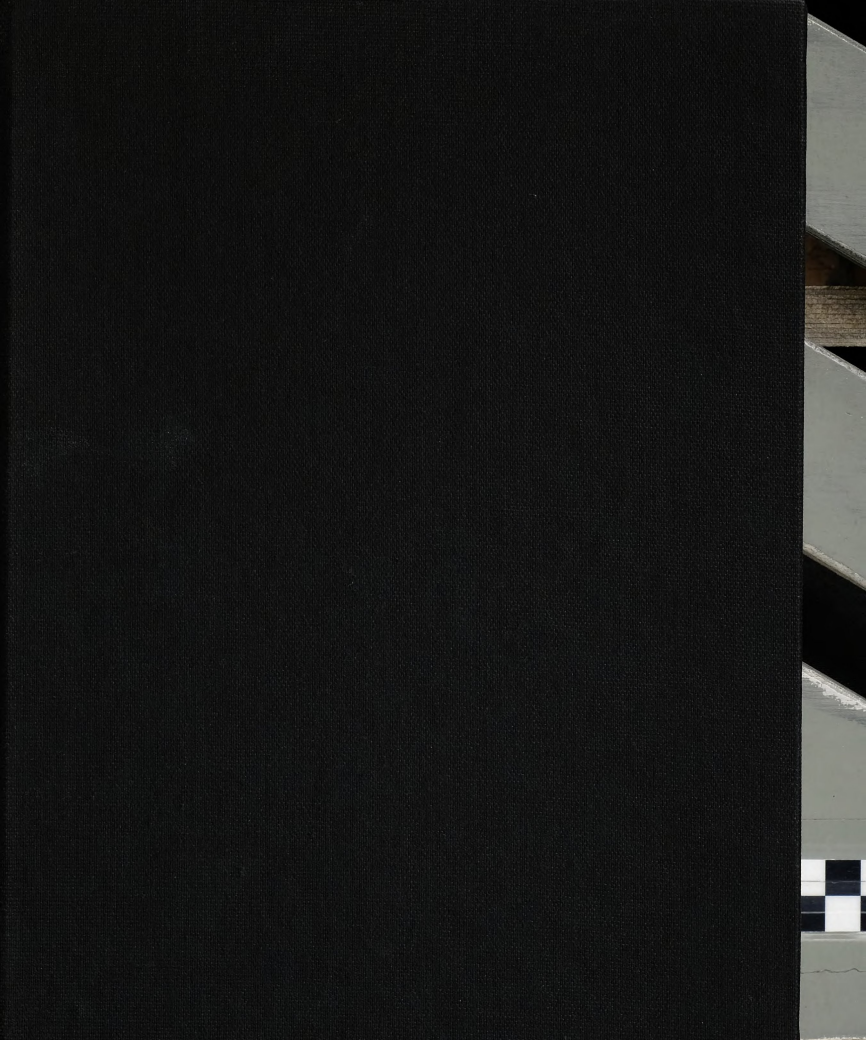

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Mark G. Evans

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IN VIVO, ULTRASTRUCTURAL, AND IN VITRO STUDIES ON THE PATHOLOGIC EFFECTS OF 2,2',4,4',5,5'-HEXABROMOBIPHENYL AND 3,3',4,4',5,5'-HEXACHLOROBIPHENYL

By

Mark G. Evans

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ABSTRACT

IN VIVO, ULTRASTRUCTURAL, AND IN VITRO STUDIES
ON THE PATHOLOGIC EFFECTS OF
2,2',4,4',5,5'-HEXABROMOBIPHENYL AND
3,3',4,4',5,5'-HEXACHLOROBIPHENYL

By

Mark G. Evans

Female Sprague-Dawley rats were partially hepatectomized, initiated with diethylnitrosamine (DEN), and fed diets containing 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB), or 3,3',4,4',5,5'-hexachlorobiphenyl (345-HCB) to determine the tumor promoting ability of these compounds in a two-stage hepatocarcinogenesis system. Tumor promoting ability was assessed by measuring hepatic foci positive for gamma glutamyl transpeptidase (GGT) activity. Dietary concentrations of 10 or 100 mg/kg of 245-HBB caused increased numbers of GGT-positive hepatic foci. Likewise, dietary concentrations of 0.1 or 1.0 mg/kg 345-HCB caused increased numbers of GGT-positive hepatic foci. When 245-HBB and 345-HCB were fed simultaneously, an additive effect on tumor promoting ability was observed at dietary concentrations of 10 mg/kg 245-HBB

and 0.1 mg/kg 345-HCB. However, an inhibitory effect on tumor promoting ability occurred when dietary concentrations of 100 mg/kg 245-HBB and 1.0 mg/kg 345-HCB were fed simultaneously.

Freeze-fracture studies revealed that less hepatocytic membrane was occupied by gap junctions in hepatic nodules from rats that were DEN-initiated and fed dietary concentrations of 10 mg/kg 245-HBB plus 0.1 mg/kg 3,3',4,4',5,5'-hexabromobiphenyl than in surrounding non-nodular hepatic parenchyma. However, numbers of nuclear pores were not significantly different between nodular and non-nodular areas of liver from similarly treated rats.

In in vitro studies, 245-HBB inhibited gap junction-mediated intercellular communication in WB-F344 (rat epithelial) cells in a dose-dependent manner in the metabolic cooperation assay, the fluorescence redistribution after photobleaching assay, and the scrape-loading/dye transfer assay. When the scrape-loading/dye transfer assay was combined with a technique in which fluorescence intensity was measured, quantitation of dose-responsiveness was similar to that found with the metabolic cooperation assay. In addition, Firemaster BP-6 (FM) did not inhibit intercellular communication more than its major congener, 245-HBB, in the metabolic cooperation assay.

Results from these studies further characterize the carcinogenic and toxicologic properties of FM, 245-HBB, and 345-HCB. Furthermore, these results demonstrate the usefulness of the scrape-loading/dye transfer assay for in vitro assessment of dose-dependent inhibition of intercellular communication by 245-HBB.

DEDICATION

To my Family

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure/Page

Figure/Page

Figure/Page

Figure/Page

LIST OF ABBREVIATIONS

245-HBB.....2,2',4,4',5,5'-hexabromobiphenyl

345-HBB.....3,3',4,4',5,5'-hexabromobiphenyl

345-HCB.....3,3',4,4',5,5'-hexachlorobiphenyl

PBB.....polybrominated biphenyl(s)

PCB.....polychlorinated biphenyl(s)

FM......Firemaster BP-6

DEN.....diethylnitrosamine

2-AAF...2-acetylaminofluorene

GGT.....gamma glutamyl transpeptidase

DDT.....dichlorodiphenyltrichloroethane

3-MC....3-methylcholanthrene

PB......phenobarbital

D-ALAS..delta-amino-levulinic acid synthetase

MC......metabolic cooperation

HGPRT...hypoxanthine guanine phosphoribosyl transferase

SL/DT...scrape-loading/dye transfer assay

FRAP....fluorescence redistribution after photobleaching

ACAS....anchored cell analysis and sorting

6-CFDA..6-carboxyfluorescein diacetate

6-TG....6-thioguanine

Pk-C....protein kinase C

PBS.....phosphate buffered saline

DMSO....dimethlysulfoxide

TPA.....12-O-tetradecanoylphorbol-13-acetate

LY......lucifer yellow

RD......rhodamine dextran

CHAPTER 1

TUMOR PROMOTING EFFECTS OF 2,2',4,4',5,5'-HEXABROMOBIPHENYL AND 3,3',4,4',5,5'-HEXACHLOROBIPHENYL IN AN INITIATION/PROMOTION HEPATOCARCINOGENESIS SYSTEM IN RATS

CHAPTER 1

TUMOR PROMOTING EFFECTS OF 2,2',4,4',5,5'-HEXABROMOBIPHENYL AND 3,3',4,4',5,5'-HEXACHLOROBIPHENYL IN AN INITIATION/PROMOTION HEPATOCARCINOGENESIS SYSTEM IN RATS

INTRODUCTION

Certain environmental contaminants resist egradation and continue to be a source of further human xposure. The polybrominated biphenyls (PBB's) and olychlorinated biphenyls (PCB's) are examples of such ompounds. The PCB's have been used extensively for arious applications because of their varied physical roperties and chemical stability and have worldwide nvironmental distribution (Mackay et al., 1983, Tanabe t al., 1983, Murphy et al., 1983). The PBB's are less idely distributed, but were involved in a chemical ccident in Michigan in the early 1970's in which cattle ccidentally ingested the compound, contaminating meat nd milk products consumed by Michigan residents (Kay, 977; Jacobs et al., 1978). Thus, residents of Michigan

have a high likelihood of carrying detectable body burdens of both PCB's and PBB's.

Strong evidence that PBB's or PCB's cause cancer in people is lacking (Brown and Jones, 1981; Stross et al., 1981). However, experimental studies in laboratory animals suggest that these compounds have tumor promoting (i.e., epigenetic) activity (Jensen et al., 1982a, 1982b, 1983; Kimura and Baba, 1973; Kimura et al., 1976; Aishizumi, 1976; Pereira et al., 1982; Deml and Oesterle, 1982; Hirose et al., 1981; Preston et al., 1981). It is of special concern that simultaneous exposure to combinations of these environmental toxicants may have additive, synergistic, or inhibitory effects on tumor promotion.

The first objective of the following studies was to determine the tumor promoting effects of 2,2',4,4',5,5'-hexabromobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl by using a two-stage hepatocarcinogenesis assay, the Pitot model (Pitot et al., 1978a). A second objective was to determine the tumor promoting ability of these compounds when fed simultaneously to rats by using the same assay. Information from this study may shed light on the additive, synergistic, or inhibitory tumor promoting effects when animals are concomitantly exposed to more than one environmental toxicant.

LITERATURE REVIEW

History and Uses of PCB's and PBB's

Commercial preparations of polychlorinated biphenyl (PCB) and polybrominated biphenyl (PBB) are formulations made by the chlorination and bromination, respectively, of biphenyl. Several PCB and PBB preparations have been widely utilized in most industrial countries. Most commercial producers have marketed PCB formulations with a variable chlorine content because the degree of biphenyl chlorination determines the properties of these industrial mixtures. However, only one PBB formulation, namely Firemaster BP-6, made by Michigan Chemical Company of St. Louis, Michigan, has been widely used for industrial purposes. Firemaster BP-6 was used as a flame retardant additive for polymeric resins, while the PCB's have been used for many varied applications because of their wide range of physical properties and their chemical stability with various organic compounds. The PCB's have been used extensively as hydraulic fluids, adhesives, heat transfer agents, flame retardants, plasticizers, wax extenders, lubricants, "dedusting" agents, organic diluents/extenders, and

dielectric fluids in electrical capacitors and transformers. The PCB's were detected in the environment during the late 1960's and early 1970's resulting in a voluntary ban on all "open" uses of these compounds, but their use a dielectric agents ("closed" use) was permitted until the late 1970's. It is estimated that 14 billion pounds of PCB's were manufactured in the United States from 1930 to 1975, and total U.S. production of PBB's from 1970 to 1976 was about 130 million pounds (Brinkman and de Kok, 1980). Currently, industrial applications of PBB and PCB have been stopped, and production of these compounds ceased during the 1970's. However, PCB's are still present as dielectric fluids in older transformers and capacitors (Pomerantz et al., 1978; Brinkman and de Kok, 1980; Rappe and Buser, 1980).

Commercial PBB's and PCB's are prepared with various catalysts and experimental conditions. The commercial products are complex mixtures of isomers and congeners, and halogen substitution occurs on the phenyl ring with no apparent preference for ortho or para positions.

The PBB and PCB formulations are similar with respect to their average number of bromine and chlorine atoms per biphenyl. However, there are two major differences in the composition of Firemaster BP-6 and Aroclor 1260, a commercial PCB preparation. First, 2,

4, 5, 2',4',5'-hexabromobiphenyl is the major PBB congener of Firemaster BP-6, whereas no single PCB congener predominates in Aroclor 1260. Second, there is no homology in the relative concentrations of structurally similar PCB's and PBB's in the commercial mixtures (Ballschmitter and Zell, 1980; Mullin et al., 1983; Moore and Aust, 1978).

Environmental persistence and stability of PCB's and PBB's is attributed to their resistance to breakdown by acids, bases, heat, reducing agents, and oxidizing agents. Furthermore, chemical stability of these compounds is partially dependent on the degree of halogenation as well as on the specific pattern of substitution (Brinkman and de Kok, 1980; Huntzinger et al., 1974). Analysis of PCB residues taken from the environment indicates that the more heavily chlorinated congeners are more persistent. This may be due to preferential microbial breakdown of the less chlorinated compounds (Ballschmitter et al., 1978).

Environmental Distribution of PCB's and PBB's

Residues of PCB's have been detected in atmospheric samples taken from both industrialized regions and remote arctic and antarctic locations (Mackay et al., 1983; Harvey and Steinhauer, 1974; Tanabe et al., 1983; Atlas and Giam, 1981; Murphy et al., 1983). These

results imply that atmospheric transport processes contribute to the distribution of PCB's in the environment. Similar residues have also been found in lake, river, and ocean sediments which provide a reservoir for the gradual release of PCB's into water, aquatic animals and plants, and eventually into the biota (Kauss et al., 1983; Sullivan et al., 1983). The PCB's have also been detected in fish (Zabik et al., 1982; Brunn and Manz, 1982; Wickstrom et al., 1981) and various wildlife species (Olsen et al., 1980; Barbehenn and Reichel, 1981; Passivirta and Linko, 1980). An extensive study of the Great Lakes ecosystem demonstrated the preferential bioconcentration of PCB residues in the food chain. The lowest average levels were found in the water and sediments, and highest levels were in the adipose tissue of carnivores such as the herring gull (International Joint Commission, Great Lakes Water Quality, 1977). The levels of PCB's in the environment are gradually diminishing due to their limited use and regulated storage and disposal (Passivirta and Linko, 1980).

The PBB's are not frequently detected in the environment because their industrial production and distribution was limited. Soils of contaminated Michigan farms and the areas adjacent to the Michigan Chemical Company, where Firemaster BP-6 was manufactured, contain detectable levels of PBB's (Kay, 1977; Carter, 1976;

Jacobs, et al., 1978). Low PBB levels have been detected in fish taken from waters near the Michigan Chemical Company manufacturing site (Hesse and Powers, 1978; Filonow et al., 1976).

Toxic Effects of PCB's and PBB's in Animals

Acute Effects

The PCB's and PBB's have similar toxic properties, and there are several generalizations that can be made about acute toxic effects. These toxic properties are dependent on the sex, strain, and age of the experimental animal, and there is wide variation in species' sensitivity to these commercial compounds. Generally, the onset of clinical signs due to the toxicity of PCB's and PBB's occurs between one and three weeks following initial exposure to the compound. The LD_{50} values for commercial PCB preparations for rats, rabbits, and mice are between one and ten grams/kg. (Damstra et al., 1982; Matthews et al., 1978). In contrast, mink are exquisitely sensitive to the acute toxic effects of PBB's and PCB's (Ringer et al., 1981). In general, the more highly chlorinated formulations of PCB's appear to be more toxic than the less chlorinated products (Kimbrough, 1974; Fishbein, 1974).

The LD_{50} values for commercial PBB's have not been rigorously established. However, like the PCB's, PBB's

are relatively nontoxic to rats, mice, and cattle, but are highly toxic to mink. Typical clinical signs in acute PBB or PCB toxicosis include weight loss that is somewhat due to decreased food intake, thymic atrophy, and hepatomegaly (Gartoff et al., 1977; Kimbrough et al., 1978; Kimbrough, 1974; McConnell and Moore, 1979; Roberts et al., 1978).

Dermal Effects

The skin lesions in animals and man in PCB and PBB toxicosis are distinctive. The most common dermal lesion is chloracne. The rabbit ear is especially sensitive to the toxic effects of halogenated aromatic compounds, and changes include hyperplasia and hyperkeratosis of the epidermis and hair follicle epithelium (Vos and Beems, 1971; Vos and Notenboom-Ram, 1972; Hass et al., 1978; Needham et al., 1982; Patterson et al., 1981). Nonhuman primates also have typical dermal and ocular lesions after dietary exposure to PCB's and PBB's (McConnell and Moore, 1979; Allen et al., 1978; Lambrecht et al., 1978; Allen et al., 1974; Altman et al., 1979; McConnell et al., 1979; Barsotti et al., 1976; Allen et al., 1979). These clinical signs can develop with diets of less than 50 mg/kg body weight of the halogenated biphenyls. Neonatal primates suckling PCB-exposed dams also have similar dermal lesions (Allen et al., 1979; Allen and Barsotti, 1976), but this may be

due to exposure in utero as well as intake of PCB-contaminated breast milk. Ingestion of PBB-contaminated feed by cattle in Michigan caused hyperkeratosis (Jackson and Halbert, 1974; Kay, 1977; Carter, 1976; Fishbein, 1974). These clinical signs are similar to hyperkeratosis (i.e., "X-disease") seen in cattle following exposure to chlorinated naphthalenes (Olafson, 1947; Bell, 1953). Interestingly, rats do not have acne or dermal lesions after exposure to PCB's or PBB's. However, hairless mice acquire dermal lesions associated with ingestion of these compounds (Inagami and Koga, 1969; Knutson and Polland, 1982; Puhvel et al., 1982).

Mink are especially susceptible to PBB and PCB toxicoses but do not have dermal lesions upon exposure to these compounds. However, the ferret, a closely related animal, had hyperkeratosis and excessive nail growth following ingestion of 20 mg/kg Aroclor 1242 (a commercial preparation of PCB's) for several months (Bleavins et al, 1982). Dermal responses to PCB's and PBB's by some animals are similar to those seen in people exposed to high levels of these compounds, but the mechanism of action responsible for this effect is not understood.

Hepatic Effects

The PCB's and PBB's cause toxicity to the liver in many animal species. The severity of the hepatotoxicity

differs according to the age and sex of the animal, the dose given, the duration of exposure, and the species tested (Matthews et al., 1978; Parkinson and Safe, 1981; Kimbrough 1974; Fishbein 1974; McConnell and Moore, 1979). Minimal hepatic damage has been reported in guinea pigs and monkeys, but moderate to severe liver damage has been seen in chickens, rabbits, rats, and mice. It is of interest that the guinea pig and monkey represent two species in which minimal liver damage occurs, although these two species are highly susceptible to other toxic effects of PCB's and PBB's. The most commonly observed gross lesion in PCB or PBB toxicosis in several animal species is hepatomegaly (Parkinson et al., 1980; Gupta et al., 1983a, 1983b; Allen et al., 1973; Kimbrough et al., 1972a; Kimbrough et al., 1973; Kimbrough et al. 1972b; Jonsson, 1981; Bruckner et al., 1974).

Moderate to severe liver damage was observed in rabbits after either dermal or dietary exposure to PCB's (Vos and Beems, 1971). Hepatomegaly and severe subcapsular and midzonal necrosis were evident. Hepatomegaly and necrotizing hepatitis have been seen in chickens exposed to commercial PCB and PBB preparations (Vos and Koeman, 1970; Ringer, 1978). Mink given these compounds in the diet had hepatomegaly and necrotizing hepatitis (Ringer et al., 1981; Aulerich et al., 1973). Dairy cattle given Firemaster BP-6 at a rate of 25

grams/day for one to two months and calves fed Firemaster FF-1 for two to twelve weeks had hepatomegaly (Moorehead et al., 1978; Durst et al., 1978; Robl et al., 1978). Nonhuman primates had hepatic lesions after exposure to PCB's and PBB's, but gastric lesions were also observed in these species (Allen et al., 1978; Allen et al., 1974; McConnell et al., 1979; Allen et al., 1979; Allen and Barsotti, 1976; Allen and Norbach, 1973; Becker et al., 1979). Studies in fish indicated that PCB's and PBB's cause hepatotoxicity in these species as well (Klaunig et al., 1979).

