into products capable of binding to microsomal protein and RNA. One or more of the products were mutagenic to bacteria, and in one phenolic product most of the deuterium from the oxidized position was retained, indicating an NIH shift and therefore prior epoxide formation.

Shimada (1976) carried out similar investigations with polychlorinated biphenyl mixtures (PCBs) averaging three and five chlorines per molecule. These mixtures were activated by liver microsomes into metabolites capable of binding to microsomal protein. The binding required O_2 and NADPH, and could be partially inhibited by including cysteine or reduced glutathione in the incubation mixtures, thus implicating the formation of epoxides as the reactive intermediates. The radioactive PCBs were also found to covalently bind in vivo to liver macromolecules.

Studies in this laboratory have demonstrated that rat liver microsomes can metabolize some polybrominated biphenyl (PBB) components in

the presence of O_2 and NADPH. Control or 3-methylcholanthrene (MC)-induced microsomes were not found to catalyze this reaction whereas microsomes induced by either phenobarbital (Pb) or PBBs were capable of doing so. Of the approximately twelve major components present in PBBs, only two were shown to be metabolized (Dannan, 1978); one is 2,2',4,5,5'-pentabromobiphenyl (peak 1, Figure 1), and the other is an unidentified hexabromobiphenyl (peak 3). It is possible, however, that the slow metabolism of other components could have been overlooked, because substrate disappearance rather than product appearance was monitored, and the disappearance of several percent of any congener over the course of the incubations could not have been detected.

If the metabolites formed from PBB congeners include epoxides (arene oxides), then these molecules would be expected to be electrophilic and capable of binding covalently to protein, RNA, and DNA. The experiments described in this Chapter were designed to examine the possibility that higher brominated biphenyls could covalently bind to protein and DNA as a consequence of their activation by microsomal enzymes. The effect of microsomal induction by PBBs on the DNA binding of a known carcinogen was also examined.

MATERIALS AND METHODS

Materials

Benzo[a]pyrene, polyethylene glycol (PEG, approximate molecular weight 400), MC, sodium dodecyl sulfate, bovine serum albumin (fraction V), butylated hydroxytoluene, highly polymerized salmon testes DNA, sodium salt (Type III), DL-trisodium isocitrate (Type I), highly

purified pig heart isocitric acid dehydrogenase (Type IV), and $NADP^+$ were purchased from Sigma Chemical Co., St. Louis, Missouri. Pb sodium U.S.P. was obtained from Merck and Co., Rahway, New Jersey. Dimethyl-POPOP (1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene), PPO (2,5-diphenyloxazole), and Triton X-100 were all scintillation grade, and were purchased from Research Products International Corp., Elk Grove, Illinois. ^{14}C-PBBs were obtained from New England Nuclear, Boston, Massachusetts. Specific activity was 9.34 mCi/mmole. The gas chromatographic profile is shown in Figure 17. [6-^3H]-benzo[α]pyrene (batch 4) was purchased from Amersham/Searle Corp., Arlington Heights, Illinois, and was used without further purification. Specific activity was 29 Ci/mmole. Unicoat precoated silica gel G TLC plates, 250 μm thick, were purchased from Analtech, Inc., Newark, Delaware. Gold shield U.S.P. quality 200 proof ethanol was obtained from IMC Chemical Group, Inc., Terre Haute, Indiana. PBBs were manufactured by the Michigan Chemical Corp., St. Louis, Michigan as the flame retardant Firemaster. The sample used was obtained from a feed mixing plant in Michigan soon after the contamination of the Michigan food supply was discovered, and is most probably Firemaster FF-1, lot 7042. All other chemicals used were reagent grade. All aqueous solutions were prepared with water which had been distilled and passed through a mixed bed resin ion exchange column.

Animals

Outbred male Sprague-Dawley rats were purchased from Spartan Research Animals, Haslett, Michigan. Rats averaging 100 g body weight were given i.p. injections of 20 mg MC/kg (in 2 ml PEG/kg) thirty-six and twenty-four hours before sacrifice. Control rats averaged 120 g

when sacrificed. Rats averaging 150 g were used for pretreatment with Pb (50 mg/kg i.p. per day for four days, sacrificed on day 5) and with PBBs (90 mg/kg i.p. once, then sacrificed seven days later). Injection volumes were 1 ml/kg for Pb (in water) and 2 ml/kg for PBBs (in PEG). Rats were housed in wire cages with no bedding underneath. They had free access to feed and water, but had no feed the night before sacrifice.

Isolation of Microsomes

Rats were sacrificed by decapitation, and microsomes were isolated from the perfused livers as described by Pederson and Aust (1970). Microsomes were rehomogenized in 0.3 M sucrose, 0.1 M tetrasodium pyrophosphate-HCl pH 7.5, repelleted by ultracentrifugation at 105,000 x g for ninety minutes, then resuspended in 0.05 M Tris-HCl pH 7.5 (at 25°), 50% glycerol, and 0.01% (w/v) butylated hydroxytoluene, and stored at -20° under argon (Welton and Aust, 1974a) until use.

Preparation of Reaction Materials

DNA was denatured before use by dissolving it in deionized water overnight, sonicating it, placing it in a boiling water bath for five minutes, then rapidly cooling the solution in an ice bath.

An appropriate aliquot of ^{3}H-benzo[a]pyrene was dried under argon to remove benzene, then redissolved in 2 mM benzo[a]pyrene in acetone, to a final specific activity of 15 mCi/mmole.

Incubation Conditions

Incubation conditions used to determine the extent of binding to macromolecules were nearly identical to those used in this laboratory

to assay aminopyrine demethylation (see Chapter 3), and to quantitate the in vitro metabolism of PBB congeners (Dannan, 1978). Incubation mixtures contained 50 mM Tris-HCl pH 7.5 (at 37°), 5 mM $MgCl_2$, 0.5 mM $NADP^+$, 5 mM DL-isocitrate, 0.005 mM $MnCl_2$, 0.5 units of isocitrate dehydrogenase, 0.08 mM ^{3}H-benzo[a]pyrene or approximately 0.008 mM ^{14}C-PBBs (50 µg), and 10 mg of control or MC-, Pb- or PBB-induced microsomal protein, in a final volume of 10 ml. Control incubations were identical except that $NADP^+$ was excluded. For the determination of binding to DNA, 20 mg of DNA in water replaced an equal volume of Tris buffer. Reactions were initiated by the addition of substrate (PBBs in 50 µl acetone, or benzo[a]pyrene in 500 µl acetone). Reaction vessels (50 ml glass centrifuge tubes) were vortexed, then placed in a Dubnoff metabolic shaker for one hour at 37°.

The addition of benzo[a]pyrene in acetone was as reported by King et al. (1975) for both the assay of benzo[a]pyrene hydroxylation and for an investigation into the covalent binding of benzo[a]pyrene metabolites to DNA as catalyzed by microsomes. Likewise, brominated biphenyl congeners are metabolized in vitro at the same concentrations of microsomal protein and PBBs (Dannan, 1978) as were used in these experiments.

Binding of ^{14}C-PBBs to Microsomal Macromolecules

Reactions were terminated by the addition of 20 ml ethanol. The turbid suspensions were vortexed, then centrifuged. Supernatants were remover, and ten 10 ml ethanol extractions were made. The ethanol was vigorously pipetted into the centrifuge tubes to help dissociate the pellets, and the tubes were vortexed each time. Little if any radioactivity was removed by the last four extractions. When the last

ethanol extractions had been made, the macromolecular residues were transferred to scintillation vials for radioactivity analyses.

Binding of ^{14}C-PBBs and ^{3}H-Benzo[a]pyrene to DNA

Incubation reactions containing DNA were terminated by the addition of 10 ml water-saturated phenol and 0.5 ml of 10% (w/v) sodium dodecylsulfate. Following centrifugation, the upper (aqueous) phases were transferred to clean 50 ml clinical centrifuge tubes. After adding 0.5 ml of 2 N NaCl, 20 ml of ethanol was added, and the tubes were centrifuged. Pellets were redissolved in 4.5 ml of 0.05 M Tris-HCl pH 7.5 (at 37°), and incubated at 37° for fifteen minutes. After adding 0.5 ml of 2 N NaCl, 10 ml of ethanol was added to reprecipitate the DNA, and the tubes were centrifuged. This cycle was performed four times, and little if any radioactivity was found in the last two washes. The final DNA pellets were transferred to scintillation vials to determine the amount of bound radioactivity.

Analysis of ^{14}C-PBBs for Metabolites

The ethanol washes from the extraction of ^{14}C-PBBs from microsomal protein (no DNA present) were pooled in culture tubes, dried under N_2 at 55°, dissolved in 4 ml of chloroform-methanol (2:1), then extracted with 0.8 ml of an aqueous salt solution (0.04% $CaCl_2$, 0.34% $MgCl_2$, and 0.58% NaCl) (Folch et al., 1956). Aliquots of both the aqueous and organic phases were removed for scintillation counting, and 100 µl of each organic phase was spotted on silica gel G TLC plates. The plates were developed in benzene-ethyl acetate (12:1), and when dry, the distribution of radioactivity was determined by scraping 1 cm portions of the silica gel into scintillation vials.

Other Methods

Protein was assayed by the method of Lowry et al. (1951), standardized with bovine serum albumin using $E_{cm}^{1\%}$ at 280 nm equal to 6.6 (Rutter, 1967). The scintillation fluid contained 2.5 g PPO and 0.1 g dimethyl-POPOP per liter, dissolved in toluene-Triton X-100 (2:1 v/v). Radioactivity was determined with a Packard Model 3310 Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Illinois). Gas chromatography was performed as described in Chapter 1.

RESULTS

The gas chromatographic profile of the ^{14}C-PBB preparation is shown in Figure 17. It can be seen that two congeners comprise approximately 98% of this mixture. While these are also the two most prominent congeners of the Firemaster PBB mixture (Figure 17), Firemaster contains 17% by weight other components. The first and third Firemaster peaks to elute from the gas chromatograph are the only two which are noticeably metabolized (Dannan, 1978), and it should be emphasized that these two congeners are virtually absent from the ^{14}C-PBB mixture. Conclusions can be drawn from these experiments only with regard to the two major Firemaster congeners, 2,2',4,4',5,5'-hexabromobiphenyl (peak 4) and 2,2',3,4,4',5,5'-heptabromobiphenyl (peak 8).

^{14}C-PBBs were aerobically incubated with rat liver microsomes in order to determine whether PBBs or their metabolites could covalently bind to microsomal macromolecules. Results are presented in Table 3. It can be seen that the amount of covalent binding was extremely small (less than 0.05% of the substrate was bound), regardless of what type

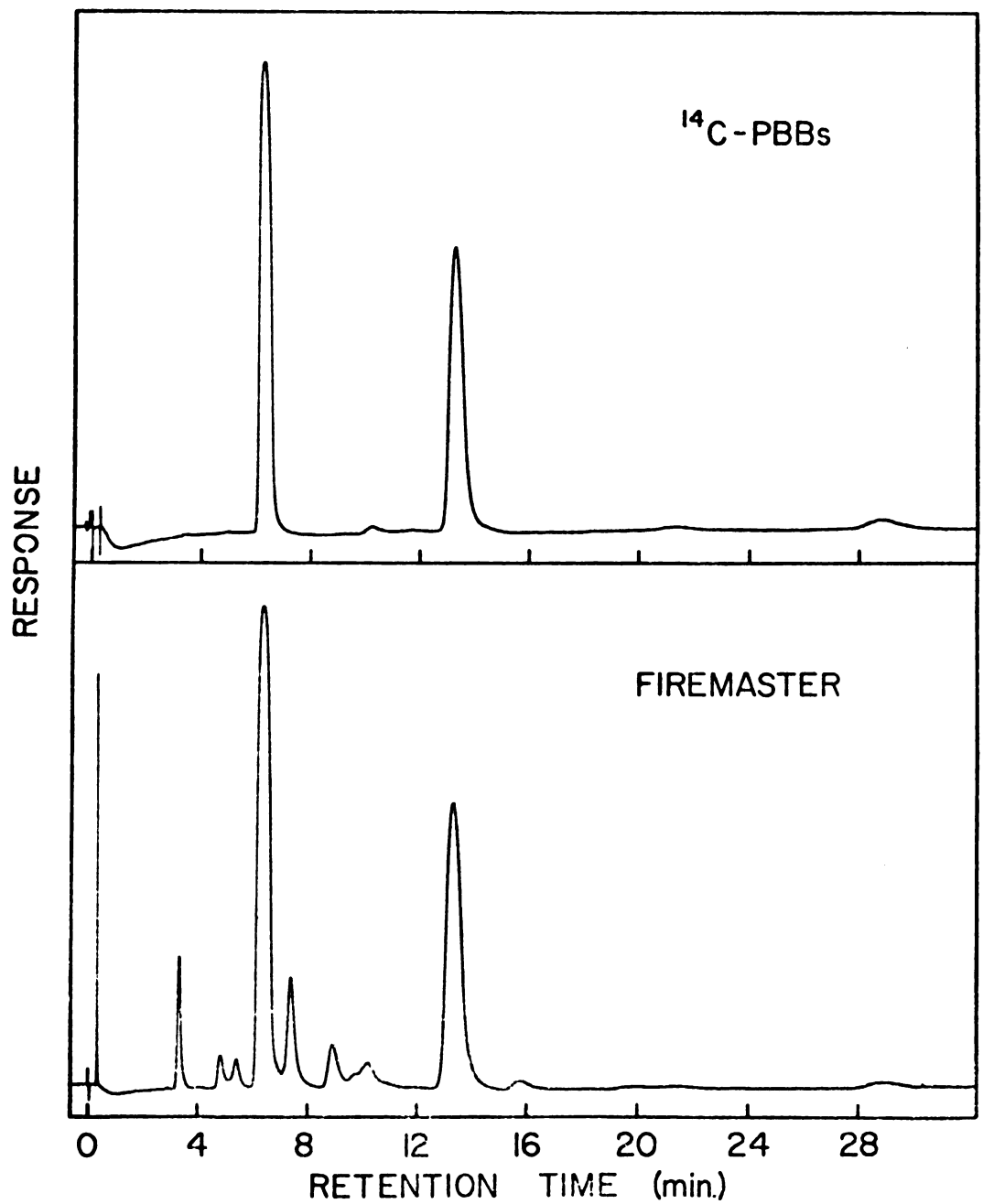


Figure 17. GAS CHROMATOGRAPHIC PROFILES OF ^{14}C-PBBs AND FIREMASTER PBBs

Three hundred ng of each sample was injected.

Table 3

EFFECTS OF MICROSOMAL ENZYME INDUCTION ON THE IN VITRO NADPH-DEPENDENT COVALENT BINDING OF ^{14}C-PBB METABOLITES TO MICROSOMAL MACROMOLECULES

Fifty μg of ^{14}C-PBBs were aerobically incubated with 10 mg of rat liver microsomal protein for one hour at 37° in the presence of NADPH. Reaction mixtures were then exhaustively extracted with ethanol to remove unbound substrate and metabolites, and the amount of radioactivity remaining associated with the microsomal macromolecules was determined. Microsomes were isolated from control rats, or from rats pretreated with MC, Pb, or PBBs. When incubations were carried out in the absence of NADPH, approximately 25 ng of PBBs remained associated with the macromolecules, regardless of the type of microsomes used.

Type of Microsomes	Quantity of PBBs Bound (ng)	(% of substrate)	Rate of Binding (fmole/min. mg protein)
Control	4.3	0.0086	11
MC	10.	0.021	27
Pb	22	0.044	58
PBBs	22	0.044	59

of microsomes were used. The extent of binding was dependent on how the microsomes had been induced; microsomes induced by Pb or PBBs bound five times the amount of radioactivity as was bound by control microsomes. Microsomes induced by MC bound more than twice the control microsome amount of radioactivity.

Denatured exogenous DNA was included in another set of in vitro microsomal incubations in order to determine whether PBBs or their metabolites could covalently bind to the nucleic acid. Positive results would indicate that these ^{14}C-PBB components are potentially mutagenic and carcinogenic. Parallel incubations were also carried out using ^{3}H-benzo[a]pyrene in place of ^{14}C-PBBs. Benzo[a]pyrene is carcinogenic when metabolically activated, and its metabolites are known to covalently bind to DNA (Grover and Sims, 1968). The benzo[a]pyrene experiments served both as a positive control for the ^{14}C-PBB binding experiments, and allowed the effect of PBB induction on the DNA binding of a secondary agent to be examined. The results of these experiments are shown in Table 4.

