COMPARATIVE EFFECTS OF FIREMASTER BP-6, 2,2',4,4',5,5'-HEXABROMOBIPHENYL (HBB) AND 3,3',4,4',5,5'-HBB ON LIPOPROTEINS AND SELECTED SERUM ENZYMES AND HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYMES IN RATS

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A DISSERTATION

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

DOCTOR OF PHILOSOPHY

Department of Pathology

1981

ABSTRACT

COMPARATIVE EFFECTS OF FIREMASTER BP-6, 2,2',4,4',5,5'-HEXABROMOBIPHENYL (HBB) AND 3,3',4,4',5,5'-HBB ON LIPOPROTEINS AND SELECTED SERUM ENZYMES AND HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYMES IN RATS

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Young male rats were fed diets containing 0, 0.1, 1, 10 or 100 ppm of Firemaster (FM) BP-6, 2,2',4,4',5,5'-hexabromobiphenyl (HBB) or 3,3',4,4',5,5'-HBB for 10 and 30 days. Effects on serum lipoproteins, serum enzymes and hepatic microsomal drug-metabolizing enzymes were compared. At dietary levels of 100 ppm, FM BP-6 and 2,2',4,4',5,5'-HBB caused elevations in total serum cholesterol at 10 and 30 days, occurring mainly in the high density lipoprotein (HDL) fraction. Similarly, an intermediate dietary level (1 ppm) of 3,3',4,4',5,5'-HBB produced a significant elevation in serum cholesterol which occurred in the HDL fraction. Rats given 100 ppm 3,3',4,4',5,5'-HBB for 10 days had dramatic decreases in total serum cholesterol, as did those fed 10 ppm for 30 days. Decreases were mainly in the high density fraction. Rats fed dietary levels of 100 ppm 3,3',4,4',5,5'-HBB died within 20 days. Mild to moderate decreases in serum triglycerides resulted from the higher dietary concentrations of FM BP-6 and both congeners. Decreases were mostly in the very low density lipoprotein and to a lesser degree in the low density fractions. Measurement of serum alanine aminotransferase, aspartate aminotransferase (AST), gamma

glutamyl transpeptidase, alkaline phosphatase (ALP) and sorbitol dehydrogenase (SDH) indicated that only SDH was consistently elevated at the higher dietary concentrations of all 3 compounds. This indicates that SDH may be a sensitive indicator of altered hepatocellular permeability in the rat. Serum AST values were significantly elevated from control values by the highest dietary concentrations of both the 10- and 30-day 3,3',4,4',5,5'-HBB treatments. Serum ALP levels in each 10-day treatment and the pair-fed controls to the 10-day, 100 ppm 3,3',4,4',5,5'-HBB group were significantly lower than those of control rats. Results of assays for hepatic microsomal drug-metabolizing enzymes clearly indicated that 2,2',4,4',5,5'-HBB is a phenobarbital-type inducer, 3,3',4,4',5,5'-HBB is a 3-methylcholanthrene-type inducer and FM BP-6 is a mixed-type inducer. The possible relationships between the lipid alterations and the induction patterns are discussed.

DEDICATION

To My Wife

Dolores J. Kunze

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INTRODUCTION

There is a definite need for concern about the production and subsequent release of man-made substances into the environment. Regardless of whether the release of an agent is deliberate or accidental, the end result is often the same. Animals, including human beings, are exposed to a variety of substances either through direct contact or frequently through their food chains. Unfortunately, the short- and long-term effects of many agents are simply not known.

In 1973, livestock throughout Michigan were given feeds that had been accidentally contaminated with a commercial mixture of polybrominated biphenyls (PBB). At the time that the compound was finally identified in the feeds, only a small amount of very inadequate research had been attempted to determine the toxic potential of the brominated biphenyls. Now, 8 years later, much has been and is still being done to correct for these inadequacies.

Many of the initial studies concentrated on describing the various physical, gross and histopathologic effects of the parent compound, Firemaster BP-6 (FM), in both farm and laboratory animals. With the identification of some of the individual congeners in FM, efforts were started to determine which components are responsible for the various toxic effects attributed to the parent mixture. Additionally, techniques such as electron microscopy, microsomal enzyme assays and gas chromatography were employed to further describe and understand the actions of the compounds in biologic systems.

Several research areas have received only peripheral attention in most PBB experiments. Included in these are evaluations of the effects of the compounds on various serum enzymes and lipoproteins. The objective of this study was to evaluate the effects of FM and 2 PBB congeners on serum enzymes, lipoproteins and hepatic microsomal enzymes in rats.

These determinations were selected for several reasons. The PBBs are known to produce hepatic alterations in rats. The bank of serum enzymes was chosen to determine which, if any, would best reflect changes in hepatocellular integrity or cholestasis. Furthermore, since the liver is the most important organ in the metabolism of lipoproteins and since lipid-soluble compounds such as PBB have to be transported in the blood either bound to proteins or in lipoprotein particles, the composition and relative amounts of the lipoprotein fractions were carefully evaluated.

The selection of the compounds was also an important aspect of the experiment. The parent compound, FM, was used as the standard against which the effects of the other 2 congeners were compared. The congener 2,2',4,4',5,5'-hexabromobiphenyl (HBB) is quantitatively the most important component of the mixture and is a phenobarbital(PB)-type hepatic microsomal enzyme inducer. The third compound, 3,3',4,4',5,5'-HBB, does not occur in FM but was used because of its structural characteristics and known 3-methylcholanthrene(MC)-type microsomal enzyme induction.

Through these experiments it was hoped that some additional knowledge and understanding about the biochemical effects of these compounds would be gained. An attempt was made to evaluate and compare certain biochemical determinations from rats fed FM with those from rats fed

a congener with either PB- or MC-type effects. Specific alterations detected by these assays in the FM-treated animal hopefully could be attributed to the actions of the PB- or MC-type compound.

OBJECTIVES

Rats were fed diets containing various concentrations of either Firemaster BP-6 (FM), 2,2',4,4',5,5'-hexabromobiphenyl (HBB) or 3,3',4,4',5,5'-HBB. These compounds are mixed-, phenobarbital- and 3-methylcholanthrene(MC)-type hepatic microsomal enzyme inducers, respectively.

The objectives of this research project were:

1. To evaluate the effects of the different compounds on selected serum enzymes and lipids.

2. To evaluate the effects of the treatments on the relative amounts and composition of the major lipoprotein fractions.

3. To attempt to identify the mode of serum transportation for the 2 purified compounds.

4. To describe the pattern of hepatic microsomal enzyme induction for each compound.

5. To identify future areas of investigation needed to describe the mechanisms associated with altered lipid metabolism.

LITERATURE REVIEW

Historical Perspective

With the controversy surrounding the accidental introduction of polybrominated biphenyls (PBB) into the food chains of livestock and, subsequently, that of human beings still raging, it is often difficult to think of the problem in terms other than those of a current crisis. If one considers the use of PBB to be an extension of the industrial use of polychlorinated biphenyls (PCB), these compounds, the polyhalogenated biphenyls (PHB), have been around for at least 100 years. In a review article discussing polychlorinated polycyclic compounds, Kimbrough (1974) cited a reference which attributed the first description of the synthesis of PCBs to *Liebig's Annalen* in 1881.

It was not until the 1930s, however, that PCB, because of its unique physical properties, was used extensively for various industrial applications. The compounds are very stable, nonflammable and have excellent dielectric and plasticizing properties (Hammond, 1972; Kimbrough, 1974). They have been used as dielectric fluids in capacitors and transformers, as hydraulic and heat exchange fluids, as plasticizers, adhesives, textile coatings and components of paints and varnishes, and as components in insulation coatings around electric wires and cables (Hammond, 1972; Kimbrough, 1974). Production of PCB peaked in 1970 and began declining in 1971 after the only United States producer voluntarily limited sales of the compound (Nisbet and Sarofim, 1972).

Industrial production of PBB in the United States began in 1970 and, like the PCB, was limited to one company (Kerst, 1974). The PBB compounds are relatively inert, water insoluble and highly heat stable. The commercial product, Firemaster BP-6 (FM), was incorporated into materials because of its flame-retarding properties. Greater than 80% of the product was incorporated into housings and components for business machines and industrial and electric equipment (Kerst, 1974). Production of FM was stopped in 1974 (DiCarlo et al., 1978).

Environmental Contamination

Both PCB and PBB are present in the environment. For PCB the major routes of entry are suspected to be leaks from transformers, heat exchangers and hydraulic systems, spills and losses during manufacturing, vaporization or leaching from PCB-containing formulations and disposal of PCB-containing fluids (Nisbet and Sarofim, 1972). Higher concentrations occur in waterways and seas (and in their associated animal life and sediments) around industrialized areas than in undeveloped areas of the world (Hammond, 1972). In addition to these important routes, several well documented incidences resulting in the high level contamination of waterways, animal feeds and human food have occurred (Nisbet and Sarofim, 1972; Kimbrough, 1974; Van Houweling et al., 1977). Perhaps the most serious accident occurred in Japan in 1968, in which more than 1000 people ingested PCB-contaminated rice oil (Kuratsune et al., 1972). Polybrominated biphenyls are ubiquitous, having been identified in ecosystems around the globe (Risebrough and deLappe, 1972) and in approximately 1/3 of the samples of adipose tissue taken from people throughout the United States (Yobs, 1972).

Unlike the widespread problems with PCB, the release of high levels of PBB into the environment has been essentially confined to one incident. The events and their consequences have been described by numerous authors (Dunckel, 1975; Carter, 1976; Getty et al., 1977; Kay, 1977). During the summer of 1973, 500 to 1000 pounds of FM were accidentally shipped to a large feed mixing mill in Michigan. Firemaster was inadvertently mixed into feed in place of magnesium oxide. From this mill highly contaminated feeds were shipped to numerous distribution sites in Michigan and subsequently sold to local farmers. These feeds not only contaminated animals that ate it and meat, eggs and milk they produced, but also bins in which it was stored and barns and pastures in which the animals were kept. Contamination of farm families and the general public that consumed tainted products was inevitable. Approximately 8 to 9 months elapsed before PBB were identified in samples of contaminated feed. An extensive sampling, quarantine and disposal program was begun. One report (Van Houweling et al., 1977) indicated that 30,000 cattle, 6000 swine, numerous sheep and poultry, and several hundred tons of feed and several tons of dairy products were destroyed.

Incidents such as this should not recur, since the production of PBB in the United States has been stopped. But because of the environmental stability of the compound (Jacobs et al., 1978) and its tendency to accumulate and persist in adipose tissue (Fries, 1978; Tuey and Matthews, 1980), research examining chronic effects of PBB will continue.

Structure of the Compounds

Polychlorinated and polybrominated biphenyls share the same basic formula, as illustrated in Figure 1. For either PCB or PBB, substitutions with their respective halogens on rings A and B can produce 210 different

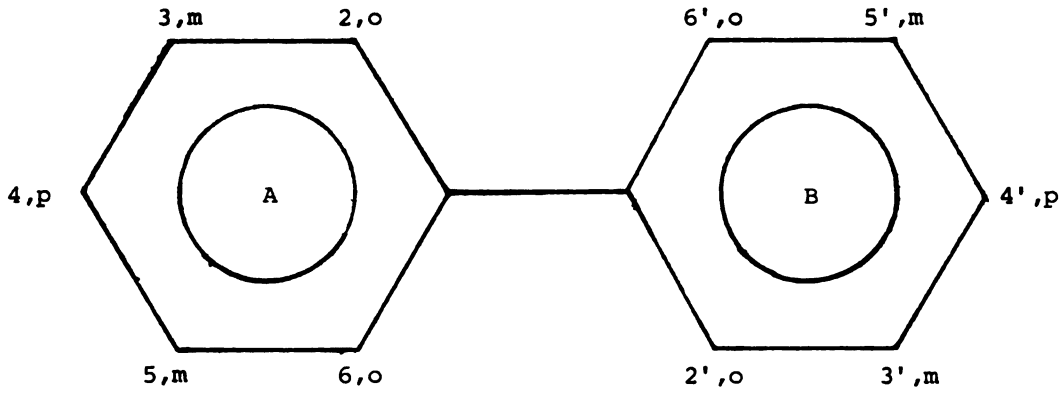

Figure 1. Basic formula for polyhalogenated biphenyls. Note numbered positions on biphenyl rings and letters indicating ortho (o), meta (m) and para (p) positions.

possible congeners for each class of compounds (Cook, 1972). Commercial preparations of PCB are formulated based upon their average chlorine content by weight. Examples of the preparations contain 21, 42, 48, 54, 60, 62 and 68% chlorine (Nisbet and Sarofim, 1972). Each preparation consists of numerous different congeners. Analyses of preparations containing 42, 54 and 60% chlorine have been shown to have 45, 69 and 78 congeners, respectively (Cook, 1972; Kimbrough, 1974).

The Firemaster mixture is not as complex as the different PCB mixtures. It contains approximately 30 different compounds, of which 13 are major PBB congeners (Moore et al., 1979; Moore et al., 1980). If each congener had similar biologic effects, then the complexity of these compounds would be interesting but not too significant. As will be discussed later, however, the various congeners differ greatly in their toxic potential.

Hepatic Microsomal Drug-Metabolizing Enzymes

Before the functional characteristics of the various compounds can be discussed, it is necessary to understand the biologic systems responsible for their disposition. In addition to its many homeostatic functions, the liver has evolved as the major organ for the detoxification and excretion of drugs and foreign compounds (xenobiotics) (Kappas and Alvares, 1975). This system is physically located in the membrane of the endoplasmic reticulum (ER) (Ullrich, 1978). Through disruption of the ER, microsomes can be produced and isolated *in vitro*. Variously called the monooxygenase, mixed function oxidase (MFO) and hepatic drug-metabolizing microsomal enzyme system, one of its important biologic functions is to convert foreign, hydrophobic compounds into hydrophilic

forms which can be more easily excreted by the body (Ingelman-Sundberg, 1980). To accomplish this, various reactions are catalyzed by the MFO system, including epoxidation, hydroxylation, oxidation, dealkylation, and desulfuration (Ullrich, 1978). While most xenobiotics are less toxic after being metabolized by the MFO system, some may form reactive intermediates, compounds which, if not detoxified by endogenous mechanisms, may react with proteins and nucleic acids and predispose to mutagenesis and carcinogenesis (Ullrich, 1978).

The microsomal enzymes can be induced by many compounds. There are, however, 2 basic patterns of microsomal enzyme induction. One pattern is produced by administration of the drug phenobarbital (PB) and the other by 3-methylcholanthrene (MC). Drugs that induce microsomal enzymes similar to those induced by one of these 2 compounds are classified as either PB-type or MC-type enzyme inducers (Conney, 1967). In 1967, the induction characteristics of over 200 drugs, insecticides, carcinogens and other chemicals had been described. The only common denominator that vaguely unites this diverse group of compounds is their solubility in lipid at a physiological pH (Conney, 1967).

The effects of PB and the compounds with similar induction characteristics are extensive and can be evaluated by various sophisticated techniques. The most obvious physical effect of the administration of PB-type compounds is a marked increase in liver size and weight. Individual hepatocytes are swollen and there is usually an increase in the smooth endoplasmic reticulum (SER). Phenobarbital has an anabolic effect on the liver with a dramatic increase in the content of microsomal protein per gram of liver (Conney, 1967).

All of these changes, however, are but reflections of important biochemical alterations occurring in the ER in response to these PB-type

compounds. There can be marked increases in the enzymes responsible for the metabolism of these compounds. There are at least 7 forms of the terminal enzyme, cytochrome P-450, with varying degrees of substrate specificity. These primarily catalyze oxidation reactions (Ingelman-Sundberg, 1980). Induction of the MFO system by PB-type compounds is reflected in increases in microsomal protein, cytochrome P-450, cytochrome P-450 reductase and microsomal enzyme activities, including aminopyrine N-demethylase, epoxide hydratase and ethylmorphine-N-demethylase (Dent et al., 1976a; Dannan et al., 1978b).

How PB-type compounds increase the protein and enzyme content of hepatic ER is not known. Researchers speculate that a cytosolic receptor for these compounds may be required (Poland and Glover, 1977). Suspected mechanisms of action include *de novo* synthesis of protein, decreased catabolism of existing microsomal enzymes and enhanced formation of enzymes at the ribosomal level. The time required for daily doses of PB to exert its maximal effects on microsomal enzymes in the rat is 3 days (Conney, 1967).

A very important characteristic of those compounds that have PB-type induction patterns is that, in spite of their enzyme induction and ability to increase the size of the liver, they generally are considered to be nontoxic (Moore et al., 1980). This is in sharp contrast to polycyclic aromatic hydrocarbon compounds that are MC-like and induce AHH activity. Toxicity of these latter compounds is characterized by lethality, chloracne, hyperkeratosis, lymphoid organ involution, hepatic lesions, teratogenicity and species-specific diseases such as chick edema syndrome (Poland et al., 1979).

Those compounds with MC-type activity include polycyclic aromatic hydrocarbons, which by definition are compounds with "3 or more fused

benzene rings in linear, angular or cluster arrangements and contain only carbon and hydrogen" (Zedeck, 1980). Other compounds which have MC-type activity are certain halogenated aromatic hydrocarbons, which include the biphenyls, dibenzo-p-dioxins, dibenzofurans, azo- and azoxybenzenes and naphthalenes (Poland et al., 1979). The MC-type compounds do not stimulate the incorporation of protein into the ER as actively as PB-type compounds (Conney, 1967). Histopathologic changes in the livers of animals exposed to MC-type compounds have included cellular swelling, vacuolization, lipid accumulation, necrosis and increases in the rough ER (RER) with little change in the SER (Kociba et al., 1978; Gasiewicz et al., 1980).

Compounds with MC-type activity induce the production of a unique terminal cytochrome, cytochrome P-448. This enzyme derives its name from the wavelength of absorption for the carbon monoxide (CO) difference spectrum maximum (Ullrich, 1978). Microsomal enzyme activities thought to be catalyzed by this cytochrome include benzo[α]pyrene hydroxylase (aryl hydrocarbon hydroxylase, AHH) and ethoxycoumarin-o-deethylase (Dent et al., 1976a; Dannan et al., 1978b). Glutathione-s-transferase, DT-diaphorase, ornithine decarboxylase and δ-amino levulinic acid synthetase are examples of other hepatic enzymes that are induced by MC-type compounds (Poland and Glover, 1980). Enzyme activity after the administration of MC-type compounds can double within 3 to 6 hours and can be maximally induced within 24 hours (Conney, 1967). This is in contrast to the 3 to 5 days for maximum induction cited by the same author for PB-type compounds.