Neurologic and Behavioral Effects

The neurotoxicity and behavioral effects of PCB's and PBB's have been studied in several animal species. Chicks had impaired and irreversible avoidance response when fed diets of 200 mg/kg of Aroclor 1254 for seven days (Kreitzer and Heinz, 1974). Coturnix quail had reduced biochemical adaptation to stress when fed Aroclor 1254 (Deiter, 1974). Pheasant chicks whose mothers had been exposed to 50 ppm of Aroclor 1254 for 17 weeks also had impaired behavioral responses (Dahlgren and Linder, 1971). Additional studies suggest that several avian species are highly sensitive to PCB-mediated neurotoxicity (Ulfstrand et al., 1971; Karlsson et al., 1974; Peakall and Peakall, 1973), and various fish species exposed to Aroclors had alterations in

levels of various neurotransmitters (Fingerman and Shortt, 1983; Fingerman and Russell, 1980). Rats exposed chronically or subchronically to Firemaster BP-6 have many neurotoxic and behavioral changes including muscular impairment, irritability, decreased maze performance, and reductions in cognitive ability (Tilson and Cabe, 1979).

Immunologic Effects

The target for PCB and PBB toxicosis in the immune system is lymphoid tissue (Vos et al., 1980). Studies in poultry revealed that exposure to PCB's caused decreased splenic and bursal weights (Flick et al., 1965; Vos and Koeman, 1970). Results from studies by Harris et al. (1976) found that the offspring of chickens fed commercial PCB's had decreased splenic and bursal weights dependent on the degree of chlorination of the compounds fed. Other studies indicate that ducklings fed Aroclor 1254 were more susceptible to duck hepatitis virus. This was thought to be associated with decreased immunocompetence and was not accompanied by other signs of PCB toxicosis (Friend and Trainer, 1970).

Chronic dietary exposure of guinea pigs to commercial PCB's reduced numbers of circulating lymphocytes and other leukocytes, suppressed delayed hypersensitivity reactions to tuberculin, and decreased circulating antibody titers to tetanus toxoid (Vos and

van Driel Grootenhuis, 1972). Results of other studies in mice and rats indicate a variety of immunotoxic effects of PCB's and PBB's, including thymic atrophy, splenic atrophy, decreased resistance to host infections, decreased antibody response, depressed T-cell responsiveness to mitogens, and diminished delayed hypersensitivity reactions (Loose et al., 1977; Silkworth and Loose, 1978; Loose et al., 1978; Smith et al., 1978; Thomas and Hinsdill, 1978; Luster et al., 1978). Results from studies in other species including monkeys, dogs, rabbits, and pigs indicate that diverse immunotoxic effects occur following exposure to halogenated biphenyls (Allen and Lambrecht, 1978; Farber et al., 1978; Koller and Thigpen, 1973; Thomas and Hinsdill, 1980; Howard et al., 1980). However, the mechanisms of immunotoxicosis by halogenated biphenyls is currently unknown.

Reproductive Effects

Results from several experiments have established that primates fed two to five mg/kg of Aroclor 1248, a commercial PCB preparation, had reproductive toxicosis. (Allen et al., 1978; Lambrecht et al., 1978; Allen et al., 1974; Barsotti et al.,1976; Allen et al., 1979; Allen and Barsotti 1976). The exposed primates had increased frequency of abortions, resorptions, and low birth weights. Primates fed Firemaster FF-1, a

commercial PBB preparation, at a rate of 0.3 ppm for seven months had prolonged menstrual cycles and diminished progesterone levels. Offspring of these animals had low birth weights (Lambrecht et al., 1978; Allen and Lambrecht, 1978).

Rats and mice also suffer from reproductive toxicosis when fed commercial PCB's and PBB's. Toxic effects include decreased numbers of live births from PCB-fed rats, decreased survivability, and decreased successful matings in rats fed Aroclor 1254. The PBB's were fetotoxic and embryotoxic in rats in a dose-dependent manner, and commercial halogenated biphenyls were potent reproductive toxins in mink (Spencer, 1982; Hansen et al., 1975; Kihlstrom et al., 1975; Beaudoin, 1977; Aulerich and Ringer, 1979; Corbett et al., 1975; Ringer et al., 1981).

Birds also suffer reproductive toxicosis from PCB's and PBB's. Hens fed a diet containing Aroclor 1254 for 39 weeks had decreased egg production (Corbett et al., 1975). Reduced hatchability was seen in chickens fed Aroclors 1232, 1242, or 1248 at a dose of 10 mg/kg for six weeks (Britton and Huston, 1972; Bush et al., 1974; Lillie et al., 1975; Ax and Hansen, 1975). Other commercial PCB's and PBB's cause similar reproductive toxicoses. Peakall (1975) reviewed the role of PCB's as a cause of eggshell thinning. However, this phenomenon has been related to exposure to a broad spectrum of

organochlorine environmental pollutants, including DDT and DDE. Herring gulls of the lower Great Lakes have experienced eggshell thinning, but the role of PCB's here has not been established (Gilbertson, 1983). The mechanisms by which PCB's and PBB's cause reproductive toxicities is unknown but may be associated with altered levels of steroid hormones or altered steroid metabolism, which may have a deleterious effect on reproduction and on the development of sexual characteristics (Lincer and Peakall, 1970; Nowicki and Norman, 1972; Platonow and Funnell, 1972).

Porphyrinogenic Effects

People exposed to halogenated aromatic hydrocarbons in industrial settings have frequently been affected by hepatic porphyria, a disorder characterized by altered porphyrin metabolism (Strik et al., 1979). Porphyrin compounds are synthesized by many enzyme-catalyzed steps in which delta-levulinic acid is converted to heme. Heme is, in turn, an important component of several enzymes, including cytochrome P-450-dependent mono-oxygenases.

Many reports clearly implicate PCB's and PBB's as porphyrinogenic in many animal species and various mammalian cells (Gupta et al., 1983a; Nonaka et al., 1979; Strik et al., 1979; Vos and Koeman, 1970; Vos and Notenboom-Ram, 1972; Fulfs and Abraham, 1976; Goldstein

et al., 1975; Strik, 1973, 1978; Vos et al., 1971). Acute administration of PCB's induced delta-aminolevulinic acid synthetase (d-ALAS) in rats and significantly increased total liver porphyrin concentrations within one week after exposure to the compounds (Grote et al., 1975). Chronic dietary exposure of female rats to PCB's also increased levels of d-ALAS. However, increases in hepatic and urinary porphyrins were not seen until several months after initial exposure to these toxins.

Commercial PBB's have similar porphyrinogenic activity in rodents, mice, and birds. The feeding of Firemaster BP-6 for six months to male and female rats and mice yielded a dose-dependent increase in hepatic porphyrins, and significant elevations were seen at doses as low as 0.3 mg/kg in male and female Fischer 344/N and male B6C3F1 mice (Gupta et al., 1983a).

The mechanism by which these compounds cause porphyria in laboratory animals has not been elucidated. Many environmental chemicals induce d-ALAS and inhibit uroporphyrinogenic decarboxylase. These effects may be major factors in the mechanisms of action of these toxins. Porphyria induced by polyhalogenated aromatic hydrocarbons is species-specific, with effects noted in adult rats, mice, human beings, and some avian species. Interestingly, young rats, mink, and guinea pigs are less susceptible or do not have this lesion at all

(Strik et al., 1979). There appears to be an association between species' susceptibility to hepatic lesions (such as hepatomegaly) and porphyria caused by PCB's and PBB's.

Metabolism of PCB's and PBB's

The PCB's, as well as many chlorinated organic pesticides, drugs, xenobiotics, and carcinogens, are potent inducers of hepatic and extrahepatic drug-metabolizing enzymes. Historically, microsomal enzyme inducers were exemplified by two main groups, specifically phenobarbital (PB) and 3-methylcholanthrene (3-MC) inducers (Conney, 1967; Gillette et al., 1972; Parke, 1975; Lu et al., 1976; Snyder and Remmer, 1979; Imai and Sato, 1966). Pretreatment of laboratory animals with chemicals which induce PB-type enzyme activity causes increased levels of hepatic and extrahepatic phase I (microsomal) and phase II (cytosolic and microsomal) drug metabolizing enzymes. These induced enzymes include several cytochrome P-450-dependent microsomal mixed function oxidase (MFO, or monooxygenase) enzymes, which catalyze N- and O-dealkylation, aromatic and aliphatic C-oxidation, glutathione S-transferases, glucuronyl transferases, and epoxide hydrolase. Additionally, 3-MC induces a similar range of enzyme activities. However, there are

differences in substrate and/or oxidation site specificities between PB- and 3-MC-induced microsomal enzymes. Several studies show that there are several microsomal cytochrome P-450 isoenzymes (Lu and Levin, 1974; Guengerich, 1979; Ryan et al., 1982). Many individual PCB isomers and congeners have PB- or 3-MC-type inducing properties. Therefore, the mixed type induction of enzymatic activity seen with PCB's is dependent on the activity of the individual congeners present within these mixtures.

Several experiments have shown that certain congeners of PCB's are metabolized by animals into hydroxylated intermediates (Hutzinger et al., 1972; Sundstrom et al., 1976; Safe, 1980). Furthermore, feeding of commercial PCB mixtures to laboratory animals has demonstrated the preferential excretion of the less chlorinated isomers (Burse et al., 1974; Burse, 1976). In addition, gas chromatographic and mass spectrometric analyses have demonstrated that the mono- and dihydroxylated PCB metabolites are eliminated in urine and feces (Safe et al., 1975). However, the pathologic effects of these metabolites has not been thoroughly examined.

Some PCB and PBB congeners are not metabolized to any appreciable extent even though they are potent enzyme inducers. The congeners 2,2',4,4',5,5'-hexabromobiphenyl and 3,3',4,4',5,5'-hexachlorobiphenyl

are examples of such agents (Dannan et al., 1978; Mills et al., 1985). Enzyme induction, toxicity, and the rate and type of metabolism appear to be correlated with the location of bromine or chlorine of the phenyl rings.

Results from in vitro studies indicate that certain PCB congeners are also metabolized by microsomal enzymes that require both oxygen and NADPH as cofactors for activity (Shimada, 1976; Shimada and Sato, 1978; Shimada et al., 1981). These in vitro studies demonstrated that specific activity of the microsomal enzymes was dependent not only on PCB-pretreatment of the animal from which the cells were obtained but also on substrate structure. The metabolism of commercial PCB mixtures was improved by the addition of PB and 3-MC microsomal enzymes. Shimada and co-workers (1981) have shown that PCB's are also metabolized by cytochrome P-450 from rabbit and rat livers and that the PB-inducible enzymes from these species were the more potent metabolic catalysts. The metabolism of PCB's includes hydroxylation (i.e., Phase I metabolism), conjugation with thiols and other water soluble derivatives, and binding to macromolecules, especially proteins (Furukawa and Matsumura, 1976; Furukawa et al., 1979).

The effects of PCB's as inducers of rat hepatic drug-metabolizing enzymes have been rigorously studied. These inductive effects occur in livers of males and females, fetal, neonatal, immature, mature, and

senescent rats. Moreover, PCB-induced rat microsomal enzymes readily metabolize other polycyclic aromatic hydrocarbons (Jacob et al., 1981; Gingell et al., 1981; Biggar et al., 1980; Jacob et al., 1982).

The metabolism of PBB preparations, such as Firemaster BP-6, has not been fully elucidated, but compounds in the mixture are preferentially eliminated after dietary administration to rodents (Wolff and Selikoff, 1979). In addition, gas chromatographic analyses of PBB's in human tissues indicate that specific components within the Firemaster mixture are degraded (Wolff and Aubrey, 1978).

Dent and co-workers first demonstrated that Firemaster BP-6 was a mixed type (i.e., PB and 3-MC) microsomal enzyme inducer in rats (Dent et al., 1976, 1978a). Like PB given with 3-MC, and similar to commercial PCB's, the commercial PBB's induce several MFO enzymes, including N-demethylases, O-dealkylases, PAH-hydroxylases, and steroid hydroxylases, as well as several phase II metabolic enzymes. The commercial PBB mixtures induce drug-metabolizing enzymes in many animal species and in both liver and nonliver tissues (Dent et al., 1977a, 1977b, 1978b; Safe et al., 1978; Moore et al., 1978a, 1978b; McCormack et al., 1978; Kluwe and Hook, 1981).

Preneoplastic Hepatic Changes as End Points for Carcinogenicity Assays

Historically, the assessment of cancer risk of certain compounds has depended mainly on traditional histopathologic examination. The accepted end point in carcinogenicity testing is the histologically identified tumor. One disadvantage of this end point is the lengthy time period for these tumors to occur in the animal. Therefore, many efforts have been made to detect early biochemical or morphological markers which may be predictive of cancer lesions. During the past two decades, a number of characteristic cellular changes that regularly precede the development of tumors has been observed, especially in the liver. Such changes have been designated as "preneoplastic lesions." These altered areas of preneoplastic cells usually form well-delineated foci and appear prior to the development of tumors. Hepatic preneoplasia has been exhaustively studied, especially in rats and mice, using various models (Peraino et al., 1983; Farber, 1984a; Ward, 1984). In rats, potentially preneoplastic hepatic foci are used as end points in carcinogenicity testing.

A preneoplastic cell may be defined as a phenotypically altered cell which has no observable neoplastic nature, but which has a greater than normal

chance of becoming a benign or malignant tumor. Some workers contend that hyperplasia can be regarded as an early stage in tumor development (Farber and Cameron, 1980). But the use of the term "hyperplasia" in the context of carcinogenesis becomes confusing. By definition, the term hyperplasia is an increase in the number of tissue-specific cells caused by extracellular growth-stimulating factors (such as hormones), while the term "neoplasia" implies an autonomous increase in the number of cells independent from such extracellular stimuli. A dilemma for pathologists is that early stages of neoplastic change may be characterized by a proliferation of cells which cannot be histologically distinguished from normal cells. While these lesions have the potential to become tumors without additional exposure to carcinogens or growth-stimulating factors, they are often classified as hyperplastic. It is probable that proliferating chemically-induced precursor lesions, whether classified as hyperplastic or preneoplastic, are already composed of irreversibly altered cells (Symposium of Rodent Liver Nodules, 1982).

Several phenotypic patterns are seen histologically prior to the appearance of chemically-induced hepatic adenomas (also called "neoplastic nodules") and carcinomas in rats (Sasaki and Yoshida, 1935; Firminger, 1955; Reuber, 1965; Schauer and Kunze, 1976). Treatment of rats with nitrosamines induced focal hepatic lesions

characterized by excessive storage of glycogen (Bannasch, 1968). Others have shown a reduction in activity of the microsomal enzyme glucose-6-phosphatase (Gossner and Friedrich-Freska, 1964; Friedrich-Freska et al., 1969). Results of light microscopic and ultrastructural studies have shown that rats treated with certain diethylnitrosamines underwent a series of hepatic changes. First, clear to acidophilic glycogen-filled hepatocytes were seen. Later, basophilic cells were seen that were low in glycogen (Bannasch, 1968). These cells represent "altered foci" which persist after withdrawal of the carcinogen and may progress to adenomas and hepatocellular carcinomas (Schauer and Kunze, 1968; Scherer, 1984). The classification of "foci of altered hepatocytes" is seen as different from "neoplastic nodules" by several groups (Squire and Levitt, 1975; Stewart et al., 1980; Bannasch et al., 1985).

Altered hepatic foci may develop spontaneously in aged untreated rats. This may be due to small amounts of carcinogens in food or in the environment (Burek, 1978; Ward, 1984). Certain rat strains have an unusually high incidence of spontaneous altered hepatic foci, suggesting a genetic predisposition toward their development (Ward, 1981).

Many biochemical markers are used to identify carcinogen-induced altered foci in rat hepatocytes.

Examples of enzymes which show a decreased activity in such foci include glucose-6-phosphatase, membrane-bound adenosine triphosphatase (Schauer and Kunze, 1968), acid and alkaline nucleases (Taper et al., 1971; Taper et al., 1983), and glycogen phosphorylase (Scherer and Emmelot, 1976; Hacker et al., 1982). Enzymes that increase in activity in these foci include gamma-glutamyl-transpeptidase (GGT) (Kalengayi and Desmet, 1975; Hanigan and Pitot, 1985), glucose-6-phosphate dehydrogenase (Hacker et al.,1982; Klimek et al., 1984), epoxide hydrolase (Enomoto et al., 1981; Kuhlman et al., 1981), uridine-diphosphate-glucuronyl transferase (Fischer et al., 1983; Sato et al., 1984), various isoenzymes of cytochrome P-450 (Schulte-Hermann et al., 1984; Buchmann et al., 1985), and glutathione transferases (Sato et al., 1984; Buchmann et al., 1985). Other alterations in preneoplastic hepatic tissue include resistance to experimental hemosiderosis (Williams et al., 1976; Williams and Watanabe, 1978), increased glutathione activity (Deml and Oesterle, 1980), and diminished lipid peroxidation (Benedetti et al., 1984).

Of special interest is GGT. It has been considered a reliable indicator to assess preneoplastic changes in the liver. (Hanigan and Pitot, 1985). However, others have found it to be lacking in some types of preneoplastic foci in rat liver (Butler et al., 1981;

Moore et al., 1983; Rao et al., 1984; Bannasch et al., 1985). Furthermore, an increase in the amount of GGT in periportal areas of the liver has been reported to occur with increasing age of the rat (Kitigawa et al., 1980a) or after partial hepatectomy (Bone et al., 1985). Some workers favor glutathione S-transferase placental form (GST-P) as a marker because it may be more accurate than GGT (Sato et al., 1984; Tatematsu et al., 1985; Thamavit et al., 1985).

Most phenotypic markers used for identifying preneoplastic cells are not stable. This represents a continuing problem in the evaluation of preneoplastic cellular changes. Therefore, given certain experimental conditions, these foci may phenotypically resemble persistent preneoplastic lesions, but may disappear after termination of the treatment. This phenomenon has been called "reversion-linked phenotypic instability" (Bannasch et al., 1985). Other changes that describe reversion-linked phenotypic instability of carcinogen-induced focal hepatic lesions have been described by numerous authors as "reversion," "remodeling," "neodifferentiation," or "maturation". (Kitigawa, 1971, 1976; Farber, 1976; Ito et al., 1976; Williams and Watanabe, 1978; Ogawa et al., 1979; Tatematsu et al., 1983). It is of interest that phenobarbital leads to a

more stable expression of altered hepatic foci. However, this is currently poorly understood (Moore et al., 1984).

Farber (1984b) has stated that 95-98% of the chemically-induced nodular hepatic lesions are reversible, while only 1-3% persist and may progress to hepatocellular carcinomas. To definitively determine if such nodules will persist, some workers have recommended that the administration of the test compound should be stopped before termination of the experiment whenever foci with a disputed significance develop (Bannasch et al., 1982).

The morphological transitions between altered hepatic foci, hepatic adenomas, and hepatocellular carcinomas have been addressed by several workers (Farber, 1973; Williams and Watanabe, 1978; Bannasch et al., 1982). These observations indicate that altered foci give rise to adenomas and that these, in turn, may progress to hepatocellular carcinomas. However, it is also probable that carcinomas can develop directly from altered foci without going through an intermediate adenomatous stage (Bannasch, 1976; Williams, 1976).

Studies assessing cellular functional changes also indicate a close correlation between hepatic altered foci, nodules or adenomas, and hepatocellular carcinomas. Several workers have demonstrated a decrease or increase in the activity of many enzymes using

enzyme histochemical methods in these lesions (Farber, 1980; Pitot and Sirica, 1980; Williams, 1980). Others have shown in both rats and mice that hepatocellular foci, nodules, and carcinomas do not accumulate iron in experimentally produced hepatic siderosis (Lipsky et al., 1979; Williams et al., 1979; Nigam et al., 1981).