No radioactivity could be detected in DNA following incubations with ^{14}C-PBBs. The type of microsomes used or the presence or absence of NADPH in the incubation mixture made no difference. The data presented in Table 4 for PBBs represent the limit of sensitivity of the assay, and were calculated assuming that 10 counts per minute above background had been observed. The samples were all below this level of radioactivity.

When ^{3}H-benzo[a]pyrene was substituted for ^{14}C-PBBs, significant quantities of radioactivity were found to be covalently bound to DNA. Pb induction of microsomes had no effect on the extent of benzo[a]pyrene

Table 4

EFFECTS OF MICROSOMAL ENZYME INDUCTION ON THE IN VITRO NADPH-DEPENDENT COVALENT BINDING OF ^{14}C-PBB AND ^{3}H-BENZO[α]PYRENE METABOLITES TO DNA

Fifty µg (80 nmoles) of ^{14}C-PBBs or 200 µg (800 nmoles) of ^{3}H-benzo[α]pyrene were aerobically incubated with 10 mg of rat liver microsomal protein for one hour at 37° in the presence of NADPH and 20 mg of denatured DNA. DNA was then isolated and exhaustively extracted with ethanol to remove unbound substrates and metabolites, and the amounts of radioactivity remaining associated with the DNA were determined. Microsomes were isolated from control rats, or from rats pretreated with MC, Pb, or PBBs. When incubations were carried out in the absence of NADPH, approximately one ng of benzo[α]pyrene metabolites were bound to the DNA, regardless of the type of microsomes used. Results with ^{14}C-PBBs were independent of NADPH.

Substrate	Type of Microsomes	Quantity of Substrate Bound			Rate of Binding (fmole/min. mg prot.)
		ng	fraction of substrate	residues/10^7 bases	
^{14}C-PBBs	All	<0.3	$<7\times10^{-6}$	<.11	<0.9
	Control	5.5	27×10^{-6}	3.7	36
^{3}H-Benzo-	MC	86	430×10^{-6}	58	570
[α]pyrene	Pb	5.1	25×10^{-6}	3.4	33
	PBBs	33	160×10^{-6}	22	220

metabolite binding, while PBB and especially MC induction greatly increased the microsome-catalyzed covalent binding of benzo[a]pyrene metabolites to DNA (Table 4).

The ethanol-soluble components from the incubations of ^{14}C-PBBs with microsomes (in the absence of DNA) were examined for the presence of metabolites. The TLC system used was one with which the hydroxylated products of chlorinated biphenyls were shown to have markedly smaller R_fs than did the unmetabolized parent compounds (Ghiasuddin et al., 1976). As shown in Figure 18, no radioactivity could be detected between the origin and the unmetabolized PBBs. The profile shown is from PBB-induced microsomes; identical results were obtained with the other three types of microsomes. An average of 99% of the applied radioactivity was recovered from each TLC plate.

DISCUSSION

PBBs are a complex mixture of chemicals whose biological effects and metabolic fates are not well understood. Technical mixtures of PCBs have been shown to be carcinogenic to mice (Ito et al., 1973; Kimbrough and Linder, 1974) and rats (Kimbrough et al., 1975). One likely mechanism for the expression of carcinogenicity would be the metabolic activation of PCB components by one or more of the microsomal cytochrome P450 hemoproteins into epoxides. Epoxides are usually reactive electrophiles, which can covalently bind to protein, RNA and DNA. The binding of chemicals to DNA can lead to mutations and cancer. Because of the demonstrated carcinogenicity of PCB mixtures, and because 4-chlorobiphenyl and PCBs can covalently bind to

Figure 18. DISTRIBUTION OF RADIOACTIVITY FOLLOWING THIN LAYER CHROMATOGRAPHY OF THE ORGANIC EXTRACTS OF MICROSOMAL INCUBATIONS WITH ^{14}C-PBBs

Fifty μg of ^{14}C-PBBs were aerobically incubated with 10 mg of rat liver microsomal protein for one hour at 37° in the presence or absence of NADPH. Reaction mixtures were then exhaustively extracted with ethanol. The lipids were dissolved in chloroform-methanol (2:1) and extracted with Folch's salt solution. The organic phases were spotted on silica gel G TLC plates and developed in benzene-ethyl acetate (12:1). The distributions of radioactivity in the plates were then examined.

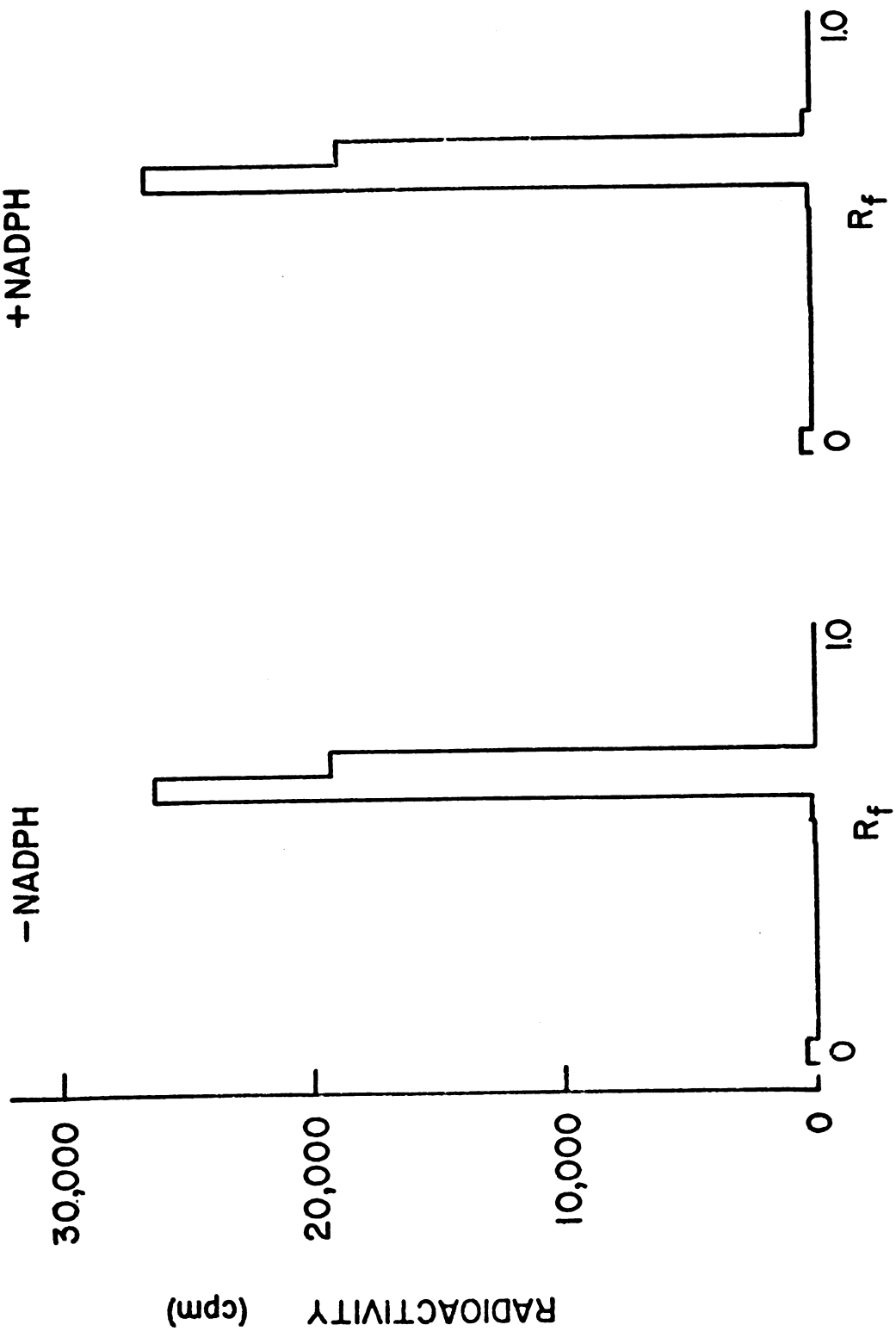

+NADPH
−NADPH
RADIOACTIVITY (cpm)
30,000
20,000
10,000
0
0
1.0
R_f
0
1.0
R_f

Figure 18.

macromolecules (Wyndham et al., 1976; Shimada, 1976), it was of interest to determine whether PBBs could be activated into metabolites capable of covalently binding to protein and more importantly DNA. And, although it appears to be absent from Firemaster, the possibility of binding was given additional credence by the observations of Safe et al. (1976) and Kohli and Safe (1976) that 4,4'-dibromobiphenyl could be metabolized via an epoxide intermediate.

The experiments reported here were performed with one serious constraint, namely, that the composition of the ^{14}C-PBBs was far simpler than that of the Firemaster PBB mixture which contaminated much of the Michigan food chain. The gas chromatographic profiles of these two mixtures are shown in Figure 17. Unfortunately, the two congeners (peaks 1 and 3) known to be metabolized in vitro by rat liver microsomes (Dannan, 1978), as well as most other congeners, were virtually absent from the ^{14}C-PBBs, and by virtue of their demonstrated ability to be metabolized, peaks 1 and 3 would be the most likely candidates to be metabolized into DNA-binding derivatives. However, these are not available in radioactive form, and these experiments were done with the only available radioactive PBBs. The ^{14}C-PBB mixture is comprised almost exclusively of 2,2',4,4',5,5'-hexabromobiphenyl (peak 4) and 2,2',3,4,4',5,5'-heptabromobiphenyl (peak 8), the two major congener in the Firemaster mixture of PBBs, and conclusions based on the experiments reported here can only be drawn with regard to these two components.

The results presented in this Chapter demonstrate that trace quantities of ^{14}C-PBBs (less than 0.05% of the substrate added) were associated with microsomal macromolecules following metabolic activation

by microsomal enzymes and subsequent exhaustive organic extractions. Whether this radioactivity originated in the two major components or in the other trace components cannot be ascertained. Most of the other Firemaster components can be seen in this ^{14}C-mixture when extremely large quantities are analyzed by gas chromatography (not shown), and one or more of these could be responsible for this low level of binding. Although only a very small amount of binding could be detected, these incubations were performed under nearly the identical conditions as used by Dannan (1978) to show the metabolism of PBB peaks 1 and 3 and the extremely rapid (nmole/min. mg protein) metabolism of 2,2'-dibromobiphenyl.

Due to the nature of the organic extraction procedure, it was very difficult to be confident that all non-covalently bound radioactivity was being extracted. The microsomal pellets remained compact despite the vigorous addition of the ethanol and repeated mixing of the contents. However, the apparent inducibility of the binding supports the conclusion that the binding was covalent, because if the radioactivity were simply being trapped, one would expect the same amount of radioactivity to be found in each pellet ragardless of the presence of NADPH or the type of induction of the microsomes. The greatest binding was seen in microsomes induced by Pb or PBBs, and MC induction also increased the binding. This finding is in rough agreement with the results of Dannan (1978), who showed that microsomed induced by Pb or PBBs could metabolize certain PBB congeners, while control or MC-induced microsomes were metabolically inactive. The binding is presumed to be to protein, since most of the RNA is known to be removed by the washing procedure used in preparing the microsomes (Welton and Aust, 1974a).

While it appears that a small amount of binding to protein can occur, no binding of ^{14}C-PBBs to DNA could be detected when DNA was included in the reaction mixtures. The DNA used in these experiments was denatured, because King et al. (1975) had shown denaturation to increase the covalent binding of benzo[a]pyrene 2.5-fold. The type of microsomes used had no effects on the results; no binding was seen whether control or MC-, Pb-, or PBB-induced microsomes were used. It can therefore be concluded that the two major components of PBBs (Firemaster), which together comprise 83% by weight of this mixture (Chapter 1), are not metabolically activated into electrophilic DNA-binding metabolites. Whether the remaining PBB components can be bioactivated into DNA binding, and therefore potentially mutagenic and carcinogenic metabolites, remains to be determined. Kimbrough et al. (1977) have obtained evidence that PBBs cause neoplastic lesions in rats, so this possibility must be seriously considered.

The experiments with ^{3}H-benzo[a]pyrene served as a positive control for the analogous experiments with PBBs. Although the observed extent of benzo[a]pyrene metabolite binding to DNA was somewhat lower than has been reported in the literature (Grover and Sims, 1968; Gurtoo and Bejba, 1974; King et al., 1975), these incubations demonstrated that the negative results obtained with ^{14}C-PBBs and DNA under the same conditions were not due to some error or fault in the experimental design.

Benzo[a]pyrene is a well characterized planar aromatic compound which is activated by microsomal drug metabolizing enzymes into a potent diol epoxide intermediate capable of binding to DNA (Sims et al., 1974). The DNA incubations with ^{3}H-benzo[a]pyrene, in addition to serving as a positive control for the analogous PBB-binding experiment,

demonstrate that the induction of microsomal drug metabolizing enzymes by PBBs can greatly enhance (by six-fold) the amount of benzo[a]pyrene metabolites covalently binding to DNA. The increased binding catalyzed by MC-induced microsomes, and the small effect of Pb-induced microsomes, confirms the results of Gurtoo and Bejba (1974). The results with all four types of microsomes on the binding of metabolites to DNA also parallel the results presented in Chapter 3 on the rates of benzo[a]-pyrene hydroxylation as measured fluorimetrically.

The enhanced binding of benzo[a]pyrene metabolites seen with PBB induced microsomes suggests that exposure to PBBs may increase the mutagenic and carcinogenic potentials of planar aromatic hydrocarbons administered secondarily. While PCBs have been shown to decrease the carcinogenic potentials of 3'-methyl-4-dimethylaminoazobenzene, N-2-fluorenylacetamide, and diethylnitrosamine (Makiura et al., 1974), they increase the carcinogenicity of α- and β-1,2,3,4,5,6-hexachlorocyclo-hexane (Ito et al., 1973). Direct studies of the effects of PBBs on the carcinogenicity of secondary agents have not been reported.

This investigation has determined only the extent of binding of the two major PBB components to protein and DNA, due to the unavail-ability of the other radioactive congeners. It is hoped that the congeners known to be metabolized will be available in the future in radioactive form so that their binding to protein, RNA, and DNA can be determined.

CHAPTER 3

EFFECTS OF PURE POLYBROMINATED BIPHENYL CONGENERS ON RAT LIVER MICROSOMAL DRUG METABOLIZING ENZYMES

ABSTRACT

2,2',4,4',5,5'-Hexabromobiphenyl (HBB_6) and 2,2',3,4,4',5,5'-heptabromobiphenyl (HBB_7) comprise 56 and 27%, respectively, of the Firemaster mixture of polybrominated biphenyls (PBBs). The effects of HBB_6, HBB_7, and of the suspected trace component 2,2'-dibromobiphenyl (DBB) on liver microsomal drug metabolizing enzymes were examined. Rats were injected i.p. with 90 mg/kg of these compounds, and sacrificed at intervals up to twenty-two days later. HBB_6 and HBB_7 increased liver weights, and strongly induced microsomal protein, NADPH-cytochrome P450 reductase, cytochrome P450, aminopyrine demethylation, and epoxide hydratase. Both caused only small inductions in benzo[α]-pyrene hydroxylation and *p*-nitrophenol-UDP-glucuronyltransferase, and neither shifted the cytochrome P450 spectral maximum from 450 nm. These results, and the results of SDS-polyacrylamide gel electrophoresis, demonstrate that HBB_6 and HBB_7 affect microsomes in a manner very similar to phenobarbital, and that their inductions are distinct from those caused by either 3-methylcholanthrene or PBBs. DBB had little if any effect on any parameter examined, a result which demonstrates that not all brominated biphenyls are microsomal inducers.

While HBB_6 and HBB_7 are both strictly phenobarbital-type inducers of liver microsomal drug metabolizing enzymes, PBBs cause a mixed-type induction of these enzymes. Seventeen percent by weight of Firemaster remains uncharacterized; one or more of these components must be

responsible for the 3-methylcholanthrene-like aspects of the induction caused by the PBB mixture.