The mechanism for induction by MC-type compounds has been extensively researched and reviewed (Poland and Glover, 1977; Poland et al., 1979; Poland and Glover, 1980). They observed that some strains of mice do

not respond to MC while others do but that both respond to 2,3,7,8-tetrachloro-p-dioxin (TCDD). If given in sufficient quantities, TCDD has approximately 30,000 times the potency of MC. Poland and his associates hypothesized that there is a cytosolic receptor for MC-type compounds. Apparently, through mutations this receptor varies in its affinity for its substrate. If binding does occur, the substrate-receptor complex is thought to move to the nucleus and initiate the expression of genes that regulate AHH and numerous other enzyme activities through *de novo* synthesis of protein. The structure of the receptor and, therefore, its affinity for a substrate is controlled by the Ah locus in mice. This locus may not only regulate the production of the putative TCDD receptor and, because of this, AHH activity, but also numerous other hepatic enzymes. This model predicts that the toxicity of MC-type compounds depends upon their ability to bind to a receptor and to initiate the expression of a group of genes. These genes control the *de novo* synthesis of AHH and numerous other enzymes. How enzyme induction is related to toxicity is not known.

The basic configuration of the polyhalogenated biphenyls (PHB) is not unlike that of TCDD. It is not surprising that PHB are known inducers of AHH activity (Poland and Glover, 1977). Also, because of the structural similarities between PCB and PBB, these 2 classes of compounds are generally considered to have similar biologic effects (Dent et al., 1976a; Kay, 1977). The order of potency for halogen substitutions is bromine > chlorine > fluorine (Poland and Glover, 1977).

Metabolism and Structure-Activity Relationships

Mixtures of PCB and PBB that have both PB- and MC-type effects are called "mixed" inducers (Goldstein et al., 1977; Dannan et al., 1978b).

With numerous different compounds in the commercial mixtures and the possibility that potent contaminants might also be included, attempts to understand the effects of individual congeners had to await their identification, isolation and purification. In 1976 and 1977, the commercial PBB mixture, FM, was clearly shown to be a mixed-type inducer (Dent et al., 1976a; Dent et al., 1976b; Dent et al., 1977). As the induction pattern of FM was being described, other researchers were isolating and identifying the major congener as 2,2',4,4',5,5'-HBB (Sundstrom et al., 1976). Approximately 54 to 68% of the FM mixture by weight consists of this congener. Presently, 13 major congeners have been detected in FM and the structures of 9 of these are known (Moore et al., 1980). The obvious task is to determine which compounds are responsible for the numerous treatment effects produced. Fortunately, through observations with both PCB and PBB, some understandings of the relationships between structure and activity have evolved.

The structural features that permit the metabolism of a PBB congener have been reviewed (Moore et al., 1980). Rapid, *in vitro* metabolism of PBB congeners occurs if both or one of the <u>para</u> positions (4,4') is unoccupied and there is an adjacent unoccupied carbon (Dannan et al., 1978a). Of the 13 major FM congeners, only 2 meet these requirements. Of the congeners which are metabolized, the rates of metabolism are generally increased by <u>ortho</u> substitutions and decreased by increasing the number of bromines on the molecules. A recent report by Purdy and Safe (1980), however, indicated that there is some metabolism of 2,2',4,4',5,5'-HBB *in vitro*.

Metabolism of a compound may or may not be a desirable fate. If the metabolite is less toxic than the original compound, then metabolism is desirable. If the metabolite is a reactive molecule that, for example,

binds to DNA or produces membrane destruction through lipid peroxidation, then metabolism may be biologically detrimental. Few attempts have been made to identify possible PBB metabolites and fewer still have been undertaken to determine the possible biologic effects of these products. Metabolites of PCB and PBB have generally been shown to be mono- and dihydroxylated forms of their corresponding parent congeners (Safe et al., 1975; Kohli et al., 1978; Safe et al., 1978). Potentially harmful metabolites from PHB congeners have been identified, however. An arene oxide was identified as an intermediate molecule in the metabolism of 4-bromobiphenyl. This metabolite could possibly bind to cellular molecules and produce toxic effects (Kohli et al., 1978). In this same study, 4-bromobiphenyl was shown to be mutagenic in the *Salmonella typhimurium* TA 1538 assay using microsomes from rats treated with PCB. Another study using FM and 2,2',4,4',5,5'-HBB failed to demonstrate either initiating or promoting activity for the compounds using a mouse strain sensitive to skin tumors (Haroz and Aust, 1979).

The metabolism of polycyclic aromatic hydrocarbons was recently reviewed (Zedeck, 1980). These compounds are generally metabolized to dihydrodiols, phenols and glutathione conjugates. These products require the formation of epoxide intermediates. Certain epoxides are potent carcinogenic and mutagenic compounds. These compounds are electrophilic and are known to bind with sites on DNA and RNA (Ingelman-Sundberg, 1980). The PHB compounds are presumed to produce their toxic effects through mechanisms similar to those attributed to the polycyclic aromatic hydrocarbons (Poland and Glover, 1977). A thorough understanding of the enzyme induction patterns produced by the PHB compounds and of the effects of the parent compounds and their metabolites on cellular components is imperative.

The ability of specific PHB compounds to produce either PB- or MC-type effects follows certain structural guidelines. PHB compounds that have at least 1 unsubstituted para position either do not induce or are very poor inducers of microsomal enzymes (Goldstein et al., 1977; Moore et al., 1980). The rapid metabolism of these compounds may preclude significant enzyme induction (Goldstein et al., 1977). In FM, the only compounds identified with less than 2 para bromines are 2,2',4,5,5'-HBB and 2,2',3,4',5,5'-HBB.

The structural requirements needed for PHB molecules to have MC-type activity have been extensively investigated. One important requirement for this activity is that the molecule be able to assume a planar configuration (Poland and Glover, 1977; Goldstein et al., 1977; Moore et al., 1980). This appears to be essential if the compound is to bind with the putative TCDD-receptor in the cytosol of target cells (Poland and Glover, 1977). Nonplanarity, or at least energy barriers to planarity, result from increased halogen substitutions at ortho (2,2',6,6') positions (Moore et al., 1980).

The ability to assume planarity, however, is not by itself adequate for MC-type activity. Biphenyl, for example, has no MC-type activity (Poland and Glover, 1977). Para or meta substitutions are obviously required. Surprisingly, the compounds 3,3',5,5'-tetrabromo- and tetrachlorobiphenyl do not have MC-type activity (Poland and Glover, 1977). Halogenated biphenyl compounds that have been identified as having MC-type activity include 3,3',4,4',5-pentachlorobiphenyl, 3,3',4,4'-tetrachlorobiphenyl and 3,3',4,4',5,5'-hexabromo- and hexachlorobiphenyl (Goldstein et al., 1977; Poland and Glover, 1977; Yoshimura et al., 1979). From these studies some observations have been made. For a compound to be an MC-type inducer, the ability to

assume planarity and the presence of 2 para halogens may be essential (Moore et al., 1980). The para halogens do not appear to be adequate by themselves, however, since 4,4'-dichlorobiphenyl failed to induce MC-type activity (Poland and Glover, 1977). Meta substitutions may be required in addition to the para ones to impart MC-activity.

Pure MC-type inducers have not been identified in the FM mixture (Moore et al., 1980). However, several components of FM have been investigated for both MC- and PB-type activities. Of these, 2,3',4,4',5,5'-HBB (Dannan et al., 1978b) and 2,3,3',4,4',5-HBB (Robertson et al., 1981) have mixed-type effects and 2,3',4,4',5-pentabromobiphenyl is suspected of being a mixed inducer (Moore et al., 1980). The ability of a biphenyl molecule with 1 ortho substitution to have MC-type activity requires that the energy barrier to rotation be small enough to allow the molecule to at least briefly assume a planar configuration and bind to the TCDD receptor.

Several general comments can be made about the structural characteristics needed for a compound to be a PB-type inducer. The PHB compounds have at least 1 and usually 2 ortho halogens. These by themselves are not sufficient, however, since 2,2'-dibromobiphenyl is not a microsomal enzyme inducer (Moore et al., 1979). Meta and para substitutions are apparently also needed for PB-type induction (Moore et al., 1980).

Specific Effects of the PHB Compounds

Physical

The earliest indication that a compound may be toxic is often reflected by the general appearance of the exposed animal, by alterations in body weight gains, or by gross changes in specific organs. Polyhalogenated biphenyls are no exceptions and their physical and gross effects

have been described in various laboratory animals, livestock and human beings. These will be briefly reviewed.

Toxic effects produced by these compounds are associated with their ability to induce AHH activity. Compounds that are PB-type microsomal enzyme inducers are generally considered to be nontoxic (Moore et al., 1980). Of the numerous compounds known to be MC-type microsomal inducers, TCDD is the most potent. Toxic effects associated with it include slow wasting and death, chloracne and hyperkeratosis, chick edema disease, hepatic lesions, teratogenicity and embryotoxicity and lymphoid involution (Poland et al., 1979).

In a 2-year study involving the feeding of TCDD to male and female rats at various dietary levels, the major gross findings included increased mortality, emaciation and decreased weight gain, liver toxicity, thymic and splenic atrophy, icterus, some focal hemorrhages and pulmonary congestion and edema (Kociba et al., 1978). Total parenteral nutrition (TPN) can prevent the weight loss in rats associated with TCDD toxicity, but it (TPN) does not prevent and may increase the toxicity of the compound (Gasiewicz et al., 1980). Gross findings in this study again included icterus, thymic atrophy and enlarged livers.

For PCB, numerous experiments have evaluated the effects of the commercial mixtures on various animal species. In rats, the predominant gross finding produced by chronic exposure to 100 parts per million (ppm) of several commercial mixtures was an increase in liver weights (Allen et al., 1976). These animals gained weight similarly to controls and appeared healthy. At a higher dietary concentration of a commercial PCB mixture, feed intake and weight gain were decreased while liver weights were increased in rats (Garthoff et al., 1977). Other animal species are more susceptible to the potential toxic effects of the PCB

compounds. Mink, for example, can be killed by less than 4 ppm PCB in the diet with the physical and gross changes being similar to those described in the rat at high levels of exposure (Platonow and Karstad, 1973; Aulerich and Ringer, 1977). Monkeys fed PCB mixtures at different concentrations and for different time periods had physical and gross alterations that included death, weight loss, alopecia, eyelid edema, chloracne, enlarged livers, ulceration of the gastric mucosa and thymic atrophy (Abrahamson and Allen, 1973; Allen et al., 1974).

In 1968 in Japan, more than 1000 people consumed various quantities of a rice oil contaminated with approximately 2000 to 3000 ppm of a commercial PCB mixture (Kuratsune et al., 1972). The clinical symptoms and physical findings included chloracne, swelling of eyelids, increased pigmentation of the skin, jaundice, malaise, fever and various neurological signs. Ten years after the exposure most of the initial symptoms, many of which were related to disorders of the skin and mucous membranes, had improved. Unfortunately, other complaints, including dullness, headache, joint swelling and pain, retarded growth in children and bronchitis-like symptoms had replaced the initial symptoms (Urabe et al., 1979).

For PBB, the clinical signs and physical and gross findings are generally similar to those reported for PCB. This should be expected based on the shared structural and chemical characteristics of these classes of compounds. Since the major release of PBB into the environment occurred in livestock feed, the first important description of the effects of the PBB mixture was in cattle (Jackson and Halbert, 1974). Physical effects attributed to the compound were death, anorexia, weight loss, abortion, decreased milk production, abnormal hoof growth, lameness, hematomas, abscesses, enlarged livers and thickening and

wrinkling of the skin. Other studies with cattle have confirmed many of these findings (Moorehead et al., 1977; Cook et al., 1978). Moorhead et al. (1977) also noted thymic atrophy as an important gross finding in their cattle given FM. A herd health study by Mercer et al. (1976) failed to identify any significant physical or clinical effects between control and exposed herds. Many of the apparent differences between studies could be related to the amount and duration of exposure to the PBB compounds.

In rats exposed to the FM mixture, the major physical and gross findings have included decreased weight gain, increased liver weight, mottled copper-colored appearance to the liver and atrophy of the thymus and spleen (Sleight and Sanger, 1976; Garthoff et al., 1977; Harris et al., 1978; Gupta and Moore, 1979). The most consistent finding in these studies was the increased liver weights. At oral doses of 100, 300 and 1000 mg/kg/day, the mortality rate in rats during a chronic study was high (Gupta and Moore, 1979).

Studies in rats with specific PBB-congeners have also been conducted. Moore et al. (1978) examined the effects of 2,2',4,4',5,5'-HBB on rats. The only reported gross findings were swollen livers with rounded lobes. Render (1980) fed FM and 2 purified congeners to rats for 10 days. These were the PB-type inducer 2,2',4,4',5,5'-HBB and the MC-type inducer 3,3',4,4',5,5'-HBB. Physical signs of toxicity were confined to rats fed high levels of 3,3',4,4',5,5'-HBB. Rats fed 100 ppm became depressed, anorectic, emaciated and died within 20 days. Within the 10-day feeding period, rats fed 10 and 100 ppm ate less, gained less weight, had decreased thymic weights and, as in animals fed the other compounds, had increased liver weights when compared to controls.

Other experimental animals have been used in studies with PBB. Important physical findings cited in these studies are as follows:

monkeys - weight loss, alopecia, subcutaneous edema and liver enlargement (Allen et al., 1978); mink - death, anorexia, weight loss, unthriftiness and fatty livers (Aulerich and Ringer, 1979); quail - subcutaneous edema of neck and shoulders in chicks and increased liver and thyroid weights(Ringer and Polin, 1977); pigs - decreased weight gain and increased liver weights (Ku et al., 1978).

Histopathology

In rats exposed to TCDD, the most consistent histopathologic changes have been confined to the thymus and liver (Gasiewicz et al., 1980). Thymic atrophy was related to a decrease in the number of cortical lymphocytes. In the livers of treated rats there was diffuse swelling and enlargement of hepatocytes with some loss of architectural pattern, vacuolization and necrosis of cells in the midzonal and centrilobular regions. More severe microscopic changes included extensive necrosis, vacuolization, cystic space formation filled with inflammatory debris, bile duct proliferation, fatty change and an increased incidence of foci of hepatocellular alterations (Kociba et al., 1978; Gasiewicz et al., 1980). Ultrastructural changes in the livers of rats given TCDD have included an increase in the rough endoplasmic reticulum (RER) with an inconsistent change (occasionally increased) in the smooth endoplasmic reticulum (SER), an increase in cytoplasmic lipid droplets and an increase in lysosomal activity with residual body formation (Kociba et al., 1978). At high levels of exposure, the RER formed concentric arrays and degenerating mitochondria were present (Gasiewicz et al., 1980).

Microscopic and ultrastructural changes reported for animals exposed to PHB are generally similar to those described for TCDD. Rats that were given various levels of PCB had hepatocellular

swelling, focal areas of degeneration and necrosis, cytoplasmic vacuolization and cyst formation, fatty infiltration and bile duct proliferation (Kimbrough et al., 1972; Allen et al., 1976; Kasza et al., 1976). All of these studies, including another by Norback and Allen (1972), noted important ultrastructural alterations in the livers. These included a proliferation of the SER with a decrease and disorganization of the RER, the presence of granular cytoplasmic inclusions and a loose "motheaten" appearance to the cytoplasm. Concentric membrane arrays developed in the cytoplasm and smooth membranes encircled mitochondria and lipid droplets.

Histopathologic and ultrastructural changes in livers from monkeys and mink fed PCB mixtures included necrosis, cellular swelling, lipid accumulation and proliferation of the SER (Platonow and Karstad, 1973; Allen et al., 1974; Allen, 1975). Thymic atrophy due to cortical hypoplasia has also been observed in monkeys fed PCB (Abrahamson and Allen, 1973). In liver biopsy samples of Japanese victims of the "Yusho" incident, the amount of RER was reduced while the SER was increased. Mitochondria were reported to be heterogeneous and contained filamentous inclusions in the matrix (Kuratsune, 1972).

Histopathologic descriptions of abnormalities produced by PBB are extensive and this review, concentrating on the primary organs affected, will be confined to changes described in the liver and thymus. Microscopic changes in the livers of rats and mice fed the FM compound have included cellular swelling and vacuolization (frequently oil red O positive), increased cellular pleomorphism, areas of inflammation and necrosis and, at very high levels, bile duct proliferation and associated fibrosis (Sleight and Sanger, 1976; Kimbrough et al., 1978; Gupta and Moore, 1979; Kimbrough et al., 1980). Thymic alterations consisted of

marked atrophy, cortical hypoplasia and a loss of demarcation between cortical and medullary regions (Gupta and Moore, 1979). Rats given intraperitoneal (IP) injections of the congeners 2,2',4,4',5,5'-HBB and 2,2',3,4,4',5,5'-heptabromobiphenyl had swollen and vacuolated hepatocytes (Moore et al., 1978; Moore et al., 1979).

Rats fed 3,3',4,4',5,5'-HBB had cortical atrophy of the thymus and an associated cortical infiltration of macrophages. Livers of these rats had swollen hepatocytes with prominent nucleoli, loss of sinusoidal spaces and midzonal to centrilobular vacuolization (Render, 1980). Hypercellularity of portal areas related to proliferation of bile duct cells was present in rats fed 100 ppm of this congener for 20 days.

Ultrastructural evaluations of livers from rats and mice given FM and octabromobiphenyl have detected mitochondrial swelling and degeneration, increases in SER and peripheral displacement and decreases in RER, myelin body formation (paired, smooth membrane arrays often surrounding lipid droplets), cytoplasmic vacuolization and increases in lysosomes and a reduction in glycogen (Lee et al., 1975; Sleight and Sanger, 1976; Corbett et al., 1978; Kimbrough et al., 1980). Rats fed 2,2',4,4',5,5'-HBB had an increase in hepatocellular SER and those fed 3,3',4,4',5,5'-HBB had a marked increase in SER and an increase in individualized, double membrane RER often located around mitochondria (Render, 1980). Other ultrastructural changes noted by Render (1980) in hepatocytes of rats fed 3,3',4,4',5,5'-HBB were increased amounts of lipid droplets, disorganization of the RER and numerous free ribosomes in the cytoplasm.

Similar hepatic changes have been reported in monkeys (Allen et al., 1978) and mink (Aulerich and Ringer, 1979). Although histopathologic

alterations in cattle fed PBB have been described in the liver (primarily fatty change), significant renal changes also occur. These include extreme dilatation of collecting ducts and convoluted tubules with pronounced epithelial degeneration (Jackson and Halbert, 1974; Moorhead et al., 1977; Cook et al., 1978).

Biochemistry of PHB Toxicity

Hepatic Microsomal Drug-Metabolizing Enzymes

Earlier in this review the induction of hepatic microsomal enzymes by various compounds was briefly discussed. It was stressed that certain compounds are similar to phenobarbital in their ability to induce microsomal enzymes while others are similar to 3-methylcholanthrene. Compounds which induce AHH activity are considered to be toxic. Numerous variables can be measured which reflect the type of induction a compound may have. These include measurement of microsomal protein, amount of cytochrome P-450, measurement of the carbon monoxide (CO) difference spectrum and metabolism of numerous substrates to evaluate the induction of microsomal enzyme activities. The effects of TCDD, PCB and PBB on these variables will be summarized.