There appears to be a dose-dependent relationship for the induction of altered foci in rat livers. Several studies indicate that quantitative correlations occur between the size and number of foci and the dose or duration of treatment with the carcinogen. However, some have questioned this relationship due to large discrepancies between the number of foci appearing early during hepatocarcinogenesis and final tumor yield (Scherer and Emmelot, 1975; Emmelot and Scherer, 1980; Scherer, 1984; Kaufman et al., 1985). These data may suggest that only a small number of foci have the potential for progression to malignancy.

An elementary interpretation of carcinogen testing using rat liver is generally agreed upon. If a test compound induces significantly more altered hepatic foci in treated animals than in untreated control animals, then the test chemical has carcinogenic potential. Because of possible reversion-linked phenotypic instability of these lesions, "stop" experiments may allow a better distinction between reversible and irreversible preneoplastic lesions (Bannasch et al.,

1982). All hepatocarcinogens tested to date have induced some level of focal hepatic lesion prior to the development of hepatic tumors. However, it remains unclear if this is always true. Therefore, the absence of hepatic foci after the administration of a test chemical does not necessarily preclude potential carcinogenicity (Bannasch et al., 1982).

Initiation and Promotion in Carcinogenesis

The process of carcinogenesis consists of multiple steps. The concepts of initiation and promotion are central to an understanding of chemically-induced carcinogenesis. Initiation is defined as an irreversible event involving a biochemical change in DNA. Cell proliferation is required to "fix" the biochemical lesions in DNA, and the altered DNA becomes a permanent property of the cell and its progeny (Craddock, 1976; Cairne, 1975; Ying et al., 1975). Nearly all initiators are in the form of procarcinogens and must be metabolically activated, usually by the cytochrome P-450 dependent monooxygenase system, to a form that has a high affinity for the genome (Czygan et al., 1973; Guengerich, 1977; Miller and Miller, 1969). However, some initiators are direct-acting and may cause alkylation or acylation of DNA without prior metabolic activation (Miller and Miller, 1981). There is cogent

evidence that mutation is a major consequence of initiation (Quintanilla et al., 1986).

Promotion is a reversible epigenetic event that causes preferential mitogenic selection of initiated cells to become phenotypically similar to neoplastic cells upon repeated exposure to the promoter. The process of initiation may predispose a cell to the effects of promotion, but promoters generally have little tumorigenic effect on non-initiated cells. Complete carcinogens are defined as compounds that have both initiating and promoting ability.

Historically, early researchers were able to distinguish between chemicals that were initiators or promoters (Rous and Kidd, 1941; Mottram, 1944; Berenblum, 1941). Classically, induction of mouse skin tumors was used to show that small doses of an initiator followed by repeated doses of a promoter caused papillomas first and carcinomas later (Berenblum and Shubik, 1949). The administration of only a single dose of initiator did not cause tumors, nor did repeated administrations of only a promoter (Boutwell, 1964). Moreover, tumors did not develop if the promoter was applied first followed by application of initiator (Williams et al., 1981). Currently, many initiation-promotion assays are used involving several different organ systems, including liver, thyroid gland, lung, colon, skin, mammary gland, stomach, kidney, pancreas,

urinary bladder, and thymus gland (Berenblum, 1979; Hicks et al., 1975, 1977; Leonard et al., 1982; Miyata et al., 1985).

In Vivo Carcinogenic Effects of PBB's and PCB's

The carcinogenicity of PBB's and PCB's is dependent upon several factors including the sex, strain, and species of the test animal as well as the composition of the commercial PBB or PCB formulation. Generally, these compounds are not regarded as initiators (i.e., genotoxins). However, several reports using different carcinogenicity testing systems indicate that PBB's and PCB's have promoting (i.e., epigenetic) activity (Kimura and Baba, 1973; Aishizumi, 1976; Kimura et al., 1976; Pereira et al., 1982; Deml and Oesterle, 1982; Hirose et al., 1981; Jensen et al., 1982a, 1982b; Preston et al., 1981; Jensen and Sleight, 1986). Strong evidence that PBB's or PCB's cause cancer in people is lacking (Brown and Jones, 1981; Stross et al., 1981).

Commercial PBB and PCB mixtures and specific congeners of PBB's and PCB'c have been identified as tumor promoters. Firemaster BP-6 has been shown to enhance the development of enzyme-altered foci in DEN-initiated partially hepatectomized rats (Jensen et al., 1982a, 1983). Using a similar assay, the PBB congener 2,2',4,4',5,5'-hexabromobiphenyl, the major congener of

Firemaster BP-6, caused increased numbers of enzyme-altered foci when compared to controls. (Jensen et al. 1982a).

Another PBB congener, 3,3',4,4',5,5'-hexabromobiphenyl was found to be hepatotoxic (Render et al., 1982) as well as a tumor promoter when fed at a concentration of 1.0 mg/kg to DEN-initiated partially hepatectomized rats (Jensen et al., 1982b; Jensen et al., 1983). This congener may have a different mechanism of tumor promoting action than the nonhepatotoxic congener 2,2',4,4',5,5'-hexabromobiphenyl (Jensen et al., 1983).

Simultaneous exposure to more than one PBB congener has been shown to have a synergistic effect on tumor promotion. Initiated and partially hepatectomized rats fed a combination of 10 mg/kg 2,2',4,4',5,5'-hexabromobiphenyl plus 0.1 mg/kg 3,3'4,4',5,5'-hexabromobiphenyl had a more than additive number of enzyme-altered foci and hepatic nodules when compared to similarly treated rats fed either one congener or the other (Jensen and Sleight, 1986). Interestingly, Firemaster BP-6 had a greater ability to enhance development of enzyme-altered foci than 2,2'4,4',5,5'-hexabromobiphenyl, the major congener of the Firemaster BP-6 mixture (Jensen et al., 1982b). These results suggest that additive or synergistic interactions of congeners in this commercial mixture may have been responsible for its greater tumor promoting ability.

Two-Stage Models for Hepatic Tumor Induction in Rats

Carcinogen-Promoter Model

Peraino and co-workers (1971, 1973a, 1973b, 1977) introduced this model in which rats were given 2-acetylaminofluorene (2-AAF) for 18 days. One week later, rats were fed a diet containing 0.05% phenobarbital, a known tumor promoter in rats, for eight months. Test chemicals could be assessed by substituting either the initiator or promoter. For example, in place of 2-AAF, diethylnitrosamine (Weisberger et al., 1975), 3'-methyl-4-dimethylaminobenzene (Kitigawa and Sugano, 1978), and 2-methyl-4-dimethylaminoazobenzene (Kitigawa et al., 1979) have been used as alternative initiators. This model provided a new approach to analysis of the promoting effects of chemicals.

Partial Hepatectomy + Promotion Model

This model was proposed as a combination of the two stages of carcinogenesis (Pitot, 1977; Pitot et al., 1978a, 1978b). Rats are given a single dose of DEN by intubation 24 hours after partial hepatectomy. Eight weeks later, a group of the animals are given phenobarbital (0.05%) for 24 weeks as a positive control. Modifications of this model include using

benzo(a)pyrene as an initiator (Kitigawa et al., 1980b). Phenobarbital can be replaced with a choline-deficient diet for the promotional phase (Shinozuka et al., 1979). Another modification uses dimethylhydrazine as the initiator and orotic acid as the promoter (Laurier et al., 1984). Results from these studies helped to clarify the conceptual distinction between the two stages of liver carcinogenesis in rats.

Selective Pressure Model

The selective pressure model was introduced by Solt and Farber (1976, 1977). The initiator was DEN injected intraperitoneally, and two weeks later animals were placed on a basal diet containing 0.02% 2-AAF. After one week of 2-AAF feeding, the DEN-treated animals were partially hepatectomized. One week thereafter, animals were returned to the carcinogen-free basal diet for eight months. The advantage of this model is the relatively rapid and marked induction of enzyme-altered foci. Furthermore, these foci are essentially synchronous and, therefore, are easy to follow for tumor sequence studies.

MATERIALS AND METHODS

Protocol

The Pitot model of two-stage (initiation/promotion) hepatocarcinogenesis was used for this study (Pitot et al., 1978). Outbred female Sprague-Dawley rats (Charles River Corporation, Portage, MI) weighing about 190-210 grams were acclimated for one week. Rats that were to be hepatectomized were anesthetized with ether (Mallinckrodt Inc., Paris, KY), and two-thirds of the liver was ligated and removed (Higgins and Anderson, 1931). Twenty-four hours later, diethylnitrosamine (DEN) (Sigma Chemical Co., St. Louis, MO) was administered intraperitoneally at a dose of 10 mg/kg body weight. Thirty days after the partial hepatectomy, rats were randomly assigned into groups (Table 1-2, page 47). Diets were prepared by adding phenobarbital (PB), 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB), or 3,3', 4,4',5,5'-hexachlorobiphenyl (345-HCB) in corn oil to a commercial ground diet for rats (Wayne Lab Blox, Allied Mills, Inc., Chicago, IL). Amounts of each compound in the diet are listed in Table 1-2. Water was available

the diet are listed in Table 1-2. Water was available free choice. Negative controls included rats not hepatectomized or given DEN but maintained either on the same basal diet or treatment diets. Rats were kept in standard clear plastic cages within laminar flow units (Contamination Control Inc., Lansdale, PA) at 22^0 C with a 12 hour light/dark cycle.

Test Chemicals

The 245-HBB used in this study was isolated from a commercial PBB mixture (Firemaster BP-6, Michigan Chemical Co., St. Louis, MI). The 345-HCB was obtained from a commercial laboratory (RFR Corporation, Hope, RI). Isolation of 245-HBB and purification of both congeners was performed using chromatographic methods by personnel in the Department of Biochemistry, Michigan State University (Moore and Aust, 1978). Greater than 99% purity of each congener was obtained.

Necropsy, Tissue Collection, and Histologic Techniques

Rats were maintained on diets for 150 days, after which they were anesthetized by ether (Mallinckrodt Inc., Paris, KY), killed using decapitation, and necropsied. The brain, kidneys, spleen, liver, thymus gland, and thyroid glands were removed and weighed (Mettler Instrument Corp., Highstown, NJ). Five sections of liver, taken from the same portions of hepatic lobes from each rat, were mounted on corks (Slee International

Inc., London, England) and frozen by immersion into isopentane cooled with liquid nitrogen. Representative sections of liver for histological evaluation were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at a thickness of six μm , and stained with hematoxylin and eosin. Other samples of liver and body fat were collected for chemical analysis, wrapped in aluminum foil, and stored at -20 ^{0} C. Sections of liver for microsomal enzyme analysis were placed into cold 1.15% KCl containing 0.2% nicotinamide.

Histochemical Staining

Frozen and cork-mounted sections of liver were cut at a thickness of eight μm using a cryostat (Slee International Inc., London, England) and stained for gamma glutamyl transpeptidase (GGT) activity (Rutenberg et al., 1969). The eight μm sections of liver were placed on a cover glass, fixed in 100% acetone for 15 minutes, air-dried, and incubated for 15 minutes in a solution of one ml of gamma-glutamyl-4-methoxy-2-naphthylamide (2.5 mg/ml) (Vega Biochemicals, Tuscon, AZ), five ml of 0.1 M Tris buffer at pH 7.4 (Sigma Chemical Co., St. Louis, MO), 10 mg glycylglycine (Sigma Chemical Co., St. Louis, MO), 10 mg Fast Blue BBN (Sigma Chemical Co., St. Louis, MO), and 14 ml of 0.85% saline solution. The section was then washed in 0.85% saline for two minutes and transferred to a 0.1 M cupric

sulfate solution (Sigma Chemical Co., St. Louis, MO) for another two minutes. The section was washed a second time for two minutes with 0.85% saline, rinsed in distilled water, counterstained with hematoxylin (Gills Hematoxylin No. 3, Polysciences Inc., Warrington, PA) for 15 minutes, air-dried, and mounted on a glass slide.

The histochemically stained slide was placed onto a Leitz Prado Projector (Ernst Leitz Wetzlar GMBH, Wetzlar, West Germany), magnified 90 X, and outlines of GGT-positive foci were traced. An approximately equal area of liver was evaluated (2.5-3.5 cm^2) from each rat. The area of each GGT-positive focus was determined with a planimeter (Lasico L-30, Los Angeles Scientific Co., Inc., Los Angeles, CA), and the number of GGT-positive foci/cm^3 was obtained by using the formula of Scherer (1981).

Microsomal Enzyme Assays

Hepatic microsomes were isolated and stored by established techniques (Moore et al., 1978; Welton and Aust, 1974) by personnel in the Department of Biochemistry, Michigan State University. Liver in cold KCl was weighed, homogenized, and centrifuged once at 10,000 xg for 20 minutes, followed by 90 minutes at 105,000 xg, using the supernatant of the first centrifugation for the second centrifugation. Microsomes were washed and stored at -20 ^{0} C in 0.05 M Tris-HCl at

pH 7.5 with 50% glycerol and 0.01% butylated hydroxytoluene.

Analysis for 245-HBB and 345-HCB in Liver and Fat

Concentrations of 245-HBB and 345-HCB in liver and body fat were determined using a technique by Thompson (1977) by personnel in the Department of Pathology, Michigan State University. Liver and fat samples from two rats within each group were analyzed. Samples of 0.5 grams were washed with petroleum ether, ground with washed ignited sand (Mallinckrodt Inc., Paris, KY), and dehydrated by adding 10-20 grams of granular anhydrous sodium sulfate (Mallinckrodt Inc., Paris, KY). For determining 245-HBB concentrations, fat and liver samples had 15 ml of hexane distilled in glass (J.T. Baker Chemical Co., Phillipsburg, NJ) added, and the mixture was brought to a boil over an 80 ^{0} C water bath. The mixture was then filtered into a flask. Hexane washes and further filtrations were repeated for a total of four extractions. Tissues for 345-HCB analysis were treated similarly except that extraction was done with toluene distilled in glass (J.T. Baker Chemical Co., Phillipsburg, NJ) instead of hexane.

The sample was added to a column filled with 1.5 grams of activated magnesium silicate (Florisil, 60-100 mesh, Fischer Scientific Co., Cleveland, OH) topped with two cm of granular anhydrous sodium sulfate. The

columns had been prewashed with acetone followed by hexane. After adding the sample, the column was repeatedly washed with hexane. The eluant was condensed to 0.5 ml and 2,2,4-trimethylpentane (Burdick and Jackson Laboratories, Inc., Muskegon, MI) was added to create a total volume of two ml.

A volume of two µl of eluant was injected into a gas chromatograph (GC Model 3700, Varian Instrument Division, Palo Alto, CA). For 245-HBB, injector temperature was 280 ^{0} C, column temperature was 250 ^{0} C, and detector temperature was 310 ^{0} C. For 345-HCB, column temperature was 300 ^{0} C and detector temperature was 350 ^{0} C. The carrier gas was nitrogen at a rate of 30 ml/minute. Tracings from the gas chromatograph were recorded and compared to standards.

Concentrations of 245-HBB and 345-HCB were determined from lipid samples using a 20 ml aliquot of each hexane or toluene extracted sample, respectively. The solvent was evaporated and the sample was placed in a preweighed foil container and dried under vacuum. Following drying, the remaining lipid was weighed, and the percentage of lipid in the original sample was determined.

Statistical Analysis

Data were analyzed using the one-way analysis of variance (Steel and Torrie, 1980a). Multiple

comparisons were analyzed using a Student-Newman-Keul's test (Steel and Torrie, 1980b). Differences between groups were considered significant at the $P \leq 0.05$ level.

RESULTS

Body Weight Gains and Organ Weights

The effects of diets containing 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB), 3,3',4,4',5,5'- hexachlorobiphenyl (345-HCB), and combined 245-HBB/345-HCB on body weight gain, thymic weight, and hepatic weight are shown in Table 1-1. Rats that were DEN-initiated, partially hepatectomized, and fed diets containing 1.0 mg/kg 345-HCB or a combination of 100 mg/kg 245-HBB plus 1.0 mg/kg 345-HCB had a significant decrease in total body weight gain when compared to similarly treated rats fed a basal diet. Hepatic weights were significantly increased in DEN-initiated, partially hepatectomized rats fed diets containing 100 mg/kg 245-HBB or 1.0 mg/kg 345-HCB, as well as in those rats fed a combination of 100 mg/kg 245-HBB and 1.0 mg/kg 345-HCB. No significant changes in the weights of brain, thymus gland, kidneys, thyroid glands, or spleen were found in treated rats when compared to control rats.

Histopathologic Evaluation of the Liver

Livers from rats fed diets containing 500 mg/kg of phenobarbital or 10 mg/kg 245-HBB had hypertrophy of

Table 1-1. Effects of Dietary 245-HBB and 345-HCB on body weight gains, thymic weight, hepatic weight, and histologic structure of livers of rats.

Chemical mg/kg diet	Body wt. gain	Absolute thymus wt.	Absolute liver wt.	Histology of liver
Basal diet	88±47	.33±.10	7.5±1.0	Normal
500mg PB[a]	74±17	.24±.03	8.7±1.4	Hepatocyte hypertrophy in CL[b] region
10mg 245-HBB	87±16	.28±.08	8.8±1.0	Hepatocyte hypertrophy in CL region
100mg 245-HBB	75±21	.21±.05	10.1±2.1[c]	Hepatocyte hypertrophy in CL region; altered foci
0.1mg 345-HCB	89±17	.24±.03	8.1±1.2	Mild macro/ microvesiculation; altered foci
1.0mg 345-HCB	73±24[c]	.30±.09	13.9±2.7[cd]	Moderate macro/micro-vesiculation; inflammation; altered foci
10mg 245-HBB+ 0.1mg 345-HCB	75±29	.24±.06	7.5±0.8	Moderate macro/micro-vesiculation; inflammation; altered foci
100mg 245-HBB+ 1.0mg 345-HCB	53±18[c]	.21±.07	11.3±4.9[c]	Severe macro/ microvesiculation; inflammation; altered foci

Data in grams as mean ± SD for 6 rats. Rats had partial hepatectomy and diethylnitrosamine 30 days before dietary treatment. [a] Phenobarbital. [b] Centrolobular. [c] Significant difference ($P \leq 0.05$) from basal diet group. [d] Significant difference ($P \leq 0.05$) from all groups except group fed 100 mg/kg 245-HBB plus 1.0 mg/kg 345-HCB.

hepatocytes in centrolobular regions of hepatic lobules. Histologically altered foci and hepatocytic hypertrophy in centrolobular regions were seen in sections of liver from rats fed 100 mg/kg 245-HBB. Livers from rats fed diets containing 0.1 mg 345-HCB had mild diffuse microvesicular and macrovesicular changes (presumably due to fatty change) and occasional altered foci. Livers from rats given 1.0 mg/kg 345-HCB or a combination of 10 mg/kg 245-HBB plus 0.1 mg/kg 345-HCB had moderate micro- and macrovesicular changes, moderate hepatocyte hypertrophy in centrolobular regions, and occasional altered foci (Figure 1-1). Hepatic changes were the most severe in rats fed a combination of 100 mg/kg 245-HBB with 1.0 mg/kg 345-HCB. Livers from these rats had severe micro- and macrovesiculation, severe hepatocyte hypertrophy in centrolobular regions, mild to moderate inflammatory changes in periportal areas, multifocal altered foci (Figure 1-2), and mild hyperplasia of biliary epithelium.

Preneoplastic changes seen in livers from these rats included foci of altered cells (Institute of Laboratory Animal Resources, National Research Council, 1980). These islands of hepatocytes had cells which were mildly basophilic and slightly enlarged with abundant cytoplasm. They contained enlarged and occasionally

Figure 1-1. Photomicrograph of the centrolobular and midzonal region of the liver from a rat fed a diet containing 1.0 mg/kg 345-HCB for 150 days after a partial hepatectomy and administration of 10 mg/kg diethylnitrosamine intraperitoneally. Notice diffuse hepatocyte hypertrophy, macrovesicular and microvesicular changes, loss of sinusoidal space, and mild inflammatory cell infiltrates (H & E, 160 X).

