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THE TOXIC AND CARCINOGENIC EFFECTS OF
N-NITROSODIMETHYLAMINE AND N-NITROSOPYRROLIDINE
IN THE LIVER AND NASAL CAVITY OF RATS

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By

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ABSTRACT

THE TOXIC AND CARCINOGENIC EFFECTS OF N-NITROSODIMETHYLAMINE AND N-NITROSOPYRROLIDINE IN THE LIVER AND NASAL CAVITY OF RATS

By

Charles Rangga-Tabbu

Female Sprague-Dawley rats were each given an intraperitoneal dose of N-nitrosodimethylamine (NDMA) or N-nitrosopyrrolidine (NPYR) to assess the development and fate of lesions in hepatic and nasal tissues. Tissues were collected from rats killed at 6 or 12 hours and 1, 3, 10 or 30 days after dosing. Olfactory epithelium and adjacent Bowman's glands were specifically targeted by each chemical. Lesions were seen as early as 6 hours and were most extensive by 3 days. At the high doses regeneration was not complete by 30 days. To test for additive effects, NDMA and NPYR were given simultaneously and tissues were collected at 3 and 30 days. Acute hepatic necrosis resulted from exposure to a high dose of NDMA and from simultaneous exposure to medium doses of each chemical. Additive effects of lower doses were seen in nasal tissues. Immunohistochemical staining for glutathione S-transferase placental form (GST-P) revealed single GST-P positive hepatocytes as early as 1 day and altered hepatocellular foci (AHF) by 30 days. Identification of GST-P positive cells shortly after exposure to known carcinogens provides a means of detecting exposure to tumor initiating agents such as NDMA or NPYR which may occur in

food. In a second experiment, weanling rats were initiated with NDMA or NPYR and promoted with a single oral dose of polybrominated biphenyls. Development of preneoplastic lesions was assessed by GST-P staining of liver and nasal tissues from rats killed after 30, 120 or 180 days of promotion. Rats initiated with either NDMA or NPYR and promoted with PBB had significant increases in AHF by 180 days. Appreciable numbers of AHF were evident in nonpromoted rats given either compound. These results suggest that short term exposure to an environmental chemical such as PBB can act as a hepatic tumor promoter in rats initiated with NDMA or NPYR. Furthermore, short term exposure to NDMA or NPYR even without exposure to known promoters may have long term hepatocarcinogenic effects. Preneoplastic lesions were not detected in nasal tissues during the course of these experiments.

Dedicated to

Mbak Rien and Felix Febriyano

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INTRODUCTION

INTRODUCTION

Epidemiologic studies have indicated that a majority of human cancers are associated with environmental factors (Doll and Peto, 1981) such as chemical contaminants, food products, cigarette smoking, ultraviolet light and occupational exposure. Although the extrapolation from animal testing to human beings is difficult (Lijinsky, 1988), in vivo studies of chemical carcinogenesis are still useful in predicting the risk of human populations to environmentally related cancer.

The present studies are concentrated on three carcinogens; N-nitrosodimethylamine (NDMA), N-nitroso-pyrrolidine (NPYR) and polybrominated biphenyls (PBBs). N-Nitrosodimethylamine and NPYR are two highly carcinogenic environmental chemicals (Lijinsky and Reuber, 1981; Preussmann and Stewart, 1984; Ishinishi et al., 1988) which are present at low concentration in various food products (Spiegelhalder et al., 1980; Song and Hu, 1988; Ishinishi et al., 1988). Both compounds have been shown to be metabolized in the liver (Brambilla et al., 1981; Streeter et al., 1990) and nasal mucosa of rats (Brittebo et al., 1981; Bermudez, 1986) and their injurious effects are suggested to be related to their metabolites (Brittebo and Tjalve, 1983; Alldrick et al.,

1985). Acute exposure of rats to NDMA has been found to produce lesions, mainly in the liver (Shank, 1975) and kidney (Hard et al., 1984). On the other hand, lesions due to acute exposure to NPYR have not been well characterized.