INTRODUCTION

Polybrominated biphenyls (PBBs) have been shown to be potent inducers of microsomal drug metabolizing enzymes in rat liver, kidney, and mammary gland (Troisi, 1975; Dent et al., 1976a,b, 1977a,b,d; Moore et al., 1976, 1978; McCormack, 1977, 1978). Inducers of microsomal drug metabolizing enzymes can be divided into two categories, one of which is exemplified by phenobarbital (Pb), while the other is exemplified by the polycyclic aromatic hydrocarbon 3-methylcholanthrene (MC). Each of these prototype compounds by itself causes a distinct and characteristic pattern of microsomal induction. When both Pb and MC are administered to animals together, the properties of both Pb- and MC-induced microsomes can be observed, and the results are termed a mixed-type induction. In liver, PBBs cause a mixed-type induction of microsomal drug metabolizing enzymes.

At least thirty different components are synthesized when PBBs are manufactured (Chapter 1), including brominated naphthalenes (Kay, 1977) and possibly brominated dibenzofurans. It is not known which of these components are capable of inducing microsomal enzymes, nor is it known what pattern of induction would result from such treatments. Also unknown is why PBBs cause a mixed-type induction. It is possible that some of its components are Pb-type inducers while others are MC-type inducers. A second possibility is that the mixed-type induction results from the parent compounds causing one type of induction while

the metabolites produced in vivo cause the second type. Also a possibility is that one or more of the components, such as an asymmetric bifunctional bromobiphenyl congener, could by itself be capable of causing a mixed-type induction.

The experiments described in this Chapter were designed to determine the effects of components found in PBBs on liver microsomal drug metabolizing enzymes in rats. The major component of the Firemaster mixture of PBBs has been identified as 2,2',4,4',5,5'-hexabromobiphenyl (HBB_6) (Sundström et al., 1976a; Jacobs et al., 1976). HBB_6 comprises 56% by weight of this mixture (Chapter 1). The second most prominent congener was identified as 2,2',3,4,4',5,5'-heptabromobiphenyl HBB_7, and shown to comprise 27% by weight of the PBB mixture (Chapter 1). Also, a trace component has been tentatively identified as 2,2'-dibromobiphenyl (DBB). In the experiments reported here, rats were given a single i.p. injection of these three congeners in order to determine their effects on liver microsomal drug metabolizing enzymes. While PBBs are a mixed-type inducer of these enzymes, HBB_6 and HBB_7 were found to be strictly Pb-type inducers, and DBB was without effect. The remaining 17% by weight of Firemaster must contain one or more components capable of causing the MC-like effects seen in response to the PBB mixture.

MATERIALS AND METHODS

Materials

Benzo[α]pyrene, polyethylene glycol (PEG, approximate molecular weight 400), sodium dodecyl sulfate (SDS), bovine serum albumin

(fraction V), butylated hydroxytoluene, DL-trisodium isocitrate (Type I), highly purified pig heart isocitric acid dehydrogenase (Type IV), $NADP^+$, type WN-3 neutral chromatographic alumina, horse heart cytochrome c (Type VI), tetrasodium NADPH (Type I), disodium NADH (Grade III), Tween 80, ammonium UDP-glucuronate, and glycine were purchased from Sigma Chemical Co., St. Louis, Missouri. Aminopyrine, 2-bromobiphenyl, 3-bromobiphenyl, 2,2'-dibromobiphenyl and coomassie brilliant blue G-250 (xylene brilliant cyanin G) were obtained from K and K Rare and Fine Chemicals, Plainview, New York. Aminopyrine was recrystallized twice from hexane before use. Styrene oxide, 2,4-pentanedione, *p*-nitrophenol, and 4,4'-dibromobiphenyl were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wisconsin. Biphenyl and 4-bromobiphenyl were purchased from Eastman Kodak Co., Rochester, New York. Acrylamide, N,N'-methylenebisacrylamide, ammonium persulfate, and N,N,N',N'-tetramethylethylenediamine were obtained from Canalco, Rockville, Maryland. Triton X-100 was purchased from Research Products International Corp., Elk Grove Village, Illinois, agar was from Difco Laboratories, Detroit, Michigan, pyronin B was from Hartman-Leddon Co., Philadelphia, Pennsylvania, and glass-distilled non-spectro hexane, suitable for pesticide analysis, was from Burdick and Jackson Laboratories, Muskegon, Michigan. The hexane was purified before use by passing it over basic alumina. [7-^{3}H]-Styrene oxide, lot number 777-215, was purchased from New England Nuclear, Boston, Massachusetts. Specific activity was 29.2 m Ci/mmole. The PBB mixture was made by the Michigan Chemical Corp., St. Louis, Michigan, as the flame retardant Firemaster. The sample was obtained from a feed mixing plant shortly after the accidental contamination of the Michigan food chain was discovered, and is most probably Firemaster

FF-1, lot 7042. All other chemicals used were reagent grade. All aqueous solutions were prepared with water which had been distilled and passed through a mixed bed resin ion exchange column.

Preparation of Brominated Biphenyls

HBB_6 was isolated from Firemaster by column chromatography and recrystallization as described in Chapter 1. HBB_7 was similarly isolated from a higher molecular weight mixture of PBBs. It contained 2% HBB_6, but otherwise was pure (Chapter 1). DBB was purified before use by dissolving 0.7 g in 10 ml of hexane, and eluting it with hexane from a column containing 100 g of neutral alumina. Fractions containing DBB were pooled, dried on a rotary evaporator, and recrystallized from hexane. The final DBB preparation melted at 79-79.5°. Gas chromatographic analyses were made as described in Chapter 1, using an electron capture detector.

Animals

Outbred male Sprague-Dawley rats were purchased from Spartan Research Animals, Haslett, Michigan. After a three day acclimatization period, they were injected i.p. with 90 mg DBB, HBB_6, or HBB_7/kg body weight, or with an equal volume of solvent (2 ml PEG/kg). The experiments with HBB_6 and with DBB and HBB_7 were done separately, each with their own set of control animals. Rats injected with HBB_6 averaged 180 g when injected, and those treated with DBB and HBB_7 averaged 155 g. All animals were kept in wire cages without bedding. Three rats from each group were sacrificed at intervals up to twenty-two days later. Rats were given free access to feed and water except for the night before sacrifice, when feed was removed.

Necropsy Examination

Each rat was necropsied immediately after the liver was removed. Specimens of liver, kidney, spleen, thymus, heart, lung, stomach, small intestine, pancreas, and brain were fixed in 10% buffered formalin, embedded in paraffin, and sectioned six μm thick. Tissue sections were stained with hematoxylin and eosin, and liver sections were also stained with oil red O. I thank Dr. Stuart Sleight for these analyses.

Isolation of Microsomes

Liver microsomes were isolated and washed individually from each rat. Liver microsomes were also isolated and washed from the pooled livers of rats pretreated with maximally effective doses of MC, Pb, or PBBs. All procedures were as described in Chapter 2. One of the HBB_7 samples from day 4 was lost during homogenization, and one from day 14 was improperly injected and showed no evidence of induction.

Aminopyrine Demethylation

Aminopyrine demethylation was assayed by a slight modification of the methods of Aust and Stevens (1971) and of Gnosspelius et al. (1969/70), in order to improve the linearity of the assay with both time and protein. Reaction mixtures (5 ml total volume) contained 50 mM Tris-HCl, pH 7.5 (at 37°), 5 mM $MgCl_2$, 0.5 mM $NADP^+$, 5 mM DL-sodium isocitrate, 0.005 mM $MnCl_2$, 0.25 units of isocitrate dehydrogenase, 20 mM aminopyrine, and 2.5 mg of microsomal protein. Reaction mixtures were prepared on ice, adding the aminopyrine last. Reactions were then initiated by placing the beakers (20 ml) in a rapidly agitating Dubnoff metabolic shaker at 37°. At 1, 4, 7, and 10 minutes of incubation, one ml aliquots were removed and pipetted into test tubes containing one ml

of 10% (w/v) trichloroacetic acid (TCA). After waiting ten minutes, two ml of Nash reagent (2 M ammonium acetate, 0.05 M acetic acid, and 0.02 M 2,4-pentanedione) (Nash, 1953) was added to each test tube. Mixtures were heated at 60° for ten minutes to develop the color, cooled to room temperature, and centrifuged for fifteen minutes. The absorbance of the supernatant was measured at 412 nm using a Coleman Jr. Spectrophotometer equipped with a flow cell. The formaldehyde concentration was calculated using an extinction coefficient of 7.08 x 10^3 M^{-1} cm^{-1} and the dilution factor of 4 (Pederson, 1973). The apparent rate of formaldehyde formation from incubations in the absence of aminopyrine was used as the blank. These blanks varied with the quantity of microsomal protein present in the incubation but the values were independent of the type of microsomal protein used.

Benzo[α]pyrene Hydroxylation

Benzo[α]pyrene hydroxylation was assayed fluorimetrically by the method of Gielen et al. (1972). Reaction mixtures contained 50 mM potassium phosphate, pH 7.2, 0.36 mM NADPH, 0.39 mM NADH, 3 mM $MgCl_2$ 0.08 mM benzo[α]pyrene in methanol (final methanol concentration of 4%), 0.6 mg bovine serum albumin/ml, and 250 μg of microsomal protein. The stock solution of benzo[α]pyrene in methanol was found to be stable indefinitely, but only when stored in complete darkness. Reaction volumes were one ml, contained in ten ml Erlenmeyer flasks. Assays were begun at one minute intervals, in sets of ten. The benzo[α]pyrene was added within the minute before the incubation was begun. Vigorous and complete mixing of the substrate was found to be essential to obtaining reproducible results, which were normally within 10%. Reactions

were initiated by placing the flasks in a rapidly agitating Dubnoff metabolic shaker at 37°, and were terminated ten minutes later by the addition of one ml of ice cold acetone. Blanks consisted of incubations without microsomes, or of reactions to which the benzo[α]pyrene was added only after the addition of cold acetone. Neither gave detectable fluorescence.

The stopped reaction mixtures were placed on ice to facilitate precipitation, then 3.25 ml of hexane was added to each. Flasks were incubated at 37° for an additional ten minutes, then one ml of each 3.3 ml organic phase was transferred to a culture tube. A separate set of culture tubes was kept for this assay, and the tubes were acid washed between uses. The Teflon-lined plastic caps were purchased specifically for this assay and were never allowed to be mixed with other caps in the lab which were often contaminated with coomassie blue. The hexane extracts were in turn extracted with three ml of 1 N sodium hydroxide, and the fluorescence of the alkali-soluble products were determined. Since the fluorescence was found to decay with a half-time of ninety minutes in the sodium hydroxide, a protocol was developed to extract each sample immediately before the fluorescence determination. Three ml of 1 N sodium hydroxide was added to the one ml hexane aliquot, and the tube was capped. After thirty seconds, the tube was shaken vigorously for one minute, then placed in a table top centrifuge for 2 and 1/2 minutes. The organic phase was aspirated off, and the fluorescence of the aqueous phase was determined in an Aminco-Bowman Spectrophotofluorometer using an excitation wavelength of 396 nm and an emission wavelength of 520 nm. In this way, with two people working, one sample could be assayed every 2 and 1/2 minutes.

There appeared to be no loss of fluorescence when the hexane extracts were kept for several hours; when thirty duplicate assays were performed in the reverse order of that in which the samples had originally been run, no differences in the duplicate values could be attributed to the sequence in which the determinations had been made.

The fluorescence of quinine sulfate was used to calibrate the assay, as described by Rickert and Fouts (1970), who found that "0.036 nmole of 3-hydroxybenzo[α]pyrene/ml in 1 N NaOH gives a fluorescence equal to that given by 0.3 μg quinine sulfate per ml in 0.1 N H_2SO_4 at an excitation wavelength of 400 nm and emission of 522 nm."

Epoxide Hydratase

Epoxide hydratase was assayed radiochemically with styrene oxide as the substrate, as described by Oesch et al. (1971). Reaction mixtures contained 0.1 M Tris-HCl, pH 9.0 (at 37°), 0.02% (w/w) Tween 80, 8 mM ^{3}H-styrene oxide (approximately 40,000 cpm/assay) in acetonitrile (final acetonitrile concentration of 4%), and approximately 500 μg of microsomal protein. The reactions were linear to at least 900 μg of protein. CAUTION: in addition to being radioactive, the substrate is carcinogenic, and despite a boiling point of 194°, it is also quite volatile.

Incubations were for twenty minutes at 37°, following a 20-25 second preincubation. Reactions were initiated by the addition of styrene oxide, and terminated by adding ten ml of petroleum ether (boiling range 30-60°) with vortexing. The aqueous phase reaggregated within five minutes, then the tubes were placed in a dry ice-acetone bath for five or more minutes. The organic phase was poured off, and

a second ten ml petroleum ether extraction was performed. Two ml of ethyl acetate was added with vortexing, and after phase separation, 0.5 ml of the organic phase was placed in a scintillation vial. Radioactivity was determined as described in Chapter 2. Blanks contained no microsomes, but were incubated at 37° for twenty minutes before extractions were performed.

UDP-Glucuronyltransferase

UDP-glucuronyltransferase was assayed spectrophotometrically by quantitating the conversion of *p*-nitrophenol to a colorless glucuronic acid conjugate.

The initial assays were run on HBB_6-induced microsomes by a modification of the method of Grote et al. (1975). Reaction concentrations were the same, except that KCl was omitted. The reaction was assayed at 25° in cuvettes having a 0.5 cm light path, at 403 nm. Rates were linear with protein from 250 to 600 µg/ml of assay mixture. Reaction rates were sigmoidal, and the length of the lag phase seemed to vary from day to day and from person to person. For these reasons, a second assay for the transferase was tested, found to be more rapid and reproducible, and was therefore used to assay the microsomes isolated from rats tested with DBB and HBB_7.

The recommended procedure is by Lucier, et al. (1977). Reactions contained 0.8 mM *p*-nitrophenol, 1.4 mM UDP-glucuronic acid, 10 mM $MgCl_2$, 150 mM Tris-HCl pH 7.4 (at 37°), 0.2 µl of Triton X-100 per mg of microsomal protein, and approximately 500 µg of microsomal protein, in a volume of 1.4 ml. Reaction mixtures were prepared at room temperature, by adding the Triton and microsomes to a small volume of Tris buffer.

After mixing, the microsomes were preincubated batchwise with the detergent at 37° for five minutes. A solution containing *p*-nitrophenol, Tris, and $MgCl_2$ was then added (0.9 ml). Reactions were initiated by adding the UDP-glucuronic acid, vortexing, and placing the test tubes in a 37° water bath. After fifteen minutes, five ml of 0.2 M sodium glycine pH 10.4 was added, and the absorbance at 403 nm was determined using cuvettes with a 0.5 cm path length. Blanks contained a range of protein (0-700 μg), but no UDP-glucuronic acid. Blank readings were a function of the quantity of microsomal protein present but were independent of the type of microsomes added. Rates were calculated using the empirically-determined extinction coefficient of 18.0 μmole/cm^2 at pH 10.4. The results were quite reproducible, and the assay is very close to being linear with protein at any one time of incubation, although reaction rates are not linear with time. When microsomes having elevated transferase activities are assayed, the quantity of protein added should be decreased to about 300 μg or less.

SDS-Polyacrylamide Gel Electrophoresis

Procedures for preparing gels and performing electrophoresis were similar to those of Fairbanks et al. (1971), with several exceptions (Welton and Aust, 1974b). The concentration of SDS in both the electrophoresis buffer and gel was lowered from 1% to 0.1%, the samples did not contain dithiothreitol or any other reducing agent, and the samples were not boiled prior to electrophoresis. Also, electrophoresis was carried out at 0-4° in the dark when gels were to be stained for hemoproteins.

A vertical slab gel apparatus similar to that described by Studier (1973) was used. The glass plates were cleaned in concentrated sulfuric acid, while the plexiglass spacers were cleaned with detergent. The pieces were assembled using pinch-type paper clamps and sealed with hot 1.5% agar. Gel solutions were added to the mole with a Pasteur pipet, and polymerization usually occurred within ten minutes.