Figure 1-2. Photomicrograph of a focus of hepatocellular alteration from a rat fed a diet containing a combination of 100 mg/kg 245-HBB plus 1.0 mg/kg 345-HCB for 150 days after a partial hepatectomy and administration of 10 mg/kg diethylnitrosamine intraperitoneally. The hepatocytes in this focus have hypertrophied, and a cell in the center of the focus is trinucleate. Notice the macrovesicular and microvesicular changes in the surrounding parenchyma (H & E stain, 160 X).

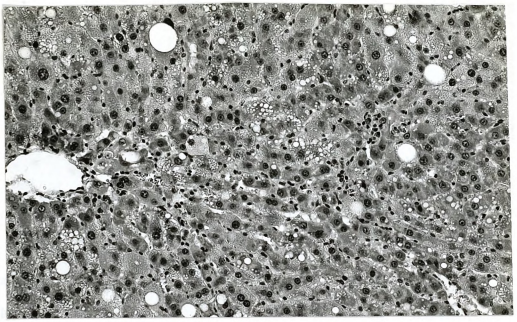

Figure 1-1

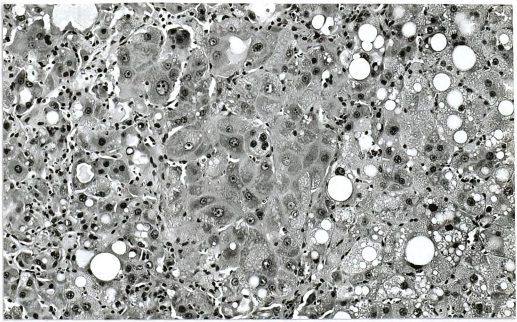

Figure 1-2

multiple nuclei (Figure 1-2), often with multiple nucleoli. One hepatic nodule was seen in a rat given 500 mg/kg phenobarbital, but hepatic nodules were not observed in rats fed 245-HBB or 345-HCB. Hepatocellular carcinomas were not observed in any rats in this study.

GGT-positive Foci

The numbers of GGT-positive foci for rats in each group are shown in Table 1-2. The typical appearance of a GGT-positive focus is seen in Figure 1-3. Rats neither partially hepatectomized nor given DEN that were then fed diets containing 245-HBB, 345-HCB, or a combination of 245-HBB with 345-HCB had fewer GGT-positive foci when compared to partially hepatectomized and DEN-initiated rats fed the same diets. Rats that were partially hepatectomized, given DEN, and fed either 245-HBB, 345-HCB, or a combination of 245-HBB with 345-HCB had significantly greater GGT-positive foci/cm^3 in their livers when compared to other DEN-treated partially hepatectomized rats fed only a basal diet.

The DEN-treated partially hepatectomized rats with the greatest number of GGT-positive foci were fed a combination of 10 mg/kg 245-HBB and 0.1 mg/kg 345-HCB. The number of GGT-positive foci for this group was nearly a summation of the GGT-positive foci for rats fed

Table 1-2. Experimental Design and Number of GGT[c]-positive Foci per Cubic Centimeter of Liver.

Group No.	Treatment	Chemical mg/kg diet	No. rats per group	GGT[+] foci/cm^3 mean ± SD
1	PH[a] + DEN[b]	Basal diet	6	48±28
2	None	Basal diet	3	0±0
3	PH + DEN	500 PB [d]	6	2840±1297 [e]
4	PH + DEN	10 mg 245-HBB	6	1695±1800 [e]
5	None	10 mg 245-HBB	3	66±38
6	PH + DEN	100 mg 245-HBB	6	1146±536 [e]
7	None	100 mg 245-HBB	3	61±40
8	PH + DEN	0.1 mg 345-HCB	6	295±192 [e]
9	None	0.1 mg 345-HCB	3	9±7
10	PH + DEN	1.0 mg 345-HCB	6	1343±1090 [e]
11	None	1.0 mg 345-HCB	3	76±46
12	PH + DEN	10 mg 245-HBB+ 0.1 mg 345-HCB	6	1852±629 [e,f]
13	None	10 mg 245-HBB+ 0.1 mg 345-HCB	3	61±35
14	PH + DEN	100 mg 245-HBB+ 1.0 mg 345-HCB	6	612±220 [e,g]
15	None	100 mg 245-HBB+ 1.0 mg 345-HCB	3	87±69

[a] Partial hepatectomy. [b] Diethylnitrosamine. [c] Gamma glutamyl transpeptidase. [d] Phenobarbital. [e] Significantly different ($P \leq 0.05$) from group 1. [f] Significantly different ($P \leq 0.05$) from group 8. [g] Significantly different ($P \leq 0.05$) from group 12.

Figure 1-3. Photomicrograph of a histochemically stained section of liver from a rat fed a diet containing 10 mg/kg 245-HBB for 150 days after a partial hepatectomy and administration of 10 mg/kg diethylnitrosamine intraperitoneally. Notice the well-defined focus of hepatocytes that have positive staining for gamma glutamyl transpeptidase activity in their cytoplasms (Gamma glutamyl transpeptidase stain, 160 X).

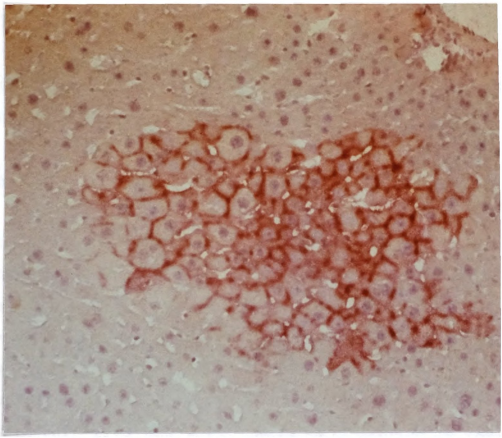

Figure 1-3

10 mg/kg 245-HBB alone and rats fed 0.1 mg/kg 345-HCB alone. However, rats receiving a combination of 100 mg/kg 245-HBB plus 1.0 mg/kg 345-HCB had only about half as many GGT-positive foci when compared to rats receiving either 100 mg/kg 245-HBB alone or 1.0 mg/kg 345-HCB alone.

Concentrations of 245-HBB and 345-HCB in Liver and Fat

Concentrations of 245-HBB and 345-HCB in liver and adipose tissue are shown in Table 1-3. Rats receiving exclusively 100 mg/kg of 245-HBB in the diet had approximately seven and 11 times more 245-HBB in their livers and adipose tissue, respectively, than rats receiving 10 mg/kg 245-HBB. Similarly, rats receiving exclusively 1.0 mg/kg 345-HCB in the diet had approximately five and eight times more 345-HCB in their livers and adipose tissue, respectively, than rats receiving 0.1 mg/kg 345-HCB. The amount of 245-HBB was similar in livers of rats fed either 10 mg/kg 245-HBB alone or 10 mg/kg 245-HBB in combination with 0.1 mg/kg 345-HCB. Likewise, the amount of 245-HBB was similar in adipose tissue of rats fed either 10 mg/kg 245-HBB alone or 245-HBB in combination with 0.1 mg/kg 345-HCB. Generally, 245-HBB reached higher concentrations in adipose tissue than in liver, regardless of whether the

Table 1-3. Concentrations of 245-HBB and 345-HCB in Liver and Adipose Tissue of Rats Fed a Basal Diet or Diets Containing Phenobarbital, 245-HBB, 345-HCB, or combined 245-HBB/345-HCB for 150 Days.

Chemical (mg/kg) in diet	Tissue Concentration (mg/kg)[a]					
	Liver			Adipose Tissue		
	% Fat	HBB[b]	HCB[c]	% Fat	HBB	HCB
Basal diet	4±1	0±0	0±0	82±2	0±0	0±0
500 mg PB[d]	4±1	0±0	0±0	84±1	0±0	0±0
10 mg 245-HBB	6±2	12±1	0±0	83±3	300±94	0±0
100 mg 245-HBB	3±1	88±39	0±0	71±9	3117±1252	0±0
0.1 mg 345-HCB	6±1	0±0	7±1	81±2	0±0	3±2
1.0 mg 345-HCB	11±2	0±0	38±29	77±1	0±0	25±5
10 mg 245-HBB plus 0.1 mg 345-HCB	4±1	9±3	4±1	81±4	289±92	2±1
100 mg 245-HBB plus 1.0 mg 345-HCB	17±6	1283±442	42±4	74±7	4220±1149	37±5

[a] Values are expressed on lipid basis and represent the mean ± SD for 3 rats, 2 of which received a partial hepatectomy plus 10 mg/kg body weight diethylnitrosamine intraperitoneally. [b] 2,2',4,4',5,5'-hexabromobiphenyl. [c] 3,3',4,4',5,5'-hexachlorobiphenyl. [d] Phenobarbital.

concentration of 245-HBB in the diet was 10 mg/kg or 100 mg/kg. Conversely, 345-HCB reached relatively higher concentrations in hepatic tissue than in adipose tissue regardless of the concentration of 345-HCB (0.1 mg/kg or 1.0 mg/kg) in the diet.

Microsomal Enzyme Assays

Concentrations of hepatic cytochrome P-450 and activities of hepatic enzymes aminopyrine demethylase and ethoxyresorufin-o-deethylase are shown in Table 1-4. Rats given 245-HBB alone or 345-HCB alone in the diet had an apparent dose-related increase in the concentration of cytochrome P-450. Rats fed diets containing 345-HCB either alone or in combination with 245-HBB had a downward shift in carbon monoxide difference spectra of cytochrome P-450 when compared to rats fed either the basal diet or 245-HBB. When compared to rats fed a basal diet, aminopyrine demethylase activity was most increased in rats fed 100 mg/kg 245-HBB alone and was somewhat increased in those rats fed a combination of 100 mg/kg 245-HBB with 1.0 mg/kg 345-HCB. Ethoxyresorufin-o-deethylase activity was increased in rats fed 1.0 mg/kg 345-HCB alone or in combination with 100 mg/kg 245-HBB. The activity of this enzyme was relatively low in rats fed only a basal diet, 10 mg/kg 245-HBB, or 100 mg/kg 245-HBB.

Table 1-4. Effects of 245-HBB and 345-HCB on the Concentration of Cytochrome P-450 and the Activity of Aminopyrine Demethylase and Ethoxyresorufin-o-deethylase in Rat Liver.

Chemical (mg/kg) in diet	Cytochrome P-450[a]	alpha max[b]	Aminopyrine demethylase[c]	Ethoxyresorufin-o-deethylase[c]
Basal diet	1.06±0.23	449.6	4.42±0.13	0.73±0.13
10 mg/kg 245-HBB	2.28±0.39	449.2	6.62±1.61	0.49±0.03
100 mg/kg 245-HBB	2.44±0.14	449.5	12.30±0.56	1.53±2.30
0.1 mg/kg 345-HCB	2.32±0.25	448.7	4.97±1.24	33.90±4.45
1.0 mg/kg 345-HCB	3.52±0.09	448.5	7.29±2.24	54.00±11.98
10 mg/kg 245-HBB plus 0.1 mg/kg 345-HCB	2.21±0.21	448.5	5.89±0.58	29.10±6.06
100 mg/kg 245-HBB plus 1.0 mg/kg 345-HCB	3.84±0.80	448.5	8.33±1.01	57.60±1.25

Data are expressed as mean ± SD for three rats from each group, analyzed as pooled samples.

[a] nmols/mg protein

[b] nanometers

[c] nmols/mg protein/minute

Other Findings

Formalin-fixed tissues in their containers were examined with ultraviolet light to detect the presence of porphyrins. Rats fed a combination of 10 mg/kg 245-HBB plus 0.1 mg/kg 345-HCB tested relatively strongly for porphyrins, and rats fed a combination of 100 mg/kg 245-HBB plus 1.0 mg/kg 345-HCB tested relatively weakly for porphyrins. However, porphyrins were neither further characterized nor quantified. Porphyrinogenic activity was not detected in other treatment groups or controls.

DISCUSSION

Compounds that enhance the development of foci positive for gamma glutamyl transpeptidase (GGT) in the livers of initiated and partially hepatectomized rats are considered tumor promoters (Pitot et al., 1978a; Leonard et al., 1982). Dietary concentrations of 10 or 100 mg/kg of 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB), 0.1 or 1.0 mg/kg 3,3',4,4',5,5'-hexachlorobiphenyl (345-HCB), a combination of 10 mg/kg 245-HBB plus 0.1 mg/kg 345-HCB, or a combination of 100 mg/kg 245-HBB plus 1.0 mg/kg 345-HCB caused significantly increased numbers of GGT-positive foci in the livers of these rats when compared to initiated and partially hepatectomized rats fed basal diets.

A dose-dependent increase in the number of GGT-positive foci was seen in initiated and partially hepatectomized rats fed 345-HCB. Rats fed 1.0 mg/kg 345-HCB alone had approximately a five-fold increase in GGT-positive foci when compared to rats fed 0.1 mg/kg 345-HCB alone. Conversely, initiated and partially hepatectomized rats fed only 100 mg/kg 245-HBB had no significant difference in the number of GGT-positive foci when compared to rats fed 10 mg/kg 245-HBB. In contrast, the results of a previous study using an

identical protocol showed a significant dose-dependent increase in the number of GGT-positive foci in rats fed 100 mg/kg 245-HBB when compared to rats fed 10 mg/kg 245-HBB (Jensen et al., 1982b).

Small numbers of GGT-positive foci occurred in the livers of rats fed various concentrations of 245-HBB and 345-HCB that had not undergone diethylnitrosamine (DEN) administration or partial hepatectomy. These foci may arise if rats had been previously exposed to low levels of initiators from the environment (Pitot et al., 1980; Pitot and Sirica, 1980; Williams et al., 1981). Alternatively, these compounds could act as both initiators and promoters and thus behave as complete carcinogens, but evidence for PBB's or PCB's acting as complete carcinogens is generally lacking (Garthoff et al., 1977). However, in one study a large single dose of Firemaster BP-6 caused hepatocellular carcinomas in rats not previously initiated (Kimbrough et al., 1981). Nevertheless, results from the current study confirm the tumor promoting ability of these compounds.

Initiated and partially hepatectomized rats fed combinations of 245-HBB plus 345-HCB are of particular interest. A combination of 10 mg/kg 245-HBB plus 0.1 mg/kg 345-HCB caused greatly increased numbers of GGT-positive foci. This number was nearly a summation of the GGT-positive foci in rats fed exclusively 10 mg/kg 245-HBB and exclusively 0.1 mg/kg 345-HCB. Therefore,

the effect of feeding this combination of compounds may best be described as an additive tumor promoting effect. This is in contrast to the results of a similar study in which 245-HBB and another polybrominated congener, 3,3',4,4',5,5'-hexabromobiphenyl (345-HBB), had a synergistic, rather than additive, effect on GGT-positive foci when fed to DEN-initiated partially hepatectomized rats at a concentration of 10 mg/kg 245-HBB plus 0.1 mg/kg 345-HBB (Jensen and Sleight, 1986). Given the structural similarity and the nearly identical toxic effects of 345-HCB and 345-HBB, it is of interest that these compounds did not have the same tumor promoting effect, whether additive or synergistic, when fed in combination with 10 mg/kg 245-HBB. Perhaps this is due to the different halogens in 345-HBB and 345-HCB. Moreover, comparison of these studies is hampered by the fact that concentrations of 345-HBB and 345-HCB were prepared in mg/kg (or ppm) concentrations rather than molar concentrations. Therefore, the number of moles of bromine in rats fed 1.0 mg/kg of 345-HBB would be different from the number of moles of chlorine in rats fed 1.0 mg/kg 345-HCB. Subtle differences in halogen concentration in these compounds may be responsible for their slightly different tumor promoting ability. However, the mechanism of action of tumor promotion for either of these compounds is unknown.

Jensen and Sleight (1986) found an inhibitory effect of the formation of GGT-positive foci in rats fed a combination of 100 mg/kg 245-HBB plus 1.0 mg/kg 345-HBB compared to the number of such foci in rats fed either 100 mg/kg 245-HBB alone or 1.0 mg/kg 345-HBB alone. These results are in agreement with the results of the present study in which 345-HCB was substituted for 345-HBB. The mechanism of inhibition of tumor promotion by diets containing a combination of 100 mg/kg 245-HBB plus 1.0 mg/kg 345-HCB is unknown. One possible explanation is that this combination of toxicants caused a suppression of body weight gains. Perhaps available dietary nutrients were not utilized as efficiently in these rats as in rats given only 245-HBB or 345-HCB. Such alterations of body growth may have negative effects on tumor formation, since long-term dietary restrictions have been shown to decrease the incidence of naturally-occurring tumors in rodents (Tucker, 1979). However, the reasons that one combination of polyhalogenated hydrocarbons in the diet caused an additive effect on tumor promotion while different concentrations of the same toxicants in the diet caused an inhibitory effect on tumor promotion remain to be determined.

SUMMARY-CHAPTER 1

Conclusions from the previously described studies include the following:

1) The compounds 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB) and 3,3',4,4',5,5'-hexachlorobiphenyl (345-HCB) have tumor promoting ability in a two-stage hepatocarcinogenesis assay.

2) A diet containing a combination of 10 mg/kg 245-HBB plus 0.1 mg/kg 345-HCB caused an apparent additive effect on tumor promotion as determined by measuring hepatic gamma glutamyl transpeptidase- (GGT-) positive foci in a two-stage hepatocarcinogenesis assay.

3) A diet containing a combination of 100 mg/kg 245-HBB plus 1.0 mg/kg 345-HCB caused an inhibitory effect on tumor promotion as determined by measuring GGT-positive hepatic foci.

As determined by the preceding experiments, 245-HBB and 345-HCB behave as tumor promoters of experimental hepatocarcinogenesis in rats. However, the results of such animal studies are difficult to extrapolate to determine human health risks from exposure to such

environmental toxicants. Unfortunately, people have been exposed to relatively high doses of polybrominated and polychlorinated biphenyls. The effect of these exposures on the development of cancer in people is currently unknown.

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CHAPTER 2

QUANTITATIVE ALTERATIONS OF GAP JUNCTIONS AND NUCLEAR PORES IN CHEMICALLY-INDUCED HEPATIC NODULES IN RATS

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INTRODUCTION

Gap junctions are ultrastructural channels found in the plasma membrane of most cells that permit the intercellular sharing of metabolites. They are responsible for "cell-cell communication" and are quantitatively altered in certain pathologic conditions, including malignant neoplasia (Schindler et al., 1982; Alroy, 1979; Inoue and Skoryna, 1979; McNutt and Weinstein, 1971; Martinez-Palomo, 1975; Swift et al., 1983) and hyperplasia (Yancey et al., 1981; Yee and Revel, 1978).

Hepatic neoplasia has several identifiable stages, and precursor (i.e., "preneoplastic") lesions have been described (Farber, 1984). The hepatic nodule is thought to be a precursor to the hepatocellular carcinoma. Gap junctions are decreased in number in hepatocellular carcinomas (Swift et al., 1983), but it is unknown if they are decreased in number in hepatic nodules.

Another ultrastructural channel, the nuclear pore, permits the sharing of low molecular weight metabolites between the nuclear and cytoplasmic compartments within a cell (deRobertis, 1983; Paine et al., 1975). Quantitative alterations in nuclear pores in neoplastic cells have not been rigorously studied, but some reports suggest that they are diminished in number in neoplastic cells (Codd et al., 1981; Czerniak et al., 1984).

The first objective of the following study was to determine if the surface area occupied by gap junctions differs between cells from normal liver and cells comprising hepatic nodules. A second objective was to compare the numbers of nuclear pores within nuclear membranes of cells in normal liver with cells in hepatic nodules.