Dual exposure to NDMA and NPYR, which may be present in various food products, is possible and may result in additive or synergistic effects. Development and fate of morphologic lesions in the nasal cavity due to single or dual acute exposure to NDMA and NPYR have not been well characterized.

Since NDMA and NPYR are known to be carcinogenic (Preussmann and Stewart, 1984) and mutagenic (Guttenplan, 1987), they may be able to induce preneoplastic lesions in the liver and nasal cavity. Subsequent exposure to environmental chemicals such as polybrominated biphenyls (PBBs), which are known as liver tumor promoters (Jensen et al., 1983; Jensen and Sleight, 1986) may increase the risk of development of tumors.

Polybrominated biphenyls have been known as an environmental contaminant in Michigan since 1973 (Carter, 1976) and may get into the human population through the food chain (Lilis et al., 1978) or by occupational contact (Williams et al., 1984). Since PBBs are highly persistent in the environment as well as in animal (Fries, 1985) or human tissues (Tuey and Matthews, 1980), this chemical mixture may become a long term health problem for human beings. The role of PBBs as a promoter in the liver and nasal cavity following

initiation with NDMA and NPYR has not been established.

Studies on mechanisms of tumor development in humans and animals indicate a multistage process (Farber and Cameron, 1980; Scherer, 1984). The natural development of these stages may be monitored by the analysis of preneoplastic lesions following initiation which are further developed during the promotion and progression stages (Farber and Sarma, 1987). Among various immunohistochemical markers that have been developed to identify these preneoplastic lesions, glutathione S-transferase placental form (GST-P) is considered to be the most reliable (Satoh et al., 1985). Although the role of GST-P as a marker enzyme in liver carcinogenesis has been well characterized (Sato, 1988), its role as a marker in nasal cavity carcinogenesis is unknown.

The present experiments were designed to characterize the development of acute lesions in the liver and nasal cavity of rats and to determine if GST-P is useful as a specific marker enzyme in preneoplastic lesions in both organs after single or combination exposure to NDMA and NPYR. An additional objective was to characterize the development of preneoplastic lesions in the liver and nasal cavity after initiation with NDMA or NPYR and promotion with PBBs. Early detection of the carcinogenic process may be important in characterizing the sequential development of carcinogenesis and in the preventive or therapeutic intervention during the early phases of neoplastic disease processes.

LITERATURE REVIEW

LITERATURE REVIEW

N-Nitrosamines

N-Nitrosamines are a group of environmental chemicals most of which have been shown to be mutagenic and carcinogenic for various laboratory animals (Bogovski and Bogovski, 1981; Lijinsky and Reuber, 1981; Guttenplan, 1987). These compounds are widely distributed in the environment, including various food products, alcoholic beverages (Spiegelhalder et al., 1980), tobacco and tobacco smoke (Brunnemann et al., 1977; Hoffmann et al., 1979), rubber materials (Spiegelhalder and Preussmann, 1983), cosmetic products (Fan et al., 1977), pesticide products (Zweig et al., 1980), tanned leathers (Rounbehler et al., 1979), emissions from diesel engines (Goff et al., 1980), and ambient air of industrial areas (Spiegelhalder and Preussmann, 1987). N-Nitrosamines have been reported at levels of a few µg/kg in cured meat (Sen et al., 1979), cheese, sausage (Spiegelhalder et al., 1980), sea foods (Yu and Henderson, 1987), and milk products (Libbey et al., 1980).

The estimated amount of daily human exposure to these compounds through food products is about 0.1 to 1.2 µg (Spiegelhalder et al., 1980; Bartsch and Montesano, 1984) and

by the occupational route is approximately 5 to 180 µg (Bartsch and Montesano, 1984) per day. However, simultaneous exposure to several different N-nitrosamines that might be present in the environment as well as continuous exposure for a long period might contribute in the development of cancer. Results of epidemiologic studies have indicated a correlation between consumption of certain N-nitrosamine-containing foods and development of tumors (Yu and Henderson, 1987; Song and Hu, 1988; Siddiqi et al., 1988; Sarkar et al., 1989).