Compounds which have PB-type activity as opposed to MC-type are reported to have a greater positive effect on the incorporation of protein into microsomes (Conney, 1967). This is evident in the data of Dannan et al. (1978b) by comparing the microsomal protein content from livers of PB- and MC-treated rats. Different microsomal proteins are induced by these treatments, as shown by polyacrylamide gel electrophoresis patterns. These proteins are thought to be either the various induced species of cytochrome P-450 or their apoproteins (Welton and Aust, 1974; Haugen et al., 1976; Toftgard et al., 1980).

Often paralleling the changes in microsomal protein content, therefore, is the amount of cytochrome P-450 in the hepatic microsomes. The effects of TCDD on microsomal protein content have been reported to be negligible while the cytochrome P-450 content has been increased (Gasiewicz et al., 1980; Poland and Glover, 1977). Parent PCB and PBB mixtures, however, can produce dramatic increases in both microsomal protein content and cytochrome P-450 (Babish and Stoewsand, 1977; Garthoff et al., 1977; Poland and Glover, 1977; Moore et al., 1979; Dannan et al., 1978b).

The 2 major congeners in FM, 2,2',4,4',5,5'-HBB and 2,2',3,4,4',5,5'-heptabromobiphenyl, are strictly PB-type inducers and both produce marked increases in microsomal protein and P-450 content. These responses are similar to those caused by the FM mixture but greater than those produced by injections of MC (Moore et al., 1978; Moore et al., 1979). The 2,3',4,4',5,5'-HBB congener, which has mixed-type induction effects, also increases microsomal protein and P-450 content (Dannan et al., 1978b).

Another method of evaluating the type of enzyme induction in microsomes is to measure the wavelength of the carbon monoxide (CO) difference spectrum for the reduced microsomes (Ullrich, 1978). Substances that have PB-type activity induce terminal P-450 enzymes which have a maximal difference at 450 nm. The MC-type inducers, however, stimulate production of different terminal cytochromes that have a maximal absorption at 448 nm. Thus, a shift in the absorption spectrum from 450 nm towards 448 nm would indicate exposure to an MC-type compound and could be associated with overt signs of toxicity. For the PBB congeners 2,2',4,4',5,5'-HBB and 2,2',3,4,4',5,5'-heptabromobiphenyl, the cytochrome P-450 spectral maximum is not shifted from 450 nm (Moore et al.,

1978; Moore et al., 1979). The FM mixture, which is a mixed-type inducer, however, has been shown to shift the CO difference spectrum towards or to 448 nm (Dent et al., 1976a; Babish and Stoewsand, 1977). Both TCDD and 3,3',4,4'-tetrachlorobiphenyl shift the CO difference spectrum towards 448 nm (Poland and Glover, 1977).

Different forms of cytochrome P-450 catalyze different reactions and have varying degrees of substrate specificity (Ullrich, 1978). Different substrates can be used to detect the ability of microsomal mixed function oxidase (MFO) systems to catalyze a given type of reaction and this ability can be used to categorize the induction as PB- or MC-like. The ability to demethylate aminopyrine (amiopyrine demethylase activity, AD) and to hydroxylate benzo[α]pyrene (arylhydrocarbon hydroxylase activity, AHH) are frequently used to reflect PB- and MC-type activities, respectively (Conney, 1967). The most potent MC-type inducer known, TCDD, has been shown to have AHH and not AD activity (Poland and Glover, 1977; Gasiewicz et al., 1980). Both FM and 2,3',4,4',5,5'-HBB will induce both activities (Dannan et al., 1978b) and 2,2',4,4',5,5'-HBB and 2,2',3,4,4',5,5'-heptabromobiphenyl primarily induce AD activity (Moore et al., 1978; Moore et al., 1979). By using these methods to categorize compounds as PB- or MC-type inducers, extremely practical information about their mechanisms of action and suspected toxicity can be obtained.

Serum Enzymes

Several serum enzyme determinations are often included in the battery of tests used to evaluate the effects of a suspected toxic compound. Most are selected because an elevation in their serum level tends to reflect either hepatic or cholestatic disorders. Changes

reported for these enzymes in animals exposed to PCBs or PBBs are inconsistent. Perhaps much of the variability is related to use of different congeners or mixtures, duration and level of exposure and different species and sexes of animals employed in the experiments.

Alanine aminotransferase (ALT, SGPT) and aspartate aminotransferase (AST, SGOT) are enzymes that catalyze the interconversion of amino groups from their respective amino acids to α-oxoacids. Both occur in relatively high concentrations in hepatic tissue but high levels of AST are also present in cardiac and skeletal muscle (Kachmar and Moss, 1976). Intracellular distribution of these 2 enzymes differs in that AST is located in both the cytoplasm and mitochondria while ALT is found primarily within the cytoplasm. While species differences in tissue specificity occur, elevated levels of either enzyme within the serum are frequently interpreted as an indication of altered hepatocellular integrity.

Sorbitol dehydrogenase (SDH) is a cytosolic enzyme that is reported to be very liver specific (Kachmar and Moss, 1976). It functions in the catalysis of the interconversion of sorbitol and fructose. Elevated serum levels should reflect altered hepatocellular integrity and should not be associated with primary dysfunctions of other organ systems.

Gamma-glutamyltranspeptidase (GGT) is an enzyme found in high concentrations on the renal brush border of the kidney and in plasma membranes enriched in bile canaliculi and biliary duct epithelial cells of the liver (Shaw and Newman, 1979; Huseby, 1979). While the enzyme was once thought to function in the translocation of amino acids into cells via the γ-glutamyl cycle, the suggested function now is extracellular catabolism of glutathione to L-glutamate and L-cysteinylglycine

(McIntyre and Curthoys, 1979; Shaw and Newman, 1979). Species differences in organ levels of GGT are marked. Human liver and kidney have approximately 10 times the tissue levels as do the same organs in rats (Shaw and Newman, 1979), and guinea pigs are reported to have much higher hepatic GGT levels than rats (Huseby, 1979). Certain drug treatments, such as PB, increase GGT levels in guinea pigs and rats, although in the latter species prolonged treatment was required. The elevated serum levels are thought to involve the induction of microsomal drug-metabolizing enzymes (Huseby, 1979). In clinical situations, the enzyme can be elevated in many types of liver disease, but the highest levels result from intra- or posthepatic biliary obstruction (Kachmar and Moss, 1976). In the dog, for example, GGT appears to be more specific and sensitive than alkaline phosphatase for biliary obstruction (Noonan and Meyer, 1979).

Serum alkaline phosphatase (ALP) is a mixture of various isoenzymes primarily derived from liver, bone, intestinal tract and placenta. The enzyme is apparently involved in the transportation of metabolites across cell membranes, including lipid transportation in the intestines and calcification processes in bone (Kachmar and Moss, 1976). In the liver, ALP is located in hepatic cell sinusoidal membranes, microvilli of bile canaliculi and in the endothelial cells of the portal and central veins (Wolf, 1978).

Righetti and Kaplan (1971) reported that the major source of ALP in the fasted rat is the bone isozyme. These rats, however, were young, weighing approximately 200 grams, and a high serum level of the bone isoenzyme could be expected in young animals. Fishman et al. (1962) indicated that ALP in the serum of a normal, well nourished rat is almost all of intestinal origin. Fishman observed a decline in serum

ALP in the rat related to a decrease in the intestinal isoenzyme following bile duct ligation. He reasoned that the lack of bile prevented adequate intestinal fat emulsification leading to a decrease in fatty acid uptake by the intestinal mucosa and a corresponding decreased release of intestinal ALP into the lymphatics. Another study showed that bile duct ligation produced an elevation in the serum of a high weight isoenzyme similar to that induced on the canalicular surfaces of the hepatocytes (Toda et al., 1980). These authors reasoned that the induced isoenzyme is solubilized from hepatic membranes by bile salts. The bile with this elevated ALP activity cannot flow through the obstructed biliary tract and may seep into sinusoidal blood.

After feeding, sharp increases in serum ALP occur in rats because of the increased activity of the intestinal isoenzyme (Saini and Posen, 1969). The lifespan of injected intestinal ALP in rats is very short, with most disappearing from the circulation within 2.5 hours (Saini and Posen, 1969).

Elevated ALP levels in the absence of bone disease are usually associated with hepatic disease. The enzyme is said to be a sensitive indicator of intra- or extrahepatic cholestasis (Wolf, 1978). Induction of the enzyme with resulting increased serum levels can occur with cholestasis, glucocorticoid therapy and with certain drugs, including primidone, phenobarbital and dieldrin (Hoffmann, 1977).

In 2 separate experiments during which rats were fed TCDD, increases occurred in serum values of ALP, GGT,ALT and AST (Gasiewicz et al., 1980; Kociba et al., 1978). These changes were compatible with both hepatocellular leakage and cholestasis. There were no increases reported in ALP, ALT, AST or lactate dehydrogenase values from 2 studies

in which rats were fed commercial preparations of PCB at high levels (up to 500 ppm) for as long as 5 weeks (Garthoff et al., 1977; Kasza et al., 1976). The "Yusho" victims previously described were subjected to very extensive clinical examinations. Even though the average amount of PCB ingested by each victim was 2 grams (Kuratsune et al., 1972), only slight increases in ALP were detected in the serum of severely affected individuals. Lactate dehydrogenase, ALT and AST values were normal in all (Kuratsune, 1972). Even though histopathologic, ultrastructural and microsomal enzyme alterations were demonstrated in liver samples from most of these studies, the changes were apparently not sufficient to produce or sustain hepatocellular leakage.

In one study in which rats were fed FM, there was no reported increase in ALT or AST (Garthoff et al., 1977). Similarly, there were no increases in serum levels of AST or ALP in the serum of pigs fed the commercial PBB mixture (Ku et al., 1978). An increase in AST was reported in cows fed high levels of PBB for 2 months (Moorhead et al., 1977). While it would be difficult to make conclusions from these sparse data, there does not appear to be any firm evidence that moderate levels of PHB exposure produce consistent liver changes that can be detected via serum enzyme determinations.

Lipoproteins

The basic knowledge of lipoprotein metabolism will not be discussed in this review. Recent review articles have been written by Small (1977), Kane (1977), Tall and Small (1978), Miller (1979), Witztum and Schonfeld (1979) and Albers and Warnick (1981). Relatively few of the numerous research projects examining the effects of PHB have studied possible alterations in lipoprotein metabolism. Many exogenous and

endogenous factors have been identified that stimulate lipoprotein synthesis. These include exercise (Hartung et al., 1980), alcohol (Belfrage et al., 1977), estrogenic hormones, insulin (Nikkila, 1978) and numerous drugs and compounds, including chlorinated hydrocarbon pesticides such as lindane, DDT, Aroclor (PCB) and Kepone (Carlson and Kolmodin-Hedman, 1972; Kato et al., 1978; Ishikawa et al., 1978), TCDD (Poli et al., 1980), phenobarbitone (Durrington, 1979), glutethimide (Bolton et al., 1980) and clofibrate (Nikkila, 1978). Although there are rare exceptions, the consistent trend for these factors is to increase cholesterol concentrations through elevations in the high density lipoprotein fraction (HDL).

Theories advanced for the HDL cholesterol increases include enhanced hepatic cholesterol synthesis and incorporation into HDL particles(Ishikawa et al., 1978; Durrington, 1979; Bolton et al., 1980; Poli et al., 1980), decreased cholesterol catabolism in the liver (Poli et al., 1980) and increased very low density lipoprotein (VLDL) catabolism via lipoprotein lipase with elevated HDL particle formation (Belfrage et al., 1977; Nikkila, 1978). Kato and Yoshida (1980) recently explored the theory that hypercholesterolemia produced by PCB administration to rats was related to enhanced hepatic microsomal cholesterol synthesis. They were able to demonstrate an increased *in vivo* rate of cholesterol synthesis and an elevated level of β-hydroxy-β-methylglutaryl Coenzyme A reductase (HMG-CoA), the rate limiting enzyme in cholesterol synthesis, in livers of rats given PCB. The possibility that other factors, such as decreased catabolism, might be involved was not pursued.

As indicated, significant increases in serum cholesterol have been reported in rats fed TCDD (Gasiewicz et al., 1980; Poli et al., 1980). In the study by Poli et al. (1980), increases were localized to the

HDL fraction. The total amount and distribution of serum triglyceride were not affected in this study (Poli et al., 1980) but serum triglyceride values were decreased in the other study (Gasiewicz et al., 1980). In addition to those experiments already cited, increased serum cholesterol levels in response to PCB administration to rats have been reported by Allen et al. (1976) and Garthoff et al. (1977). Total serum triglyceride values were only measured in one of these studies (Allen et al., 1976) and was reported to transiently increase.

Relatively few studies have examined the possibility of serum lipid alterations in association with PBB administration. While one study using rats reported an increase in total serum cholesterol (Garthoff et al., 1977) and another detected altered lipoprotein electrophoresis patterns (Sleight et al., 1978), an experiment with monkeys (Allen et al., 1978) and a survey of contaminated dairy herds (Mercer et al., 1976) revealed decreased serum cholesterol levels. No changes in serum cholesterol were detected in swine fed PBB, although altered lipoprotein values were reported (Howard et al., 1980). As indicated earlier, these inconsistencies may be species, dose or time related. Additional studies are needed to define lipid alterations resulting from such environmental contaminants.

MATERIALS AND METHODS

Experimental Design

The experimental design is depicted in Table 1. Rats were fed diets containing various amounts (0, 0.1, 1, 10 or 100 ppm) of either FM, 2,2',4,4',5,5'-HBB or 3,3',4,4',5,5'-HBB. There were 6 rats in each group. Separate 10-day and 30-day exposure periods were conducted for each compound. Each experiment involved feeding one compound at the 4 concentrations for one time period. This resulted in 6 separate experiments, each with its own set of controls.

The 30-day, 100 ppm group for the compound 3,3',3,3',4,5'-HBB could not be included because of the toxicity of the compound. During a trial experiment with 2 rats at this exposure level, one rat died on day 20 and the other was moribund and was euthanatized the same day. A paired-feeding experiment was included for the rats fed 100 ppm of 3,3',4,4',5,5'-HBB for 10 days. This was needed to determine if changes in the rats fed this compound at this concentration were dose related or associated with reduced feed intake. Animals fed this concentration (100 ppm) for 10 days ate an average of 13 g of feed/day/rat. Their controls (0 ppm) ate 23 g of feed/day/rat. Accordingly, during the paired-feeding experiment, 6 rats were fed 13 g of feed/day/rat, 6 were fed 23 g/day/rat and 6 were given feed *ad libitum*.

Table 1. Experimental design

Compound	Conc. in Diet (ppm)	Exposure Periods (days)									
		10					30				
		0	0.1	1	10	100	0	0.1	1	10	100
FM		-[a]	-	-	-	-	-	-	-	-	-
2,2',4,4',5,5'-HBB		-	-	-	-	-	-	-	-	-	-
3,3',4,4',5,5'-HBB		-	-	-	-	+[b]	-	-	-	-	ND[c]

[a] Six animals per group

[b] Four animals per group

[c] ND = not determined

Animals, Housing, Feed

Outbred, male, Sprague-Dawley rats were purchased from Spartan Research Animals, Haslett, MI. They weighed between 250 and 300 g. All animals were acclimated for at least 2 to 3 days before an experiment was begun. During this period, the rats were fed a commercial rat feed (Wayne Lab-Blox, Allied Mills, Inc., Chicago, IL). The same feed, ground into fine granules, was also used in all experimental trials. During the experiments, the rats were kept in plastic cages, 3 animals per cage. Animals were housed in separate cages during the paired feeding experiment. Water was available during all phases of an experiment.

Bedding consisted of heat-treated wood chips (Northeastern Products Corporation, Warrensburg, NY). Once an experiment was started, the cages were placed in a laminar flow, filter chamber (Contamination Control, Inc., Lansdale, PA). This was necessary to prevent dissemination of the PBB compounds throughout the animal room. The chamber was cleaned and all prefilters were changed between experiments.

Before each experiment was begun, the total amount of feed and quantity of compound needed for that trial were calculated. Each compound was weighed and placed in warm (40 C) corn oil (Mazola^{R}). Both FM and 2,2',4,4',5,5'-HBB dissolved with stirring--the former within 1 to 2 hours and the latter overnight. The congener 3,3',4,4',5,5'-HBB never dissolved but dissipated into a fine suspension. These solutions were added to a calculated amount of ground commercial feed to produce the 100 ppm diet. Fresh corn oil was added to this mixture for a final concentration of 10 ml/kg. One part of a higher dietary concentration added to 9 parts of stock ground feed produced the next lower dietary concentration. Corn oil content was adjusted for the

desired final concentration. Control diets contained only corn oil vehicle. All feed preparations were tumbled for 15 minutes on a rotary mixer. Animals were fed from porcelain cup feeders with stainless steel caps and floating perforated discs.

Chemicals

Firemaster BP-6 used in this experiment was produced by Michigan Chemical Corporation, St. Louis, MI. The 2,2',4,4',5,5'-HBB congener was separated and purified from FM in the laboratory of Steven D. Aust, Department of Biochemistry, Michigan State University. Firemaster was dissolved in hexane, applied to a column of alumina in hexane, eluted with hexane and specific fractions were pooled, dried and recrystallized to greater than 99.9% purity (Moore et al., 1978). The 3,3',4,4',5,5'-HBB congener was purchased from RFR Corporation, Hope, RI. The congener was purified >99% in Dr. Aust's laboratory by repeated alumina chromatography.

Anesthesia and Blood Collection

Eighteen to 24 hours prior to the termination of an experiment feed was removed from all treatment groups. Each animal was anesthetized with carbon dioxide (CO_2, dry ice) in a closed plastic container. Blood (10-12 ml) was aspirated from the heart by cardiac puncture. With the animal in dorsal reumbency, an 18 gauge, 1.5 inch needle attached to a 10 ml syringe was inserted just lateral to the xiphoid cartilage. Upon aligning the needle in an anteroventral direction and applying slight negative pressure on the syringe plunger, the needle was advanced until the tip was located within a chamber of the heart and blood flowed freely into the syringe. After sample collection, rats were left in the CO_2 chamber until they died, which usually occurred within 5 minutes.

Within 10 minutes of collection, blood samples were placed in a refrigerator at 4 C and left for 2 hours. They were then removed and centrifuged for 10 minutes at 3,000 revolutions per minute (rpm). Serum was removed from the clot and placed in storage at 4 C. All enzyme and lipoprotein assays were performed within 5 days of serum collection. Beyond that time, samples were frozen (-10 C) and for additional determinations, such as the measurement of the congeners associated with specific lipoprotein fractions, these serum samples were used.

Determinations

Body and Organ Weights

The body weight of each rat was obtained at the beginning of an experiment, every other day during a trial, and immediately before blood collection and euthanasia. After each animal was euthanatized, the following organs were immediately removed and weighed on a top-loading balance (Mettler Series P, Model 163, Mettler Instrument Corp., Hightstown, NY): thymus, liver, thyroids, and spleen.