LITERATURE REVIEW

Role of Gap Junctions in Metabolic Cooperation

Gap junctions are integral protein structures of the plasma membrane found in all metazoan animals and in nearly all cells comprising organized tissues and organs (Hertzberg et al., 1981; Hooper and Subak-Sharpe, 1981; Pitts, 1980; Loewenstein, 1981). They form pore-like openings, called connexons, connecting the cytoplasms of two adjacent cells. A group of coupled cells will therefore form a compartment within which ions and small molecules are easily exchanged. The junctions have a characteristic appearance in freeze-fractured preparations and appear as an array of hexagonally packed connexons. Small molecules of less than 1000 daltons may pass through these connexons (Flagg-Newton et al., 1979; Spray et al., 1977). Examples of such molecules include cAMP (Hertzberg et al., 1981) calcium (Loewenstein, 1981), nucleotides (Hertzberg et al., 1981; Hooper and Subak-Sharpe, 1981; Pitts, 1980), and amino acids (Pitts, 1980). These molecules can pass between cells via gap junctions without entering the interstitial space.

Transfer of small molecules between cells via gap junctions has been termed "metabolic cooperation" and allows adjacent cells to communicate with one another (Subak-Sharpe et al., 1969; Hooper and Subak-Sharpe, 1981). Intercellular communication may be required for several fundamental biological events, including: (a) synchronized contraction of cells within a tissue, (b) metabolic coordination of cells within a tissue, (c) growth control, (d) differentiation and development, and (e) enzymatic regulation.

Synchronized Contraction

In tissues such as the heart, cardiac muscle cells are interfaced with each other by gap junctions allowing for electrical synchronization of cardiac tissue (De Mello, 1982). Synchronized contraction is also a feature of the gravid uterus. Approximately two days before the onset of parturition there is a 100-fold increase in the number of gap junctions in the endometrium (Garfield et al., 1978) which is apparently in response to increasing levels of estrogen. Two days after parturition, the number of gap junctions decreases and returns to pre-pregnancy levels (Garfield et al., 1980).

Metabolic Coordination of Cells

Intercellular sharing of metabolites via gap junctions may help cells in some tissues respond to hormones or growth factors. For example, the rat pancreas contains four different subsets of cells: the A (or alpha) cells which produce glucagon, the B (or beta) cells of the islets of Langerhans which produce insulin, the D (or delta) which produce somatostatin, and the PP cells which make pancreatic polypeptide (Micheals and Sheridan, 1981; Meda et al., 1981). Groups of beta cells are connected with each other via gap junctions but are connected to surrounding non-beta cells with fewer gap junctions, thus forming discrete functional domains of cells within the pancreas. With prolactin stimulation, the beta cells increase their numbers of gap junctions two-fold and increase the numbers of gap junctions with their neighboring A, D, and PP cells by 10 to 20-fold (Micheals, 1982).

Rats made hyperglycemic by chemically-induced blockage of insulin secretion had a two-fold increase in gap junctions in beta cells, while the ability of these cells to share metabolites increased nine-fold (Meda et al., 1983). Conversely, rats made hypoglycemic with a chemical that depletes beta cell insulin content had a two-fold increase in the number of gap junctions between beta cells while intercellular communication with neighboring cells increased three-fold. This experiment

is an in vivo example of how an effect of hormones or blood-borne factors can spread from the target cells to nontarget cells by the intercellular exchange of metabolites via gap junctions.

Growth Control

Certain organs depend on intact communication with surrounding cells for normal growth. An example of total metabolic dependence of an organ upon neighboring cells is the lens of the eye. The cells of the lens are not in direct contact with the blood vasculature but are nourished totally by intercellular communication via gap junctions with neighboring epithelial cells which are in intimate contact with the blood supply. Another similar example is the mammalian oocyte. Its surrounding granulosa cells appear to be required for maintenance of the oocyte in meiotic arrest (Wassarman and Letourneau, 1976). Furthermore, results of studies on metabolic cooperation indicate that the uridine used for RNA synthesis by the oocyte is obtained from neighboring cumulus cells via gap junctions (Gilula et al., 1978).

Differentiation and Development

Gap junctions occur in the Xenopus embryo as early as the four-cell stage (Hertzberg et al., 1981). Cells of the "grey crescent" are known to develop into the Xenopus eye. If a polyvalent antibody against gap

junctional protein is injected into such cells at an early stage of embryogenesis, then intercellular communication of the injected cells with surrounding cells is prevented. This may lead to abnormal differentiation and development of the injected cells (Warner et al., 1984).

Regulation of Enzyme Activities

Metabolic cooperation regulates the activities of several enzyme systems, including HGPRTase (hypoxanthine guanine phosphoribosyl transferase) (Vitkauskas and Canellakis, 1984; Sheridan et al., 1979; Vitkauskas et al., 1983), sodium and potassium ATPase (Ledbetter and Lubin, 1979; Ledbetter and Young, 1983), and some protein kinases (Fletcher et al., 1983; Murray and Fletcher, 1982). For example, the enzyme HGPRTase produces the product inosine monophosphate from its substrates hypoxanthine and phosphoribosyl phosphate (PRPP). Lesch-Nyhan cells lack HGPRTase and die when grown in hypoxanthine-aminopterine-thymidine (HAT) medium. Normally, Lesch-Nyhan cells have higher PRPP levels than other cells and increase their PRPP content three-to-four-fold when grown in HAT medium. If Lesch-Nyhan cells lacking HGPRT activity ($HGPRT^-$ cells) are co-cultured in HAT medium with normal fibroblasts ($HGPRT^+$ cells), the HGPRTase activity in the $HGPRT^+$ cells is increased three to four fold (Benke and

Dittman, 1977). By modifying the PRPP content of the $HGPRT^-$ Lesch-Nyhan cells, the HGPRTase activity in such co-cultures varies depending upon the amount of PRPP available from Lesch-Nyhan cells (Vitkauskas and Canellakis, 1984). Therefore, the increase in HGPRTase activity is associated with the equilibration of excess PRPP of the Lesch-Nyhan cells with the $HGPRT^+$ cells. However, it is unclear if the increase in HGPRTase activity is due to induction of additional enzymes or to increased activity of existing enzymes in response to higher levels of substrate. Nevertheless, metabolic cooperation appears to be central to the regulation of the activity of this enzyme.

The Nature and Structure of Gap Junctions

The physical structure of gap junctions was first described by Revel and Karnovsky (1967) when they found that gap junctions appear as a pair of apposed plasma membranes separated by a 2-3 nm gap. Studies suggest that gap junctions are assembled from protein units that have a molecular weight of about 27,000 daltons (Hertzberg et al., 1982). Cholesterol and phospholipids are the other main constituents of gap junction proteins (Hertzberg and Gilula, 1979). The inner core of the gap junction is considered to be hydrophilic (Hirokawa and Heuser, 1982; Loewenstein, 1981).

Gap junctional proteins have a high turnover rate, and different half-lives have been reported depending on the technique used. Yancey et al. (1981) reported a 19 hour half-life. However, Fallon and Goodenough (1981) reported a shorter half-life of 5.5 hours using a different technique. Cells apparently contain appreciable levels of gap junctional proteins which can be rapidly inserted into the plasma membrane when junctional contact between adjacent cells has been established (Gilula, 1984). Metabolic cooperation does not appear to be dependent upon ongoing gap junctional protein synthesis (Epstein et al., 1977).

The current structural model of gap junctions was proposed by Unwin and Zampighi (1980). Their model is composed of six closely associated junctional proteins that extend individually through the full thickness of a cell membrane to form a hemichannel that can open and close by lateral and circular movement of the proteins. The complete junctional channel is the bipartite structure formed by suitable alignment of hemichannels of two adjacent cells.

Regulation of Gap Junctions

Several substances are known to alter the permeability of gap junctions. Increased levels of intracellular calcium can inhibit intercellular electrical coupling within seconds (Rose and Loewenstein, 1975).

Several other cations, including sodium, barium, and cobalt, have similar functions when injected into individual cells (De Mello, 1984). Increased acidity, causing a corresponding increase in intracellular calcium concentrations, has also been shown to decrease gap junctional coupling (Rose and Rick, 1978).

Calcium is thought to elicit its inhibitory electrical coupling effect via calmodulin or a calmodulin-like protein (Peracchia et al., 1981). It has been hypothesized that calmodulin binds to gap junctional proteins causing conformational changes in them, resulting in partial or complete occlusion of the hemichannels within connexons. (Peracchia, 1984).

Damage to tissue may modulate gap junctions. Myocardial infarction (Nealy et al., 1976) and other heart lesions (De Mello, 1972) lead to ischemia and lowering of the intracellular pH in the cells adjacent to the afflicted area. Normal cells uncouple from the damaged cells in a phenomenon known as "healing over." This phenomenon appears to be a homeostatic mechanism by which normal cells are protected from the deleterious effects of ischemia and cell death. By stopping intercellular communication with their surrounding damaged cells, the nondamaged cells may help restore function to remaining tissue.

Another example of the effect of tissue damage on gap junctions is seen with partial hepatectomy. When

two-thirds of the liver was removed in rats there was progressive loss in the number of gap junctions in the remaining lobes of the liver undergoing hyperplasia (Yee and Revel, 1978). The number of gap junctions decreased and became minimal at 29-35 hours after partial hepatectomy. It then increased and returned to normal by 48 hours after partial hepatectomy (Meyer et al., 1981).

Gap Junctional Communication and Neoplasia

In Vivo Studies

Chemically-induced Cancers. There is limited experimental evidence to suggest that neoplastic cells have decreased numbers of gap junctions when compared to their normal tissue counterparts. Wistar rats given N-4-(5-nitro-2-furyl)-2-thiazolyl formamide (FANFT) had decreased numbers of gap junctions in urothelial cell tumors compared with normal tissue. Furthermore, the decrease in numbers of gap junctions was associated with progression of the tumors to a more malignant stage, and gap junctions were absent in the most malignant tumor cells. Additionally, in FANFT-induced urinary bladder carcinomas, there was a selective loss of larger gap junction plaques but a preservation of the numbers of smaller gap junctional plaques (Pauli and Weinstein, 1981). Rats with methylcholanthrene-induced dermal

tumors, including squamous cell carcinomas, had detectable gap junctions in all primary tumors but had no gap junctions in metastatic squamous cell carcinomas in lymph nodes and lung (Horak et al., 1984). Mice treated with tumor-promoting doses of phorbol esters had epidermal tumors with decreased numbers of gap junctions (Kalimi and Sirsat, 1984).

Janssen-Timmen et al. (1986) and Willecke et al. (1985) have described the usefulness of monoclonal antibodies to demonstrate diminished numbers of gap junctions in diethylnitrosamine-induced hepatomas and hepatocellular carcinomas in rats. Janssen-Timmen et al. (1986) found that the numbers of gap junctions in hepatocellular carcinomas were reduced by 71% when compared to normal control livers. However, they found no decrease in gap junctional numbers in most, but not all, of small ATPase-deficient preneoplastic cell populations. Perhaps those few enzyme-altered preneoplastic foci which have decreased numbers of gap junctions have the greatest potential for autonomous proliferation and may, therefore, be more likely to develop into hepatocellular carcinomas. Decreased numbers of gap junctions may contribute to inhibited cell-cell communication, a mechanism by which tumor promotion may occur (Trosko et al., 1983). Although the molecules which regulate tissue homeostasis are not known, one would predict that cells which have lost

their gap junctions are more likely to escape growth control.

Spontaneous Neoplasia. Gap junctions in progressive human cervical neoplasia have been studied, and were found to progress from normal in number to nearly zero in more invasive cervical nonsquamous epithelial tumors (Schindler et al., 1982). Gap junctions were also few in number in invasive squamous cell carcinomas of the cervix (Schindler et al., 1982). Numbers of gap junctions were decreased in urinary bladder adenocarcinomas of dogs (Alroy, 1979). In human and murine mammary adenocarcinomas, gap junctions were decreased in number, and gap junctional plaques were considered decreased in size when compared to normal mammary epithelial cells. Furthermore, the number of gap junctions was significantly decreased in non-neoplastic parenchymal cells immediately peripheral to the tumor mass (Inoue and Skoryna, 1979). No gap junctions could be demonstrated in cells comprising malignant human glioblastoma multiforme (McNutt and Weinstein, 1971), and few gap junctions could be found in human hepatocellular carcinomas (Swift et al., 1983). These studies suggest that decreased numbers of gap junctions are associated with the progression of cells toward a malignant state, and that this trait is not merely a random phenotype present in malignant cells.

There is contrary evidence that suggests that not all tumors have decreased numbers of gap junctions. For example, virally-transformed cells, such as Rous sarcoma-transformed cells, have numbers of gap junctions similar to that found in normal, non-virally-transformed cells (Pinto de Silva and Gilula, 1972). Furthermore, in spontaneous pulmonary metastases of virus-induced murine mammary adenocarcinomas, gap junctions appeared to be similar in number to that found in normal cells (Shamsuddin, 1984). In another study, spontaneous pulmonary metastases of mammary adenocarcinomas in virus-infected mice had numbers of gap junctions similar to that seen in cells of the normal mammary gland (Pitelka et al., 1980). Human benign meningiomas and astrocytomas had similar numbers of gap junctions when compared to control tissues (McNutt and Weinstein, 1971). However, in none of these studies was the functional status of gap junctions determined.

In Vitro Studies

A relationship between intercellular communication and tumor promotion was made when it was observed that tumor-promoting chemicals, used at noncytotoxic and nongenotoxic concentrations, inhibited gap junction-mediated metabolic cooperation (Yotti et al., 1979; Murray and Fitzgerald, 1979) and electrocoupling activity between cells (Enomoto et al., 1981). Chinese

hamster V79 cells treated with a tumor promoter were shown, using freeze-fracture techniques, to have fewer numbers of gap junctions on their plasma membranes than nontreated control cells (Yancey et al., 1982). Somatic cell hybrids had a good correlation between decreased junctional communication and neoplastic growth (Azarnia and Loewenstein, 1977). However, none of these studies determined if the decrease in intercellular communication was due exclusively to qualitative or quantitative changes in gap junctions.

Structure and Function of Nuclear Pores

The nuclear pore complex provides a channel of communication between the nuclear and cytoplasmic compartments of cells (deRobertis, 1983; Feldherr, 1965; Kessel, 1973; Paine et al., 1975). The complex spans the two nuclear membranes and the perinuclear space and is an octagonally symmetrical structure (Gall, 1967; Unwin and Milligan, 1982). Ultrastructural studies have revealed the nuclear pore complex to be composed of a central channel, or annulus, the diameter of which may vary from 0-400 angstroms (Gall, 1967). The central channel is flexible and may expand or contract in a manner similar to a muscle sphincter. Limited evidence indicates that the pore diameter may be influenced by

alterations in the activity of ATPase within the cell (Jiang and Schindler, 1986).

Functional studies involving transport of molecules through nuclear pores have been performed in normal and neoplastic tissues. Some findings indicate that functional transport via nuclear pores is decreased in neoplastic tissue when compared to its normal counterpart (Drews et al., 1968; Garret et al., 1973a, 1973b).

Few studies have tried to assess quantitative morphological changes of nuclear pore complexes in neoplastic tissue. Czerniak et al. (1984), using freeze-fracture techniques, found fewer nuclear pores in human urinary bladder carcinomas than in the normal tissue counterpart. Similarly, Codd et al. (1981), found fewer nuclear pore complexes in an experimentally-induced oral neoplasm than in normal tissue. These findings suggest that decreases in the number of nuclear pores per area of nuclear membrane may be associated with the transition of a cell toward neoplasia, but such changes have yet to be rigorously studied, especially in hepatocarcinogenesis systems.

MATERIALS AND METHODS

Rats

Female Sprague-Dawley rats used for tumor promotion studies (described previously in Chapter 1, Materials and Methods) were fed a combination of 10 mg/kg 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB) plus 0.1 mg/kg 3,3',4,4',5,5'-hexabromobiphenyl (345-HBB) for 140 days following partial hepatectomy and diethylnitrosamine (DEN) administration intraperitoneally (10 mg/kg body weight). Rats were then maintained on diets free of HBB until day 480 at which time they were killed with ether anesthesia and decapitation, and liver sections were taken during necropsy. Sections of six grossly visible hepatic nodules were taken from six rats, and six sections of liver were taken from the same rats' livers from non-nodular areas. The tissue section was bisected, and one half was fixed in 10% neutral buffered formalin, processed for light microscopy, and stained with hematoxylin and eosin. The other half of each liver

section was placed in 4% gluteraldehyde for two hours and processed for freeze-fracturing and electron microscopy.

Electron Microscopy

For assessment of gap junctions, samples fixed in 4% gluteraldehyde were prepared for freeze-fracturing by gradual infiltration over three hours with 25% glycerol in phosphate buffered saline (pH 7.2). Sections were cut using a vibratome (Lanar Vibratome, Series 1000, Brunswick Co., St. Louis, MO) to a thickness of two mm and "glued" (using a 1:2 solution of glycerol to 30% polyvinyl alcohol in double distilled water), to a gold replica holder (Electron Microscopy Sciences, Fort Washington, PA) followed by immersion into Freon 22 in its liquid state (- 150^0 C) for 10 seconds. Tissues were then stored on their replica holders in liquid nitrogen (- 190^0 C). Tissue replicas were freeze-fractured using a Balzer's BA-360 M freeze-fracture apparatus (Balzers, Hudson, NH), coated with platinum and carbon, cleaned for 30 minutes in 5.24% sodium hypochlorite (Clorox^R Bleach, The Clorox Co., Oakland, CA), placed onto 270-mesh honeycomb copper grids (Ted Pella, Inc., Tustin, CA), and examined using a JEOL 100-CX II scanning/transmission electron microscope (Japanese Electron Optics Laboratory, Tokyo, Japan) at an accelerating electron beam voltage of 100 kilovolts.

The magnification of the microscope was calibrated regularly using a diffraction grating replica of 2160 lines/mm.

For assessment of nuclear pores, samples were slightly overfixed (eight hours) in 4% gluteraldehyde to increase chances of transmembrane fracture. Sections were then gradually infiltrated with 25% glycerol as before and freeze-fractured as described above.

Morphometric Analysis

Gap Junctions. Total membrane surface area was estimated by calculating the amount of grid space occupied by hepatocyte cell membranes during electron microscopic examination. Selected images from the "P" faces of hepatocyte cell membranes from control sections and from hepatic nodules were recorded at a magnification of 30,000 X. The smooth portion of the hepatocyte membrane extending from the bile canaliculus toward the periphery where the cell surface is thrown into numerous irregular projections as it interfaces with another hepatocyte was sought. This has been shown to be the most likely area for the occurrence of gap junctions (Meyer et al., 1981; Yancey et al., 1981). The recorded images were analyzed, using a technique described by Yancey et al. (1981), with an Apple^{R} computer (Apple Computer Corporation, Cupertine, CA), and a digitizing tablet with which outlines of gap

junctions were traced and quantified. The total gap junctional area was measured and expressed as a percentage of total measured membrane occupied by gap junctions. A total of 6,000 μm^2 (1,000 μm^2/sample) of hepatocyte membranes was surveyed from control tissue and 6,000 μm^2 was surveyed from hepatic nodules.

Nuclear Pores. Freeze-fractured sections of control liver and hepatic nodules were examined for transmembrane fracture, and images of hepatocytic nuclear membranes were recorded at a magnification of 30,000 X. Numbers of nuclear pores per 9 cm^2 of recorded image were determined by averaging six measurements in a single electron micrograph. A total of 25-30 cells per tissue sample was used in this determination.

Statistics

A rank-sum test was used to compare the area occupied by gap junctions in sections of control livers with that from sections of hepatic nodules (Steel and Torrie, 1980). Similarly, a rank-sum test was used to determine statistical significance between the number of nuclear pores in hepatocytic nuclei from control liver sections with the number in cell nuclei of hepatic nodules. Significance was defined as $P \leq 0.05$.