Formation of N-Nitrosamines. N-Nitrosamines are the N-nitroso derivatives of secondary amines (Fajen et al., 1980). The R_1 and R_2 are alkyl or aryl moieties and they may be connected to form cyclic compounds (Preussmann and Stewart, 1984). The nitrosyl part (NO) can be derived from nitrogen oxides, such as NO, NO_2, N_2O_3 or N_2O_4 (Challis et al., 1978) or from nitrous acid or nitrite salts (Mirvish, 1975). In the laboratory, N-nitrosamines are commonly made by the reaction of secondary amines with nitrous acid at acidic conditions (Fajen et al., 1980). However, depending on the reactants and catalysts that are being used, N-nitrosation can also occur at neutral or alkaline pH (Fine, 1979). Formation of N-nitrosamines from primary and tertiary amines and quaternary ammonium compounds has also been reported (Fiddler et al., 1972; Warthesen et al., 1975). Another mode of formation of N-nitrosamines is by transnitrosation, whereby other nitro or nitroso compounds serve as the nitrosating agent (Singer et

al., 1978). In many food systems, naturally occurring secondary amines and nitrites that may be added as preservatives or produced by bacterial reduction of nitrates are the most important source of N-nitrosamines (Gray et al., 1979).

The availability of precursor amines and nitrites in the environment is a significant factor in the formation of N-nitroso compounds. Many types of amines can be found in certain agricultural products, food components and additives, drugs, industrial components and products such as rubber additives, plastics, cosmetics, and solvents (Lijinsky, 1980; Fajen et al., 1980). Nitrite is widely distributed in the environment and is used in curing of meat, poultry and fish (Roberts, 1975; Gray et al., 1979) and in industry as corrosion inhibitors (Shuker, 1988).

Endogenous synthesis of N-nitrosamines may be the most significant mode of human exposure to these compounds. Several factors are important in the endogenous formation of N-nitrosamines including the type and quantity of precursor amines in the diet (Mirvish, 1975), concentrations of dietary nitrate and atmospheric nitrogen-oxide (NO) exposure (Wagner et al., 1983), salivary nitrate reductase activity (Spiegelhalder et al., 1976), gastric pH (Kim et al., 1982), microbial colonization of the stomach and the presence of nitrosation catalysts and inhibitors in the diet (Wagner et al., 1985). N-Nitrosamines can be formed in vivo in the

stomach from precursor amines and nitrosating agents (Mirvish, 1975; Ohshima and Bartsch, 1981; Zeisel, et al., 1988). The precursor amines and nitrites may be ingested through foods, drugs or drinking water. Nitrites may be present in normal and pathological conditions in saliva (Tannenbaum et al., 1976), gastric contents (Mueller et al., 1986), intestinal contents and feces (Saul et al., 1981) and urine (Green et al., 1982; Ohshima et al., 1987). It is generally accepted that development of certain human tumors, such as gastric and esophageal tumors, can be associated with in vivo formation of N-nitrosamines (Lu et al., 1986; Hill, 1988). In addition to the in vivo formation in the stomach, reaction of NO in the lung (Tannenbaum, 1987) and nitrosation at neutral pH mediated by bacteria or macrophages at other sites of the body (Stuehr and Marletta, 1985) are the other pathways in the endogenous formation of N-nitrosamines.

Ascorbic acid has been known to inhibit in vivo and in vitro nitrosation (Archer et al., 1975) and may prevent development of gastric tumors (Mirvish, 1983). Alpha-tocopherol has also been shown to prevent nitrosation in bacon (Fidler et al., 1978).