Serum Cholesterol, Triglyceride

All cholesterol, triglyceride and serum enzyme assays were performed with centrifugal analyzers in the veterinary clinical pathology laboratory at Michigan State University. Cholesterol, triglyceride, GGT and SDH were assayed on a Gemini^{R} analyzer (Electro-Nucleonics, Inc., Fairfield, NJ) and serum AST, ALT and ALP on a Gemsaec^{R} centrifugal autoanalyzer (Electro-Nucleonics, Inc., Fairfield, NJ). All assays except for free cholesterol were available as commercial kits.

Total serum cholesterol was measured using a kinetic enzymatic procedure (cholesterol oxidase) first described by Allain et al. (1974). Free cholesterol was determined by modification of techniques described by Allain et al. (1974), Nagasaki and Akanuma (1977) and Worthington Diagnostics (1978). Modifications were made to eliminate cholesterol esterase from the reaction mixture, thus preventing the conversion of cholesterol esters to unesterified cholesterol and the inclusion of the former in final determinations. Table 2 depicts reagents and their concentrations used in free cholesterol assays. Amounts of reagent and serum used in each free cholesterol test were 0.7 ml and 10 µl, respectively.

Aqueous cholesterol standards prepared by the method of Abele and Khayam-Bashi (1979) were used for both total and free cholesterol assays. Crystalline cholesterol (99+% pure for chromatography) was obtained from Sigma Chemical Company, St. Louis, MO) and powdered sodium desoxycholate from Fisher Scientific Company, Fair Lawn, NJ.

Triglyceride concentrations were determined by a kinetic enzymatic assay using a commercial kit (Worthington Diagnostics, Freehold, NJ).

Serum Enzymes

Serum alkaline phosphatase (ALP), gamma glutamyltranspeptidase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and sorbitol dehydrogenase (SDH) were determined using standard kinetic assays available in commercial kit form (Spin Chem, Sunnyvale, CA). All serum samples were stored at 4 C and enzyme assays were performed within 4 days.

Table 2. Free cholesterol assay

Reagent	Reconstituted Reagent Concentration	Source
Cholesterol oxidase	150 U/l	ICN Nutritional Biochemicals, Cleveland, OH
Phenol	21.2 mmol/l	Mallinckrodt Chemical Works, St. Louis, MO
4-Aminoantipyrine	1.6 mmol/l	Sigma Chemical Co., St. Louis, MO
Peroxidase	5390 U/l	Sigma Chemical Co.
Tris-HCl buffer (pH 7.5)	50 mmol/l	Sigma Chemical Co.
Triton X-100	0.5 ml/l	Rohm and Hass, Philadelpha, PA

Lipoproteins

Preparation and analysis of serum lipoprotein fractions were conducted within 5 days of sample collection. Serum lipoproteins were separated into 3 major fractions, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). Portions of each serum sample (175 µl) were placed in polyallomer tubes and spun in an ultracentrifuge (Airfuge^{R}, Beckman Instruments, Inc., Palo Alto, CA) for 2.5 hours at 100,000 rpm (106,000 to 165,000 x g, minimum to maximum). After centrifugation, each tube was carefully removed and placed in a hole drilled within a metal block such that 125 µl of the serum sample was below the surface of the block and 50 µl above. Using a razor blade, the tube was sliced flush with the block and each section of tube was placed in a separate test tube. The VLDLs (density 0.95 to 1.006 g/ml) were located in the top section of the tube while LDLs (density 1.006 to 1.063 g/ml) and HDLs (density 1.063 to 1.21 g/ml) were isolated in the bottom section. Each fraction was then reconstituted with 0.195 molar NaCl to the initial sample volume (175 µl). These were stored at 4 C.

Isolation of HDLs was accomplished through selective chemical precipitation using sodium phosphotungstate (NaPhT) and magnesium chloride (Matheson, Coleman and Bell, Cincinnati, OH) by the method of Burstein et al. (1970). Into 50 ml of distilled water, 4.0 g of phosphotungstic acid (Sigma Chemical Company, St. Louis, MO) and 16 ml of 1.0 mol/l sodium hydroxide (Mallinckrodt Chemical Works, St. Louis, MO) were added. Distilled water was added to produce a final volume of 100 ml. A 2.0 ml /l solution of $MgCl_2$ was also prepared. To 1 ml of serum, 100 µl of the NaPhT and 25 µl of the $MgCl_2$ solutions were added. After brief mixing, the VLDLs and LDLs formed precipitates with the

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reagents and were sedimented by centrigugation at 6,000 rpm for 10 minutes. The supernatant fluid, which contained the HDLs, was aspirated from the tube and stored at 4 C.

In addition to the serum samples, total cholesterol and triglyceride concentrations were determined for the 3 lipoprotein fractions using the techniques previously described. Since the VLDL and HDL-LDL fractions were previously restored to their original serum concentrations, no adjustment was needed for the lipid values. For similar quantitative assays in the HDL fraction, all values were multiplied by a factor of 1.125 to correct for dilution. The LDL values were obtained by subtracting corrected HDL fractions from HDL-LDL fractions for the same animal.

Lipoprotein electrophoresis was conducted on serum samples from all experiments and on lipoprotein fractions from the 30-day experiments during which 2,2',4,4',5,5'-HBB and 3,3',4,4',5,5'-HBB were fed. All electrophoresis procedures were performed within 5 days of sample collection. Equipment and reagents used for electrophoresis procedures were produced by Helena Laboratories, Beaumont, TX. Lipoprotein samples were applied to cellulose acetate plates (Titan III XW^{R}) that had been presoaked for 24 hours in Tris-barbital-sodium barbital buffer (Electra^{R} HR Buffer) diluted to 650 µl. These were immediately placed in an electrophoresis chamber containing the same buffer and voltage was adjusted to 180 volts, maintained for 20 minutes. Plates were then removed and stained for 2.5 to 3.5 hours in a 0.2% solution of oil red O in methanol (Oil Red Om^{R}). Plates were then washed in water, soaked with glycerol and scanned with a densitometer (Quick Quant II, Helena Laboratories, Beaumont, TX).

Gas-Liquid Chromatography

Pooled serum, albumin and lipoprotein samples from 30-day, 100 ppm 2,2',4,4',5,5'-HBB and 10 ppm 3,3',4,4',5,5'-HBB experiments were analyzed by electron capture, gas-liquid chromatography (GLC). This was an attempt to measure the concentration of these specific congeners in serum and their pattern of association with different serum components. Liddle et al. (1976) described an extraction technique which involved an ethyl-hexane extraction of methanol-treated serum. To increase yield of 3,3',4,4',5,5'-HBB congener, ethyl ether-hexane solution was replaced with toluene during the extraction procedure. Extraction solutions were eluted through florisil columns, condensed and subjected to GLC analysis. The gas chromatograph (G.C. Model 3700, Varian Instrument Division, Palo Alto, CA) was operated by personnel in the Department of Pathology, Michigan State University. A brief description of the technique has been provided by Render (1980).

Albumin fractions for GLC analysis were prepared from pooled serum samples from the 2 experimental groups previously cited. These were treated with a solution of sodium sulfite (Na_2SO_3, Mallinckrodt Chemical Works, St. Louis, MO) such that a final concentration of 26.9% Na_2SO_3 was produced (Cannon et al., 1974). Precipitated globulins were sedimented by centrifugation at 3,000 rpm for 10 minutes and supernatant fluid containing the albumin fraction was aspirated and stored at -10 C until extraction could be performed.

Hepatic Microsomal Enzymes

Immediately after euthanasia, liver samples from rats in various treatment and control groups were placed in cold potassium chloride-nicotinamide solution. These pooled samples were analyzed in the

laboratory of Steven D. Aust, Department of Biochemistry, Michigan State University. Microsomes were isolated, washed and stored according to methods previously described (Pederson and Aust, 1970; Welton and Aust, 1974). The following assays were performed on the microsomes: protein content, cytochrome P-450 content, maximum wavelength of the carbon monoxide difference spectrum and aminopyrine demethylase and benzo[α]pyrene hydroxylase activities. These techniques have been previously described or referenced (Moore et al., 1978).

Statistical Analysis

Data were analyzed for significant treatment effects by a one-way analysis of variance (ANOVA). Significant differences between means were detected by Student-Newman-Keul's (SNK) test.

RESULTS

Serum Enzymes

Treatment effects on serum enzyme values are summarized in Table 3. Serum levels of SDH for 10- and 30-day periods were consistently increased by all treatments except the paired feeding experiment. These data are presented in Figures 2 and 3, respectively.

Serum values for ALP decreased with all 10-day treatments, including the paired feeding experiment. There were no significant treatment effects during the 30-day experiments on serum ALP levels. Data for the 10-day experiments are summarized in Table 4.

Only 3,3',4,4',5,5'-HBB, during both the 10- and 30-day experiments, significantly altered (increased) serum levels of AST. None of the other treatments significantly changed AST values. These data are presented in Table 5. There were no significant treatment effects on serum levels of ALT or GGT.

Serum Cholesterol

Treatment effects on serum total cholesterol, free cholesterol, and percent free to total cholesterol are presented in Table 6.

Serum total cholesterol values were significantly affected by all treatments except the 10-day FM and paired feeding experiments. During the 30-day FM and both feeding periods for 2,2',4,4',5,5'-HBB, serum cholesterol values increased, while the 10-day feeding of 3,3',4,4',5,5'-HBB significantly decreased cholesterol levels, the 30-day feeding

Table 3. Summary of treatment effects on serum enzyme activity

Treatment and Period	Days	Enzyme				
		SDH	ALP	AST	ALT	GGT
FM	10	↑	↓	-	-	-
	30	↑	-	-	-	-
2,2',4,4',5,5'-HBB	10	↑	↓	-	-	-
	30	↑	-	-	-	-
3,3',4,4',5,5'-HBB	10	↑	↓	↑	-	-
	30	↑	-	↑	-	-
Pair-fed controls	10	-	↓	-	-	-

↑Values significantly increased from control ($p<0.05$)

↓Values significantly decreased from control ($p<0.05$)

-No significant treatment effect

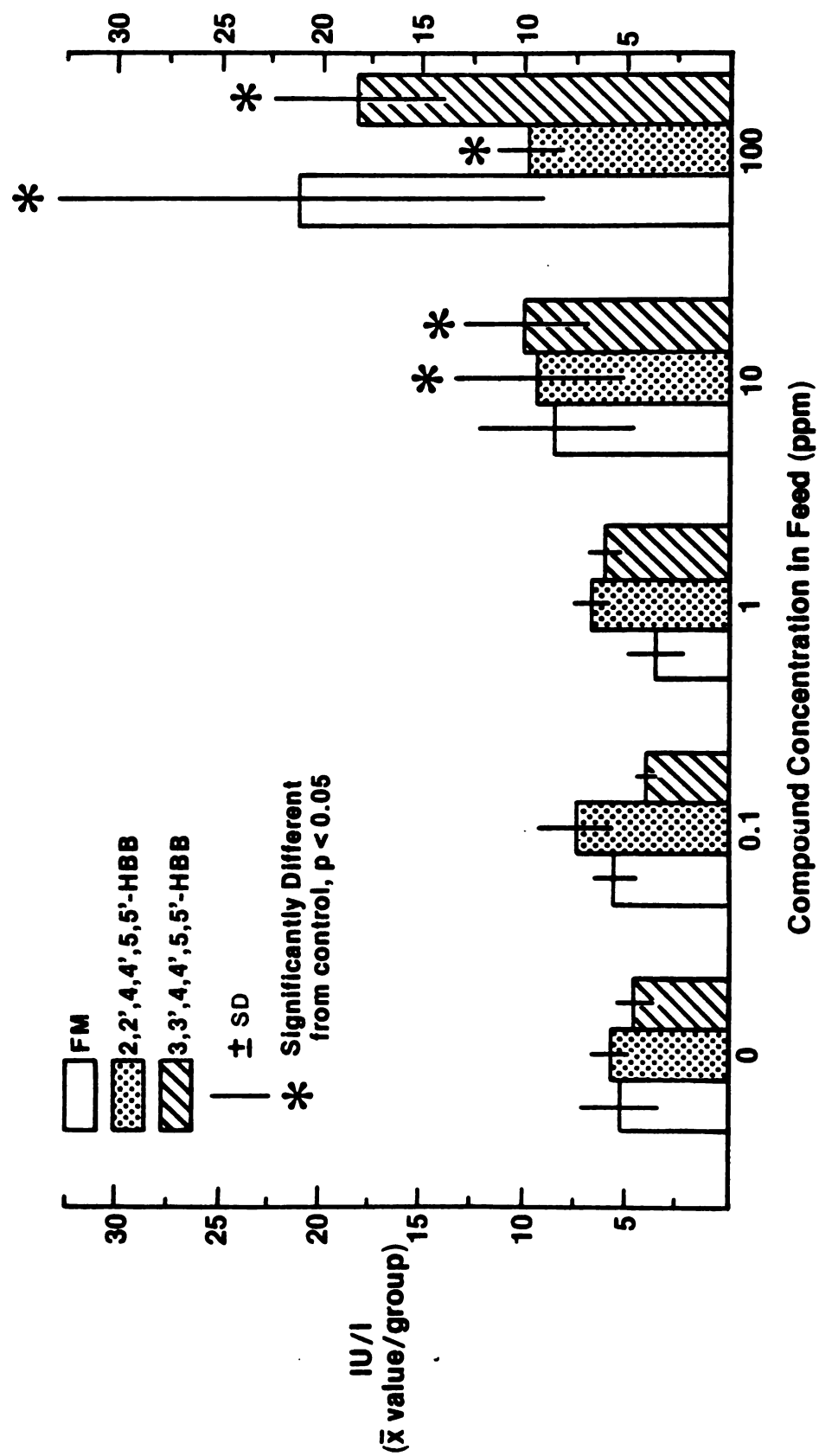


FIGURE 2. SDH ACTIVITY IN 10-DAY EXPERIMENTS

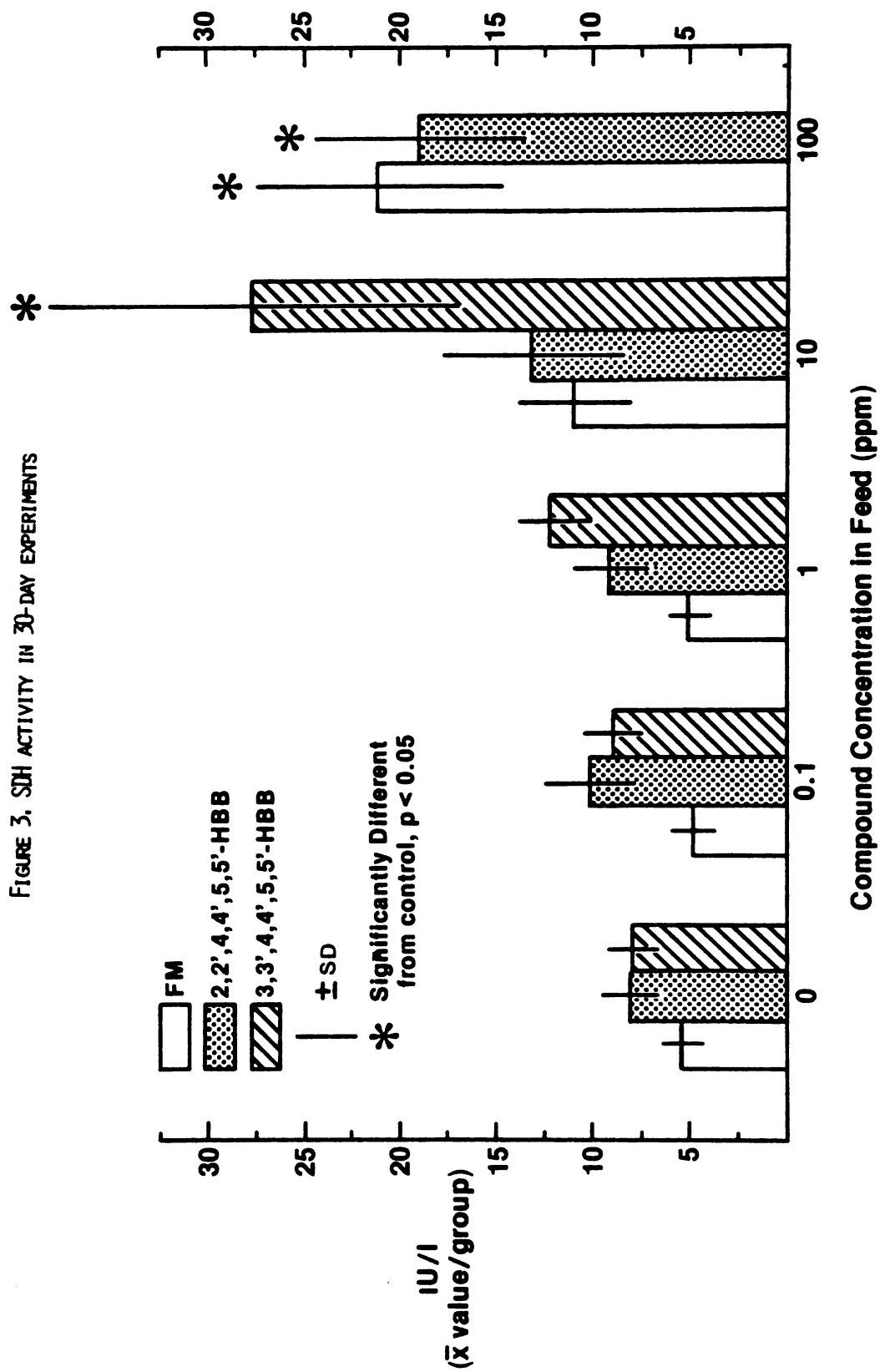


FIGURE 3. SDH ACTIVITY IN 30-DAY EXPERIMENTS

Table 4. Serum alkaline phosphatase activity in 10-day experiments

Dairy Concentration (ppm)	FM (IU/l)	Compound	
		2,2',4,4',5,5'-HBB (IU/l)	3,3,',4,4',5,5'-HBB (IU/l)
0	155 ± 29	112 ± 13	93 ± 20
0.1	137 ± 29	97 ± 16	88 ± 13
1	127 ± 29	100 ± 9	76 ± 11
10	116 ± 25	78 ± 14[b]	82 ± 9
100	92 ± 19[b]	80 ± 8[b]	60 ± 13[b]

[a]Data represent mean ± SD

[b]Values significantly different from control ($p<0.05$)

Table 5. Serum aspartate aminotransferase activity in 10- and 30-day 3,3',4,4',5,5'-HBB experiments[a]

Dietary Concentration (ppm)	Period (days)	
	10 (IU/l)	30 (IU/l)
0	51 ± 4	64 ± 5
0.1	66 ± 13	68 ± 7
1	58 ± 9	69 ± 10
10	65 ± 8	98 ± 26[b]
100	83 ± 5[b]	NE

[a]Data represent mean ± SD

[b]Value significantly different from control ($p<0.05$)

NE = no experiment

Table 6. Summary of treatment effects on serum total, free and percent free/total cholesterol

Treatment and Period	Days	Determinations		
		Total Cho.	Free Cho.	% Free/Total
FM	10	-	-	-
	30	↑	↑	-
2,2',4,4',5,5'-HBB	10	↑	↑	↑
	30	↑	↑	↑
3,3',4,4',5,5'-HBB	10	↓	↓	↓
	30	↓	-	↓
Pair-fed controls	10	-	↑	↑

-No significant treatment effect

↑Values significantly increased from control ($p<0.05$)

↓Values significantly decreased from control ($p<0.05$)

increased serum total cholesterol at moderate dietary concentrations and drastically decreased it at higher dietary concentrations. These data are presented for 10- and 30-day experiments in Figures 4 and 5, respectively.