After overnight storage at room temperature, the dried agar was removed and replaced at the sides with fresh agar. The top and bottom pieces of the mold were removed, and the gel was sealed to the slab gel apparatus with agar and metal clips. After adding about 450 ml of electrophoresis buffer per chamber, loose pieces of gel and agar were removed from the sample wells with a syringe, and bubbles were removed from below the gel by using a Pasteur pipet with a bent tip. Gels were pre-electrophoresed for thirty minutes at 30V. Electrophoresis required about seven hours in the cold (about four hours at room temperature), with currents of about 24 and 38 mA, respectively. When the tracking dye approached the bottom, the gels were removed, placed in a trough containing a small volume of water, and the positions of the dye fronts were marked with a razor blade.

Gels to be stained for protein were soaked overnight in 33% isopropanol - 10% TCA, followed by three or more hours in 10% TCA, or until they were clear. Protein stain was freshly prepared for each gel by making a 0.1% solution of coomassie brilliant blue G250 in 1 N H_2SO_4. After half an hour, the solution was filtered by gravity, and 10 N KOH was added until the color changed from green to dark blue or purple. Solid TCA was then added to a final concentration of 12%. The entire procedure for protein staining is similar to that described by

Malik and Berrie (1972). Gels were stained overnight with this solution, then destained for one hour in 0.1 N H_2SO_4. Destaining was completed with several changes of water. These procedures were performed in the dark for best results.

Gels to be stained for heme were first placed in 0.02 M Tris-HCl pH 7.4 - 50% methanol for thirty minutes. A forty-five minute staining period followed, using a freshly prepared solution containing 0.25 M sodium acetate, pH 5.3, 0.25% (w/v) benzidine dihydrochloride, 25% (v/v) methanol, and 0.75% H_2O_2 (the H_2O_2 was added immediately before staining was begun). CAUTION: benzidine is a potent carcinogen. The gel was then soaked for ten minutes in 25% methanol, then in 0.1 M Tris-HCl pH 7.5. Gels were ready to photograph or scan within an hour. All procedures were carried out in the dark.

Other Assays

NADPH-cytochrome P450 reductase was assayed by its ability to reduce exogenous cytochrome c by the method of Pederson et al. (1973). Cytochrome P450 was assayed by its reduced carbon monoxide difference spectrum in the presence of 10% glycerol (Omura and Sato, 1964). Protein was assayed by the method of Lowry et al. (1951), using bovine serum albumin as the standard as described by Rutter (1967), and using a Coleman Jr. Spectrophotometer.

Except where indicated otherwise, all spectrophotometric assays were performed with a Perkin-Elmer-Coleman 124 double beam spectrophotometer.

RESULTS

Firemaster is a mixture of at least thirty brominated biphenyls and other contaminants. As shown in Chapter 1, Figure 1, there are approximately a dozen congeners present in easily detectable quantities, and many more components can be revealed by gas chromatographic analysis of partially purified fractions (not shown). In no case, however, do the major peaks as shown behave as more than one major component when chromatographed on alumina or any other column material tested. Figure 19 shows the structures of the three congeners tested, and demonstrates that the preparations were nearly homogeneous. HBB_7 did contain 2% HBB_6, while DBB contained 2% impurities.

The animal experiments described in this Chapter were designed to characterize the consequences of a maximally effective dose of each of these three congeners on liver microsomal drug metabolizing enzymes. Previous experiments in our laboratory (Troisi, 1975) had shown that five daily injections of PBBs (90 mg/kg) resulted in a more rapid induction of microsomal enzymes than a single injection, but that the final induced levels were comparable. Other preliminary experiments have shown that a single i.p. injection of 22.5 mg/kg of either HBB_6 or PBBs caused nearly as complete an induction within seven days as did an injection of 90 mg/kg of either agent. The treatments used in these investigations are therefore assumed to have resulted in the maximum possible responses.

Figure 19. GAS CHROMATOGRAPHIC PROFILES AND STRUCTURES OF DBB, HBB_6, AND HBB_7

Each sample contained 100 ng of purified congener.

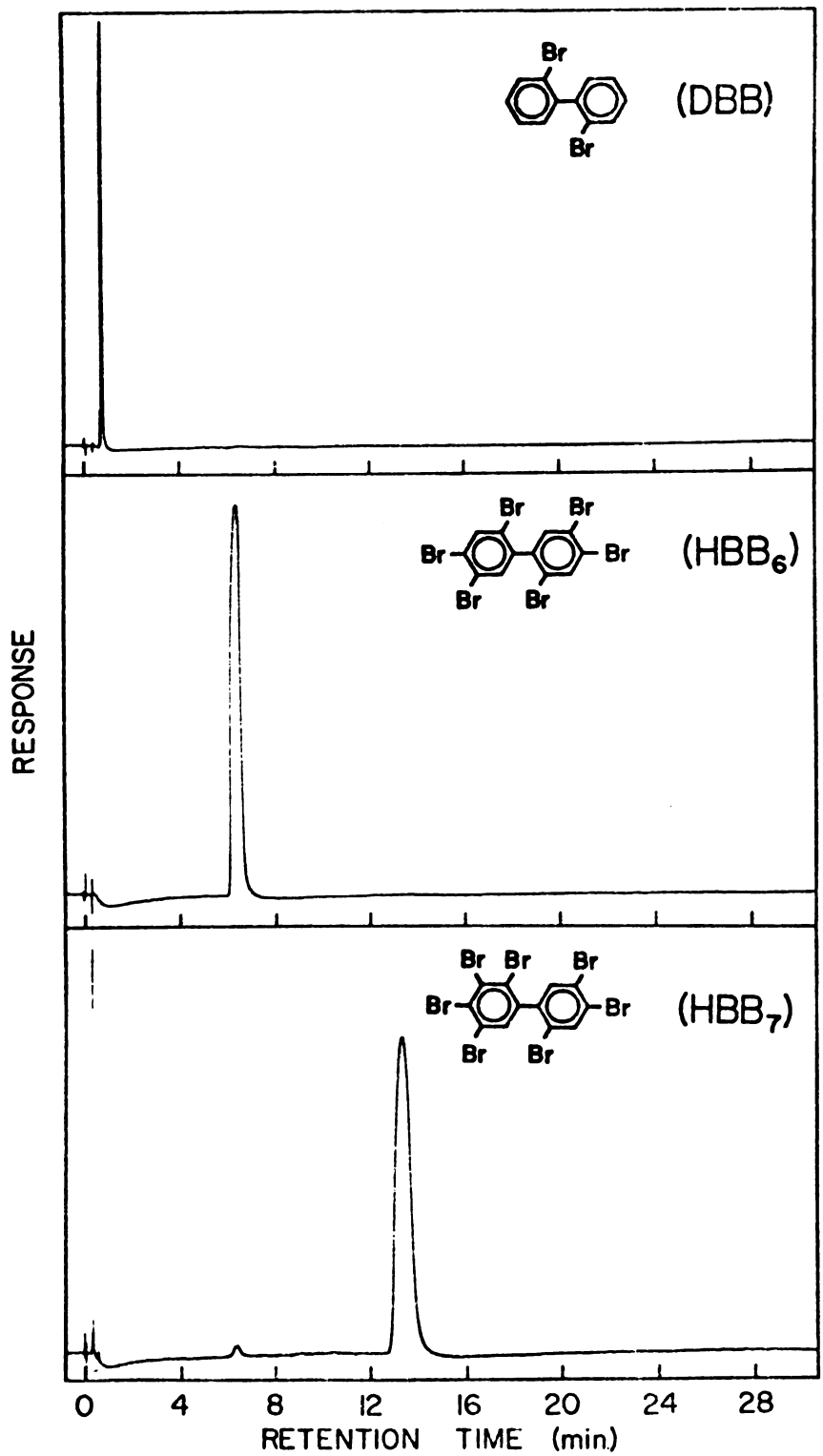

Br
Br
(DBB)
Br Br
Br
Br Br
(HBB6)
Br Br Br
Br
Br Br
(HBB7)
RESPONSE
0 4 8 12 16 20 24 28
RETENTION TIME (min.)

Figure 19

Gross examination of the rats revealed the presence of a few flecks of whitish precipitate in the peritoneal cavity of some of the rats given HBB_6. Rats injected with HBB_6 had livers that were somewhat swollen, with the edges of the lobes having a rounded appearance. Otherwise, there were no remarkable gross lesions in these animals or their controls. The pathological results from the rats given DBB or HBB_7 are not yet available.

Microscopic examination revealed lesions in the liver and lung, while none were found in the kidney, spleen, thymus, heart, stomach, small intestine, pancreas, or brain. Most of the rats, regardless of treatment, had a mild pneumonia characterized by thickening of the alveolar walls and by increased numbers of inflammatory cells, mainly lymphocytes, around the bronchioles. Beginning on day two, some of the hepatic cells in rats given HBB_6 were swollen and vacuolated (Figure 20). The affected cells were not confined to any particular region of the hepatic lobule, but the majority of them were in the midzonal region. These lesions were seen in two of three livers on day two, three of three on day four, and in two of three livers from rats killed on days seven, ten, and fourteen. Although vacuoles were occasionally seen in hepatic cells from the controls, they were much less numerous than in the treated rats, and the cells did not appear swollen (Figure 20). Examination of livers stained with oil red O did not reveal a significant increase in the quantity of fat in the livers of rats given HBB_6. These analyses were provided by Dr. Stuart Sleight.

As shown in Figure 21, liver weight to body weight ratios were increased by up to a third by HBB_6. The increase was rapid, with a 25% increase within two days after treatment, and was still pronounced

Figure 20. EFFECT OF HBB_6 ON LIVER STRUCTURE

Top: Section of liver from a rat four days after an i.p. injection of 90 mg HBB_6/kg. Note swollen and vacuolated cells.

Bottom: Section of liver from a control rat. Hematoxylin and eosin stain, X 400.

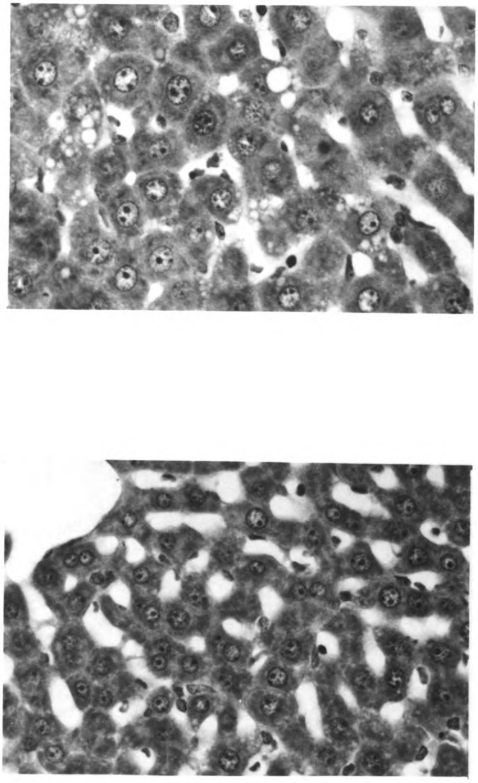


Figure 20

Figure 21. EFFECTS OF 90 mg DBB, HBB_6, AND HBB_7/kg ON LIVER WEIGHT

Rats were injected on day 0 and sacrificed at intervals up to twenty-two days later. Values shown are means ± SE (N=3). Responses to maximally effective doses of MC, Pb, and PBBs are shown for comparison.

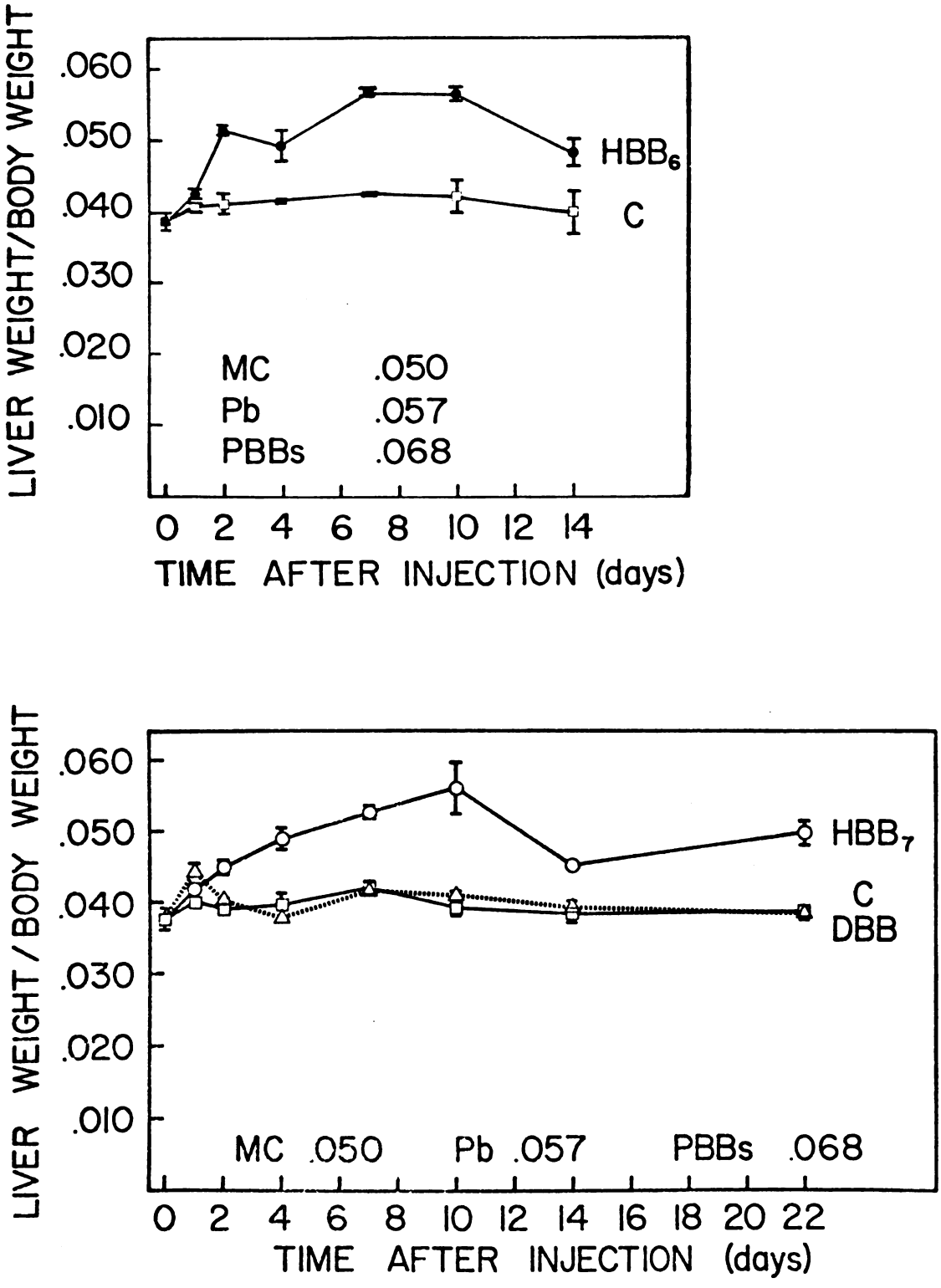

LIVER WEIGHT/BODY WEIGHT
.060
.050
.040
.030
.020
.010
HBB6
C
MC .050
Pb .057
PBBs .068
0 2 4 6 8 10 12 14
TIME AFTER INJECTION (days)
LIVER WEIGHT / BODY WEIGHT
.060
.050
.040
.030
.020
.010
HBB7
C
DBB
MC .050
Pb .057
PBBs .068
0 2 4 6 8 10 12 14 16 18 20 22
TIME AFTER INJECTION (days)

Figure 21

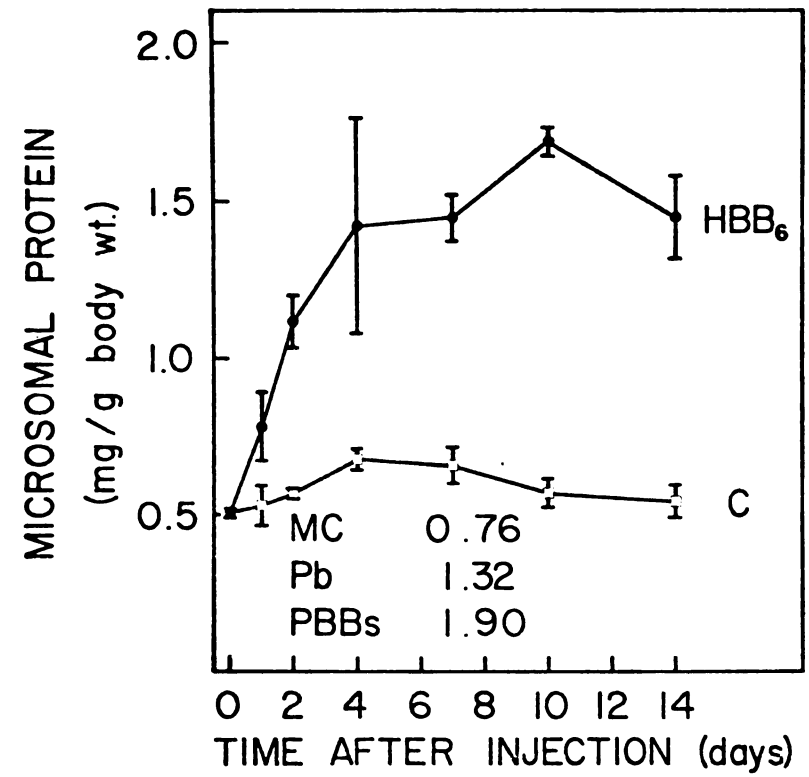
fourteen days after the injection. Except for a slight increase on day 1, DBB had no effect. HBB_7 caused a steady increase of up to 43% by day 10, and livers were still 28% heavier than controls at day 22. The effects of HBB_6 and HBB_7 were comparable to that caused by Pb, but were far less than the 60% increase seen in response to PBBs. MC was not as effective at inducing liver weight as were the other agents.