Metabolism. It is well established that N-nitrosamines require metabolic activation for conversion into ultimate carcinogens (Miller and Miller, 1981a). The metabolic activation of these compounds is mediated by cytochrome P450 (Chan et al., 1978; Lai and Arcos, 1980) and is a complicated

process which involves several pathways (Michejda et al., 1982). Some pathways result in the formation of highly reactive electrophilic intermediates, which interact with cellular nucleophilic sites, including DNA, RNA and other macromolecules. A small fraction of these interactions results in the initiation of a complex series of events which eventually lead to tumor formation (Michejda et al., 1982). The most widely accepted hypothesis for this activation is the α-hydroxylation hypothesis (Michejda et al., 1981, 1982). An enzyme-mediated step in this sequence of reaction results in the formation of α-hydroxylated N-nitrosamine (Michejda et al., 1982). The subsequent steps consist of a nonenzymatic cascade of reactions which lead to the formation of the reactive diazonium ion. This substance then reacts randomly with a variety of nucleophilic sites in cellular macromolecules.

Another product of the α-hydroxylation reaction is formaldehyde which is derived from formation of unstable methylol derivatives with amine groups (Harris, 1987). Both of the major metabolites of N-nitrosamines may act in concert in producing the toxic, mutagenic, and carcinogenic effects (Harris, et al., 1982). N-Nitrosamines may also be activated through alternative pathways, including β-hydroxylation followed by sulfate conjugation and formation of alkoxydiazenium ions (Michejda et al., 1981).

Mechanism of Toxicity and Carcinogenicity. N-Nitrosamines have been known for their ability to selectively induce a high incidence of malignant tumors in a wide variety of target organs (Schmahl and Habs, 1980; Lijinsky and Reuber, 1981). Although the biological basis of organ-specific carcinogenesis is not fully understood, several factors have been identified, including distribution of the parent carcinogen, tissue-specific bioactivation, DNA repair, cell division and inherent tissue susceptibility such as the presence of proto-oncogenes (Kleihues et al., 1987). Many of these compounds have been shown to be converted to alkylating intermediates which form covalent bonds with DNA, resulting in adduct formation (Miller and Miller, 1981a). Several of these adducts, especially O^6-alkylguanine and O^4-alkylthymine, may have the potential to initiate mutations and consequently tumor formations (Singer and Kusmierek, 1982; Singer, 1984, 1986). The mutational effects of these adducts can be associated with their persistence in DNA which presumably increases the probability that a miscoding event will take place during DNA synthesis resulting in a permanent heritable change in the base sequence (Montesano et al., 1982).

An activated N-nitrosamine has been found to cause mutations in cellular proto-oncogenes, a reaction that may be a necessary step in the multistage process of carcinogenesis (Barbacid, 1986). The effects of a major adduct O^6-alkylguanine depend on the repair process by O^6-alkylguanine-DNA-

alkyltransferase (AAT) following cell division (Pegg et al., 1987). In tissues that have significant AAT activity, the rapid repair of O^6-alkylguanine along with the very slow removal of O^4-alkylthymine may lead to a significant build-up of the relatively minor thymine adduct. This adduct might be important in the initiation of tumors (Singer, 1984; Richardson et al., 1985).

The ability of AAT to repair DNA damaged by alkylating agents and the cellular content of AAT may be important in the susceptibility of various tissues to toxic and carcinogenic effects of N-nitrosamines (Hecht and Hoffmann, 1988; Scherer et al., 1989). The DNA repair by AAT can be inhibited by electrophilic intermediates generated during metabolic activation of N-nitrosamines and by alkylating agents or alkylated DNA (Brent, 1986). The failure in DNA repair may result in mutation.

N-Nitrosamines have been demonstrated to activate human-ras oncogene in laboratory animals by causing point mutations (Zarbl et al., 1985). In addition, these compounds can cause DNA single-strand breaks and chromosomal deletion and rearrangement (Waldren et al., 1986) which may contribute to their genotoxicity.