Free cholesterol levels in serum were increased by 30-day FM, 10- and 30-day 2,2',4,4',5,5'-HBB and paired feeding experiments. Serum levels of free cholesterol for animals in the 10-day 3,3',4,4',5,5'-HBB experiment were significantly decreased, reflecting a similar decrease in total cholesterol. These changes in free cholesterol are presented in Table 7. Percent free to total cholesterol ratios generally reflected significant changes in free cholesterol content of serum samples.

Lipoprotein Cholesterol

Table 8 contains a summary of treatment effects on HDL, LDL and VLDL cholesterol concentrations. Ten- and thirty-day data for HDL cholesterol concentrations are presented in Figures 6 and 7, respectively. Both FM and 2,2',4,4',5,5'-HBB significantly elevated HDL cholesterol during both feeding periods. For both feeding periods 3,3',4,4',5,5'-HBB tended to increase HDL cholesterol at a moderate dietary level (although this was only significant in the 30-day experiment) and tended to decrease HDL cholesterol at a higher level (although only the values from the 10-day experiment were significantly different from controls).

LDL and VLDL cholesterol changes were few and unremarkable. They will not be presented in detail. Similarly, pair-fed control rats did not have any significant changes in cholesterol content of various lipoprotein fractions.

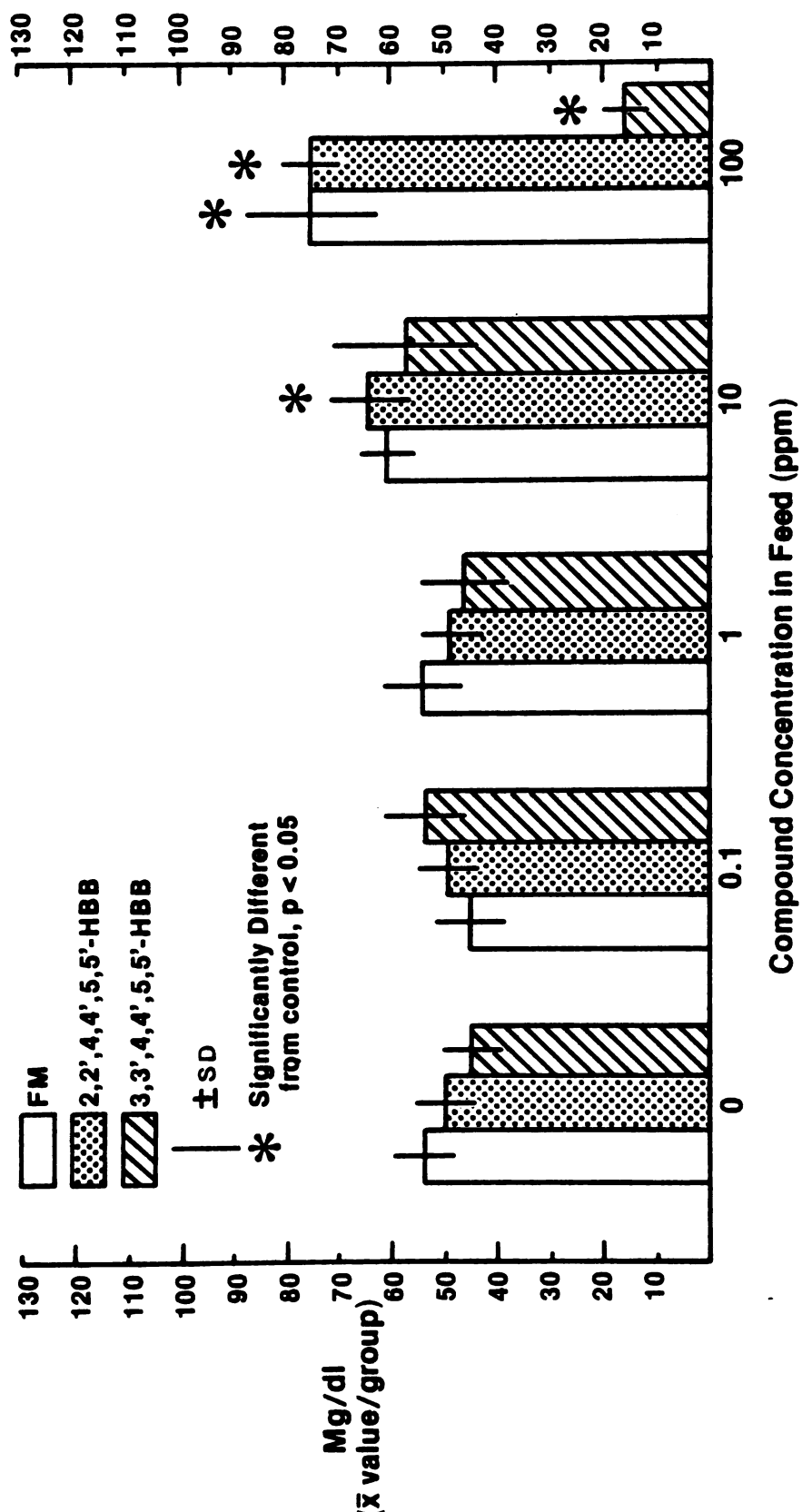


FIGURE 4. SERUM CHOLESTEROL LEVELS IN 10-DAY EXPERIMENTS

FIGURE 5. SERUM CHOLESTEROL LEVELS IN 30-DAY EXPERIMENTS

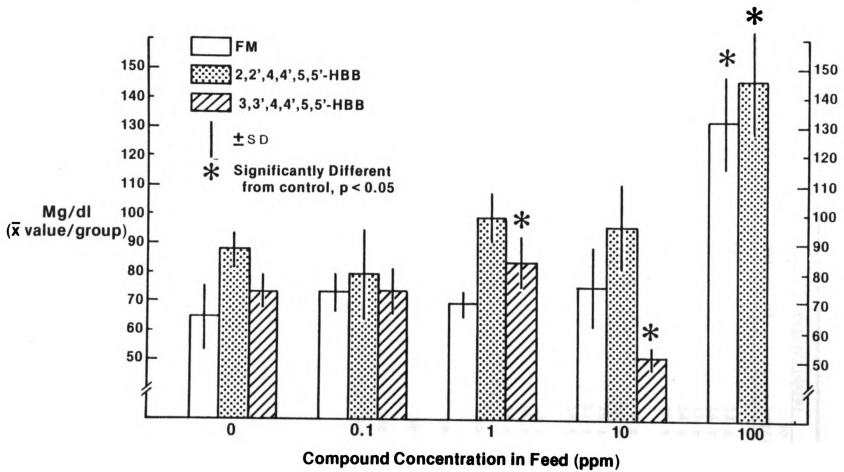


Table 7. Serum free cholesterol levels in 10- and 30-day experiments[a]

Compound	Dietary Concentration (ppm)	Period (days) 10 (mg/100 ml)	30 (mg/100 ml)
FM	0	10 ± 2	12 ± 4
	0.1	9 ± 1	14 ± 2
	1	10 ± 1	12 ± 1
	10	10 ± 2	14 ± 5[b]
	100	12 ± 5	30 ± 6[b]
2,2',4,4',5,5'-HBB	0	8 ± 2	9 ± 1
	0.1	8 ± 2	10 ± 1
	1	11 ± 2	10 ± 1
	10	10 ± 2[b]	13 ± 3[b]
	100	14 ± 2[b]	18 ± 1[b]
3,3',4,4',5,5'-HBB	0	11 ± 1	13 ± 1
	0.1	12 ± 3	14 ± 2
	1	12 ± 2	14 ± 1
	10	10 ± 2	
	100	1 ± 1[b]	ND
Pair-fed controls	*ad libitum*	12 ± 2	NE
	23 g/day	12 ± 2	NE
	13 g/day	15 ± 1[b]	NE

[a]Data represent means ± SD

[b]Value significantly different from control ($p<0.05$)

ND = not determined

NE = no experiment

Table 8. Summary of treatment effects on HDL, LDL and VLDL cholesterol levels

Compound	Period (days)	Cholesterol Determinations		
		HDL	LDL	VLDL
FM	10	↑	-	-
	30	↑	-	-
2,2',4,4',5,5'-HBB	10	↑	-	-
	30	↑	↑	↓
3,3',4,4',5,5'-HBB	10	↓	-	-
	30	↑	↓	-
Pair-fed controls	10	-	-	-

↑Values significantly increased from controls ($p<0.05$)

-No significant treatment effect

↓Values significantly decreased from control ($p<0.05$)

FIGURE 6. HDL CHOLESTEROL LEVELS IN 10-DAY EXPERIMENTS

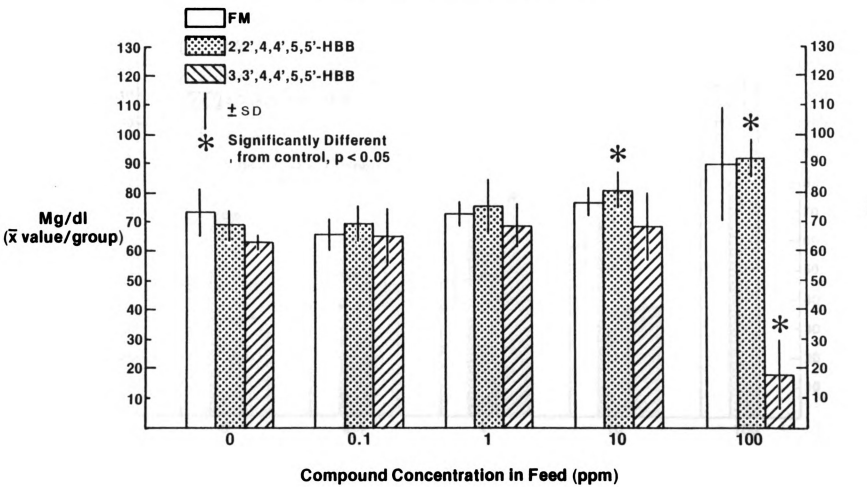
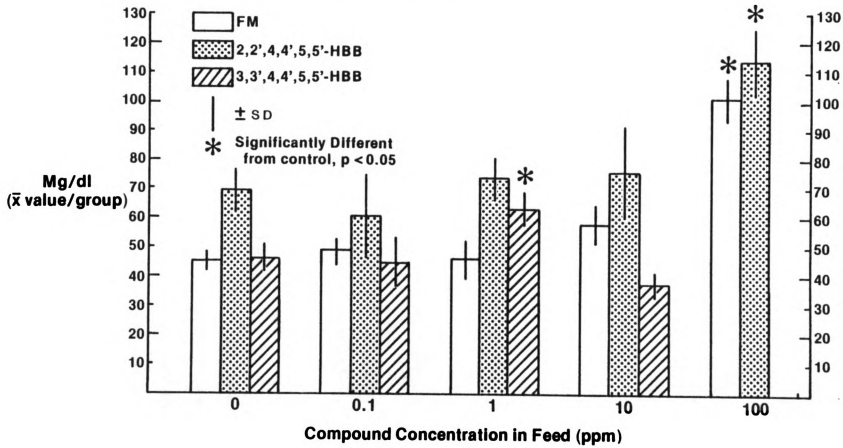


FIGURE 7. HDL CHOLESTEROL LEVELS IN 30-DAY EXPERIMENTS



Serum Triglyceride

A summary of treatment effects on serum triglyceride concentrations is presented in Table 9. A more detailed presentation of results from the triglyceride assays is given in Table 10. The predominant effect of feeding these 3 compounds to rats was to lower serum triglyceride levels. Even with the 10-day 2,2',4,4',5,5'-HBB group there was a trend, although not at significant levels, for the serum triglyceride values to be decreased with treatment. The reduced feed intake enforced during the paired feeding experiment also significantly depressed serum triglyceride values.

Lipoprotein Triglyceride

A summary of the HDL, LDL and VLDL triglyceride concentrations during the various experiments is presented in Table 11.

The treatment effects on triglyceride content of a specific lipoprotein class occurred primarily in the VLDL fraction. These data are presented in more detail in Table 12. All treatments decreased triglyceride content of the VLDLs and these changes were significant in 4 treatment groups, including the paired feeding experiment.

Lipoprotein Electrophoresis

Electrophoretic separation of serum lipoproteins was performed on all serum samples and on the lipoprotein fractions from the 30-day 2,2',4,4',5,5'-HBB and 3,3',4,4',5,5'-HBB studies. Electrophoretic patterns of various fractions in all cases were pure and showed no evidence of incomplete isolation.

Electrophoretic patterns were evaluated by measuring the relative percentage of each lipoprotein band on densitometer tracings. Since rat VLDLs tend to migrate with HDLs, it was usually impossible to detect

Table 9. Summary of treatment effects on serum triglyceride levels

Compound	Time Period (days)	Serum Triglyceride
FM	10	↓
	30	↓
2,2',4,4',5,5'-HBB	10	-
	30	↓
3,3',4,4',5,5'-HBB	10	↓
	30	↓
Pair-fed controls	10	↓

↓Values significantly decreased from control ($p<0.05$)

-No significant treatment effect

Table 10. Serum triglyceride levels in 10- and 30-day experiments[a]

Compound	Dietary Concentration (ppm)	Period (days) 10 (mg/100 ml)	30 (mg/100 ml)
FM	0	56 ± 7	69 ± 22
	0.1	44 ± 7	57 ± 11
	1	50 ± 4	64 ± 8
	10	54 ± 9	50 ± 10[b]
	100	39 ± 10[b]	32 ± 6[b]
2,2',4,4',5,5'-HBB	0	60 ± 10	74 ± 17
	0.1	76 ± 12	75 ± 20
	1	68 ± 8	72 ± 8
	10	71 ± 12	63 ± 16
	100	51 ± 7	40 ± 8[b]
3,3',4,4',5,5'-HBB	0	50 ± 10	61 ± 10
	0.1	55 ± 9	60 ± 10
	1	56 ± 11	57 ± 7
	10	52 ± 8	35 ± 9[b]
	100	16 ± 4[b]	NE
Pair-fed controls	*ad libitum*	76 ± 11	NE
	23 g/day	57 ± 14[b]	NE
	13 g/day	37 ± 6[b]	NE

[a]Data represent means ± SD

[b]Value significantly different from control ($p<0.05$)

NE = no experiment

Table 11. Summary of treatment effects on high density, low density and very low density lipoprotein triglyceride levels

Compound	Period (days)	Determinations		
		HDL	LDL	VLDL
FM	10	-	-	↓
	30	-	-	-
2,2',4,4',5,5'-HBB	10	-	-	-
	30	-	-	↓
3,3',4,4',5,5'-HBB	10	-	-	↓
	30	-	↓	-
Pair-fed controls	10	-	-	↓

-No significant treatment effect

↓Values significantly decrease from control ($p<0.05$)

Table 12. Very low density lipoprotein triglyceride levels in 10- and 30-day experiments[a]

Compound	Dietary Concentration (ppm)	Period (days) 10 (mg/100 ml)	30 (mg/100 ml)
FM	0	28 ± 4	36 ± 14
	0.1	20 ± 4	35 ± 11
	1	26 ± 2	30 ± 8
	10	22 ± 9	26 ± 9
	100	14 ± 4[b]	16 ± 6
2,2',4,4',5,5'-HBB	0	34 ± 7	32 ± 14
	0.1	42 ± 6	27 ± 12
	1	44 ± 9	23 ± 16
	10	43 ± 10	16 ± 8
	100	29 ± 8	3 ± 2[b]
3,3',4,4',5,5'-HBB	0	22 ± 5	11 ± 5
	0.1	21 ± 6	9 ± 4
	1	28 ± 8	9 ± 3
	10	26 ± 4	6 ± 2
	100	5 ± 2[b]	NE
Pair-fed controls	*ad libitum*	23 ± 10	NE
	23 g/day	10 ± 4[b]	NE
	13 g/day	2 ± 2[b]	NE

[a]Data represent means ± SD

[b]Values significantly different from control ($p<0.05$)

NE = no experiment

this band and, therefore, to assess treatment effects on VLDLs with this technique. Effects of various treatments on relative percentages of HDL and LDL fractions are presented in Table 13, with the sum of HDL and LDL bands expressed as 100%. Both 10- and 30-day treatment periods for FM and 3,3',4,4',5,5'-HBB increased the relative percentage of HDL in relationship to LDL. Neither animals fed 2,2',4,4',5,5'-HBB nor pair-fed controls had an obvious change in the ratio of HDL and LDL.

Microsomal Enzymes

Table 14 contains a summary of changes in data for cytochrome P-450 content and wavelength maximum of carbon monoxide (CO) difference spectrum of hepatic microsomes from each treatment group. All treatments except pair-fed controls produced 2- to 6-fold increases in cytochrome P-450 content of hepatic microsomes. Shifts in wavelength maximums of microsomes were towards 448 for both FM and 3,3',4,4',5,5'-HBB and essentially unchanged for 2,2',4,4',5,5'-HBB and paired feedings.

In Figure 8 the effects of treatments on aminopyrine demethylase (AD) and benzo[α]pyrene hydroxylase activities for 30-day experiments are compared. The data were converted to means and then represented as increases over controls. FM increased both enzyme activities, while 2,2',4,4',5,5'-HBB primarily increased aminopyrine demethylase activity and 3,3',4,4',5,5'-HBB, only benzo[α]pyrene hydroxylase activity. Data for 10-day experiments were similar to these findings.