RESULTS

Gap Junctions

The total hepatocyte membrane surface area examined and the percent ratio of gap junctional area to total measured membrane area in sections of control liver and hepatic nodules are listed in Table 2-1. The typical histologic appearance of a hepatic nodule is seen in Figure 2-1. There was less area occupied by gap junctions in sections of hepatic nodules (1.13%) when compared to control sections (2.81%) from the same liver in which hepatic nodules occurred.

The typical appearance of gap junctions from freeze-fracture preparation is seen in Figure 2-2. The morphology of gap junctions in control sections of liver was similar to that found in hepatic nodules. Packing, spacing, density, and shape of individual connexons were similar in gap junctions from control livers and hepatic nodules, but resolution of freeze-fracture replicas was not great enough to permit morphological assessment of the size of hemichannels within individual connexons.

Table 2-1. Membrane Surface Area Occupied by Gap Junctions in Non-nodular Sections of Liver (Control) and in Hepatic Nodules in Rats.

Rat number	Ratio of gap junctional area to total membrane area (%)	
	Control Sections	Hepatic Nodules
1	2.36	0.93
2	2.97	1.52
3	3.15	1.03
4	2.71	0.98
5	2.80	1.30
6	2.87	1.02
Mean ± SD	2.81 ± 0.30	1.13 ± 0.23 [a]

[a] Significantly different from controls ($P \leq 0.05$).

Sections of control liver and hepatic nodules were taken from rats receiving a partial hepatectomy and diethylnitrosamine (10 mg/kg body wt.) followed by 140 days dietary treatment with a combination of 10 mg/kg 245-HBB plus 0.1 mg/kg 345-HBB. Rats were then fed diets free of HBB until day 480. Sections of control liver were from non-nodular areas of livers in which hepatic nodules occurred.

The total membrane area examined was approximately 6,000 μm^2 for control liver and 6,000 μm^2 for hepatic nodules.

Figure 2-1. Photomicrograph of a hepatic nodule in a section of liver from a rat fed a diet containing a combination of 10 mg/kg 245-HBB plus 0.1 mg/kg of 345-HBB for 140 days after partial hepatectomy and administration of diethylnitrosamine (10 mg/kg body wt.) intraperitoneally. Notice light staining of cells within hepatic nodule and compression of hepatocytes near periphery of nodule (H & E, 90 X).

Figure 2-1

Figure 2-2. Recorded image of a freeze-fractured section of hepatocytic membrane from a section of hepatic nodule from a rat fed a diet containing a combination of 10 mg/kg 245-HBB plus 0.1 mg/kg 345-HBB for 140 days following diethylnitrosamine administration (10 mg/kg body wt.) intraperitoneally. Notice the gap junction (star) and its close proximity to tight junctions (arrows) (platinum and carbon coating, 67,500 X).

Figure 2-2

Nuclear Pores

The numbers of nuclear pores in control sections of liver and in sections of hepatic nodules are shown in Table 2-2. The number of nuclear pores was not significantly different when nuclei from sections of control liver were compared to nuclei from hepatic nodules. Surface area of individual nuclear pores was not quantified, but there appeared to be little variation in the size of nuclear pores from one nuclear membrane surface to another. Resolution of freeze-fractured replicas was not great enough to permit a detailed morphological assessment of individual nuclear pores or their annuli, but morphological variation appeared minimal between nuclear pores from sections of control hepatocytes and those from hepatic nodules (Figure 2-3).

Table 2-2. Numbers of Nuclear Pores in Hepatocytes from Non-nodular Sections of Liver (Control) and from Cells in Hepatic Nodules in Rats.

Rat number	Number of Nuclear Pores per 9 cm^2 [a]	
	Control Sections [b]	Hepatic Nodules [b]
1	18	20
2	25	17
3	17	22
4	19	25
5	16	19
6	21	23
Mean ± SD	19.3 ± 3.3	21.0 ± 2.9 [c]

[a] On recorded image at magnification of 30,000 X.

[b] A total of 25-30 nuclei per tissue section was examined.

[c] Not significantly different from controls ($P \leq 0.05$).

Figure 2-3. Recorded image of a freeze-fractured section of hepatocytic nuclear membrane from a section of hepatic nodule from the liver of a rat fed a combination of 10 mg/kg 245-HBB plus 0.1 mg/kg 345-HBB for 140 days after partial hepatectomy and diethylnitrosamine administration (10 mg/kg body wt.) intraperitoneally. Notice nuclear pores on the surface of the nuclear membrane (platinum and carbon coating, 30,000 X).

Figure 2-3

DISCUSSION

Freeze-fracture studies provide useful information regarding morphological and quantitative alterations in ultrastructural cellular organelles such as gap junctions and nuclear pores. However, freeze-fracture studies are limited because they do not provide information about the functional status of these structures. In addition, some workers have estimated that for quantitative studies of gap junctions in liver tissue, freeze-fracture allows examination of only approximately 7% of the the total contact area of a hepatocyte (Meyer et al., 1981). Therefore, freeze-fracture studies such as these provide useful but limited information about such organelles.

The results of this study indicate that there is less membrane surface area occupied by gap junctions in hepatic nodules than in control sections from the same livers in which hepatic nodules occurred in DEN-initiated and partially hepatectomized rats given known hepatic tumor promoters. Hepatic nodules have been proposed as precursor lesions to hepatocellular carcinomas in rats (Farber, 1984; Scherer, 1984; Schulte-Hermann, 1985; Williams, 1982). Hepatocellular carcinomas are known to have fewer gap junctions than

either normal or cirrhotic liver (Swift et al., 1983). A reduction in the number of gap junctions on cell surface membranes has been associated with nonhepatic neoplasia (Schindler et al., 1982; Alroy, 1979; Inoue and Skoryna, 1979; McNutt and Weinstein, 1971; Martinez-Palomo, 1975), hepatic neoplasia (Swift et al., 1983), and hepatic regeneration (i.e., hyperplasia) (Yancey et al., 1981; Yee and Revel, 1978). The amount of hepatocytic membrane area occupied by gap junctions in sections of control liver was similar to that reported for normal rat liver by others (Meyer et al., 1981).

It has been postulated that decreases in gap junctional surface area may alter the intercellular sharing of metabolites, decrease electrocoupling between cells, and inhibit cell-cell communication (Swift et al., 1983; Meyer et al., 1981). Diminished gap junctional function has been observed specifically during tumor promotion (Enomoto et al., 1981; Yancey et al., 1982). Blockage of intercellular communication may contribute to the autonomous proliferative behavior of neoplastic cells and has been proposed as a mechanism of tumor promotion (Trosko et al., 1982). However, the role of gap junctions in in vivo intercellular communication in cells undergoing preneoplastic or hyperplastic changes is largely unknown. Furthermore, it is unknown if there is a critical number of gap junctions that must still be functioning in a cell in

order for it not to be metabolically "blocked" from other adjacent cells. Studies in which anti-gap junction antibodies are used could allow rigorous quantitation of gap junctions under various conditions of cellular dysfunction.

Little is known about morphological or quantitative changes in nuclear pores of neoplastic or preneoplastic cells. Results from the present study indicate that there was no difference in numbers of nuclear pores in individual hepatocytes when sections of control liver were compared with hepatic nodules. These results differ from those in another study in which a decrease in numbers of nuclear pores was found in experimentally-induced oral epithelial cancer (Codd et al., 1981). Functional studies indicate that transport via nuclear pores is decreased in neoplastic tissue when compared to normal tissue (Drews et al., 1968; Garret et al., 1973a, 1973b). No reports on quantitative alterations in nuclear pores in hepatocarcinogenesis systems were found.

SUMMARY-CHAPTER 2

Conclusions from the preceding experiments include the following:

1) The numbers of gap junctions in freeze-fractured preparations of hepatic nodules induced by a two-stage hepatocarcinogenesis assay were significantly decreased when compared to non-nodular areas of liver.

2) The numbers of nuclear pores in freeze-fractured preparations of cells from hepatic nodules induced by a two-stage hepatocarcinogenesis assay were not significantly different when compared to hepatocytes from sections of non-nodular liver.

The results from these studies suggest that decreased numbers of gap junctions are associated with the development of hepatic nodules. Such a phenomenon may cause decreased intercellular communication and may be an in vivo mechanism of tumor promotion. However, the role of nuclear pores in the development of hepatic nodules is less clear.

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CHAPTER 3

THE EFFECTS OF 2,2',4,4',5,5'-HEXABROMOBIPHENYL ON INTERCELLULAR COMMUNICATION: ASSESSMENT BY THREE IN VITRO ASSAYS

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INTRODUCTION

Intercellular communication is the phenomenon of sharing metabolites, ions, and other small molecules between cells. The cellular organelles responsible for distribution of these molecules between cells are gap junctions. Thus, functional gap junctions are important for tissue homeostasis (Trosko et al., 1982; DeMello, 1982; Loewenstein, 1979).

Certain environmental toxicants behave in vivo as tumor promoters. Such compounds apparently have little genotoxicity and therefore are not categorized as "mutagens." Rather, tumor promoters seemingly have "epigenetic" activity and affect the target cell in some way other than by genomic alteration. However, the mechanisms of tumor promotion have yet to be fully elucidated.

One proposed mechanism by which tumor promotion may occur is inhibition of cell-cell communication (Saxen et al., 1976; Loewenstein, 1979; Trosko et al., 1983a). Tumor promoters may disrupt the function of gap junctions such that previously initiated cells may be liberated from the control of normal cells. Perhaps shared metabolites responsible for control of cellular growth are no longer able to enter neighboring cells and prevent expansion of initiated clones.

Cell-cell communication is measured by several different in vitro techniques. The first objective of the following studies was to assess the ability of 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB), the major congener of Firemaster BP-6 (FM), to inhibit intercellular communication in vitro using a rat liver epithelial cell line (WB-F344) in the metabolic cooperation assay at various concentrations of FM or 245-HBB. An ancillary objective of this study was to determine if 245-HBB had the same ability to inhibit cell-cell communication as FM at similar concentrations. A second objective was to assess the ability of 245-HBB to inhibit cell-cell communication using a novel technique of "Fluorescence Redistribution After Photobleaching" ("FRAP") from a technique known as Anchored Cell Analysis and Sorting (ACAS). A third objective was to determine the usefulness of a new in vitro assay, termed "scrape-loading/dye transfer," for

assessing inhibition of cell-cell communication in a dose-dependent manner. A final objective was to combine the new techniques provided by ACAS with results from the scrape-loading/dye transfer assay to rigorously quantify dose-responsiveness of inhibited intercellular communication at various concentrations of 245-HBB.

LITERATURE REVIEW

In Vitro Properties of Tumor Promoters

One property of known tumor promoters that has been recently elucidated is their ability to inhibit metabolic cooperation between cells in vitro by cell membrane interactions (Trosko et al., 1981; Yotti et al., 1979). One manner in which tumor promoters may interact with the cell membrane involves alteration of cell-cell communication via gap junctions (Loewenstein, 1979; Peracchia, 1980; Larsen, 1983). Cell-cell communication is an important determinant in the control of cellular growth, differentiation, and development, as well as tissue function and homeostasis (Bertram, 1979; Trosko et al., 1982; Andrew et al., 1981; DeMello, 1982; Gilula, 1980; Lawrence et al., 1978; Loewenstein, 1979). Contact inhibition between cells may be dependent upon functional gap junctions (Levine et al., 1965). Tumor promoters appear to disrupt gap junctional intercellular communication such that previously initiated cells may be freed from the control of normal cells. Many tumorigenic cell lines have been shown to have modified gap junctional characteristics. Therefore, inhibition

of intercellular communication may be associated with the tumorigenic process (Saxen et al., 1976; Loewenstein, 1979; Trosko et al., 1983a)

Tumor promoters may exert their cell membrane effects by two general means. One involves direct interaction with the plasma membrane of the cell (Weinstein et al., 1979). Another mechanism may be the altering of gene expression (Yamasaki, 1984) without altering the genetic material of the cell. This is consistent with the observation that most tumor promoters have little or no mutagenic potential (Trosko et al., 1983b).

While tumor promoters are not generally mutagens, such compounds have been conceptualized as "mitogens" (Trosko et al., 1983a). Two major hypotheses have emerged to explain how certain tumor promoters cause clonal expansion of previously initiated cells. First, tumor promoters may activate protein kinase C (Pk-C), a calcium-dependent phospholipid enzyme (Nishizuka, 1986). This sets off a chain reaction to phosphorylate a sequence of cellular structures. An end point of Pk-C activation has been postulated to be loss of gap junctional permeability (Castagna et al., 1982; Fujiki et al., 1984). It is not known if the activated Pk-C directly phosphorylates gap junctional proteins, rendering gap junctions impermeant, or indirectly inactivates gap junctional permeability via

phosphorylation of other membrane-bound enzymes or proteins.

A second possible mechanism for the cellular effects of tumor promoters is that they may induce a "prooxidative" state in the cell. According to this hypothesis, oxygen radical species are generated by the action of certain tumor promoters (Cerutti, 1985). The target for cellular damage by such oxygen radicals is DNA, implying that tumor promoters are somehow mutagenic. This leaves open the possibility that oxygen radicals might directly effect gap junctions or membrane components regulating gap junctional function. However, this hypothesis fails to account for the very different biological responses between tumor initiators and tumor promoters.

Many different cell types have been used in studying chemicals which could inhibit intercellular communication. Chinese hamster V79 cells (Yotti et al., 1979), various human cell types (Davidson et al., 1985; Enomoto et al., 1981; Friedman and Steinberg, 1982; Mosser and Bols, 1982), rat cells of hepatic origin (Telang et al., 1982; Walder and Lutzelschwab, 1984), and murine cell lines (Fitzgerald et al., 1983; Murray and Fitzgerald, 1979) have been used. It is important to use various cell lines because chemicals which inhibit intercellular communication may show organism or organ specificity. Therefore, employing a wide variety

of cell lines improves the chances of detecting inhibition of cell-cell communication by a wide variety of chemicals.

Assays for Measuring Gap Junctional Communication

Electrocoupling Assays

The sharing of passive electrical potential between touching cells is termed electrocoupling. These assays involve placing microelectrodes into the cytoplasm of two contiguous cells (Yamasaki et al., 1983). Pulses of current are then passed into one of the cells. The electrical potentials of the two cells are determined concurrently with respect to the external environment. The ratio of voltage change of the second cell to that of the injected cell is the coupling coefficient or coupling ratio. When cells are joined by functional gap junctional channels, the coupling coefficient is relatively high (Socolar and Loewenstein, 1978). This assay is a sensitive test for the presence of functional gap junctions. However, one limitation is that the assay only detects the passage of the very smallest molecules, namely inorganic ions, that are responsible for the intercellular spread of the electrical current. In addition, it is unknown what the effects of trauma of microinjection are to the cell membrane or to the integrity of gap junctions.

Junctional Conductance Assays

Quantitative determinations of gap junctional permeability can be measured by junctional electrical conductance. This technique requires the insertion of multiple microelectrodes with simultaneous measurements of several end points and is technically more difficult than electrocoupling. Normal values for junctional conductance for various tissues have yet to be established, making such measurements limited in their usefulness. It is further restricted for use with cells in pairs or short chains (Socolar and Loewenstein, 1978).

Freeze-Fracture Studies

Freeze-fracturing of intercellular membranes allows visualization of gap junctions. The technique requires electron microscopy, and relatively large areas of membrane must be scanned to obtain accurate estimates of the amount of area of the membrane occupied by gap junctions, making this method time-consuming. Furthermore, decreases in gap junction numbers are not positively associated with reduced electrical coupling (Meyer et al., 1981). In fact, electrical coupling appears possible with only a small amount of the membrane occupied by gap junctional channels. These channels may be difficult to locate in freeze-fractured

preparations. Another limitation of freeze-fracture studies is that they do not assess gap junctional function. In spite of these limitations, such studies have been used for qualitative and quantitative information about gap junctions.

Metabolic Cooperation Assays

Subak-Sharpe et al. (1969) first used the term "metabolic cooperation" to describe the intercellular exchange of metabolites by direct cell contact. One method for the detection of metabolic cooperation involves measurement of survival of mutant cells in the presence of a toxic precursor compound. Wild-type cells capable of metabolizing the compound to its toxic product died when cultured in the presence of the compound. However, mutant cells that lack the ability to metabolize the compound survived during exposure to the toxicant. When wild-type and mutant cells were co-cultured the mutant cells received, via gap junctions, the toxic metabolite from the wild-type cells. Gap junctional communication was thus measured as decreased survival of mutant cells in co-cultures compared to mutant cells cultured without wild-type cells (Fujimoto et al., 1971; Davidson et al., 1985; Mosser and Bols, 1982; Yotti et al., 1979; Jone et al., in press, 1987). Further evidence that metabolic cooperation was positively correlated with electrocoupling and with the

presence of gap junctions was demonstrated by Gilula et al. (1972), who measured the transfer of radiolabeled nucleotides between cells. Radioactive metabolite transfer between cells has been used by others as an assessment of intercellular communication (Mosser and Bols, 1982; Newbold et al., 1981; Davidson et al., 1985).

Several modifications and cell types have been successfully incorporated into the metabolic cooperation assay. Williams et al. (1981) have modified the metabolic cooperation assay to include primary rat hepatocytes that provide a metabolizing system for activation of certain toxicants in order for them to behave as tumor promoters. Kavanagh et al. (1986) characterized a human cancer cell line useful in the metabolic cooperation assay. Jone et al. (in press, 1987) have described the development of a rat hepatic nonparenchymal epithelial cell line, termed WB-F344, for measuring metabolic cooperation.

Dye Transfer Assays

Functional gap junctional communication can be assessed using low molecular weight fluorescent dyes as tracers (Enomoto and Hamasaki, 1984; Fitzgerald and Murray, 1980; Friedman and Steinberg, 1982). These dyes may be injected into cells or may be introduced into cells by transmembrane diffusion if the dye is of the

appropriate nonpolar ester (Rotman and Papermaster, 1966; Goodall and Johnson, 1982). Once the esterified dye is inside the cell, the dye is rapidly hydrolyzed by esterases to a fluorescent compound. These dyes are typically hydrophilic and do not readily cross into other cells by other than gap junctional transfer.

Scrape-Loading/Dye Transfer Assay. El-Fouly et al. (in press, 1987) have introduced a rapid and simple technique for measuring gap junctional communication. This assay, termed "scrape-loading/dye transfer," introduces dyes into cells in culture by creating a tear in the cell membrane without affecting cell viability or colony-forming capacity (McNiel et al., 1984). The tracer dye, Lucifer yellow, has a molecular weight of 457.2 and is a brightly fluorescent 4-aminoaphthalimide compound with a high quantum yield of 0.25 (Stewart, 1978, 1981). The quantum yield is stable from pH one to 10 and is easily detectable with epifluorescence microscopy. Lucifer yellow does not diffuse through intact cell membranes but its low molecular weight permits its transfer from one cell to another via patent gap junctions (Stewart, 1978, 1981; Lo and Gilula, 1979). Another dye, rhodamine dextran, has a high molecular weight (10,000) and is administered concurrently with Lucifer yellow. Rhodamine dextran can neither diffuse through intact plasma membranes nor pass though gap junctional channels. Rhodamine dextran emits

red fluorescence whereas Lucifer yellow emits yellow-to-apple green fluorescence. The simultaneous introduction of both dyes into cells allows the identification of primary dye-recipient cells, whose cell membranes have been torn by scraping with a wooden probe, and verifies that transfer of Lucifer yellow into contiguous cells (i.e., secondary dye-recipient cells) has occurred via gap junctions. Major advantages of the scrape-loading/dye transfer assay include low cost, rapidity, and direct visualization of results. Its greatest potential is for a quick screening assay to determine inhibition of cell-cell communication by various chemicals. Furthermore, it requires minimal metabolic and biochemical integrity of the cells. Other advantages include minimization of physiological alterations or artifacts that may be induced by other lengthy or complicated procedures. It has application for both quantitative and qualitative evaluation of cell-cell communication. However, the sensitivity of this technique is unknown.