N-Nitrosodimethylamine

N-Nitrosodimethylamine (NDMA) has been known as a potent carcinogen in many laboratory animals (Schmahl and Habs, 1980;

Preussmann and Stewart, 1984) and a mutagen in a number of in vitro tests (Bartsch et al., 1980; Guttenplan, 1987, 1989). N-Nitrosodimethylamine has also been implicated in the development of certain tumors in human beings in some epidemiologic studies (Yu and Henderson, 1987; Song and Hu, 1988; Siddiqi et al., 1988; Sarkar et al., 1989).

Distribution. N-Nitrosodimethylamine is widely distributed in the environment, including various foods and alcoholic beverages, industrial products and tobacco products. This compound is among the most commonly detected N-nitrosamine in food systems (Spiegelhalder et al., 1980; Song and Hu., 1988). N-Nitrosodimethylamine has been detected in cured meat products, such as fried bacon, smoked meat, sausage, ham, bologna, pepperoni, corned beef, and baby foods (Sen et al., 1979, 1988; Spiegelhalder et al., 1980; Ikins et al., 1986). The levels of NDMA in cured meat ranged from <0.1 to 3.0 µg/kg (Sen et al., 1988). Dairy products including cheese, yogurt, nonfat dried milk, dried butter milk and whole milk have been reported to contain NDMA (Gray et al., 1979; Libbey et al., 1980; Lakritz and Pensabene, 1981). The concentrations in cheese ranged from 1 to 5 µg/kg (Gray et al., 1979) and in milk products between <0.2 to 4.5 µg/kg (Libbey et al., 1980). N-Nitrosodimethylamine has been found in sea foods such as fish and shrimp products at levels of 0.5 to 43.9 µg/kg (Siddiqi et al., 1988; Song and Hu, 1988). Miscellaneous foods including pickled and salted vegetables,

soya sauce and fermented bean curd have been reported to contain NDMA at levels of 0.1 to 1.85 µg/kg (Siddiqi et al., 1988; Song and Hu, 1988). N-Nitrosodimethylamine has been detected in alcoholic beverages, including beer and malt at levels of 0.2 to 5.9 µg/kg (Spiegelhalder et al., 1980; Scanlan et al., 1980; Harvey et al., 1981).

N-Nitrosodimethylamine has been found in tobacco and cigarette main stream and side stream smoke (Brunnemann et al., 1977, 1980; Adams et al., 1987). The concentration of this compound in tobacco is 6.9 to 188 µg/kg (Brunnemann et al., 1977). Cigarette mainstream smoke contains 15 to 100 ng/cigarette (Adams et al., 1987).

Fine (1980) suggested that the greatest human exposure to N-nitrosamines, including NDMA, is by the industrial route. In the rubber and tire industry, NDMA was detected in the working environment at levels of 0 to 0.5 µg/m^3 (Fajen et al., 1979). In baby bottle rubber nipples, this N-nitrosamine was found at concentrations of 2.1 to 14.1 µg/kg (Gray and Stachiw, 1987).

This compound has been detected at a concentration of 47 µg/m^3 in a leather tannery (Rounbehler et al., 1979) and 0.01 to 0.04 µg/m^3 in heavily industrially polluted areas (Spiegelhalder and Preussmann, 1987). The levels of NDMA in diesel engine crankcase emissions were in the range of 0.5 to 17.2 µg/m^3 (Goff et al., 1980). This compound has also been found as an airborne contaminant in new motor cars (Rounbehler

et al., 1980).

In human beings, NDMA has been detected in saliva (Ellen et al., 1982), gastric juice (Schlag et al., 1980), urine (Ohshima et al., 1987), and blood (Gough et al., 1983).