Serum and Lipoprotein GLC Analysis for PBB

Pooled serum, lipoprotein and albumin fractions from the 30-day experiments at the 100 ppm dietary level of 2,2',4,4',5,5'-HBB and 10 ppm level of 3,3',4,4',5,5'-HBB were analyzed for PBB by gas-liquid

Table 13. Treatment effects on relative concentrations of high density/low density lipoprotein determined by lipoprotein electrophoresis[a]

Compound	Dietary Concentration (ppm)	Periods (days) 10(%)	30(%)
FM	0	81/19	82/18
	0.1	77/23	-
	1	82/18	89/11
	10	86/14	93/7
	100	94/6	97/3
2,2',4,4',5,5'-HBB	0	82/18	87/13
	0.1	87/13	84/16
	1	84/16	88/12
	10	85/15	90/10
	100	87/13	87/13
3,3',4,4',5,5'-HBB	0	75/25	80/20
	0.1	76/24	87/13
	1	70/30	92/8
	10	72/28	98/2
	100	92/8	NE
Pair-fed controls	*ad libitum*	80/20	NE
	23 g/day	82/18	NE
	13 g/day	79/21	NE

[a]Data represent mean for group

NE = no experiment

Table 14. Cytochrome P-450 content and shift in wavelength maximum of hepatic microsomes for carbon monoxide difference spectrum

Compound	Period (days)	Determinations: Cytochrome P-450, Fold Increase[a]	Determinations: Shift in λ Maximum[b] (nm)
FM	10	ND	ND
	30	2.2	450 to 449
2,2',4,4',5,5'-HBB	10	6.0	449 to 450
	30	2.8	450 to 450
3,3',4,4',5,5'-HBB	10	3.0	450 to 448
	30	3.6	450 to 449
Pair-fed controls	10	0.9	449 to 450[c]

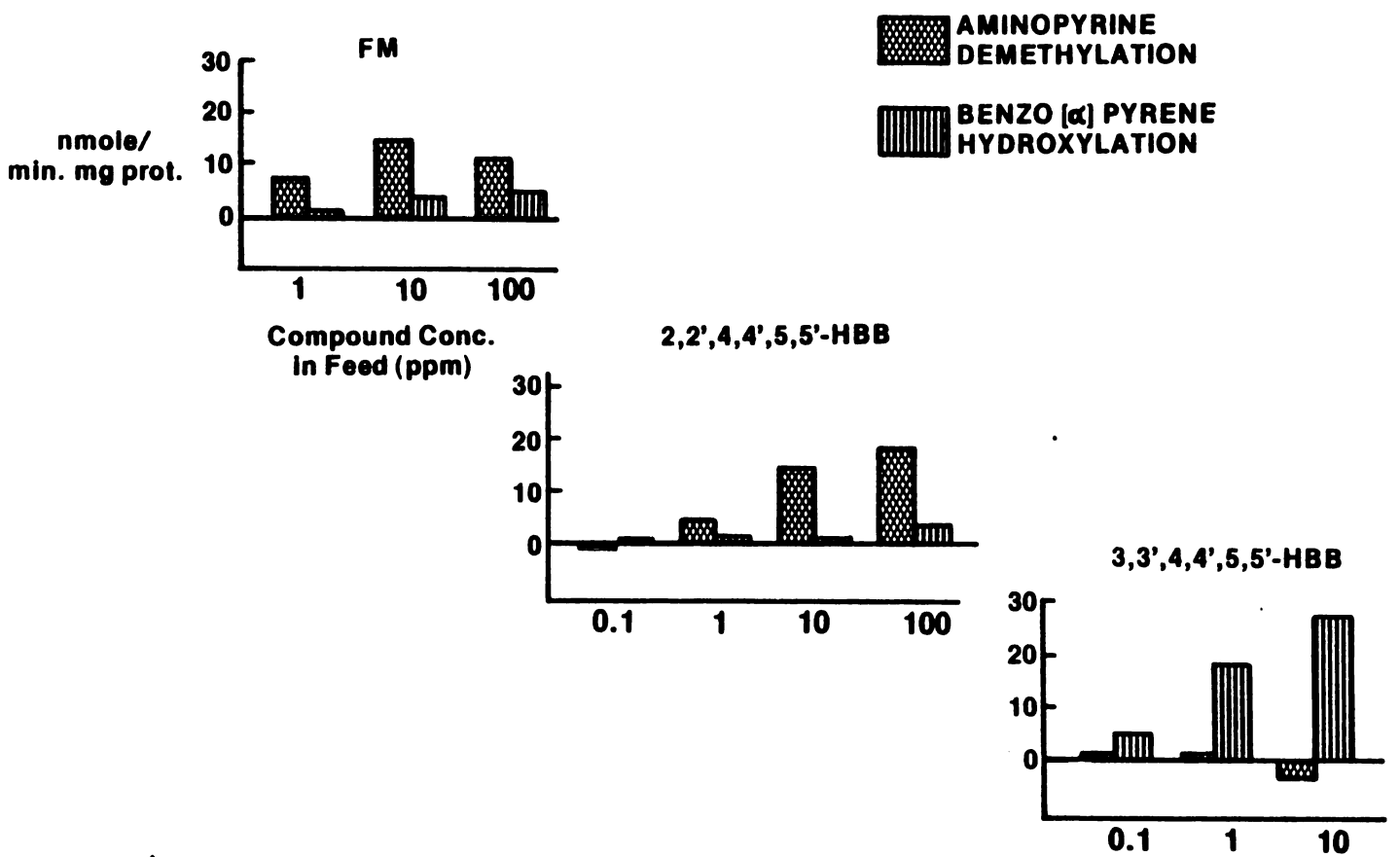
[a]Data calculated as nmol P-450/mg protein represent mean of results from highest treatment group ÷ mean of control

[b]Data represent mean for control versus mean for highest treatment group

[c]Data represent mean for group fed *ad libitum* versus group fed 13 g/day

ND = not determined

FIGURE 8. HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYME ACTIVITY IN 30-DAY EXPERIMENTS[A]



[A]DATA EXPRESSED AS INCREASES OVER CONTROLS FOR POOLED SAMPLES

chromatography. Results are presented in Table 15. Approximately 59% of 2,2',4,4',5,5'-HBB was associated with the HDL fraction, 29% with the LDL fraction and 5 to 7% with both VLDL and albumin fractions.

Body Weights

Effects of the treatments on body weight were analyzed by comparing body weights of animals at the beginning and ending of an experimental period. The results are summarized in Table 16. Treatments with FM and 2,2',4,4',5,5'-HBB did not affect weight gain by the rats. During the 10-day experiment at the 100 ppm dietary level of 3,3',4,4',5,5'-HBB, all rats lost weight from their initial level. Weight gains at other dietary concentrations for this congener during the 10-day study were not significantly different from controls. Rats fed 3,3',4,4',5,5'-HBB for 30 days at 10 ppm had a significant decrease in weight gain when compared with controls. The rats fed 13 g/day had a similar significant loss in body weight during the experiment.

Organ Weights

Liver weights were significantly increased over those of controls by higher dietary concentrations of each of the three compounds. The rats fed 13 g/day had significantly lower liver weights when compared to their controls. Data for liver weights are presented in Table 17.

Data for treatment effects on thymic, thyroid and splenic weights are summarized in Table 18. Significant decreases in thymic weights, when compared to controls, occurred in rats during 30-day treatment periods with FM and 10- and 30-day treatment periods with 3,3',4,4',5,5'-HBB. Significant changes were confined to the highest dietary level of each compound in that treatment group.

Table 15. Serum, lipoprotein and albumin associated concentrations of 2,2',4,4',5,5'-HBB and 3,3',4,4',5,5'-HBB from 30-day experiments[a]

Sample	Congener Concentration	
	2,2',4,4',5,5'-HBB (ppm)	3,3',4,4',5,5'-HBB (ppm)
Serum	3.490	0.004
HDL + LDL	3.096	ND
HDL	2.071	b
VLDL	0.221	b
Albumin	0.241	b

[a]Data represent pooled samples

[b]Congener not detected in extraction solution

ND = not determined

Table 16. Summary of treatment effects on body weight gains

Compound	Feeding Period (days) 10	30
FM	-	ND
2,2',4,4',5,5'-HBB	-	-
3,3',4,4',5,5'-HBB	↓[a]	↓
Pair-fed controls	↓[b]	NE

[a] High level group (100 ppm) lost body weight

[b] The 13 g/day group lost body weight

- No significant treatment effect

↓ Values significantly decreased from control ($p<0.05$)

ND = not determined

NE = no experiment

Table 17. Treatment effects on liver weights in 10- and 30-day experiments[a]

Compound	Dietary Concentration (ppm)	Feeding Period (days) 10 (g)	30 (g)
FM	0	10.9 ± 0.4	13.9 ± 1.4
	0.1	10.0 ± 0.9	14.9 ± 1.3
	1	10.8 ± 0.8	15.4 ± 1.7[b]
	10	12.5 ± 1.3[b]	17.8 ± 2.2[b]
	100	16.7 ± 0.8[b]	24.5 ± 2.2[b]
2,2',4,4',5,5'-HBB	0	11.2 ± 0.3	14.3 ± 1.4
	0.1	10.9 ± 0.6	14.7 ± 1.1
	1	11.6 ± 0.6	13.9 ± 0.8
	10	12.7 ± 1.4[b]	17.6 ± 2.0[b]
	100	14.3 ± 1.1[b]	22.3 ± 1.1[b]
3,3',4,4',5,5'-HBB	0	10.3 ± 0.9	12.2 ± 1.3
	0.1	10.2 ± 0.7	12.4 ± 0.5
	1	11.3 ± 1.0[b]	14.5 ± 1.4[b]
	10	12.0 ± 0.7[b]	17.0 ± 1.4[b]
	100	13.5 ± 1.4[b]	NE
Pair-fed controls		11.8 ± 2.0	NE
	23 g/day	10.1 ± 0.8	NE
	13 g/day	8.1 ± 0.3[b]	NE

[a]Data represent mean ± SD

[b]Values significantly different from control ($p<0.05$)

NE = no experiment

Table 18. Summary of treatment effects on thymic, thyroid and splenic weights

Compound	Periods (days)	Organ		
		Thymus	Thyroid	Spleen
FM	10	-	↑	-
	30	↓	↑	-
2,2',4,4',5,5'-HBB	10	-	-	-
	30	-	-	-
3,3',4,4',5,5'-HBB	10	↓	-	↓
	30	↓	↑	-
Pair-fed controls	10	-	-	↓

- No significant treatment effect

↑ Values significantly increased from controls ($p<0.05$)

↓ Values significantly decreased from controls ($p<0.05$)

Significant changes in thyroid gland weights also developed in only FM and 3,3',4,4',5,5'-HBB treated rats. With 10- and 30-day FM experiments, significant increases in thyroid gland weights occurred in the 100 ppm groups. The significant increase noted for the 30-day experiment with 3,3',4,4',5,5'-HBB was detected in both 1 and 10 ppm groups. Decreased splenic weights were associated with rats fed 100 ppm of 3,3',4,4',5,5'-HBB for 10 days and those fed 13 g/day for 10 days. The decreases were statistically significant when compared with splenic weights of control rats.

DISCUSSION

The underlying theme of this project was to investigate the effects of certain polybrominated biphenyl compounds on various biochemical processes through the clinical analysis of serum samples. Specific serum enzyme activities and lipoprotein concentrations were determined. Also, effects of the compounds on hepatic drug-metabolizing enzymes and body and organ weights were evaluated to help assess their toxic properties.

Serum Enzymes

Serum enzymes were selected to reflect possible hepatic alterations that might develop in response to treatments with PBB. These enzymes can be divided into 2 groups, those which through elevated serum levels reflect hepatocellular leakage (ALT, AST, SDH) and those that indicate cholestasis (ALP, GGT). One conclusion from this research project is that SDH was a consistent, sensitive indicator of hepatocellular damage and leakage. It was significantly elevated above control values by all 3 compounds at both time periods. Pair-fed controls (13 g/day) did not have significant alterations in serum SDH levels. This indicates that starvation and enforced feed restriction did not contribute to elevated serum SDH values.

Both SDH and AST occur in the cytosol of hepatic cells, but a significant amount of the cellular component of AST is also associated with mitochondria. AST was significantly elevated by 10- and 30-day treatments with 3,3',4,4',5,5'-HBB. Render (1980) reported 10-day feedings of

3,3',4,4',5,5'-HBB produced histopathologic and ultrastructural changes that were more severe than those of either FM or 2,2',4,4',5,5'-HBB. Some of these alterations included enlarged hepatocytes, lipid vacuolization, proliferation of the SER, disorganization of the RER and an increased number of free ribosomes and lipid droplets. Increased hepatocellular permeability and the subsequent release of AST into the serum could have accompanied these and perhaps other subtle structural alterations. Since histopathologic changes with FM and 2,2',4,4',5,5'-HBB were less dramatic, there appears to be no direct correlation between serum SDH elevations and hepatic structural changes.

Both ALT and SDH are primarily located in the cytosol of hepatic cells and yet there was no significant change in serum ALT levels related to any of the treatments. Failure of this enzyme to be elevated by these compounds indicates that in the rat ALT may be an insensitive indicator of increased hepatocellular permeability. Of those examined, SDH would appear to be the enzyme of choice to detect hepatocellular leakage in the rat. Elevated serum levels of AST were detected only in animals fed high dietary concentrations of 3,3',4,4',5,5'-HBB. This enzyme may be elevated in the serum of rats with hepatic damage but indications are that moderate to extensive hepatocellular change is needed.

As was discussed in the review section, elevated serum levels of ALP and GGT are frequently associated with cholestasis. Neither enzyme increased in any of these experiments. In fact, ALP was significantly decreased in each 10-day experiment, including the pair-fed controls. Although GGT was detected in many serum samples, it was not uncommon for the serum level to be below detectable limits. This appears to be consistent with the findings of Caisey and King (1980), who did not

detect GGT in the serum of normal rats. Huseby (1979) reported the hepatic levels of GGT in rats to be much lower than those of human beings and guinea pigs.

Gamma glutamyltranspeptidase may be an inappropriate choice as an indicator of cholestasis in the rat. It is also possible, however, that bile duct obstruction was simply not a factor in these experiments and that elevated serum values would develop under proper circumstances. To establish the usefulness of this enzyme in the rat to detect cholestasis, experiments such as bile duct ligation would be needed. As an additional complicating factor, certain compounds, of which PB is an example, increase serum GGT levels in rats and guinea pigs through hepatic microsomal enzyme induction (Huseby, 1979). It may be difficult to determine whether an increase, therefore, is related to physical bile duct obstruction or solely to enzyme induction.

The decreases detected in serum levels of ALP are very interesting. The reason that significantly lower ALP values are associated with all 10-day experiments, including the pair-fed controls, and not with the 30-day studies is not apparent. Identification of various ALP isoenzymes in rats and their relative contribution to total serum concentrations would help resolve this point.

Both Fishman et al. (1962) and Toda et al. (1980) identified the intestinal isoenzyme as the major form of ALP in the serum of rats. Both reported a decline in serum ALP concentrations after bile duct ligation but in the study by Toda et al. (1980) serum levels initially increased during the 24-hour period immediately after the procedure. This increase was associated with the appearance of a high molecular weight isoenzyme in the serum and on hepatocyte membranes along canaliculi. Chemically induced cholestasis with α-naphthalisothiocyanate

(ANIT) also initially increased serum ALP levels, but the increase was associated with a low molecular weight hepatic isoenzyme that is located along the sinusoidal surfaces of hepatocytes.

During this study restricted feed intake alone significantly decreased serum ALP levels. According to Fishman et al. (1962), there is a well established direct correlation between fat content in the diet and serum levels of ALP. Lam and Mistilis (1973) made several important observations concerning the function of intestinal ALP in the rat. They reported that the enzyme does not contribute to intraluminal digestion of triglycerides, does not aid in the transportation of fatty acids across the mucosal brush border and is not physically bound to lipoprotein particles released into lymph. They did discover that increased levels of intestinal ALP in serum after a fatty meal were associated with increased ALP content in the microsomal fraction of intestinal mucosal cells and that there was a quantitative relationship between intestinal ALP and triglycerides in lymph. They suggested that intestinal ALP is either directly involved in the synthesis of triglycerides or lipoprotein particles or may be involved in the transportation of lipoprotein particles across plasma membranes into lymph by reverse pinocytosis. Regardless of the actual function of the intestinal isoenzyme, it is not difficult to understand why rats fed a restricted diet would have reduced serum levels.

If the significant decrease in serum ALP values associated with the 10-day 3,3',4,4',5,5'-HBB experiment were also caused by decreased food consumption, how can the similar reduced ALP levels in the animals fed FM and 2,2',4,4',5,5'-HBB be explained? Render (1980), in reviewing the feed intake of these same rats, noted that while FM generally failed to alter consumption levels from those of controls, 2,2',4,4',5,5'-HBB

actually significantly increased intake at the 1 and 10 ppm dietary concentrations. Obviously, variations in feed intake did not produce the lower levels of serum ALP in either of these experiments. Fishman et al. (1962) suggested that the decline in serum levels of intestinal ALP occurs after bile duct ligation because, without bile, fats are not properly emulsified or digested. Pursuing this scenario, the decrease in fatty acids and other absorbable products of fat digestion in the intestinal lumen would decrease the absorption of these compounds and decrease the amount of triglyceride and associated ALP released into the lymph. For this mechanism to contribute to the decreased serum ALP levels in the 10-day experiments, there may be some degree of cholestasis produced by the compounds. The data do not preclude this possibility, since serum ALP levels in the rat, as earlier noted, will actually decrease 48 hours after bile duct ligation.

If all this were true, then why do the animals in the 30-day experiments all have ALP levels indistinguishable from control values? There are several possibilities. Assuming that a decrease in intestinal ALP is responsible for the initial decline and that this condition persists through the 30-day studies, normal levels may be produced by an increase in serum levels of an hepatic isoenzyme. This could be related to cholestasis or to enzyme induction by PHB compounds. It is also possible that there is some compensatory mechanism in the intestinal mucosal cells that with time increases production of this ALP isoenzyme.

Serum Cholesterol

Except for rats in the 10-day experiments with FM and the pair-fed controls (13 g/day), serum cholesterol values were significantly affected by all other treatments. For rats fed FM for 30 days and

2,2',4,4',5,5'-HBB for 10 and 30 days, there were significant increases in serum levels of cholesterol at the higher dietary concentrations. In rats fed 3,3',4,4',5,5'-HBB for 30 days there was a significant increase in total serum cholesterol in the 1 ppm group and, as in the 10-day experiment, a significant decrease in serum cholesterol at the highest dietary level. These changes in serum cholesterol were almost totally produced by alterations in HDL cholesterol. Variations in VLDL and LDL cholesterol levels, although they did occasionally occur, were of minor importance.

Cholesterol in animal tissue is of exogenous and endogenous origin. Under normal dietary conditions, approximately 80% of the cholesterol added to the total body pool each day is synthesized by various tissues. Approximately 20% is derived from the diet (Mayes, 1979). Numerous tissues, including the adrenal cortex, skin, intestines, testes and aorta, can make cholesterol from acetyl-CoA precursors. In the rat the liver is the predominant organ in cholesterol biosynthesis (Mayes, 1979). Within cells, synthesis of cholesterol begins within the cytoplasm and is concluded in the endoplasmic reticulum. The important regulatory step in the complicated sequence of events occurs at the conversion of β-hydroxy-β-methylglutaryl-CoA to mevalonate by the enzyme β-hydroxy-β-methylglutaryl-CoA reductase (HMG-CoA reductase).