The effects of bromobiphenyl congeners on liver microsomal protein are shown in Figure 22. The induction by HBB_6 was apparent within one day of injection, and continued to increase sharply for several days thereafter, until the amount of protein was nearly triple the control value by day 10. The content of microsomal protein in response to HBB_6 was only slightly greater than that caused by Pb. HBB_7 caused a persistent induction of up to 2.8-fold, a level somewhat greater than that caused by Pb. Both HBB_6 and HBB_7 were less effective inducers than the mixture from which they were derived. MC induced microsomal protein far less than did these four agents, and DBB had no effect on the quantity of microsomal protein.

The data in Figure 22 have been placed on a body weight basis in order to compensate for the small variations in the body weight of the rats on a given day and for the growth of the rats over the course of the experiment. The data in the figures which follow are all expressed as units per mg of microsomal protein. Thus, for example, while cytochrome P450 was induced 2.6-fold in microsomes by HBB_6 (Figure 24), because the microsomes were also induced (Figure 22), the treated rats actually contained 5-8 times the amount of hepatic cytochrome P450 as did the controls.

Figure 22. EFFECTS OF 90 mg DBB, HBB_6, AND HBB_7/kg ON MICROSOMAL PROTEIN

Rats were injected on day 0 and sacrificed at intervals up to twenty-two days later. Values shown are means ± SE (N=3). Responses to maximally effective doses of MC, Pb, and PBBs are shown for comparison.


MICROSOMAL PROTEIN
(mg/g body wt.)
2.0
1.5
1.0
0.5
HBB6
C
MC 0.76
Pb 1.32
PBBs 1.90
0 2 4 6 8 10 12 14
TIME AFTER INJECTION (days)

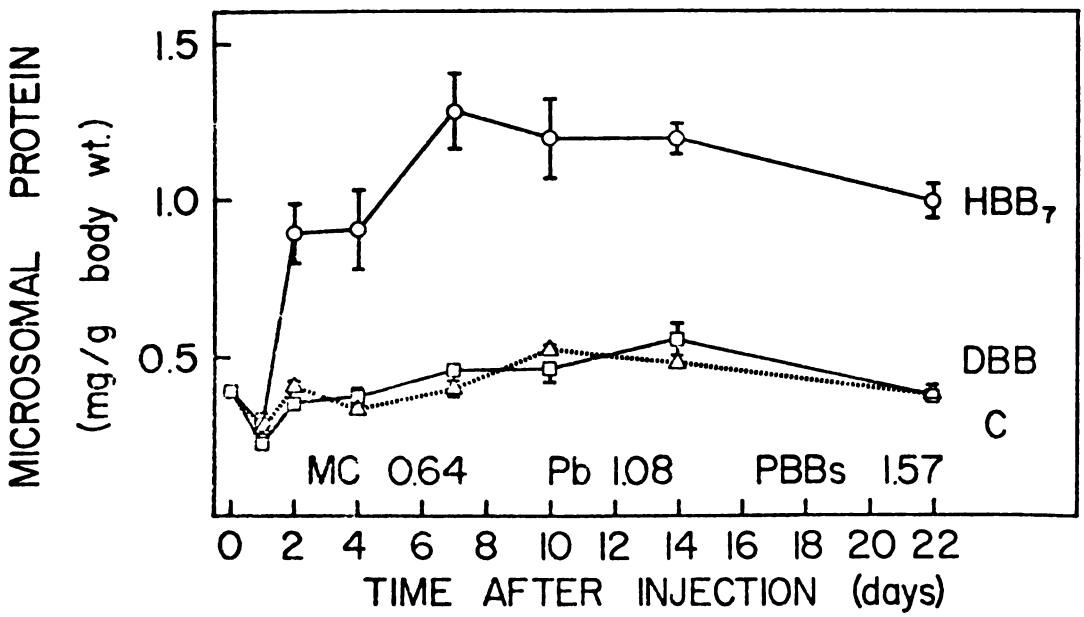

MICROSOMAL PROTEIN
(mg/g body wt.)
1.5
1.0
0.5
HBB7
DBB
C
MC 0.64 Pb 1.08 PBBs 1.57
0 2 4 6 8 10 12 14 16 18 20 22
TIME AFTER INJECTION (days)

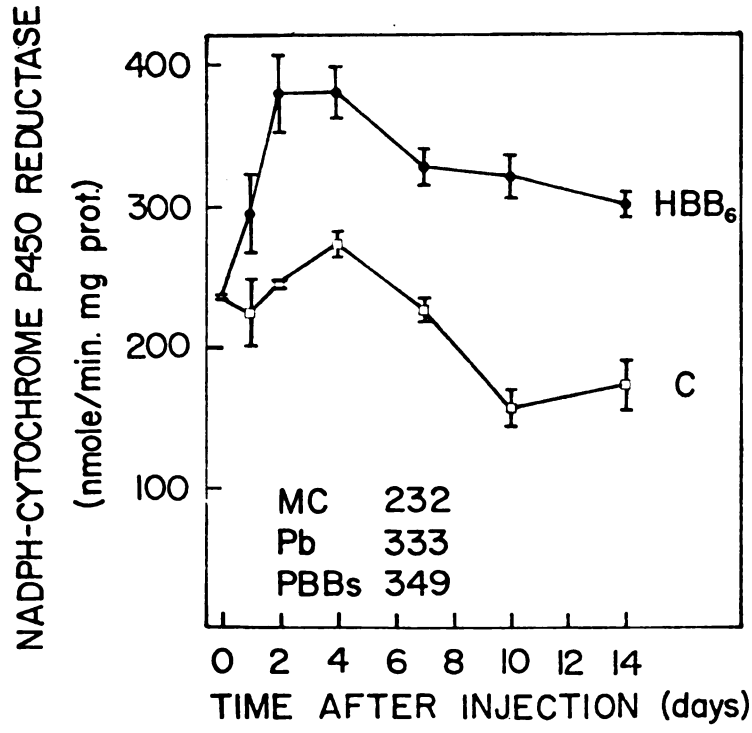
Figure 22

The microsomal mixed-function oxidase system consists of NADPH-cytochrome P450 reductase plus the cytochrome P450 hemoproteins. Figure 23 shows that HBB_6 and HBB_7 induced the reductase to about the same extent as was caused by Pb or PBBs, and that MC had little effect on this activity. The only effect of DBB was to increase the reductase at day 1. The induction of cytochrome P450 content by HBB_6 reached a maximum by day 2, and stayed essentially the same through the end of the study (Figure 24). The magnitude of the induction by HBB_6 was nearly identical to that caused by Pb, but markedly less than that caused by the PBB mixture. The extent of induction in response to HBB_7 was nearly the same as that caused by HBB_6, however, the differences in magnitude between the effects of Pb, HBB_7 and PBBs were not as pronounced. Induction by MC was smaller, and it shifted λ_{max} in the spectral assay to 448 nm. PBBs also shifted the spectral maximum, to 449.5 nm. Neither HBB_6, HBB_7, DBB, nor Pb caused any shift from 450 nm, and DBB had no effect on the specific content of the cytochrome P450 hemoproteins.

Pb and MC are the two classically distinct inducers of microsomal drug metabolizing enzymes, and aminopyrine demethylation can be used as a measure of the extent of Pb-type induction (Conney, 1967). Benzo-[α]pyrene hydroxylation (arylhydrocarbon hydroxylase) is similarly a measure of the MC-type induction (Parke, 1975). Figure 25 shows that HBB_6 strongly induced aminopyrine demethylation, but to a level only two-thirds of that produced by Pb or PBBs. HBB_7 also strongly induced this activity, but to a level comparable to that produced by Pb and PBBs. The levels attained in response to both HBB_6 and HBB_7 showed no signs of diminishing when the studies were concluded. Neither MC nor

Figure 23. EFFECTS OF 90 mg DBB, HBB_6, AND HBB_7/kg ON NADPH-CYTOCHROME P450 REDUCTASE

Rats were injected on day 0 and sacrificed at intervals up to twenty-two days later. Values shown are means ± SE (N=3). Responses to maximally effective doses of MC, Pb, and PBBs are shown for comparison.


NADPH-CYTOCHROME P450 REDUCTASE
(nmole/min. mg prot.)
400
300
200
100
HBB6
C
MC 232
Pb 333
PBBs 349
0 2 4 6 8 10 12 14
TIME AFTER INJECTION (days)

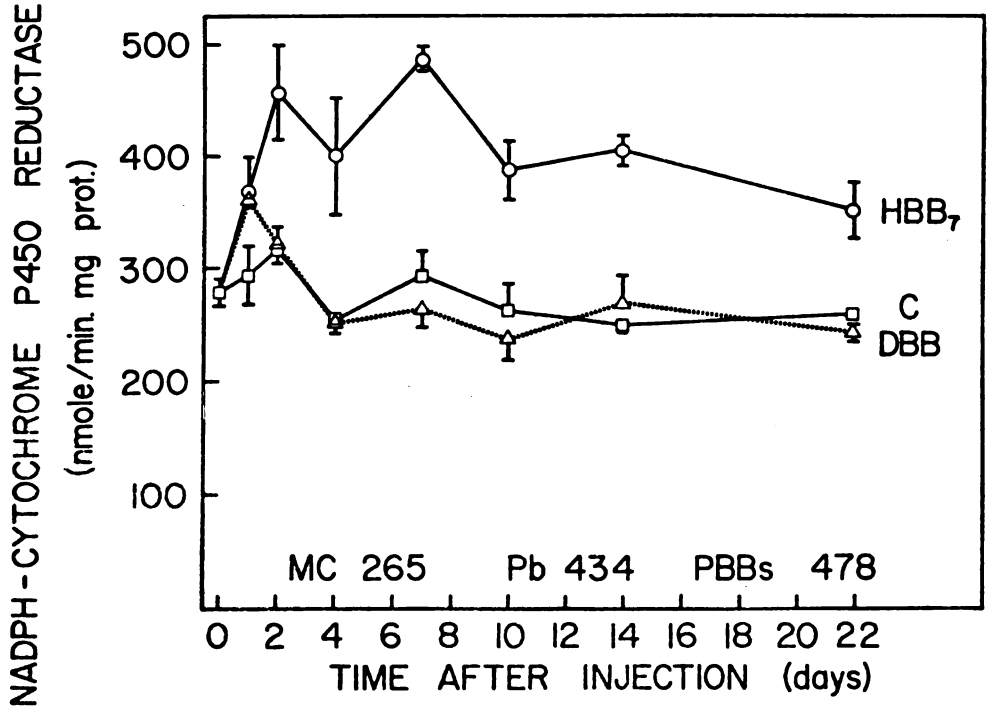
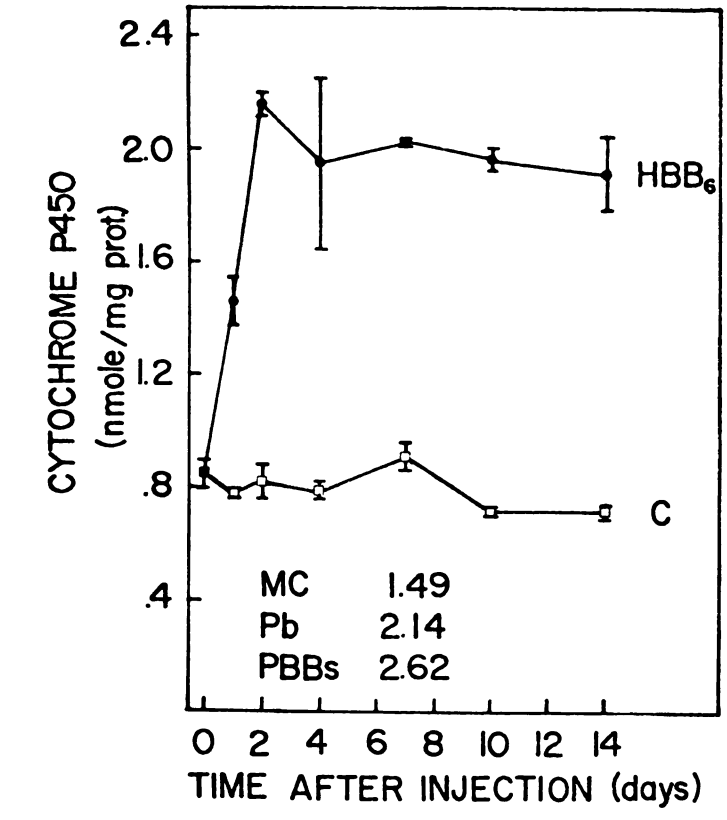

NADPH-CYTOCHROME P450 REDUCTASE
(nmole/min. mg prot.)
500
400
300
200
100
HBB7
C
DBB
MC 265
Pb 434
PBBs 478
0 2 4 6 8 10 12 14 16 18 20 22
TIME AFTER INJECTION (days)

Figure 23

Figure 24. EFFECTS OF 90 mg DBB, HBB_6, AND HBB_7/kg ON CYTOCHROME P450

Rats were injected on day 0 and sacrificed at intervals up to twenty-two days later. Values shown are means ± SE (N=3). Responses to maximally effective doses of MC, Pb, and PBBs are shown for comparison.