Although the levels of NDMA in various food and industrial products and environments occur only in the range of few µg/kg (Fajen et al., 1979; Spiegelhalder et al., 1980; Song and Hu, 1988), in tobacco products the levels may exceed 100 µg/kg (Brunnemann et al., 1980). These amounts are much lower than the effective dose that produces tumors in laboratory animals. For rats an effective dose is about 50 to 100 µg/kg of NDMA in the diet or drinking water (Terracini et al., 1967). Since this compound is ubiquitous in the environment, daily exposure of human beings throughout their life time is very likely. Therefore, its role in the formation of cancers must be taken into account. In addition, NDMA may be present in combination with other N-nitrosamines with the same target organ which possibly enhances its toxic and carcinogenic effects.

Chemistry and Formation. N-Nitrosodimethylamine belongs to a group of symmetric dialkyl N-nitrosamines (Preussmann and Stewart, 1984) and is volatile and nonpolar (Eisenbrand et al., 1970; Fan et al., 1978). This compound is a yellow, oily liquid (Magee and Barnes, 1967) of low viscosity and is soluble in water, alcohol, ether, other organic solvents and lipids (International Agency for Research on Cancer, 1978).

N-Nitrosodimethylamine is photochemically reactive (Fridman et al., 1971) and is rapidly decomposed by aluminium nickel alloy and aqueous alkali (Lunn et al., 1983).

This compound may be preformed in the environment or formed endogenously in the human body from precursor amines and nitrosating agents. Exogenous formation of NDMA in the environment is a complex process and is not well understood. Precursor amines, including monomethylamine, dimethylamine and trimethylamine, are widely distributed in the environment and occur in foods (Yang, 1982; Kawabata et al., 1982; Zeisel and DaCosta, 1986), beverages (Mangino et al., 1981), industrial environments (Fine et al., 1976; Fine and Rounbehler, 1981), pesticide products (Ross et al., 1977), and soil (Golovnya et al., 1982). Nitrosating agents including nitrite and nitrosating gaseous (NO, NO_2, N_2O_3 and N_2O_4) are also present in the environment (Mergens and Newmark, 1981; Shuker, 1988). Nitrite and its precursor (nitrate) are found in various foods (White, 1975; Yang, 1982) and are commonly used in food processing (Roberts, 1975; Aidjanov and Sharmanov, 1982). Nitrite and nitrate have been detected in soil (Tate and Alexander, 1975), drinking water (Haenzel et al., 1976), and tobacco (Andersen et al., 1982). Nitrosating gases are present in polluted atmospheres (Spiegelhalder and Preussmann, 1987; Shuker, 1988). In particular, nitric oxide is an important byproduct of fossil fuel combustion (Tannenbaum, 1987).

Endogenous formation of NDMA has been well established

(Tannenbaum, 1980; Zeisel et al., 1988; Perciballi and Hotchkiss, 1989). The in vivo formation of this compound in human beings occurs mostly in the stomach (Tannenbaum, 1980; Zeisel et al., 1988), as the acidic conditions favor formation of nitrous anhydride and nitrosyl compounds which nitrosate amines to form NDMA (Mirvish, 1975). In addition, in vivo formation of NDMA has been reported in the oral cavity (Ellen et al., 1982), stomach (Walters et al., 1982) and urinary tract (Ohshima et al., 1987). Dimethylamine, a precursor of NDMA, is present in various foods (Kawabata et al., 1982; Zeisel and DaCosta, 1986). This secondary amine can be formed in the intestinal tract by the action of normal bacterial flora (Zeisel et al., 1983) and it may be synthesized endogenously by mammals (Zeisel et al., 1985). Monomethylamine is found in fish and vegetables and it is rapidly nitrosated in the stomach (Huber and Lutz, 1984). Trimethylamine is present in foods (Patterson and Motram, 1974; Singer and Lijinsky, 1976) and has been known to form NDMA (Lijinsky et al., 1972; Huber and Lutz, 1984). Nitrite, the other precursor of NDMA, is present in saliva (Tannenbaum et al., 1976), gastric juice (Mueller et al., 1986), feces (Saul et al., 1981) and urine (Green et al., 1982; Ohshima et al., 1987). Nitrate can undergo bacterial reduction to nitrite at particular sites within the body (Leach, 1988). Therefore, nitrate may indirectly provide the major source of endogenous nitrite.