Fluorescence Redistribution After Photobleaching. Another dye transfer technique, termed "fluorescence redistribution after photobleaching" ("FRAP"), involves labeling of cells in tissue culture with 6-carboxyfluorescein diacetate (Wade et al., 1986). All cells in the culture are internally labeled by this stain. Upon contact with the cell cytoplasm the dye is

hydrolyzed and a hydrophilic fluorescein derivative is maintained in the cell (Rotman and Papermaster, 1966). Any labeled cell may be photobleached by a laser beam whose width is approximately equal to the diameter of the cell. Alternatively, the dye may be photobleached by a series of laser pulses with each pulse having a diameter of about one μm. Following photobleaching, the bleached dye molecules from one cell and the nonbleached dye molecules from an adjacent contacting cell may be redistributed via gap junctions. The dye and labeling conditions do not affect cell viability and all measurements can be performed at room temperature. However, leaching of the dye may occur through the cell membrane after a certain period of time, depending on the cell type used and the culture conditions employed.

The FRAP technique requires sophisticated and expensive equipment. In the original assay, a tissue culture plate of labeled cells was placed on a high speed computer-controlled two dimensional stage of an instrument known as ACAS 470 (Anchored Cell Analysis and Sorting, Meridian Instruments, Okemos, MI). The stage moves the culture plate in a defined manner above the objective lens of an inverted epifluorescence microscope. The objective lens of the microscope focuses an argon ion laser beam (excitation wavelength of 488 nm) to a one μm spot that excites fluorescence in individual cells. Digitized pseudoimages record the

fluorescence intensity of the cells, and this information can be stored in a computer.

Several compounds that inhibit cell-cell communication in the metabolic cooperation assay have been found to inhibit dye transfer using FRAP. Many of the compounds are tumor promoters in vivo such as dieldrin and 12-0-tetradecanoylphorbol-13-acetate (TPA). Results from FRAP studies using these compounds correlate with dye microinjection techniques (Fitzgerald et al., 1983).

A major advantage of the FRAP assay is that it has the capacity to make multiple measurements within the same cell without inducing traumatic alterations to the plasma membrane. Another advantage is its ability to measure various end points of all anchored cells types and cell configurations.

MATERIALS AND METHODS

Metabolic Cooperation Assay

Cells and Culture Methods. Cells used for this assay were WB-F344 (rat epithelial) cells, previously shown to metabolically cooperate (Jone et al., in press, 1987). These cells had been previously isolated from the livers of adult male Fischer 344 rats (Tsao et al., 1984). The WB-F344 cells (courtesy of Dr. J. W. Grisham, University of North Carolina) were maintained in vials and were frozen in liquid nitrogen. Cells had been previously biochemically characterized as either lacking activity for the enzyme hypoxanthine guanine ribosyl transferase ($HGPRT^-$) or having such activity ($HGPRT^+$). Thawed cell suspensions were entered into sterile 25 cm^2 culture flasks (Corning Glass Works, Corning, NY) and were grown in modified Eagle's medium (Gibco Inc., Grand Island, NY) with Earle's balanced salt solution (Gibco Inc., Grand Island, NY). This medium was supplemented with a 50% increase in vitamins and essential amino acids (except glutamine), a 100% increase in nonessential amino acids, 5% fetal calf serum (Gibco Inc., Grand Island, NY), insulin (10^{-6}

M/liter) (Sigma Chemical Co., St. Louis, MO), and gentomycin (12 mg/L of medium) (Quality Biologicals, Gathersburg, MD). Phenol red (10 mg/L of medium) (Sigma Chemical Co., St. Louis, MO) was used as an indicator of pH. Cells were grown in humidified incubators (Heinicke Instrument Co., Hollywood, FL) at 37^0 C with 5% CO_2. Cultures were tested routinely for contamination with Mycoplasma spp. using the Hoechst 33258 (Hoechst Inc., Philadelphia, PA) staining technique (Chen, 1977).

Three days prior to the experiment, cells in flasks were rinsed twice with 10 ml sterile phosphate buffered saline (PBS), detached with a solution of trypsin (Worthingham Diagnostic Systems, Freehold, NJ) with 2% EDTA (Sigma Chemical Co., St. Louis, MO), placed onto a warming plate (37^0 C) for five minutes, and 1/10 of the cells were placed into a new flask (i.e., 1:10 split). On the day of the experiment, the same procedure was followed and cells were split 1:2. Cell density in each flask was calculated by using a hemocytometer (American Optical Co., Buffalo, NY), and cells were dispensed into 60 mm plates (Corning Glass Works, Corning, NY) each with five ml of medium (Table 3-1). Cells were incubated as before for two to four hours, then the desired concentrations of FM or 245-HBB (Table 3-1) were added. Each treatment group consisted of ten plates. One hour later, 50 µl of 6-thioguanine

Table 3-1. Experimental Design of Metabolic Cooperation Assay using 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB) or Firemaster BP-6 (FM) as Test Chemicals with Resistant ($HGPRT^-$) and Sensitive ($HGPRT^+$) WB-F344 cells.

Group name	Volume of medium (ml)	Resistant cells/ml medium	Sensitive cells/ml medium	Volume of chemical per plate	Final [ ] per ml of medium
PE	4	100	----	25 µl DMSO	5 µl
N	3	100	4×10^5	25 µl DMSO	5 µl
AL	3	100	4×10^5	10 µl Aldrin	10 µl
TA	4	100	----	25 µl [a]	1 µg [a]
TB	4	100	----	25 µl [a]	5 µg [a]
TC	4	100	----	25 µl [a]	20 µg [a]
TD	4	100	----	25 µl [a]	40 µg [a]
MA	3	100	4×10^5	25 µl [a]	1 µg [a]
MB	3	100	4×10^5	25 µl [a]	5 µg [a]
MC	3	100	4×10^5	25 µl [a]	20 µg [a]
MD	3	100	4×10^5	25 µl [a]	40 µg [a]

[a] = Firemaster BP-6 or 2,2',4,4',5,5'-hexabromobiphenyl

n = 10 plates per group

Abbreviations: PE = Plating efficiency group
N = Negative control group
AL = Aldrin (positive control) group
T* = Cytotoxicity assay groups
M* = Metabolic cooperation assay groups
DMSO = Dimethylsulfoxide (vehicle)
[ ] = Concentration

(6-TG) (Sigma Chemical Co., St. Louis, MO) were added to each plate.

Four days after the beginning of the experiment, the medium in the flasks was discarded and replaced with fresh medium, and 50 µl of 6-TG were again added to each plate. Eight days after the beginning of the experiment the medium was discarded, cell colonies were rinsed twice with PBS, fixed and stained with a solution of 10% ethanol and 1.0% crystal violet (Sigma Chemical Co., St. Louis, MO), dried, and counted (Colony Counter, American Optical Co., Buffalo, NY).

Statistics. A one-way analysis of variance was used to determine significance between treatment and control groups (Steel and Torrie, 1980a). A Student-Newman-Keul's test was used for multiple comparisons (Steel and Torrie, 1980b). Significance was defined as $P \leq 0.05$.

Fluorescence Redistribution After Photobleaching (FRAP) Assay.

The WB-F344 cells were plated at low density (not confluent) on 35 mm plates (Corning Glass Works, Corning, NY) in two ml of medium as described above (but without phenol red) in a humidified incubator at 37^0 C and 5% CO_2. Cells were allowed to settle and attach to the plate for one hour. After that time, each plate received 1, 5, 20, or 40 µg 245-HBB/ml of medium. The 245-HBB was dissolved in DMSO as a vehicle. Cells were

allowed to incubate for 24 hours, after which the medium was decanted from the plates. Cells were rinsed twice with calcium/magnesium saline solution (made by adding one gram $CaCl_2$ and one gram $MgCl_2$ to 10 liters PBS), and two ml of calcium/magnesium saline solution were added back to the plates. Fourteen µl (seven µl/ml of medium) of 0.1 mg/ml 6-carboxyfluorescein diacetate (6-CFDA) (Molecular Probes, Inc., Eugene, OR) were slowly added to the plates, mixed with gentle agitation, and allowed to stain the cells for a period of 20 minutes in incubator conditions. After 20 minutes, the calcium/magnesium saline solution and 6-CFDA were decanted, two ml of medium (without phenol red) were added, and cells were examined by using Anchored Cell Analysis and Sorting (ACAS 470, Meridian Intruments, Okemos, MI).

Cells appearing in touching pairs and as single cells were evaluated at a magnification of 400 X using phase contrast microscopy. One cell in a touching pair was selected for photobleaching, and single nontouching cells were selected as negative controls for fluorescence redistribution. The same number of photobleaching points was selected for each photobleached cell. The digitized pseudoimages were examined, and selected cells were then marked with boxes and photobleached at photomultiplier tube voltage of 25-30%, argon ion laser power of 200 milliwatts, blast

strength of 30%, scan strength of 7%, blast time of 250 milliseconds, and a stage speed of 40.0 megahertz. Cells were then analyzed for fluorescence recovery following photobleaching. Recovery of fluorescence was monitored for a period of 15 minutes after initial photobleaching, with one post-bleaching scan every five minutes for the duration of the 15 minute period. Digitized pseudoimages were recorded and saved in the computer's memory (Model XT, International Business Machines, Boca Raton, FL), and previously selected cells were compared for return of fluorescence in 245-HBB-treated and nontreated (DMSO only) cells.

Scrape-Loading/Dye Transfer Assay

Cell and Culture Conditions. The WB-F344 cells were grown overnight to confluency in 35 mm plates in incubator conditions and using medium (without phenol red) as previously described. Final concentrations of 1, 5, 20, and 40 μg 245-HBB/ml medium (in DMSO as the vehicle) were added. After 24 hours, cells were rinsed twice with room temperature PBS. There were six plates in each treatment group. A dye mixture of 0.05% Lucifer yellow and 0.05% rhodamine dextran (Molecular Probes, Inc., Eugene, OR) was dissolved in PBS and added to the cell culture. Care was taken to not expose the dye mixture to excessive room light. A wooden probe was used to scrape several rows of cells, and the dye

solution was left on the cells for two minutes after scraping. The dye was decanted and cells were rinsed twice with PBS at 25^0 C to remove background fluorescence and any detached cells. Two milliliters of medium were added to each plate. Plates were examined for the distribution of yellow-green fluorescence (from Lucifer yellow) and red fluorescence (from rhodamine dextran) from scraped edges using a phase microscope with epifluorescence capacity and appropriate filters for fluorescence detection of the two different fluorochromes (Figures 3-1 through 3-3).

Quantitation of Fluorescence Intensity. Plates from each treatment group were examined on an Anchored Cell Analysis Sorter (ACAS 470, Meridian Intruments, Okemos, MI). Digitized pseudoimages of scraped edges were recorded at a magnification of 400 X using a photomultiplier tube voltage of 25-30% and a scan strength of 7%. Digitized pseudoimages were stored in the computer, and fluorescence intensity values were obtained for each field examined. A total of ten fields from each plate was quantified for fluorescence intensity.

Statistics. Data were analyzed using a one-way analysis of variance (Steel and Torrie, 1980a). Significance was defined at a level of $P \leq 0.05$.

Figure 3-1. Phase contrast photomicrograph of a monolayer of untreated WB-F344 cells in culture. Upper left portion of plate has been scraped with a wooden probe. Cells appear confluent and have normal conformation in unscraped portion (400 X).

Figure 3-2. Photomicrograph from same field as Figure 3-1 stained with Lucifer yellow (LY) and rhodamine dextran. Notice primary LY-loaded cells along the scraped edge have the most intense fluorescence and that the fluorescence extends four to seven cell layers beyond the primary LY-loaded cells, indicating that LY has transferred via gap junctions into the secondary LY-recipient cells (Rhodamine dextran/Lucifer yellow, B filter, 400 X).

Figure 3-3. Photomicrograph from same field as Figure 3-1 stained with Lucifer yellow and rhodamine dextran (RD). Notice intracellular loading of RD in the cell layer along the scraped edge but that RD did not transfer to layers of cells away from scraped edge, indicating that cell membranes away from scraped edge are intact (Rhodamine dextran/Lucifer yellow, G filter, 400 X).

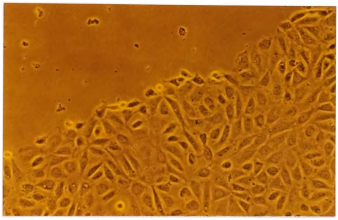


Figure 3-1

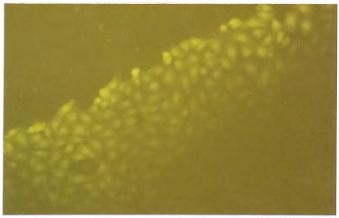


Figure 3-2

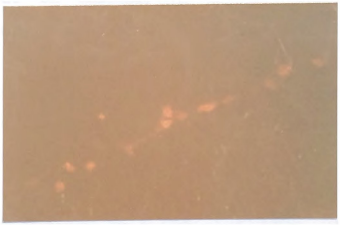


Figure 3-3

RESULTS

Metabolic Cooperation Assay

The results from the metabolic cooperation assay are depicted in Figure 3-4. The chemical 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB) inhibited metabolic cooperation at the noncytotoxic concentrations used and in a dose-dependent manner such that the highest concentration of 245-HBB (40 µg/ml medium) had a three-fold greater inhibition of metabolic cooperation than those cells receiving a concentration of 1 µg/ml medium. A similar pattern was seen when the chemical Firemaster BP-6 (FM) was tested at identical concentrations of 245-HBB, as FM caused a nearly four-fold increase in recovery of mutant (i.e., $HGPRT^-$) cells. However, the degree of inhibition of metabolic cooperation with FM was not significantly different from 245-HBB when tested at the same concentrations as FM. These results indicate that FM and 245-HBB inhibit metabolic cooperation in WB-F344 cells in a dose-dependent manner, but that one chemical does not inhibit metabolic cooperation more than the other in this in vitro testing system.

Figure 3-4. Effect of 245-HBB and FM on metabolic cooperation (MC) in WB-F344 cells. The top two (red and green) lines represent cytotoxicity curves for 100 $HGPRT^-$ cells/ml medium not co-cultured with $HGPRT^+$ cells and exposed to various concentrations of FM and 245-HBB. The bottom two (blue and red) lines represent % of $HGPRT^-$ cells recovered in the metabolic cooperation assay when 100 $HGPRT^-$ cells are co-cultured with 4 X 10^5 $HGPRT^+$ cells and exposed to various concentrations of FM or 245-HBB. In the metabolic cooperation assay at a concentration of 40 µg FM/ml medium, there was a four-fold increase in % recovery of 6-TG-resistant mutants (i.e., $HGPRT^-$ cells) when compared to 1 µg FM/ml medium. Similar concentrations of 245-HBB had a three-fold increase in recovery of 6-TG-resistant mutants. There was no statistical difference between the ability of FM and 245-HBB to inhibit metabolic cooperation when compared at the same concentrations (i.e., 1, 5, 20, and 40 µg/ml medium).

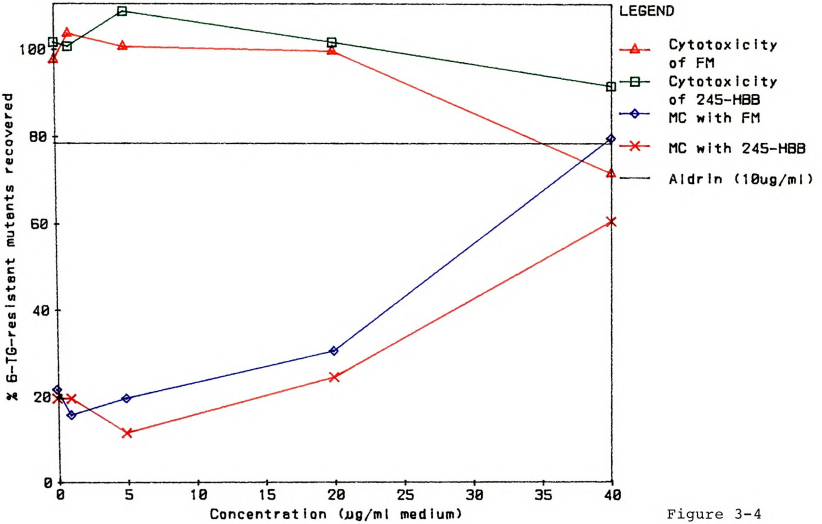

LEGEND
Cytotoxicity of FM
Cytotoxicity of 245-HBB
MC with FM
MC with 245-HBB
Aldrin (10ug/ml)
% 6-TG-resistant mutants recovered
0
20
40
60
80
100
0
5
10
15
20
25
30
35
40
Concentration (µg/ml medium)

Figure 3-4

Fluorescence Redistribution After Photobleaching (FRAP) Assay

Redistribution of the fluorescent dye 6-carboxyfluorescein diacetate (6-CFDA) into touching photobleached WB-F344 cells treated with 245-HBB was most inhibited in those cells treated with 40 µg 245-HBB/ml medium (Figures 3-5 through 3-13). Touching cells treated with lower concentrations of 245-HBB had a greater degree of return of the 6-CFDA into photobleached cells such that the lowest concentration, 1.0 µg 245-HBB/ml medium, had only slightly less return of 6-CFDA than non-treated controls. These results were similar to those obtained with cells treated with DMSO only (control group).

Scrape-Loading/Dye Transfer Assay

In those plates treated with 40 µg 245-HBB/ml medium, transfer of Lucifer yellow into confluent WB-F344 cells was totally blocked and did not spread from the primary dye-loaded cells into secondary recipient cells. (Figures 3-14 through 3-19). Lucifer yellow spread into one secondary dye-recipient layer of cells in those plates treated with 20 µg 245-HBB/ml medium. Redistribution of Lucifer yellow into secondary

Figure 3-5. Photograph of digitized pseudo-image of WB-F344 cells grown in culture, not exposed to test chemical (negative control), stained with 6-carboxyfluorescein diacetate, and analyzed for fluorescence redistribution after photobleaching with Anchored Cell Analysis and Sorting. Photobleaching of cells in boxes has not yet occurred. Notice that cells in all boxes except one (arrow) are attached to touching cells. Cell in box (arrow) is not touching other cells and is a negative control for dye return.

Figure 3-6. Photograph of above image immediately following bleaching with argon ion laser. Notice that cells in all boxes have been completely photobleached.

Figure 3-7. Photograph of same field 15 minutes following initial photobleaching of cells in boxes with argon ion laser. Notice return of fluorescence to prebleaching levels in all boxes except nontouching cell.

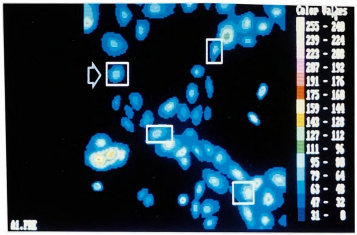


Figure 3-5

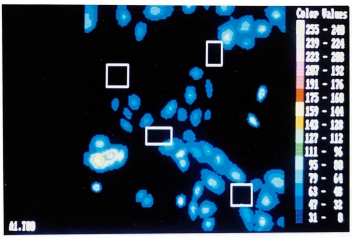


Figure 3-6

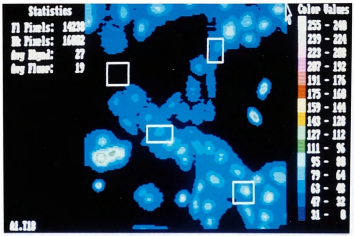


Figure 3-7

Figure 3-8. Photograph of digitized pseudo-image of WB-F344 cells grown in culture, exposed for 24 hours to 5 μg 245-HBB/ml medium, stained with 6-carboxyfluorescein diacetate, and analyzed for fluorescence redistribution after photo-bleaching. Photobleaching of cells in boxes has not yet occurred. Notice that all boxes except one (arrow) are around touching cells. Cell in box (arrow) is not touching other cells and serves as a negative control for dye transfer.