CYTOCHROME P450
(nmole/mg prot)
2.4
2.0
1.6
1.2
.8
.4
HBB6
C
MC 1.49
Pb 2.14
PBBs 2.62
0 2 4 6 8 10 12 14
TIME AFTER INJECTION (days)

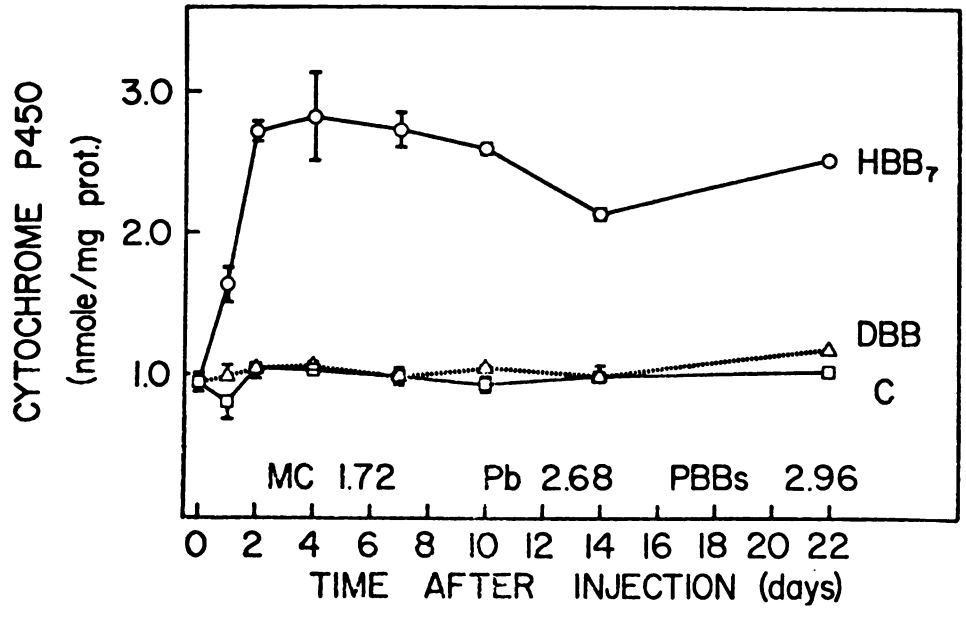
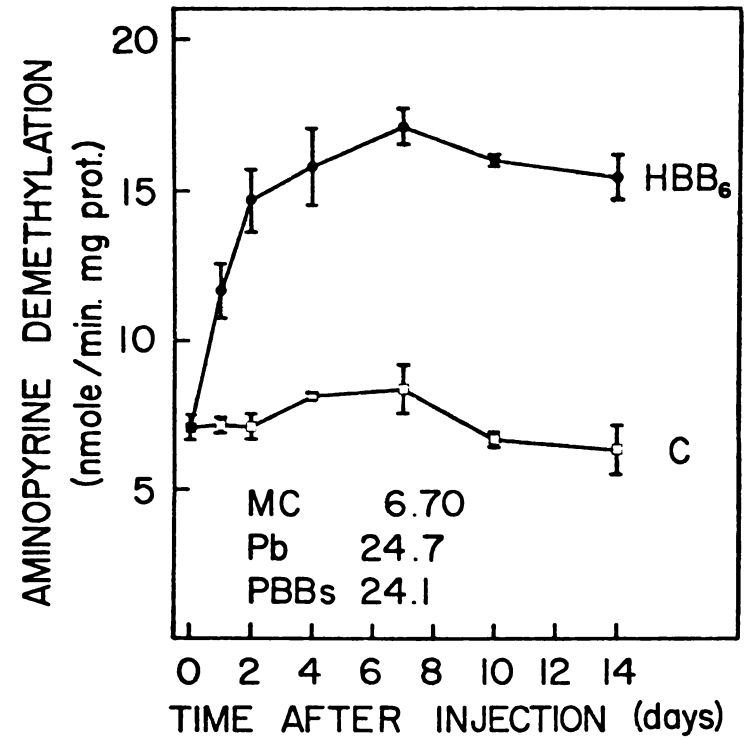

CYTOCHROME P450
(nmole/mg prot.)
3.0
2.0
1.0
HBB7
DBB
C
MC 1.72 Pb 2.68 PBBs 2.96
0 2 4 6 8 10 12 14 16 18 20 22
TIME AFTER INJECTION (days)

Figure 24

Figure 25. EFFECTS OF 90 mg DBB, HBB_6, AND HBB_7/kg ON AMINOPYRINE DEMETHYLATION

Rats were injected on day 0 and sacrificed at intervals up to twenty-two days later. Values shown are means ± SE (N=3). Responses to maximally effective doses of MC, Pb, and PBBs are shown for comparison.


AMINOPYRINE DEMETHYLATION
(nmole/min. mg prot.)
20
15
10
5
HBB6
C
MC 6.70
Pb 24.7
PBBs 24.1
0 2 4 6 8 10 12 14
TIME AFTER INJECTION (days)

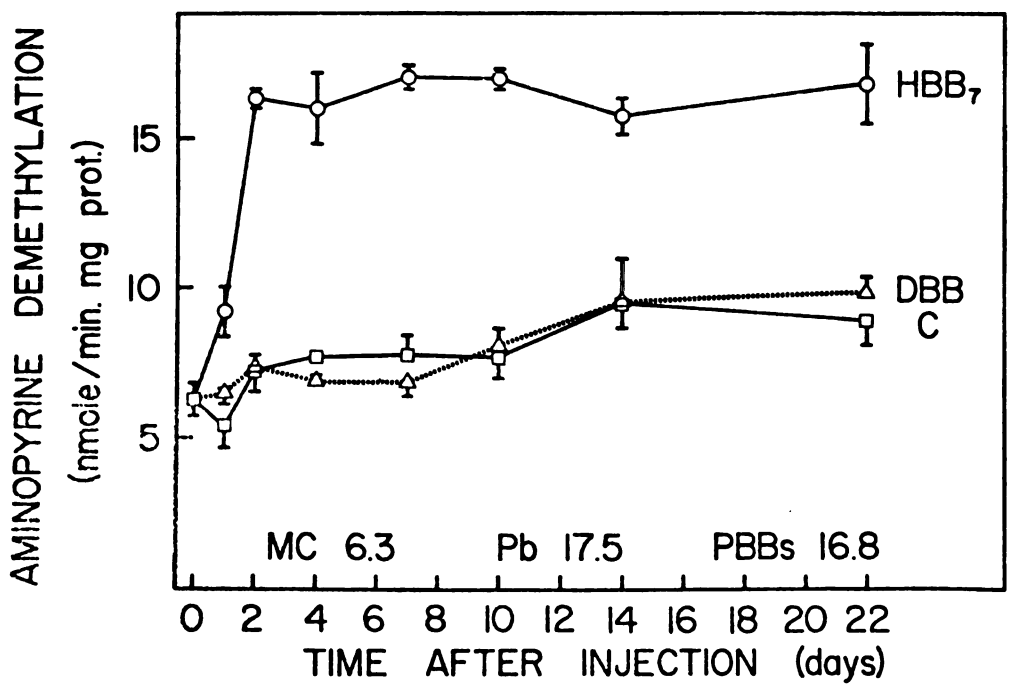

AMINOPYRINE DEMETHYLATION
(nmole/min. mg prot.)
15
10
5
HBB7
DBB
C
MC 6.3
Pb 17.5
PBBs 16.8
0 2 4 6 8 10 12 14 16 18 20 22
TIME AFTER INJECTION (days)

Figure 25

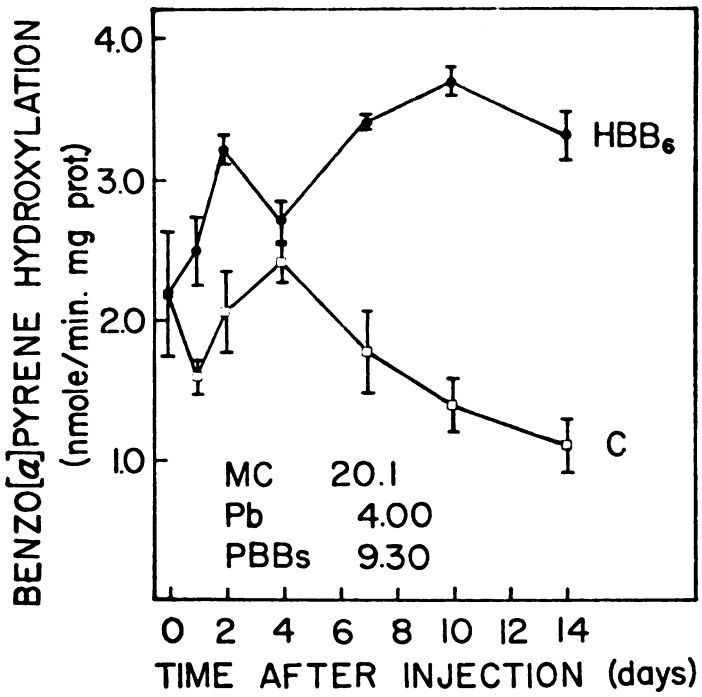
DBB had any effect on aminopyrine demethylation. Benzo[α]pyrene hydroxylation was induced ten-fold by MC, as shown in Figure 26, and while HBB_6, HBB_7 and Pb all caused a moderate increase in this activity, the level was only one-fifth of the maximum possible induction. PBBs, in contrast, caused a strong induction in benzo[α]pyrene hydroxylation, to a level half of that induced by MC. DBB had little if any effect on the specific activity of this enzyme.

SDS-polyacrylamide gel electrophoresis has proven to be a useful tool in elucidating the structure and function of microsomal proteins (Alvares and Siekevitz, 1973; Welton and Aust, 1974a,b; Haugen et al., 1976). Figure 27 shows the effects of all agents tested on the profiles of microsomal proteins (top) and cytochrome P450 hemoproteins (bottom). The gels stained for protein fall into four categories. Microsomes from DBB-pretreated rats were identical to control microsomes. Pb, HBB_6 and HBB_7 all caused the same pattern, while MC caused a unique induction pattern. The proteins induced by PBBs were those induced by the Pb-type inducers, plus those induced by MC. When heme staining was performed, similar results were seen (bottom), except that the difference between control and MC microsomes was very subtle and could not be seen in this gel. The differences can be observed when the proteins smaller than 40,000 daltons are electrophoresed off such a gel (results not shown).

A number of substrates are metabolized by the cytochrome P450 family into epoxides. These reactive products can bind covalently to a variety of nucleophiles including protein, RNA and DNA and thereby cause great cellular damage (Jerina and Daly, 1974). Epoxide hydratase converts epoxides into much less reactive dihydrodiols, and HBB_6 tripled

Figure 26. EFFECTS OF 90 mg DBB, HBB_6, AND HBB_7/kg ON BENZO[a]PYRENE HYDROXYLATION

Rats were injected on day 0 and sacrificed at intervals up to twenty-two days later. Values shown are means ± SE (N=3). Responses to maximally effective doses of MC, Pb, and PBBs are shown for comparison.


BENZO[a]PYRENE HYDROXYLATION
(nmole/min. mg prot)
4.0
3.0
2.0
1.0
HBB6
C
MC 20.1
Pb 4.00
PBBs 9.30
0 2 4 6 8 10 12 14
TIME AFTER INJECTION (days)

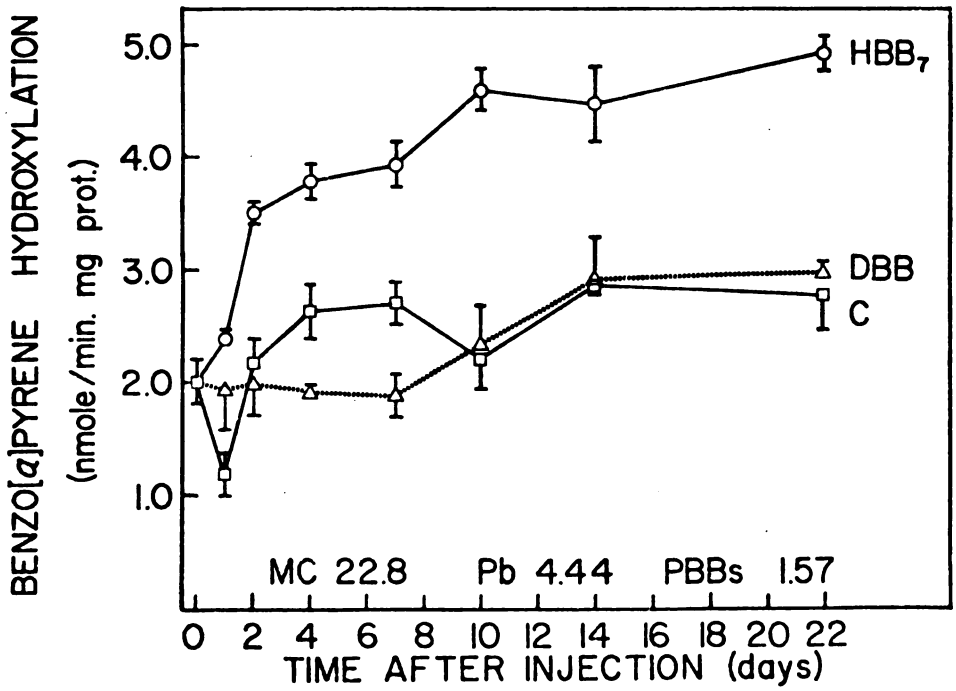

BENZO[a]PYRENE HYDROXYLATION
(nmole/min. mg prot.)
5.0
4.0
3.0
2.0
1.0
HBB7
DBB
C
MC 22.8 Pb 4.44 PBBs 1.57
0 2 4 6 8 10 12 14 16 18 20 22
TIME AFTER INJECTION (days)

Figure 26

Figure 27. EFFECTS OF POLYBROMINATED BIPHENYL CONGENERS AND OTHER XENOBIOTICS ON THE PROTEIN AND HEME PROFILES OF MICROSOMES SUBJECTED TO SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

From left to right, the microsomes are from control rats, or from rats pretreated with MC, Pb, PBBs, HBB_6, HBB_7, and DBB. Rats were injected i.p. with 90 mg/kg of the brominated agents seven days before sacrifice. MC and Pb pretreatments were likewise maximally effective. Protein staining (top) was performed on gels containing 20 µg of microsomal protein, while 120 µg of microsomal protein was applied to gels stained for heme. The broad, rapidly migrating band in the heme-stained gel represents dissociated heme.

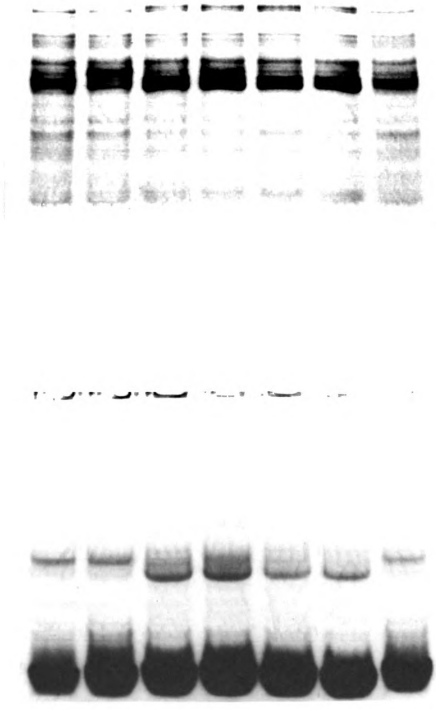


Figure 27

this activity, while HBB_7 quadrupled it. Pb and especially PBBs were also good inducers, while MC and DBB had no effect (Figure 28). Bresnick et al. (1977) have previously shown Pb to be a good inducer, and MC to have little effect on this enzyme.

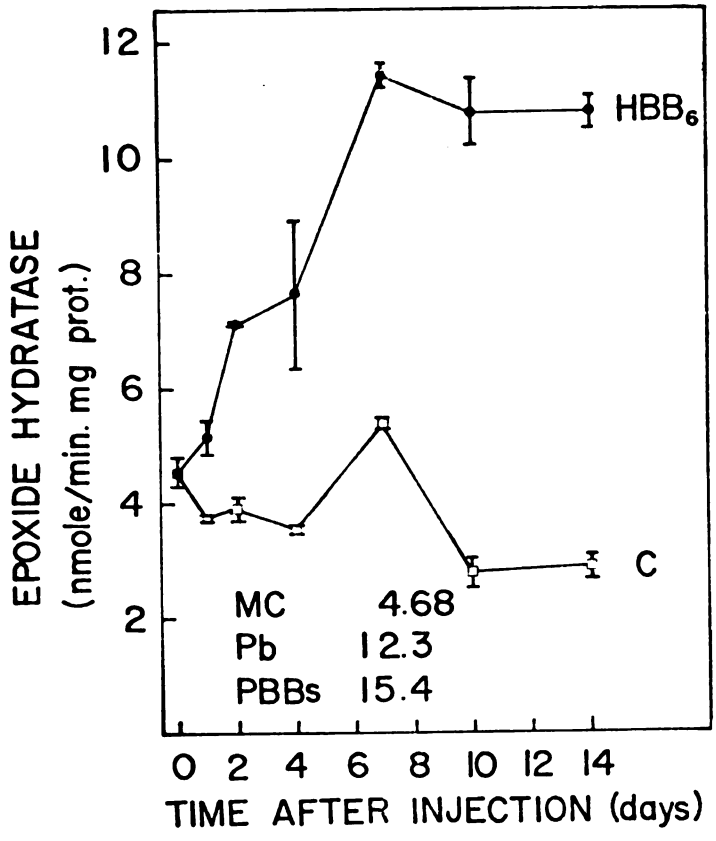
Another pathway for the further disposition of compounds metabolized by the cytochrome P450 hemoproteins involves the conjugation of a hydroxylated metabolite with glucuronic acid. Neither HBB_6, HBB_7 nor DBB had much effect on UDP-glucuronyltransferase, while PBBs were a good inducer, as shown in Figure 29. When assayed with *p*-nitrophenol as the acceptor, MC is known to induce, while Pb has no effect (Bock et al., 1973); these observations were verified by the data presented here.