The precise mechanism of endogenous synthesis of NDMA is not known. Leach (1988) proposed two different pathways of endogenous synthesis of N-nitrosamines including chemical mechanisms and biologically mediated N-nitrosation reactions. The chemical mechanisms comprise reactions of compounds derived from the protonation of nitrite in acid aqueous solution, nitrosation by gaseous nitrogen oxides, and reactions of nitrite ion. The biologically mediated N-nitrosation reactions include bacterially mediated N-nitrosation and formation of N-nitrosamines from endogenously formed nitrite.

Metabolism. The most widely accepted pathway for the activation of NDMA is α-hydroxylation (Druckrey, 1973; Michejda et al., 1982). N-Nitrosodimethylamine is metabolized by NDMA-demethylase, a cytochrome P450-dependent mixed-function oxidase (MFO) (Druckrey, 1973). This enzyme catalyses the hydroxylation of the α-carbon to form an unstable compound, hydroxymethylmethyl N-nitrosamine (Druckrey, 1973; Mochizuki et al., 1980). This compound then breaks down to methyldiazonium hydroxide by dealkylation and finally yields electrophilic intermediates, known as methyldiazonium ions (Druckrey, 1973; Michejda et al., 1981; Harris, 1987). These have been known to interact with nucleophilic sites in cellular macromolecules (Druckrey, 1973; Michejda et al., 1981; Harris, 1987). The hydroxymethylmethyl N-nitrosamine also yields formaldehyde and N_2 (Hecht and Hoffmann, 1988).

These hydroxymethylated derivatives can produce stable methylene bridges between macromolecules by a slow spontaneous secondary reaction (Harris, 1987). Formaldehyde may also be rapidly oxidized to formate, and finally to CO_2 by different enzymatic pathways (Harris, 1987). Both formaldehyde and formate can enter the one-carbon pool as N^5-, N^{10}-tetrahydrofolate derivatives and then become incorporated into a number of cellular products (Harris, 1987).

There is strong evidence that several demethylase enzymes may participate in the activation of NDMA as shown by in vitro studies with rat hepatic preparations (Kroeger-Koepke and Michejda, 1979). At present, it is evident that a microsomal monoamine oxidase participates in the activation of NDMA (Harris, 1987).

An alternative pathway in the metabolism of NDMA is through denitrosation (Keefer et al., 1987; Appel et al., 1987) which appears to be catalysed by a cytochrome P450-dependent monooxydase and results in deactivation of this compound (Rowland, 1988; Amelizad et al., 1989). It has been reported that denitrosation of NDMA results in the generation of methylamine and nitrite (Haussmann and Werringloer, 1985; Keefer et al., 1987). Streeter and co-workers (1990) indicated that denitrosation is a major metabolic pathway for NDMA elimination.

Metabolism of NDMA can be assumed as a competition between two pathways, with denitrosation diverting a

significant proportion of metabolic deactivation and demethylation activating the alkylation pathway responsible for tumor formation (Keefer et al., 1987).

Mechanism of Toxicity and Carcinogenicity. Autoradiographic studies demonstrated that NDMA is evenly distributed in the body and is able to cross the cellular membrane (Johansson and Tjalve, 1978). This indicates that the nonmetabolized compound must be relatively nontoxic to the tissues and the injurious effects are due to the metabolites (Brittebo and Tjalve, 1983). Metabolic activation of NDMA results in the formation of alkylating intermediates which can react with nucleophilic sites in cellular macromolecules (Michejda et al., 1981; Harris, 1987). The metabolic activation is mediated at least in part by cytochrome P450 (Druckrey, 1973; Malaveille et al., 1987), the levels of which may vary in different tissues and species (Hecht and Hoffmann, 1988). The reactive metabolites will form covalent binding with DNA leading to formation of promutagenic adducts, including 7-methylguanine, O^6-methylguanine and O^4-methylthymine (Devereux et al., 1988; Diaz Gomez et al., 1988; Scherer et al., 1989). Following treatment with NDMA, the O^6-methylguanine is the major adduct known to induce miscoding (Scherer et al., 1989; Fan et al., 1989) resulting in the formation of a point mutation which can lead to the activation of proto-oncogenes (Barbacid , 1986). This reaction may be a necessary step in the multistage process of tumor formation