Important physiologic variables have been identified that regulate hepatic cholesterol biosynthesis (Van Golde and Van den Bergh, 1977). These include the amount of dietary cholesterol, caloric intake of the animal and integrity of the enterohepatic circulation of bile. Increased cholesterol and bile acid pools in the body depress cholesterol synthesis, apparently by decreasing the synthesis of HMG-CoA reductase in the liver. Additionally, conversion of cholesterol to bile acids is

decreased by an increased bile acid pool. This action occurs at the committed step in bile acid formation, the conversion of cholesterol to 7α-hydroxycholesterol (Mayes, 1979). The regulatory enzyme catalyzing this step, 7α-hydroxylase, is a microsomal enzyme and can be induced in rats by PB treatment.

According to Mayes (1979), cyclic-AMP (c-AMP) can inhibit cholesterol synthesis, perhaps through a phosphorylation-dephosphorylation mechanism. Decreased caloric intake through fasting or starvation may inhibit cholesterol biosynthesis in the liver by altering intracellular levels of c-AMP. Insulin, for example, decreases intracellular levels of c-AMP in hepatocytes, while glucagon increases it. Through this mechanism, decreased insulin levels during fasting could decrease cholesterol biosynthesis.

As in the conversion of cholesterol to bile acids, the biosynthesis of cholesterol is also thought to require cytochrome P-450 enzymes. Gibbons and Mitropoulos (1973) demonstrated that cytochrome P-450 is involved in the removal of the 14α-carbon from lanosterol during cholesterol biosynthesis. They also showed that pretreatment of rats with PB substantially increased hepatic microsomal cholesterol formation from the lanosterol substrate.

The significant increases in total serum cholesterol that developed with FM, 2,2',4,4',5,5'-HBB and with intermediate levels of 3,3',4,4',5,5'-HBB can be attributed to either increased synthesis or decreased conversion to bile salts. Kato and Yoshida (1980) demonstrated that a PCB mixture fed to rats significantly increased both hepatic and serum cholesterol concentrations. These increases were associated with a greater than 5-fold elevation in hepatic microsomal levels of HMG-CoA reductase. They suggested that increased cholesterol levels were related

to microsomal enzyme induction by the PCB compounds. This is an attractive hypothesis and will remain so until researchers can demonstrate that this or other hypercholesterolemic compounds function by actually reducing cholesterol catabolism. It should be remembered that PB induces the 7α-hydroxylase enzyme, thereby increasing the conversion of cholesterol to bile salts.

The increases in total serum cholesterol may have been produced by microsomal enzyme induction and enhanced cholesterol biosynthesis. But why did the higher dietary concentrations of 3,3',4,4',5,5'-HBB decrease serum cholesterol levels? Since the restricted feeding experiment had no significant effect on serum cholesterol, it is unlikely that significant decreases were related to reduced caloric intake.

Yoshimura et al. (1979) noted that the compound 3,3',4,4',5-pentachlorobiphenyl, which was a very strong MC-type inducer, significantly increased hepatic cholesterol content but also lowered serum cholesterol levels. While these points were not discussed by the researchers, they did make several relevant observations. They administered purified PCB compounds to rats, including PB and MC. While all increased liver size, only the MC-types actually produced fatty livers, as indicated by increased total lipid, triglyceride and cholesterol content. Thus, decreased serum cholesterol may not be from decreased synthesis or increased biliary excretion but from interference with mobilization and release of lipoprotein particles. Such a mechanism could result from disruption of triglyceride, phospholipid or protein synthesis, a packaging defect in the golgi apparatus or perhaps from disruption of the endoplasmic reticulum from lipid peroxidation. It is not known if such defects could allow cholesterol biosynthesis to continue and cholesterol to accumulate within a cell.

Lipoprotein Cholesterol

Alterations in total serum cholesterol occurred almost exclusively in the HDL fraction. In this experiment 72% of the serum cholesterol in control animals was transported by HDL particles. It is not surprising that significant changes in total cholesterol would involve this fraction. Under normal circumstances, most cholesterol is released from the liver in VLDL and very small amounts in nascent HDL particles (Miller, 1979). Recently, elaborate experiments in the rat have identified the small intestine as the most important source of the major HDL apolipoproteins (A-I, A-IV) in this species (Wu and Windmueller, 1979). The small intestine in the rat may be an important source of HDL particles or perhaps these apolipoproteins are simply released with chylomicrons or VLDLs only later to dissociate and combine with HDL in circulation. If this is an important production site, the fact that bile acids and not cholesterol inhibit cholesterol synthesis in the small intestine (Mayes, 1979) assumes added importance. If there were an interruption of the normal enterohepatic circulation of bile acids, the small intestine could increase the production of HDL particles.

In addition to intestinal and hepatic production of HDL, an important component of the serum HDL pool results from peripheral metabolism of VLDL particles (Miller, 1979). With catabolism of triglycerides to fatty acids and glycerol by the enzyme lipoprotein lipase (LPL) fragments of the VLDL coat containing A-apolipoproteins are released into the circulation. These become nascent HDL particles. If a treatment were to enhance the activity of LPL (and some do), an increase in HDL cholesterol would occur at the expense of VLDLs.

Poli et al. (1980) briefly commented on hypercholesterolemias produced in rats by several treatments. They noted that in rats fed diets

high in cholesterol, serum levels of HDLs usually decreased. With TCDD exposure, however, HDL cholesterol levels increased. They believed that hypercholesterolemia from TCDD exposure results from enhanced hepatic protein synthesis. It is assumed that they are implying TCDD increases the hepatic production of the A-apolipoproteins. They did not rule out the possibility that the increase in HDL cholesterol results from decreased catabolism as can be caused by nicotinic acid in man.

The free cholesterol levels in the serum of rats during the various experiments were significantly increased by treatment with FM, 2,2',4,4',5,5'-HBB and the pair-fed controls but significantly decreased by 3,3',4,4',5,5'-HBB. Relative amounts of free to total cholesterol in the serum generally reflected significant alterations in the free cholesterol values. According to some sources, cholesterol synthesized by the liver and incorporated into HDL particles is unesterified (Mayes, 1979). This same author notes, however, that 80-90% of the cholesterol in the intestinal lymphatics is esterified. This process may occur in the mucosal cell before lipoproteins are released into lymphatics.

Using isolated, perfused livers from rats, Marsh and Sparks (1979) reported that cholesterol in HDLs just secreted from the liver is less than 50% esterified, while that of plasma is approximately 80% esterified. Kempen (1980) isolated rat hepatocytes in suspension and identified a VLDL particle as the major lipoprotein product. The molar ratio of free to esterified cholesterol in these particles was 2 to 1. Kempen did state that the presence of esterified cholesterol may have reflected contamination of his suspension with the enzyme lecithin:cholesterol acyltransferase (LCAT). This serum enzyme, which is itself produced by hepatocytes, catalyzes the transfer of the fatty acids from the second position of phosphatidylcholine to position 3 of cholesterol (Mayes,

1979). The preferred substrate for this reaction is not VLDL, however, but HDL.

The reasons for the significant changes in the absolute and relative amounts of free (unesterified) cholesterol in this current project are unknown. From the previous discussion, likely mechanisms would involve an alteration in the intracellular esterification of cholesterol or a change in the serum level or activity of LCAT. Familial and acquired LCAT deficiencies secondary to acute hepatitis and chronic cholestasis have been identified in human beings (Miller, 1979). The possibility that PHB could produce an LCAT deficiency has not been explored.

Lipoprotein Triglyceride

Except for rats in the 10-day 3,3',4,4',5,5'-HBB groups, all treatments, including pair-fed controls, had significantly reduced serum triglyceride values. These changes occurred primarily in the VLDL fraction. As for the pair-fed controls, decreased serum triglyceride values were probably related to reduced carbohydrate intake and the subsequent decrease in acetyl-CoA units available for fatty acid synthesis in the liver. While this explanation could also be used for the changes observed in rats fed 3,3',4,4',5,5'-HBB, it is not compatible with the normal or occasionally increased appetites of the rats fed FM and 2,2',4,4',5,5'-HBB.

Decreased serum triglyceride values could develop from either decreased synthesis or increased utilization. Triglyceride synthesis in the liver is associated primarily with the microsomal fraction. There are various sites in the synthesis and assimilation of triglycerides into lipoprotein particles that are susceptible to disruption by numerous compounds and physiologic processes. These agents or events

can produce fatty livers and decrease the ability of the liver to release VLDL. Starvation, which would be an important stimulus for the release of free fatty acids from adipose tissue, was only present in rats fed 100 ppm of 3,3',4,4',5,5'-HBB for 10 days. While there was an increase in the amount of fat in the livers of rats fed 3,3',4,4',5,5'-HBB, there was no indication that hepatic fatty infiltration was prominent in animals fed FM or 2,2',4,4',5,5'-HBB or in the pair-fed controls.

Some compounds are known to disrupt or block the synthesis of proteins that are essential to the formation of lipoproteins. However, these agents generally produce fatty livers. FM and 2,2',4,4',5,5'-HBB treatments appeared to enhance cholesterol biosynthesis and its incorporation into HDLs. It does not seem prudent to assume that the cellular apparatus that produced increased levels of one lipoprotein was also compromised and unable to synthesize and assemble the components of another.

Increased cholesterol synthesis can produce a deficiency in essential fatty acids (EFA). This can promote fatty liver formation by preventing the assimilation of lipoprotein particles and allowing triglycerides to accumulate in the liver (Mayes, 1979). Cholesterol apparently competes with phospholipid synthesis for the EFAs to produce cholesterol esters. This can decrease formation of phospholipids and, in addition to promoting triglyceride accumulation, result in loss of important structural lipids for cellular membranes. If elevated levels of HDL cholesterol that developed in these experiments were related to increased biosynthesis, it is possible that cholesterol competed for the available fatty acids and thereby reduced the production of triglycerides and phospholipids. Relative and absolute concentrations of free cholesterol increased in the serum of rats fed FM for 30 days and

2,2',4,4',5,5'-HBB for 10 and 30 days. Absolute elevations in the esterified cholesterol content of the serum were also present.

Earlier it was noted that increased activity of LPL could increase the serum concentration of HDLs. The other consequence of enhanced LPL activity would be the decline in VLDL concentrations. Such a mechanism is attractive in providing a single explanation for increased HDL and decreased VLDL concentrations seen in the FM and 2,2',4,4',5,5'-HBB experiments. It is doubtful, however, that LPL could account for the very high total cholesterol values encountered in some of these rats. Enhanced LPL activity would tend to shift cholesterol from the VLDL-LDL fractions to the HDL fraction and not cause a dramatic elevation in the total serum cholesterol concentrations. This process fails to explain the decreased cholesterol values produced in the high level 3,3',4,4',5,5'-HBB groups.

To summarize the discussion concerning the alteration in the serum cholesterol and triglyceride levels from these rats, there are several mechanisms that are obviously more attractive than others. Studies previously cited have provided strong evidence that PCB and halogenated hydrocarbon pesticides elevate HDL cholesterol levels through increased hepatic cholesterol synthesis. While one study did show a decrease in serum cholesterol in rats after administration of a potent MC-type PCB compound (Yoshimura et al., 1979), no explanation for the effect was offered. It may be that at such high levels the compound has sufficient hepatocellular toxicity to interfere with either cholesterol synthesis or the assimilation and release of lipoprotein particles from the liver. Also, the possibility that cholesterol catabolism was enhanced at the higher exposure levels of these compounds cannot be ruled out. Serum triglyceride values were consistently decreased by treatments. Two

plausible mechanisms for decreased triglyceride concentrations are a reduction in hepatic synthesis of VLDLs perhaps through increased competition for EFA molecules and increased peripheral catabolism of these particles via LPL. Future research efforts should concentrate on evaluating these and other possible mechanisms of action.

Serum and Lipoprotein GLC Analysis

As explained by Slalsky et al. (1979), many studies have demonstrated the binding of various xenobiotics to plasma proteins, primarily albumin, and lipoproteins. Dieldrin, DDT, DDE, benzo[α]pyrene and 3-methylcholanthrene have all been shown to bind to plasma constituents. In comparing the binding of chlordecone with that of DDT or dieldrin the former appears to have an affinity for HDL and the latter 2 for LDL. Slalsky et al. (1979) speculated that if HDL cholesterol is the preferred substrate for bile acid formation, then binding to these lipoproteins by a xenobiotic could favor its hepatic excretion.

In the serum of the 30-day, 100 ppm 2,2',4,4',5,5'-HBB group, approximately 60% of the congener was associated with the HDL fraction, approximately 30% with the LDL fraction and 5% each with VLDL and albumin. At first it would seem that the compound preferentially associates with the HDLs. But since 78% of the cholesterol in these serum samples was in the HDL fraction and since approximately 87% of the lipoproteins detected by electrophoresis are in the HDL bands, the association is probably one of convenience rather than one of preference.

The inability to detect the 3,3',4,4',5,5'-HBB congener in the serum of the 30-day, 10 ppm group can be related to 1 of 2 possibilities: either the compound was present in serum at levels below detectable limits or the compound was not adequately extracted from the samples.

Considering the high tissue levels reported by Render (1980), the latter explanation is more plausible. He alluded to difficulties encountered in extracting 3,3',4,4',5,5'-HBB from tissue samples by indicating that a modified extraction technique was employed.

Lipoprotein Electrophoresis

Lipoprotein electrophoretic evaluation of serum and individual fractions provided evidence that lipoprotein separation techniques were valid in the rat. Densitometer tracings of isolated fractions on cellulose acetate plates showed no evidence of contamination between lipoprotein classes. Evaluation of electrophoretic patterns of whole serum samples indicated that FM and 3,3',4,4'5,5'-HBB treatments increased the relative concentration of HDL bands. Pair-fed control and 10- and 30-day 2,2',4,4',5,5'-HBB experiments did not greatly alter the ratio of HDL to LDL as determined by electrophoresis. This is not contrary to the evidence that treatment with the 2,2',4,4',5,5'-HBB congener significantly increased HDLs as measured by cholesterol content. It simply indicates that the ratio between HDL and LDL was not dramatically altered. As depicted in Table 8, HDL cholesterol concentration in the serum of animals fed 2,2',4,4'5,5'-HBB for 10 and 30 days significantly increased. The LDL concentration also increased for the 30-day but not the 10-day group. The HDL to LDL ratios of the 30-day 2,2',4,4',5,5'-HBB groups (Table 13) were not changed by the treatments. There was, however, a mild increase in the HDL to LDL ratios for the 10-day groups. Electrophoretic data are compatible with the values obtained from cholesterol assays and tend to support the findings reported for these studies.

Microsomal Enzymes

Microsomal enzyme assays substantiated reports that FM is a mixed-type hepatic drug-metabolizing microsomal enzyme inducer, 2,2',4,4',5,5'-HBB is a PB-type inducer, and 3,3',4,4',5,5'-HBB is an MC-type inducer. Both FM and 3,3',4,4',5,5'-HBB produced shifts in the wavelength maximum for the CO difference spectrum from 450 nm to 449 or 448 nm, while that for 2,2',4,4',5,5'-HBB remained at 450 nm. MC-type activity is associated with a blue shift in the wavelength maximum, indicating induction of cytochrome P-448. Induction patterns were further defined by the activities of aminopyrine demethylase (AD) and benzo[α]pyrene hydroxylase (BH). FM induced moderate elevations in both activities, 2,2',4,4',5,5'-HBB almost entirely induced AD and 3,3',4,4',5,5'-HBB produced very dramatic increases in BH with minimal effects on AD.

Organ Weights, Body Weights and Toxicity

Based on these findings, one would expect 3,3',4,4',5,5'-HBB to be the most toxic of the compounds, FM to be moderately toxic and 2,2',4,4',5,5'-HBB to be relatively nontoxic. The toxicity of 3,3',4,4',5,5'-HBB during this study was demonstrated by death in rats fed a dietary concentration of 100 ppm for 20 days. Animals treated with this congener also had elevations in serum enzymes, enlarged livers, a decrease in body weight gain or actual loss of weight, a significant decrease in thymic weights and an increase in thyroid weights. Rats in the treatment groups receiving 2,2',4,4',5,5'-HBB appeared healthy, did not have reduced weight gains and had no detectable treatment effects on thymic, thyroid or splenic weights. Except for serum elevations of SDH, serum lipid alterations and enlarged livers, rats fed this PB-type compound did not appear to be adversely affected by the treatment.

Firemaster, which is a mixed-type inducer, was more toxic than 2,2',4,4',5,5'-HBB but much less toxic than 3,3',4,4',5,5'-HBB. Rats eating diets containing FM appeared healthy, gained weight similarly to controls and had no significant treatment effect on splenic weights. However, Firemaster produced alterations in serum lipid and SDH levels, increased liver and thyroid weights and decreased thymic weights.

Pair-fed controls had significantly decreased body, liver and splenic weights. Only rats fed the 100 ppm 3,3',4,4',5,5'-HBB diet for 10 days and their 13 g/day pair-fed controls had significantly decreased splenic weights. Some of this effect may have been associated with starvation and subsequent weight loss. Render (1980) reported an increase in macrophages in sections of splenic tissue from the 3,3',4,4',5,5'-HBB treatment groups which was not present in the controls or pair-fed animals.

Thyroid weights were increased by compounds that have MC-type activity, specifically FM and 3,3',4,4',5,5'-HBB. Render (1980) was unable to detect histopathologic changes in thyroid glands of rats fed the 3 compounds for the 10-day periods. Collins et al. (1977) reported that a commercial PCB mixture fed to rats produced ultrastructural changes in thyroid glands. These changes included the accumulation of lysosomal bodies and colloid droplets in follicular cells and the formation of abnormal microvilli on luminal surfaces. Serum thyroxine values were also significantly reduced. Neal et al. (1979) noted that TCDD has been reported to increase the biliary excretion of thyroxine. They were unable to demonstrate that triiodothyronine had any marked protective effect on TCDD toxicity in mice or that TCDD interfered with the normal thyroxine-dependent metamorphosis of bullfrog tadpoles to adults. If we are to assume that TCDD, PCB and PBB are exerting their

toxic effects through similar MC-type mechanisms, then there appears to be no firm evidence that changes in thyroid gland structure or peripheral thyroxine levels are important causes of toxicity.

As with thyroid weights, only those animals fed the compounds with MC-type activity, that is FM and 3,3',4,4',5,5'-HBB, had significant decreases in thymic weights. As Render (1980) and other researchers have noted, the primary histopathologic changes associated with these compounds have been a decrease in cortical size, an increase of macrophages in the cortex and loss of a distinct cortical-medullary junction. Thymic atrophy might be related to increased glucocorticoid synthesis or mimicry of glucocorticoid activity through binding of the xenobiotic to a cytosolic steroid receptor. This was investigated by Neal et al. (1979). They were unable to demonstrate that increased glucocorticoid activity, directly or through mimicry, was an important cause of TCDD toxicity.

The reasonable assumption that thymic atrophy secondary to the administration of xenobiotics could be associated with an impaired immune response has also been explored. Thomas and Hinsdill (1978) and Vos and de Roij (1972) examined the effects of PCBs on immune responses in monkeys and mice and guinea pigs, respectively. Both groups reported that these compounds produced mild immunosuppressive effects. Howard et al. (1980) reported that swine and piglets fed FM had decreased responses to mitogen stimulation but that bactericidal activity of whole blood was similar to that of controls. Neal et al. (1979) acknowledged that TCDD has been shown to have significant suppressive effects on immune competence of rodents. They felt, however, that similar toxic effects of TCDD on specific pathogen-free and germfree rats as compared to normally raised rats indicates that adverse effects

on the immune system are not the primary cause of toxicity for this compound.