DISCUSSION

PBBs have been shown by several laboratories to induce a variety of microsomal enzymes in several different tissues and species (Farber and Baker, 1974; Cecil et al., 1975; Corbett et al., 1975; Troisi, 1975; Babish et al., 1975, 1976; Farber et al., 1976; Sleight and Sanger, 1976; Moore et al., 1976, 1978; Dent et al., 1976a,b, 1977a,b, c,d, 1978; Roes et al., 1977; McCormack et al., 1977, 1978). In rat liver, this induction is of a mixed type in that it includes the properties of microsomes induced both by MC and by Pb (Troisi, 1975; Moore et al., 1976, 1978; Dent et al., 1976a,b, 1977a,b,d, 1978; McCormack et al., 1977, 1978). PBBs, however, are a complex mixture of chemicals, and it had not previously been known which components of the mixture were able to induce microsomal drug metabolizing enzymes, nor what

Figure 28. EFFECTS OF 90 mg DBB, HBB_6, AND HBB_7/kg ON EPOXIDE HYDRATASE

Rats were injected on day 0 and sacrificed at intervals up to twenty-two days later. Values shown are means ± SE (N=3). Responses to maximally effective doses of MC, Pb, and PBBs are shown for comparison.


EPOXIDE HYDRATASE
(nmole/min. mg prot.)
12
10
8
6
4
2
HBB6
C
MC 4.68
Pb 12.3
PBBs 15.4
0 2 4 6 8 10 12 14
TIME AFTER INJECTION (days)

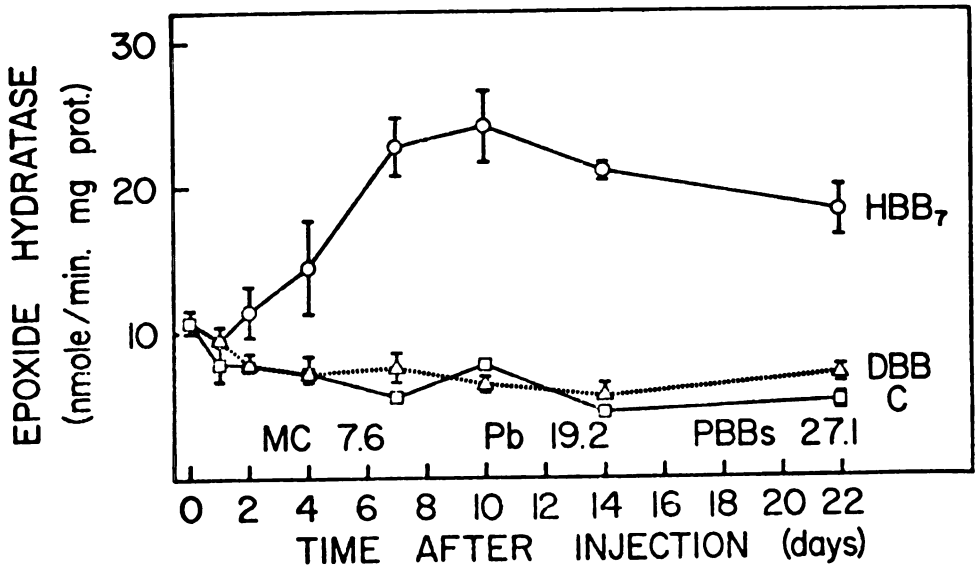

EPOXIDE HYDRATASE
(nmole/min. mg prot.)
30
20
10
HBB7
DBB
C
MC 7.6
Pb 19.2
PBBs 27.1
0 2 4 6 8 10 12 14 16 18 20 22
TIME AFTER INJECTION (days)

Figure 28

Figure 29. EFFECTS OF 90 mg DBB, HBB_6, AND HBB_7/kg ON UDP-GLUCURONYL-TRANSFERASE

Rats were injected on day 0 and sacrificed at intervals up to twenty-two days later. Values shown are means ± SE (N=3). Responses to maximally effective doses of MC, Pb, and PBBs are shown for comparison.

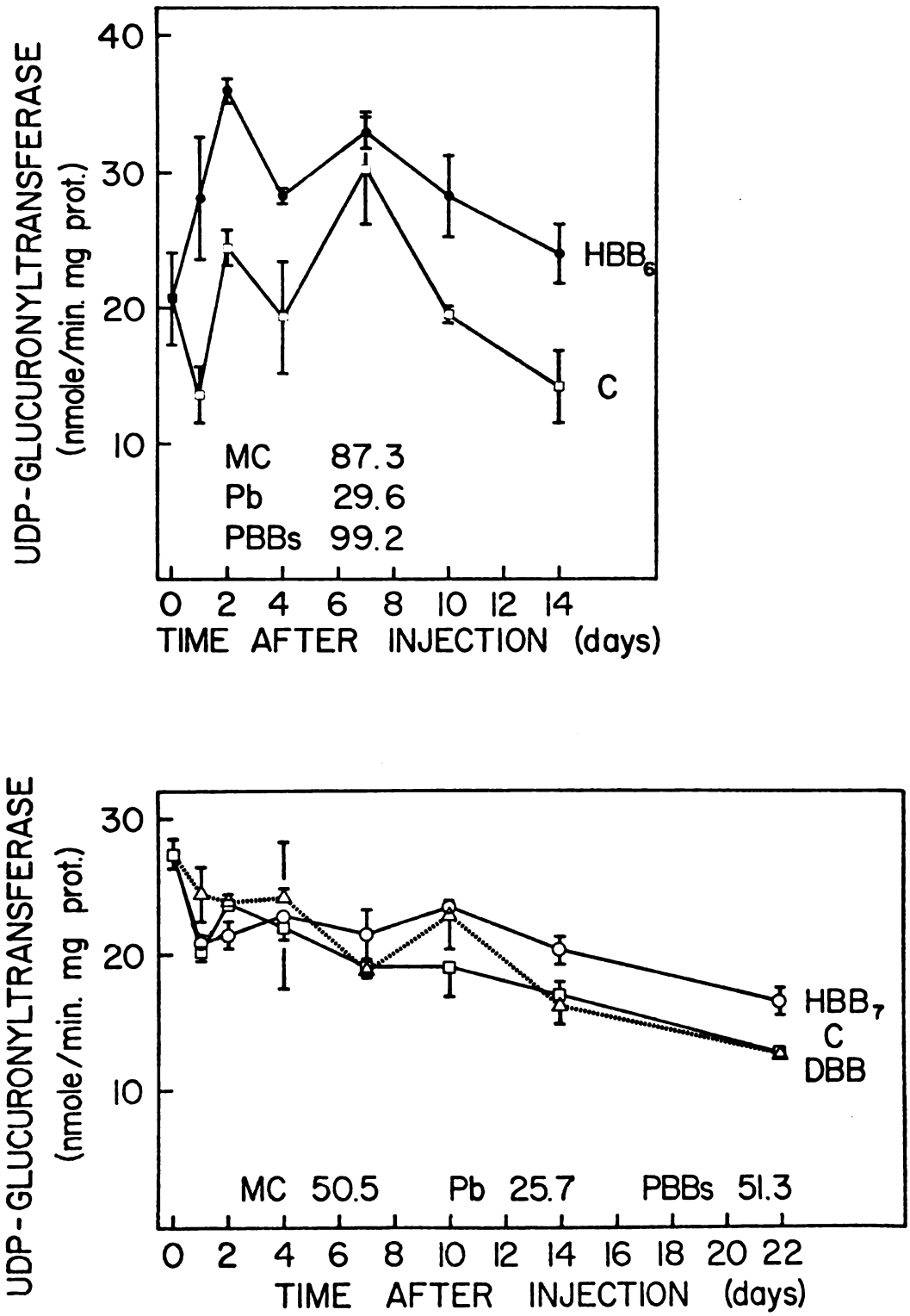

UDP-GLUCURONYLTRANSFERASE
(nmole/min. mg prot.)
40
30
20
10
HBB6
C
MC 87.3
Pb 29.6
PBBs 99.2
0 2 4 6 8 10 12 14
TIME AFTER INJECTION (days)
UDP-GLUCURONYLTRANSFERASE
(nmole/min. mg prot.)
30
20
10
HBB7
C
DBB
MC 50.5
Pb 25.7
PBBs 51.3
0 2 4 6 8 10 12 14 16 18 20 22
TIME AFTER INJECTION (days)

Figure 29

pattern of induction would result from treating animals with these purified components. The results presented in this Chapter demonstrate which enzymes can be induced by a rapidly metabolized suspected trace component of PBBs, and by the two most prominent congeners, which together comprise 83% by weight of the Firemaster mixture of PBBs.

As with any study of this sort, it is important to know that the chemicals under investigation are indeed pure, or that any contaminants present would have only minimal effects on the test animals. The results of gas chromatographic analyses of DBB, HBB_6 and HBB_7 are presented in Figure 19, and show that by this criterion they were 98, 99.9 and 98% pure, respectively. The contaminant in HBB_7 was HBB_6, but the amount present was shown by preliminary experiments to have no detectable effects on microsomal enzymes. Since PBBs might contain brominated dibenzofurans, it was important to exclude the possibility that even trace quantities of them could be present in the final preparations of HBB_6 and HBB_7. Also, the DBB (purchased commercially) was probably specifically synthesized: if the Ullman reaction was used, brominated dibenzofurans were probably present (based on the work of Moron et al. (1973) and Morita et al. (1977) with chlorinated compounds). For these reasons, the presence of oxygenated contaminants was of concern. However, since the bromobiphenyl congeners were all eluted from alumina with pure hexane before use, no such contaminants should have been present in the preparations. (While PCBs are eluted from alumina with 1% methylene chloride in hexane, 20% methylene chloride was required to elute chlorinated dibenzo-*p*-dioxins and dibenzofurans (Porter and Burke, 1971; Zitko, 1972)). The purified compounds were also recrystallized,

to minimize the possibility of contamination by brominated naphthalenes or other components.

DBB had little if any effect on rat liver microsomal drug metabolizing enzymes. Results of the pathological analysis are not yet available. DBB caused slight increases in several parameters one day after treatment, and an occasional value after that marginally differed from the corresponding control values, but it was clearly without major effect. DBB has been shown to be very rapidly metabolized in vitro (Dannan, 1978), but whether the metabolites or the parent compound caused these apparent minor effects is unknown. It can be concluded, because the rats in this study were treated with approximately five thousand times the amount of DBB as is found in PBBs, that whatever DBB is present in the Firemaster mixture is completely without effect.

The effects of the chlorinated analog of DBB, 2,2'-dichlorobiphenyl (DCB) have been investigated by other researchers. DCB had little effect on microsomal drug metabolizing enzymes in rats, although it doubled the cytoplasmic glutathione-S-epoxide transferase (Johnstone et al., 1974; Ecobichon and Comeau, 1975), presumably as a consequence of its metabolism. Another study with DBB showed it to have no effect on rat liver microsomal drug metabolizing enzymes, either as a Pb- or as an MC-type inducer (Goldstein et al., 1977).

When injected into rats, HBB_6 was found to have structural effects similar to PBBs. Results of HBB_7 treatment are not yet available. Microscopic evaluation of tissues revealed that hepatic cells were swollen and vacuolated in a majority of rats given HBB_6. Special staining did not reveal an appreciable increase in fat. A previous study with PBBs (Sleight and Sanger, 1976) revealed similar lesions, although

at extremely high levels fat deposits and necrosis were also observed. As was the case with PBBs, HBB_6 did not cause microscopically visible lesions in any other organ examined. Kimbrough et al. (1977) have also stated that the principal organ affected by PBBs in the rat is the liver, and a variety of light microscopic changes were noted. McCormack et al. (1977), however, have observed renal in addition to hepatic changes as a consequence of exposure to PBBs.

The biochemical results show that HBB_6 and HBB_7 are Pb-type inducers of rat liver microsomal drug metabolizing enzymes. Pb, HBB_6 and HBB_7 had comparable effects on every parameter examined, although HBB_7 was a slightly better inducer of microsomal protein and epoxide hydratase than was Pb, and HBB_6 did not cause as great an induction of aminopyrine demethylation as did Pb, though it was still a good inducer. Neither congener appears to be metabolized in vitro (Chapter 2, and Dannan, 1978), and while it is conceivable that metabolites of these congeners could have been formed in vivo during the course of these experiments, they did not, if present, cause any detectable shift towards an MC-type induction.

It is clear that neither HBB_6 nor HBB_7 are MC-type inducers. HBB_6 and HBB_7 had far greater effects on liver weight, microsomal protein, and cytochrome P450 than did MC, and they strongly induced NADPH-cytochrome P450 reductase, aminopyrine demethylation, and epoxide hydratase, which MC did not affect. MC, unlike HBB_6 or HBB_7, strongly induced benzo[α]pyrene hydroxylation and UDP-glucuronyltransferase. MC also shifted the cytochrome P450 spectral maximum to 448 nm, while these congeners caused no shift, and it caused patterns of microsomal

proteins and hemoproteins on SDS gels which were distinct from those caused by HBB_6 and HBB_7.

PBBs induced liver weight and microsomal protein to a greater extent than did either HBB_6 or HBB_7, and the effects of these three agents on NADPH-cytochrome P450 reductase were comparable. PBBs were somewhat more effective inducers of cytochrome P450 and epoxide hydratase than were HBB_7 or especially HBB_6. Aminopyrine demethylation was induced to comparable levels by HBB_7 and PBBs, while HBB_6 was not as effective an inducer as PBBs. These were all major effects, however, and are indicative of a Pb-type induction. The differences between PBBs and these congeners were most pronounced as revealed by the following assays. PBBs also strongly induced benzo[α]pyrene hydroxylation and UDP-glucuronyltransferase, and decreased the cytochrome P450 spectral maximum by 0.5 nm, responses which are indicative of an MC-type induction. PBBs also caused an SDS gel electrophoretic pattern of proteins and hemoproteins that was the summation of the effects of not only Pb but also MC. The strong induction of benzo[α]pyrene hydroxylation by PBBs was only half as great as that caused by MC, an observation consistent with the results of Dent et al. (1976b) for both PBBs and for MC plus Pb. Because of its effects on all parameters, PBBs are a mixed-type inducer. But because neither HBB_6 nor HBB_7 caused the latter category of MC-type changes, they are only Pb-type inducers and are therefore in a different class of inducing agents (Pb-type) than are PBBs (mixed-type). PBBs typically caused a greater induction than did HBB_6 or HBB_7; the components of PBBs responsible for the differences have yet to be identified.

Several laboratories have investigated the effects of 2,2',4,4',5,5'-hexachlorobiphenyl (HCB_6), the chlorinated analog of HBB_6, on microsomal drug metabolizing enzymes. Johnstone et al. (1974) and Ecobichon and Comeau (1975) demonstrated that HCB had effects consistent with a Pb-type induction. Stonard and Grieg (1976) showed that HCB, unlike two other hexachlorobiphenyls, was strictly a Pb-type inducer in rats. Goldstein et al. (1976, 1977) have also shown HCB to cause only a Pb-type induction in both rats and chicks, and while two other hexachlorobiphenyls were also Pb-type inducers, another was inactive, and the 3,3',4,4',5,5'-isomer was a strong MC-type inducer. Poland and Glover (1977) have also demonstrated that HCB_6 is a Pb-type inducer in rat liver. The results obtained with HBB_6 are in agreement with these studies. While it is not possible from the literature to compare the relative potencies of the two molecules, it appears that the substitution of one halogen for the other has little effect on the nature of the microsomal induction. No studies have been reported on the effects of the analogous fluorinated or iodinated compounds.