Figure 3-9. Photograph of above image immediately following photobleaching of the dye with argon ion laser. Notice that cells in all boxes have been completely photobleached. (Cell in box in lower left corner was not photobleached.)

Figure 3-10. Photogragh of same field 15 minutes following initial photobleaching of cells in boxes with argon ion laser. Notice slight return of fluorescence in three of the five boxes in which touching cells were located and no return of fluorescence in the other two boxes. These results suggest that gap junctional transfer of the dye was partially inhibited by 5 μg 245-HBB/ml medium.

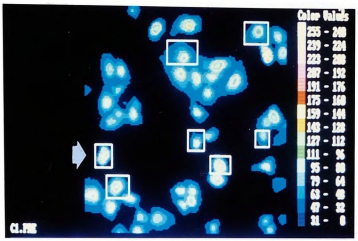


Figure 3-8

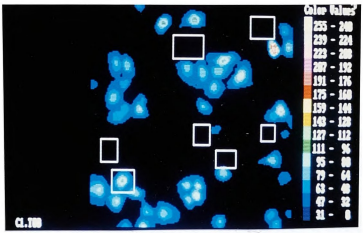


Figure 3-9

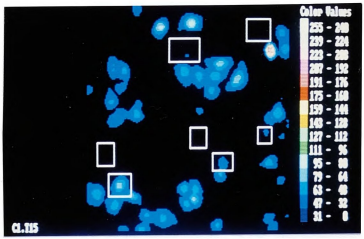


Figure 3-10

Figure 3-11. Photograph of digitized pseudo-image of WB-F344 cells grown in culture, exposed for 24 hours to 20 μg 245-HBB/ml medium, stained with 6-carboxyfluorescein diacetate, and analyzed for fluorescence redistribution after photobleaching. Photobleaching of cells in boxes has not yet occurred. Notice that cells in all boxes except two (arrows) are touching other cells. Cells in boxes (arrows) are negative controls for dye return.

Figure 3-12. Photograph of above image immediately following photobleaching of the dye with argon ion laser. Notice that cells in all boxes (except cell in upper left) have been completely photobleached. (Cell in upper left was not photobleached).

Figure 3-13. Photograph of same field 15 minutes following initial photobleaching of cells in boxes. Notice total lack of return of fluorescence in all boxes. These results suggest that gap junctional transfer of the dye was totally blocked by 20 μg 245-HBB/ml medium.

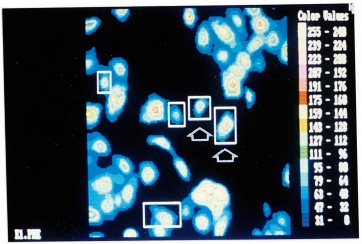


Figure 3-11

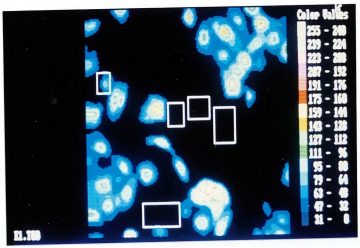


Figure 3-12

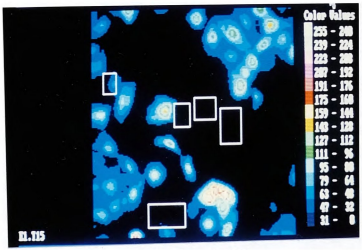


Figure 3-13

Figures 3-14 through 3-19

3-14 / 3-15

3-16 / 3-17

3-18 / 3-19

Figures 3-14 through 3-19. Series of photomicrographs of WB-F344 cells grown in culture, exposed to various concentrations of 245-HBB, and subjected to the scrape-loading/dye transfer assay. Notice that cells not exposed to any chemical (Figure 3-14-negative control) have yellow dye (Lucifer yellow) in several cell layers beneath the scraped edge. This is interpreted as noninhibition of gap junctional communication. Cells exposed to 1 µg 245-HBB/ml medium (Figure 3-15), 5 µg 245-HBB/ml medium (Figure 3-16), 20 µg 245-HBB/ml medium (Figure 3-17) and 40 µg 245-HBB/ml medium (Figure 3-18) have progressively inhibited transfer of Lucifer yellow dye into secondary recipient cells. Control cells (DMSO only) are shown in Figure 3-19, and had a similar degree of transfer of Lucifer yellow as Figure 3-14, indicating that the vehicle used (DMSO) did not interfere with transfer of Lucifer yellow (Rhodamine dextran/Lucifer yellow, B filter, 400 X.)

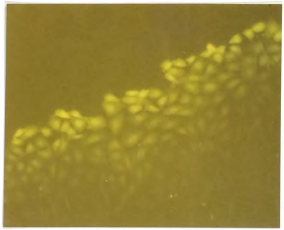


Figure 3-14

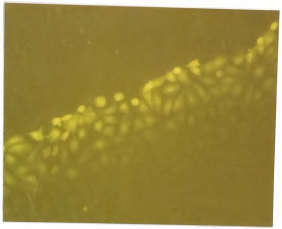


Figure 3-15

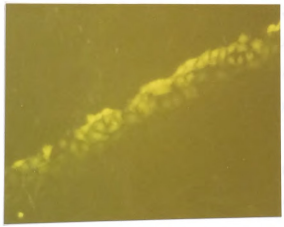


Figure 3-16

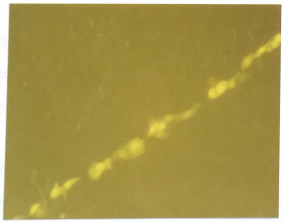


Figure 3-17

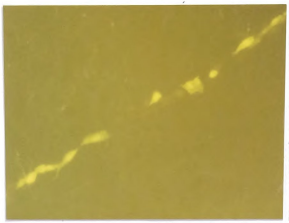


Figure 3-18

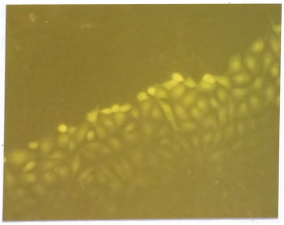


Figure 3-19

recipient cells was more noticeable in cells treated with 5 μg 245-HBB/ml medium. Cells treated with 1.0 μg 245-HBB/ml medium appeared to have only slightly less redistribution of Lucifer yellow into secondary recipient cells than cells receiving DMSO only. In all plates, the larger molecular weight dye, rhodamine dextran, was confined to the primary loaded cell layer, and in no instance did this dye redistribute into secondary recipient cells. This indicates that cell membranes remained functionally intact during the experiment, and that the concentrations of 245-HBB used were probably not high enough to cause membrane damage to the WB-F344 cells.

Results from quantitation of fluorescence from scrape-loading/dye transfer experiments are seen in Figures 3-20 through 3-26. These results confirm the visual observation of an inverse correlation between the concentration of 245-HBB and the amount of dye transfer into secondary recipient cells.

Figures 3-20 through 3-25

Figures 3-20 through 3-25. Series of photographs of digitized pseudoimages of WB-F344 cells grown in culture, exposed to various concentrations of 245-HBB, subjected to the scrape-loading/dye transfer technique, and examined with Anchored Cell Analysis and Sorting to quantify fluorescence intensity. Notice that cells not exposed to any chemical (Figure 3-20-negative control) have fluorescence in several cell layers beneath the scraped edge at the left of each photograph. This is interpreted as noninhibition of gap junctional communication. Cells exposed to 1 µg 245-HBB/ml medium (Figure 3-21), 5 µg 245-HBB/ml medium (Figure 3-22), 20 µg 245-HBB/ml medium (Figure 3-23), and 40 µg 245-HBB/ml medium (Figure 3-24) had progressively inhibited transfer of dye into secondary recipient cells. Control cells (DMSO only) are shown in Figure 3-25, indicating that the vehicle used, DMSO, did not interfere with dye transfer (Rhodamine dextran/Lucifer yellow, 400 X).

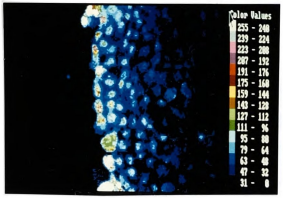


Figure 3-20

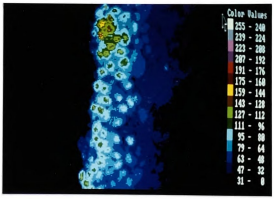


Figure 3-21

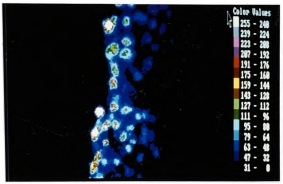


Figure 3-22

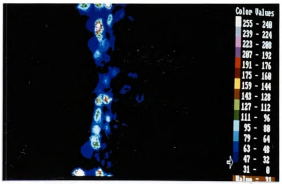


Figure 3-23

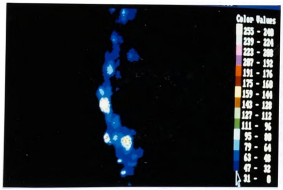


Figure 3-24

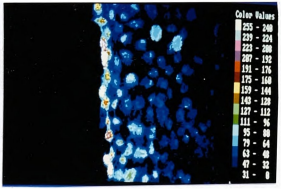
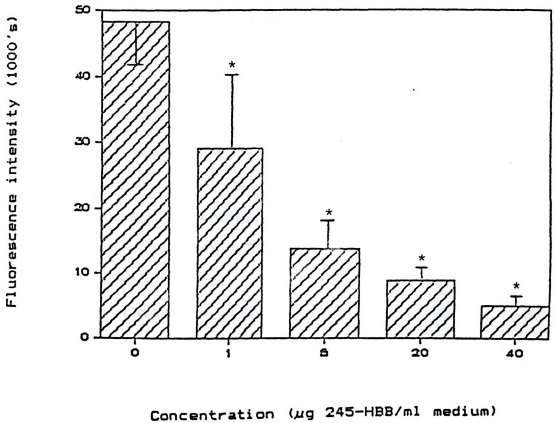


Figure 3-25

Figure 3-26. Quantitation of fluorescence intensity (relative units) in WB-F344 cells exposed to 1, 5, 20, or 40 μg 245-HBB/ml medium and subjected to the scrape-loading/dye transfer assay. All treatment groups were significantly different (*) from the nontreated control group.

Figure 3-26


Fluorescence intensity (1000's)
50
40
30
20
10
0
*
*
*
*
0
1
5
20
40
Concentration (µg 245-HBB/ml medium)

DISCUSSION

The results of the metabolic cooperation assay, the fluorescence redistribution after photobleaching assay (FRAP), and the scrape-loading/dye transfer assay indicate that 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB), at noncytolethal concentrations, blocks in vitro gap junction-mediated intercellular communication in a dose-dependent manner. These results agree with the results from previous in vitro studies in which 245-HBB, the major congener in Firemaster BP-6 (FM), was shown to inhibit metabolic cooperation in Chinese hamster V-79 cells (Trosko et al., 1981). Results from the current study are also in agreement with those from another experiment in which 245-HBB was shown to inhibit metabolic cooperation in a dose-dependent manner in Chinese hamster V79 cells (Tsushimoto et al., 1983). However, results from the present study when compared with those of Tsushimoto et al. (1983) indicate that there are differences in the slopes of the dose-response curves between WB-F344 cells and Chinese hamster V79 cells when each cell line was exposed to similar concentrations of 245-HBB. This may be due to inherent biochemical and metabolic differences between these two

cultured cells lines. However, the precise differences between these cell lines responsible for these results are unknown.

Results from this study indicate that the cytotoxic concentration for WB-F344 cells exposed to FM is probably between 20 and 40 μg/ml medium. At a concentration of 40 μg FM/ml medium, there is recovery of about 80% of 6-thioguanine-resistant (i.e., $HGPRT^-$ cells) (Figure 3-4). This concentration is in the range of cytotoxicity for these cells as indicated by the cytotoxicity curves. However, it is unusual that the number of mutant cells recovered at this concentration in the metabolic cooperation plates was greater than the number recovered from cytotoxicity plates. One explanation for this may be that at cytotoxic levels of a chemical, killing of wild type (i.e., $HGPRT^+$) cells may allow some mutant (i.e., $HGPRT^-$) cells to survive by not receiving the lethal biochemical product from a dead neighboring cell. This phenomenon could result in an increased survival of mutant cells at cytotoxic concentrations of the chemical. Another explanation is that the cell density in cytotoxicity plates is lower than in metabolic cooperation plates. Therefore, the effective concentration per cell is greater in cytotoxicity plates.

Firemaster BP-6 has been shown to be a more potent tumor promoter than 245-HBB at the same doses when both

chemicals were tested with in vivo tumor promotion studies. However, FM was not a stronger inhibitor of metabolic cooperation than 245-HBB when given at the same concentrations in the present in vitro study. Findings from previous studies with various tumor promoting agents have shown that results from metabolic cooperation assays are reasonably predictive of the tumor promoting activity of these compounds in certain in vivo initiation/promotion hepatocarcinogenesis systems (Ito et al., 1980; Tennekes et al., 1982; Tatematsu et al., 1983; Trosko et al., in press, 1987). The reason for the discrepancy between the results of previous in vivo studies and those of the present in vitro studies with FM and 245-HBB is not readily apparent.

The characterization and successful isolation of the WB-F344 cell line has been described by Tsao et al. (1984). It is an epithelial cell, isolated from the livers of adult male Fischer 344 rats, that expresses the oval cell phenotype. Oval cells are a population of nonparenchymal cells in the liver which have the capacity to proliferate and undergo phenotypic and karyotypic changes in response to hepatocarcinogens (Tsao et al., 1985). Histologically, WB-F344 cells resemble biliary epithelial cells. The WB-F344 cells are useful because they can be transformed in vitro, yet are diploid. Many other cell lines that continuously grow in culture conditions are not diploid.

The WB-F344 cells in culture may more closely resemble in vivo metabolic characteristics than many other cultured cell lines. Therefore, culture systems using this cell line may be of more value than other cell lines for predicting the in vivo effects of certain environmental toxicants.

The usefulness of the WB-F344 cell line in the metabolic cooperation assay has been recently established (Jone et al., in press, 1987). Since metabolic cooperation is dependent upon functioning gap junctions, this cell line could be capable of detecting certain chemicals which inhibit cell-cell communication in rat epithelial cells. Previous studies in which two organochlorine pesticides, dieldrin and aldrin, were used in the metabolic cooperation assay indicate that cell-cell communication was inhibited in several different cell lines when exposed to these environmental toxicants at noncytolethal concentrations (Jone et al., 1985; Kurata et al., 1982; Trosko et al., in press, 1987; Lin et al., 1986). In vivo studies have shown that these compounds are tumor promoters in rat initiation/promotion hepatocarcinogenesis systems (Ito et al., 1980; Tatematsu et al., 1983; Tennekes et al., 1982). Therefore, results from these in vitro studies have been predictive of the in vivo behavior of certain environmental toxicants and suggest that one possible mechanism of tumor promotion may be the inhibition of

gap junction-mediated intercellular communication. While in vitro studies are not a replacement for more conventional animal studies, such in vitro experiments provide another means by which to detect nongenotoxic hepatocarcinogens and hepatic tumor promoters which may be missed by traditional in vitro short term genotoxic assays.

One limitation of the metabolic cooperation assay, as with several in vitro cell systems, is that it does not totally reflect in vivo biological complexity. This limitation has been recognized by Williams and co-workers (1981) who have used cultures of primary rat hepatocytes co-cultured with other cells to enhance the metabolic capabilities of their in vitro system. The WB-F344 cells probably have their greatest potential in co-cultured cell systems with normal rat hepatocytes. Such a co-culture system would more closely resemble in vivo metabolic characteristics.

The three assays used in the present study have different strengths and weaknesses. The metabolic cooperation assay is well-established and measures gap junctional intercellular communication over a period of approximately 3-4 days. However, cell-cell communication is not measured at any intermediate times during the course of a metabolic cooperation assay. Therefore, if a test chemical blocks intercellular communication for only a short time then this transient

phenomenon would not be detected. Reversibility of inhibited cell-cell communication would not be detected in our metabolic cooperation assays.

The FRAP assays have some advantages over the metabolic cooperation assay. With FRAP, there is direct visualization of the return of dye via gap junctions into touching cells. With the metabolic cooperation assay, other factors, such as contamination of culture plates by low numbers of bacterial or fungal organisms, may influence cell survival. If not detected, even slight amounts of contamination could lead to erroneous results. The FRAP analysis is performed over a shorter time period, somewhat reducing the chances of microbial contamination.

The FRAP assay is not dependent on consistent biochemical or metabolic factors within the cell. With the metabolic cooperation assay it is assumed that certain biochemical pathways are not only present in the cells but that they are consistently functioning as well. These assumptions are not made with FRAP analysis. However, FRAP analysis is done over a relatively short duration when compared to the metabolic cooperation assay. It is possible that test chemicals which at first inhibit gap junctional communication may reverse this inhibition after only a short time. Analysis with FRAP may be of a short enough duration to allow detection of only temporary blockage of cell-cell

communication, whereas results from the metabolic cooperation assay may indicate no inhibition of communication. Perhaps testing chemicals with both assays would permit better characterization of transient inhibition of cell-cell communication.

The scrape-loading/dye transfer assay has several advantages over the other assays used in this study. It is relatively simple when compared to the FRAP and metabolic cooperation assays and gives a rapid and clear visual assessment of spread of a dye into cell layers. It is of low cost and probably has its greatest potential as a rapid screening assay to quickly assess the ability of test chemicals to inhibit gap junctional communication (El-Fouly et al., in press, 1987). It is similar to the FRAP assay in that detailed characterization of metabolic and biochemical pathways in the cells used is not needed to perform the assay.

Both the FRAP assay and the scrape-loading/dye transfer technique depend on transfer of an exogenous dye from one cell to another. The toxicity of dyes to cultured cells is unknown, but these biological tracers are assumed to be physiologically inert. However, it is possible that certain cell lines used with these dyes may be exquisitely sensitive to deleterious effects from such exogenous compounds. Nevertheless, the scrape-loading/dye transfer technique, as used in the present

study, appears to be sensitive enough to be used for the determination of dose-response curves with certain test chemicals.

The nature of the loss of gap junctional function is generally unknown. However, there are several conceivable mechanisms by which this may occur. Perhaps patency of gap junctional hemichannels is lost, as hypothesized by Loewenstein (1979). Other possible mechanisms include inhibition of assembly of gap junctional precursor proteins into the plasma membrane, inhibition of mRNA coded for production of gap junctional precursor proteins, or increased degradation of assembled gap junctional plaques. It is currently unknown which of these phenomena is most responsible for chemically-induced communication incompetence between these cultured cells.

SUMMARY-CHAPTER 3

Conclusions from the preceding experiments include the following:

1) The compound 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB) inhibits gap junctional intercellular communication in a dose-dependent manner in the metabolic cooperation assay, the fluorescence redistribution after photobleaching ("FRAP") assay, and the scrape-loading/dye transfer assay.

2) Both Firemaster BP-6 (FM) and its major congener, 245-HBB, inhibit gap junctional intercellular communication as determined by the metabolic cooperation assay. The difference between the ability of these two agents to do so was not significantly different.

3) The scrape-loading/dye transfer assay, a new technique for assessing gap junctional intercellular communication, was useful for measuring the dose-responsiveness of inhibition of cell-cell communication by 245-HBB. This technique has potential as a rapid and simple method for assessing the ability of many

environmental toxicants to inhibit intercellular communication.

Results from these studies suggest that the ability of 245-HBB to behave as a tumor promoter in vivo may be associated with the ability of this compound to inhibit intercellular communication in vitro. In addition, these results imply that the metabolic cooperation assay, the FRAP assay, and the SL/DT assay are each sensitive enough to be used for dose/response studies to measure the ability of certain environmental toxicants to inhibit gap junctional intercellular communication.

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VITA

VITA

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