As was confirmed in this study, feeding of both PB- and MC-type compounds to rats can significantly increase liver weights. Conney (1957) reported that PB-type compounds have a marked anabolic effect on the liver, significantly increasing the microsomal protein content per gram of liver. The MC compounds can also increase liver growth and the synthesis of total liver protein but have lesser effect on the amount of microsomal protein per gram of liver. Additional, PB-type compounds have been reported to stimulate division of hepatocytes and to increase cell size. Both PB and MC can stimulate the incorporation of protein into microsomal enzymes and PB has a pronounced stimulatory effect on proliferation of the SER.

Since the PB-type compounds are relatively nontoxic, it is difficult to associate increases in liver weight or protein content with toxic effects of some of these compounds. The mechanisms behind toxic effects of the MC-type compounds remain to be established. It is apparent from this research and a related project (Render, 1980) that 3,3',4,4',5,5'-HBB, when compared to FM and 2,2',4,4',5,5'-HBB, is very toxic to rats. Serum lipid and enzyme changes produced by these compounds, while reflecting alterations in basic metabolic processes and changes in hepatocellular integrity, cannot be assumed to represent direct evidence of toxicity. The causes of these alterations, particularly those produced by 3,3',4,4',5,5'-HBB, should be explored.

SUMMARY

Young male Sprague-Dawley rats were fed diets containing various concentrations of FM, 2,2',4,4',5,5'-HBB and 3,3',4,4',5,5'-HBB for periods of 10 and 30 days. Effects of these compounds on serum enzymes, serum lipoproteins, hepatic drug-metabolizing enzymes and on body weights and selected organ weights were determined.

Serum concentrations of alkaline phosphatase (ALP), gamma glutamyl-transpeptidase (GGT), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and sorbitol dehydrogenase (SDH) were measured. Only SDH was consistently, significantly elevated by the higher dietary concentrations of the 3 compounds. Aspartate aminotransferase was also significantly elevated by 10- and 30-day treatments with 3,3',4,4',5,5'-HBB. Serum ALP values were significantly decreased in rats fed each compound for 10 days and in pair-fed controls. Increases in SDH and AST are considered to be related to leakage of these enzymes from hepatocytes. Decreased serum ALP concentrations may represent a decrease in serum content of the intestinal isoenzyme.

All treatments produced significant increases in serum cholesterol primarily related to elevated levels of HDL cholesterol. The compound 3,3',4,4',5,5'-HBB also significantly decreased serum cholesterol levels at the highest dietary concentrations for 10- and 30-day treatments. Serum triglyceride concentrations were significantly decreased by all treatments, including the pair-fed controls. The mechanisms behind

these changes are not known, but elevated cholesterol values are thought by many researchers to reflect enhanced cholesterol biosynthesis in response to microsomal enzyme induction.

Microsomal enzyme assays were performed on liver samples from rats in various treatment groups. The amount of cytochrome P-450, wavelength maximum of the carbon monoxide difference spectrum and activities of aminopyrine demethylase and benzo[α]pyrene hydroxylase were measured. Firemaster was a mixed-type enzyme inducer, 2,2',4,4',5,5'-HBB, a PB-type and 3,3',4,4',5,5'-HBB, an MC-type inducer. These results are in agreement with previous reports and by themselves indicate that 3,3',4,4',5,5'-HBB should be the most toxic of the 3 compounds.

Within 20 days of the beginning of a pilot study, the 2 rats fed 100 ppm of 3,3',4,4',5,5'-HBB became depressed, anorectic and died. Rats fed a diet containing 100 ppm 3,3',4,4',5,5'-HBB for 10 days lost weight from their initial level and rats fed the same compound at 10 ppm for 30 days had significantly lower gains in body weight as compared to controls. Neither the FM mixture nor 2,2',4,4',5,5'-HBB had any significant effects on body weight gains.

While all compounds significantly increased liver weights, only FM and 3,3',4,4',5,5'-HBB treatments had any effects on thymic and thyroid gland weights. Thymic gland involution is a common consequence of MC-type toxicity, but its cause and significance are unknown. Significantly elevated thyroid gland weights were also associated with compounds that had MC-type activity. The significance of this finding in relationship to toxicity of these compounds is not known.

This research project detected alterations in serum enzymes, lipoproteins, and hepatic microsomal enzymes in rats fed various PBB compounds. Most of the serum lipid and enzyme changes had been previously

undescribed for these compounds. Furthermore, by using purified congeners with different structural and functional characteristics, the changes could be attributed to PB-type, MC-type or mixed-type activities. Biochemical mechanisms responsible for the lipid changes should be investigated and identified in future endeavors.

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APPENDICES

APPENDIX A

SERUM ENZYME STUDIES

Table A-1. Sorbitol dehydrogenase and alkaline phosphatase enzyme activities[a]

Compound	Dietary Concentration (ppm)	(days)	Determinations			
			SDH		ALP	
			10	30	10	30
			(IU/l)		(IU/l)	
FM	0		5	5	155	101
	0.1		5	5	137	93
	1		4	5	127	115
	10		8	11	116	84
	100		21	21	92	86
2,2',4,4',5,5'-HBB	0		6	8	112	75
	0.1		7	10	97	66
	1		7	9	100	72
	10		9	13	78	81
	100		10	19	80	66
3,3',4,4',5,5'-HBB	0		5	8	93	131
	0.1		4	9	88	125
	1		6	12	76	118
	10		10	28	82	135
	100		18	NE	60	NE
Pair-fed controls	*ad libitum*		7	NE	153	NE
	23 g/day		5	NE	103	NE
	13 g/day		7	NE	81	NE

[a]Data represent means for groups

NE = no experiment

Table A-2. Alanine aminotransferase and aspartate aminotransferase enzyme activities[a]

Compound	Dietary Concentration (ppm)	(days)	Determinations			
			ALT		AST	
			10	30	10	30
			(IU/l)		(IU/l)	
FM	0		26	25	56	58
	0.1		25	21	53	48
	1		22	24	52	55
	10		29	25	64	56
	100		35	25	75	62
2,2',4,4',5,5'-HBB	0		18	28	69	62
	0.1		21	29	68	66
	1		18	30	60	61
	10		19	33	71	67
	100		17	36	56	59
3,3',4,4',5,5'-HBB	0		23	29	51	64
	0.1		27	25	66	68
	1		24	25	58	69
	10		22	18	65	98
	100		17	NE	83	NE
Pair-fed controls	*ad libitum*		29	NE	67	NE
	23 g/day		28	NE	79	NE
	13 g/day		25	NE	66	NE

[a]Data represent means for groups

NE = no experiment

Table A-3. Gamma glutamyltranspeptidase enzyme activities[a]

Compound	Dietary Concentration (ppm)	(days)	Determination GGT 10	Determination GGT 30
			(IU/l)	
FM	0		1	1
	0.1		0	2
	1		0	1
	10		2	2
	100		2	1
2,2',4,4',5,5'-HBB	0		1	2
	0.1		2	3
	1		2	4
	10		1	9
	100		2	7
3,3',4,4',5,5'-HBB	0		1	9
	0.1		1	10
	1		2	5
	10		2	6
	100		2	NE
Pair-fed controls	*ad libitum*		0	NE
	23 g/day		0	NE
	13 g/day		0	NE

[a]Data represent means for groups

NE = no experiment

APPENDIX B

SERUM LIPID STUDIES

Table B-1. Serum cholesterol and triglyceride values[a]

Compound	Dietary Concentration (ppm)	(days)	Determinations: Serum Cho. 10	Serum Cho. 30	Triglyceride 10	Triglyceride 30
			(mg/100 ml)			
FM	0		74	64	56	69
	0.1		67	73	44	57
	1		73	70	50	64
	10		78	75	54	50
	100		90	132	39	32
2,2',4,4',5,5'-HBB	0		70	88	60	74
	0.1		70	81	76	75
	1		77	100	68	72
	10		82	96	71	63
	100		92	146	51	40
3,3',4,4',5,5'-HBB	0		63	73	50	61
	0.1		66	74	55	60
	1		69	86	56	57
	10		69	51	52	35
	100		18	NE	16	NE
Pair-fed controls	*ad libitum*		70	NE	76	NE
	23 g/day		83	NE	57	NE
	13 g/day		88	NE	37	NE

[a]Data represent means for groups

NE = no experiment

Table B-2. Serum free cholesterol and percent free/total cholesterol values[a]

Compound	Dietary Concentration (ppm)	(days)	Determinations: Free Cholesterol 10	Free Cholesterol 30	% Free/Total 10	% Free/Total 30
			(mg/100 ml)			
FM	0		10	12	13	18
	0.1		8	14	13	18
	1		10	12	15	18
	10		10	14	13	18
	100		12	30	13	22
2,2',4,4',5,5'-HBB	0		8	9	11	10
	0.1		8	10	12	12
	1		11	10	14	11
	10		10	13	13	13
	100		14	18	15	13
3,3',4,4',5,5'-HBB	0		11	13	18	18
	0.1		12	14	17	18
	1		12	14	18	15
	10		10	ND	13	ND
	100		1	NE	4	NE
Pair-fed controls	*ad libitum*		12	NE	12	NE
	23 g/day		12	NE	12	NE
	13 g/day		15	NE	15	NE

[a]Data represent means for groups

ND = not determined

NE = no experiment

Table B-3. Serum high density and high density + low density lipoprotein cholesterol values[a]

Compound	Dietary Concentration (ppm)	(days)	Determinations			
			HDL		HDL+LDL	
			10	30	10	30
			(mg/100 ml)			
FM	0		53	46	64	61
	0.1		45	49	59	66
	1		53	47	66	67
	10		61	58	73	70
	100		75	101	86	126
2,2',4,4',5,5'-HBB	0		50	69	61	83
	0.1		50	61	62	74
	1		49	74	68	89
	10		63	76	72	89
	100		75	113	84	138
3,3',4,4',5,5'-HBB	0		45	47	58	71
	0.1		51	45	59	73
	1		47	62	60	86
	10		57	38	58	50
	100		16	NE	19	NE
Pair-fed controls	*ad libitum*		46	NE	56	NE
	23 g/day		51	NE	66	NE
	13 g/day		58	NE	79	NE

[a]Data represent means for groups

NE = no experiment

Table B-4. Serum low density and very low density lipoprotein cholesterol values[a]

Compound	Dietary Concentration (ppm)	(days)	Determinations			
			LDL		VLDL	
			10	30	10	30
			(mg/100 ml)			
FM	0		11	21	10	3
	0.1		14	16	7	7
	1		13	19	7	3
	10		5	19	5	5
	100		11	17	11	6
2,2',4,4',5,5'-HBB	0		11	12	9	7
	0.1		12	13	10	6
	1		18	16	9	6
	10		8	12	10	2
	100		8	25	8	1
3,3',4,4',5,5'-HBB	0		13	24	5	4
	0.1		7	28	8	2
	1		13	24	9	3
	10		2	11	10	1
	100		4	NE	3	NE
Pair-fed controls	*ad libitum*		13	NE	14	NE
	23 g/day		15	NE	17	NE
	13 g/day		21	NE	9	NE

[a]Data represent means for groups

NE = no experiment

Table B-5. Serum high density and high density + low density lipoprotein triglyceride values[a]

Compound	Dietary Concentration (ppm)	(days)	Determinations			
			HDL		HDL+LDL	
			10	30	10	30
			(mg/100 ml)			
FM	0		16	12	27	33
	0.1		16	13	22	22
	1		14	14	22	34
	10		19	14	32	25
	100		17	14	26	20
2,2',4,4',5,5'-HBB	0		16	32	25	43
	0.1		23	35	30	40
	1		18	32	26	43
	10		19	27	27	36
	100		17	28	22	36
3,3',4,4',5,5'-HBB	0		17	20	29	52
	0.1		18	21	34	51
	1		16	21	28	46
	10		20	15	26	29
	100		8	NE	13	NE
Pair-fed controls	*ad libitum*		34	NE	52	NE
	23 g/day		29	NE	47	NE
	13 g/day		26	NE	39	NE

[a]Data represent means for groups

NE = no experiment

Table B-6. Serum low density and very low density lipoprotein triglyceride values[a]

Compound	Dietary Concentration (ppm)	(days)	Determinations			
			LDL		VLDL	
			10	30	10	30
			(mg/100 ml)			
FM	0		11	16	28	36
	0.1		10	10	20	35
	1		9	16	26	30
	10		13	12	22	26
	100		9	6	14	16
2,2',4,4',5,5'-HBB	0		10	7	34	32
	0.1		7	6	42	27
	1		8	11	44	23
	10		8	9	43	16
	100		5	8	29	3
3,3',4,4',5,5'-HBB	0		13	33	22	11
	0.1		15	30	21	9
	1		11	25	28	9
	10		6	14	26	6
	100		5	NE	5	NE
Pair-fed controls	*ad libitum*		18	NE	24	NE
	23 g/day		17	NE	10	NE
	13 g/day		13	NE	2	NE

[a]Data represent means for groups

NE = no experiment

APPENDIX C

HEPATIC MICROSOMAL ENZYME STUDIES

Table C-1. Microsomal enzyme assays - cytochrome P-450 and wavelength maximum

Compound	Dietary Concentration (ppm)	(days)	Determinations			
			Cytochrome P-450		λ Max	
			10	30	10	30
			(nmole/mg protein)		(nm)	
FM[a]	0		ND	0.63	ND	450
	0.1		ND		ND	
	1		ND	0.90	ND	449
	10		ND	2.20	ND	450
	100		ND	3.64	ND	449
2,2',4,4',5,5'-HBB[b]	0		0.38	0.99	449	450
	0.1		ND	1.13	ND	450
	1		0.95	1.26	449	450
	10		1.58	2.05	450	450
	100		2.29	2.80	450	450
3,3',4,4',5,5'-HBB[a]	0		0.95	0.92	450	450
	0.1		0.96	1.27	448.7	449.7
	1		1.19	2.00	448.4	449
	10		2.40	3.37	448	449
	100		2.88	NE	448	NE
Pair-fed controls[b]	*ad libitum*		0.76	NE	449	NE
	23 g/day		0.68	NE	449	NE
	13 g/day		0.69	NE	450	NE

[a]Data represent means of individual samples

[b]Data represent pooled samples

ND = not determined

NE = no experiment

Table C-2. Microsomal enzyme assays - aminopyrine demethylation and benzo[α]pyrene hydroxylation

Compound	Dietary Concentration (ppm)	(days)	Determinations			
			AD		BH	
			10	30	10	30
			(nmoles/min)		(mg protein)	
FM[a]	0		ND	5.05	ND	1.01
	0.1		ND		ND	
	1		ND	12.05	ND	2.34
	10		ND	20.0	ND	5.13
	100		ND	16.95	ND	6.95
2,2',4,4',5,5'-HBB[b]	0		7.06	13.6	0.49	1.39
	0.1		ND	12.9	ND	1.40
	1		14.9	17.1	1.80	1.93
	10		16.4	28.7	2.06	2.73
	100		28.1	32.4	2.02	3.45
3,3',4,4',5,5'-HBB[a]	0		11.2	13.6	1.2	2.69
	0.1		13.5	14.4	1.5	7.62
	1		12.9	14.4	4.3	21.1
	10		10.5	10.1	10.5	30.2
	100		10.5	NE	10.1	NE
Pair-fed controls[b]	*ad libitum*		14.4	NE	1.24	NE
	23 g/day		15.8	NE	1.14	NE
	13 g/day		15.3	NE	1.45	NE

[a]Data represent means of individual samples

[b]Data represent pooled samples

ND = not determined

NE = no experiment

APPENDIX D

ORGAN WEIGHTS

Table D-1. Hepatic and thymic weights[a]

Compound	Dietary Concentration (ppm)	(days)	Determinations			
			Liver		Thymus	
			10	30	10	30
			(g)		(g)	
FM	0		11	14	0.68	0.77
	0.1		10	15	0.63	0.84
	1		11	15	0.66	0.68
	10		12	18	0.72	0.71
	100		17	24	0.66	0.53
2,2',4,4',5,5'-HBB	0		11	14	0.69	0.58
	0.1		11	15	0.71	0.48
	1		12	14	0.76	0.56
	10		13	18	0.69	0.55
	100		14	22	0.81	0.50
3,3',4,4',5,5'-HBB	0		10	12	0.66	0.85
	0.1		10	12	0.66	0.83
	1		11	14	0.70	0.82
	10		12	17	0.51	0.42
	100		13	NE	0.32	NE
Pair-fed controls	*ad libitum*		12	NE	0.67	NE
	23 g/day		10	NE	0.69	NE
	13 g/day		8	NE	0.54	NE

[a]Data represent means for groups

NE = no experiment

Table D-2. Thyroid and splenic weights[a]

Compound	Dietary Concentration (ppm)	(days)	Determinations: Thyroid 10 (g)	Thyroid 30 (g)	Spleen 10 (g)	Spleen 30 (g)
FM	0		0.014	0.018	0.76	1.01
	0.1		0.013	0.020	0.82	1.19
	1		0.015	0.023	0.84	1.09
	10		0.014	0.023	0.84	0.97
	100		0.018	0.027	0.87	0.85
2,2',4,4',5,5'-HBB	0		0.015	0.023	0.82	1.18
	0.1		0.015	0.025	0.87	0.99
	1		0.015	0.027	0.94	0.89
	10		0.017	0.027	0.89	0.86
	100		0.016	0.028	0.82	0.93
3,3',4,4',5,5'-HBB	0		0.013	0.015	0.87	0.92
	0.1		0.013	0.016	0.83	1.08
	1		0.012	0.018	0.89	0.90
	10		0.014	0.019	0.76	0.71
	100		0.011	NE	0.56	NE
Pair-fed controls	*ad libitum*		0.014	NE	0.84	NE
	23 g/day		0.013	NE	0.81	NE
	13 g/day		0.011	NE	0.67	NE

[a]Data represent means for groups

NE = no experiment

APPENDIX E

BODY WEIGHT GAINS

Table E-1. Body weight gains[a]

Compound	Dietary Concentration (ppm)	(days)	Determination: Δ Body Weight 10 (g)	Determination: Δ Body Weight 30 (g)
FM	0		43	ND
	0.1		32	ND
	1		34	ND
	10		34	ND
	100		29	ND
2,2',4,4',5,5'-HBB	0		39	22
	0.1		46	33
	1		50	27
	10		43	28
	100		47	38
3,3',4,4',5,5'-HBB	0		28	176
	0.1		25	173
	1		30	178
	10		18	134
	100		-34	NE
Pair-fed controls	*ad libitum*		44	NE
	23 g/day		48	NE
	13 g/day		-6	NE

[a]Data as g represent means for groups

ND = not determined

NE = no experiment