There are no reports in the literature on the effects of 2,2',3,-4,4',5,5'-heptachlorobiphenyl, the HBB_7 analog. The only congener with seven chlorines which has been examined is 2,3,3',4,4',5,5'-heptachlorobiphenyl, which surprisingly had no effect on either MC- or Pb-inducible drug metabolizing enzymes (Goldstein et al., 1977).

DBB was investigated in part because it appears to be a trace component of PBBs, but mainly because of its physico-chemical properties, as will be discussed later. When HBB_6 is isolated from Firemaster by column chromatography, very small quantities of several compounds with very short (less than one minute) gas chromatographic retention times

are eluted before HBB_6. The major one of these has a gas chromatographic retention time identical to that of DBB, but different from the retention times of biphenyl, 2-, 3-, or 4-bromobiphenyl, or 4,4'-dibromobiphenyl. Firemaster contains only about 200 ppm of this component, and gas chromatographic-mass spectrometric analysis of these early eluting peaks unfortunately was insufficiently sensitive to confirm the presence of a dibromobiphenyl.

While biphenyl appears to not be a planar molecule in solution (d'Annibale et al., 1973; Niederberger et al., 1973; Lunazzi and Macciantelli, 1975), the existence of a large energy barrier to free rotation about the axis of the molecule appears improbable, and the molecule should be able to attain a planar state relatively easily. HBB_6 and HBB_7 are both Pb-type inducers, and each undoubtedly has a twisted structure because of the steric effect of the bromines at the 2 and 2' positions (adjacent to the bridge carbon) in each molecule. In fact, the energy barrier to rotation caused by having this substitution pattern may be sufficient for the 2,2'-dibrominated compounds to exist as enantiomers, as is clearly the case for other ortho substituted biphenyls (Eliel, 1962). (Whether the enantiomers differ in their biological effects is unknown).

DBB was studied to determine whether bromination of the 2 and 2' positions, and the consequent twisting of the molecule, was alone sufficient to induce drug metabolism. DBB also offered the possibility of studying a bromobiphenyl with numerous (eight) unsubstituted positions, a property which may be expected to facilitate metabolism. Dannan (1978) has since showed that DBB is indeed rapidly metabolized in vitro by rat liver microsomes, although it should be pointed out

that a large number of unsubstituted positions is alone not sufficient to guarantee metabolism, since 4,4'-dibromobiphenyl was not metabolized under the same conditions.

Poland and Glover (1977) have very recently examined several aspects of the induction caused by two additional brominated congeners. 3,3',5,5'-Tetrabromobiphenyl was incapable of inducing benzo[α]pyrene hydroxylation in chick embryo liver, but it was not examined for its ability to induce Pb-responsive enzymes. 3,3',4,4',5,5'-Hexabromobiphenyl was an excellent inducer of benzo[α]pyrene hydroxylation in chick embryo, mouse and rat liver, and it caused only a weak induction of aminopyrine demethylation in rat liver. It can thus be classified as an MC-type inducer. Because of the absence of bromines adjacent to the bridge carbons, both 3,3',5,5'-tetrabromobiphenyl and 3,3',4,4',5,5'-hexabromobiphenyl would be expected to maintain the same solution conformation as biphenyl.

Based on the studies presented in this Chapter, and on the more limited investigations by Poland and Glover, several conclusions can now be drawn about the relationships between the structures of brominated biphenyl congeners and their effects on microsomal drug metabolizing enzymes. Biphenyl itself is not an inducer (Ecobichon and Comeau, 1975; Goldstein et al., 1977). As evidenced by the lack of effects of DBB, bromination at the 2 and 2' positions, and the concomitant twisting of the molecule, is alone insufficient to cause induction. HBB_6 is a Pb-type inducer, and so bromination at some or all of the 4,4',5, and 5' positions, alone or in combination with the 2 and 2' bromines, must be necessary for this molecule to exert its effects on drug metabolizing enzymes. HBB_7 differs from HBB_6 in that it has one additional bromine,

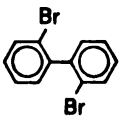
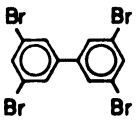
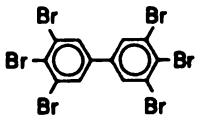
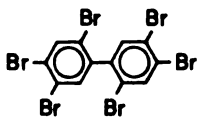
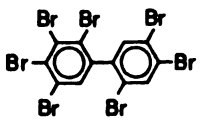
at the three position. Because both molecules cause the same responses in microsomes, it is obvious that this extra bromine is unnecessary for the induction and does not affect the type of induction. A planar configuration, or at least the lack of a major energy barrier to planarity (i.e., the absence of 2,2',6, and 6' bromines), is alone insufficient to cause an MC-type induction, since 3,3',5,5'-tetrabromobiphenyl is not an MC-type inducer although 3,3',4,4',5,5'-hexabromobiphenyl is. Bromination at the 4 or at both the 4 and 4' positions must be necessary for 3,3',4,4',5,5'-hexabromobiphenyl to be an MC-like inducer.

All three congeners which have been established as microsomal inducers contain bromines at the 4,4',5, and 5' positions, although it is not known whether all four are necessary. Nor is it known whether these four alone are sufficient to cause induction. It is clear, however, that the presence of these four bromines in more highly brominated biphenyls does not by itself determine the nature of the induction, since the 2,2'-derivative of this core structure (HBB_6) is a Pb-type inducer, and the 3,3'-derivative is an MC-type inducer. The effects of these five pure bromobiphenyl congeners on microsomal drug metabolizing enzymes are summarized in Table 5.

The effects of a large number of chlorinated biphenyls and structurally related compounds on microsomal drug metabolizing enzymes have been examined. As a result of testing twenty-two chlorinated biphenyls, Goldstein et al. (1977) concluded that biphenyls chlorinated symmetrically in both the meta and para positions (with respect to the bridge carbons) are MC-type inducers. Biphenyl congeners chlorinated in both the para and ortho positions are Pb-type inducers, regardless of the chlorination in the meta position. Congeners which are chlorinated in

Table 5

SUMMARY OF THE EFFECTS OF PURE POLYBROMINATED BIPHENYL CONGENERS ON THE TYPE OF INDUCTION OF MICROSOMAL DRUG METABOLIZING ENZYMES

COMPOUND	TYPE OF INDUCTION	
	Phenobarbital	3-Methylcholanthrene
	—	—
	?	—
	—	+
	+	—
	+	—

only one ring, or are chlorinated in both rings but not in the para position, have very little activity as inducers of liver microsomal enzymes. Results to date with brominated biphenyls are consistent with these conclusions, although it cannot yet be concluded that these rules fully apply to brominated biphenyls.

Poland and Glover (1977) studied the effects of sixteen halobiphenyls and a number of isostereomers on the induction of benzo[α]pyrene hydroxylation, and concluded that the presence of at least two adjacent halogens in the meta and para positions of each benzene ring, as well as the absence of halogens at the ortho positions, were required for MC-type induction. The requirement for a lack of ortho substitution was attributed to the concomitant loss of planarity. The most potent MC-type inducers are the most planar ones, particularly those with two bonds between benzene rings. Again, results with brominated biphenyls are consistent with these observations.

The metabolism of drugs, other xenobiotics, and some steroids would be expected to be altered as a consequence of the inductions such as have been observed with HBB_6, HBB_7, and PBBs (Conney and Burns, 1972), however, the biological consequences of these inductions, either alone or in combination with other environmental chemicals, are unclear. Of particular interest are increases in the enzyme or enzymes which catalyze the hydroxylation of benzo[α]pyrene, enzymes which also catalyze the metabolic activation of a wide variety of other polycyclic aromatic hydrocarbons into carcinogenic derivatives (Jerina and Daly, 1974; Grover and Sims, 1974). One might expect that the induction of benzo[α]pyrene hydroxylation by PBBs could enhance the chemical carcinogenicity of other agents. Indeed, treatment of mice with polychlorinated

biphenyls was found to enhance the carcinogenicity of the α and β isomers of 1,2,3,4,5,6-hexachlorocyclohexane (Ito et al., 1973). However, polychlorinated biphenyls were found to decrease the carcinogenicity of 3'-methyl-4-dimethylaminoazobenzene, N-2-fluorenylacetamide, and diethylnitrosamine in rats (Makiura et al., 1974). Further investigations will be required to elucidate the mechanisms underlying such interactions.

Similar experiments concerning the interactions between PBBs or their components with other chemical carcinogens have not been reported, although several related experiments have been performed. Crawford and Safe (1977) found that when liver microsomes induced by PBBs were incubated in vitro with 4-chlorobiphenyl, the rate of NADPH-dependent covalent binding to microsomal macromolecules was twelve times greater than when control microsomes were used. The binding of 4-chlorobiphenyl metabolites to DNA was not examined. Results presented in Chapter 2 demonstrate that rat liver microsomes induced by PBBs catalyzed the covalent binding of benzo[α]pyrene metabolites to DNA at a rate six-fold greater than was observed with control or Pb-induced microsomes. While neither experiment, particularly the first, allows any direct conclusions to be drawn concerning the effects of PBBs on chemical carcinogenesis, both support the idea that induction of microsomal drug metabolizing enzymes may enhance the carcinogenic potential of certain other chemicals.

The experiments described in this Chapter have determined the effects of three pure brominated biphenyls on rat liver microsomal drug metabolizing enzymes. DBB, a suspected trace component of PBBs, is at best a marginal, transient inducer, and in no way accounts for the induction caused by PBBs. HBB_6 and HBB_7 account for 56 and 27%,

respectively, of PBBs (Firemaster). Both are Pb-type inducers and contribute heavily to the Pb-like aspects of the mixed-type induction caused by PBBs. It is probable that other PBB components also contribute to the Pb-like aspects of the induction caused by PBBs. Both HBB_6 and HBB_7 cause long lasting effects. However, it is clear that neither compound is responsible for all the biological effects caused by an identical dose of PBBs. In several cases, the effects of PBBs were far greater than those resulting from the purified congeners, despite the fact that the rats injected with PBBs received only 56 and 27%, respectively, of the amount of HBB_6 and HBB_7 received by the rats injected with the pure chemicals. The remaining biological effects caused by PBBs but not by HBB_6 and HBB_7 must result from one or more of the components in the 17% by weight of Firemaster which has not yet been fully characterized.

PBB congeners which have been identified in this fraction include 2,2',4,5,5'-pentabromobiphenyl, 2,3',4,4',5-pentabromobiphenyl, 2,2',3,4,4',5'-hexabromobiphenyl, 2,3',4,4',5,5'-hexabromobiphenyl, and 2,2',3,3',4,4',5,5'-octabromobiphenyl (Chapter 1). However, the effects of these compounds on microsomal drug metabolizing enzymes are not known. Of particular interest are 2,3',4,4',5-pentabromobiphenyl and 2,3',4,4',5,5'-hexabromobiphenyl, because they contain a 3,4-dibromo and a 3,4,5-tribromo substitution pattern, respectively, and because 3,3',4,4'-tetrachlorobiphenyl, 3,3',4,4',5,5'-hexachlorobiphenyl, and 3,3',4,4',5,5'-hexabromobiphenyl are all MC-type inducers of microsomal enzymes (Goldstein et al., 1977; Poland and Glover, 1977). These two components of the Firemaster mixture may well be bifunctional molecules, with the 2,4,5-tribrominated ring acting as a Pb-type

inducer (as it does in HBB_6), and the 3,4-dibrominated or 3,4,5-tribrominated ring acting as an MC-type inducer. In this way, these compounds might account for the MC-like aspects of the mixed-type induction caused by PBBs.

Also a candidate for the MC-type inducer in PBBs is 3,3',4,4',5,5'-hexabromobiphenyl. It is not known whether this congener is present in PBBs, or if it is, whether the quantity present is sufficient to account for the MC-like aspects of the mixed-type induction caused by PBBs. Dr. John A. Liddle of the Center for Disease Control has found that 3,3',4,4',5,5'-hexabromobiphenyl constitutes, at most, one part per million of Firemaster (personal communication).

In a preliminary experiment, an MC-type inducer was shown to exist in a polar PBB fraction. PBBs were applied to a column of alumina, and most were eluted with hexane. A polar fraction was then obtained by washing the column with acetonitrile. This fraction induced benzo[α]-pyrene hydroxylation, and shifted the cytochrome P450 spectral maximum to 449 nm. It is possible, however, that MC-type or mixed-type inducers were also present in the other fractions, but that their activity was masked by the presence of much larger quantities of Pb-type inducers.

The results presented in this Chapter raise an important question regarding the routine assay used to monitor PBB contamination of the environment, which only measures HBB_6 concentrations. If low molecular weight brominated biphenyls, naphthalenes, dibenzofurans, terphenyls, or other such compounds turn out to have significant biological effects distinct from those of HBB_6 (or HBB_7), then this assay may prove to be an invalid measure of contamination, because the absorption, distribution, and excretion rates of these classes of compounds would likely be

different from those of the major congeners. It is important to identify which of the less prominent components cause the MC-like aspects of the mixed-type induction caused by the PBB mixture. These studies are now in progress in this laboratory.

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APPENDIX

APPENDIX

LIST OF PUBLICATIONS

In Press

Robert W. Moore, Ann F. Welton, and Steven D. Aust. Detection of Hemoproteins in SDS-Polyacrylamide Gels, in Methods in Enzymology, Vol. 51 - Biomembranes C - Biological Oxidations (S. Fleischer and L. Packer, eds.), Academic Press, New York.

Robert W. Moore, John V. O'Connor, and Steven D. Aust. Identification of a Major Component of Polybrominated Biphenyls as 2,2',3,4,4',5,5'-Heptabromobiphenyl. Bull. Environ. Contam. Toxicol.

Robert W. Moore, Stuart D. Sleight, and Steven D. Aust. Induction of Liver Microsomal Drug Metabolizing Enzymes by 2,2',4,4',5,5'-Hexabromobiphenyl. Toxicol. Appl. Pharmacol.

Robert W. Moore, Ghazi A. Dannan, and Steven D. Aust. Induction of Drug Metabolizing Enzymes in Polybrominated Biphenyl-Fed Lactating Rats and their Pups. Environ. Health Perspect.

Ghazi A. Dannan, Robert W. Moore, and Steven D. Aust. Studies on the Microsomal Metabolism and Binding of Polybrominated Biphenyls (PBBs). Environ. Health Perspect.

In Preparation

Robert W. Moore, Stuart D. Sleight, and Steven D. Aust. Effects of 2,2'-Dibromobiphenyl and 2,2',3,4,4',5,5'-Heptabromobiphenyl on Liver Microsomal Drug Metabolizing Enzymes. To be submitted to Toxicol. Appl. Pharmacol.

Robert W. Moore, and Steven D. Aust. Purification and Characterization of Polybrominated Biphenyl Congeners. To be submitted to J. Am. Chem. Soc.

Abstracts

R.W. Moore, F.O. O'Neal, L.C. Chaney, and S.D. Aust. Specificity of Antibody to the Cytochrome P-450 Hemoprotein Induced by Phenobarbital. Fed. Proc. 34, 623 (1975).

Robert W. Moore, Ghazi Dannan, and Steven D. Aust. Induction of Drug Metabolizing Enzymes in Rats Nursing from Mothers Fed Polybrominated Biphenyls. Fed. Proc. 35, 708 (1976).

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Robert W. Moore, Ghazi A. Dannan, and Steven D. Aust. Effects of Firemaster and Selected Pure Brominated Biphenyls on Microsomal Drug Metabolizing Enzymes. Presented at the Workshop on Scientific Aspects of Polybrominated Biphenyls, East Lansing, Michigan, October 24 and 25, 1977.

Ghazi A. Dannan, Robert W. Moore, and Steven D. Aust. Studies on the Microsomal Metabolism and Binding of PBBs. Presented at the Workshop on Scientific Aspects of Polybrominated Biphenyls, East Lansing, Michigan, October 24 and 25, 1977.

Robert W. Moore and Steven D. Aust. Effects of 2,2'-Dibromobiphenyl (DBB) and 2,2',3,4,4',5,5'-Heptabromobiphenyl (HBB_7) on Microsomal Drug Metabolizing Enzymes. Toxicol. Appl. Pharmacol., in press, to be presented at the Seventeenth Annual Meeting of the Society of Toxicology, San Francisco, California, March 13-16, 1978.