(Barbacid, 1986). There is a correlation between the persistence of O^6-methylguanine in tissues and formation of tumors following exposure to NDMA (Montesano and Hall, 1984; Yaros, 1985). However, results of several studies with methylating carcinogens indicated that the persistence of O^6-methylguanine in replicating cells may be important but not sufficient for tumor development (Singer, 1984; Pegg, 1984). The effectiveness of this compound to induce tumors is associated with the DNA repair enzyme, O^6-alkylguanine-DNA alkyltransferase (AAT) (Pegg, 1984; Singer, 1984; Scherer et al., 1989). Therefore, the levels of AAT and cytochrome P450 may play an important role in the sensitivity of different species and individual organs (Pegg, 1984; Singer, 1984; Hecht and Hoffmann, 1988; Scherer et al., 1989).

Other metabolites derived from metabolic activation of NDMA may have roles in the induction of cellular injuries and tumors. Metabolites such as formaldehyde have been known to induce multiple effects, including DNA-protein cross links, DNA single-strand breaks, inhibition of DNA repair and cytotoxicity (Harris, 1987). It may be assumed that the major metabolites of NDMA, such as methyldiazonium ion and aldehydes, may act together to produce the toxic, mutagenic and carcinogenic effects.

Toxicity and Carcinogenicity in the Rat. N-Nitroso-dimethylamine induces cellular injuries and tumors in many laboratory animals, including rats (McCracken et al., 1973;

Sato et al., 1973; Schmahl and Habs, 1980; Bogovski and Bogovski, 1981; Ishinishi et al., 1988). In rats, the acute lethal dose $(LD)_{50}$ by the intraperitoneal route is 43 mg/kg of body weight (BW) (Heat, 1962). Although the major lesion in rats is centrolobular hepatic necrosis (Shank, 1975), lesions in the kidney have also been reported (Hard and Butler, 1971; Hard et al., 1984). The toxic effects in kidney include cytoplasmic accumulation of granular and membranous debris and occasionally cell necrosis.

Several studies have demonstrated that NDMA is a potent immunotoxin capable of suppressing both humoral (Holsapple et al., 1984; Kaminski et al., 1989) and cell-mediated immune responses (Holsapple et al., 1985). The target cells for NDMA mediated immunosuppression include B and T cells (Holsapple et al., 1985; Johnson et al., 1987). The immunosuppressive effect of NDMA is suspected to contribute in the development of tumors, at least in part, by inhibition of the immune surveillance capability of the host (Kaminski et al., 1989).

The carcinogenicity of NDMA has been demonstrated in rats by different routes. Oral administration of this compound resulted in the development of carcinomas, hemangioendothelial sarcomas (Lijinsky and Reuber, 1984), and cholangiocellular tumors of the liver (Terracini et al., 1967), kidney tumors (Hard and Butler, 1971) and occasionally adenocarcinomas and squamous cell carcinomas of the lung (Lijinsky and Reuber, 1984). Subcutaneous or intramuscular administration of NDMA

induced kidney tumors, mainly nephroblastomas, adenomas and clear cell carcinomas (Ito, 1973) and hepatocellular carcinomas (Terracini and Magee, 1964). Intraperitoneal injection of this compound resulted in the development of kidney tumors (Hard and Butler, 1971) and squamous cell carcinomas of the nasal cavity (Noronha and Goodall, 1972). Oral or intraperitoneal administration of NDMA in pregnant rats during the last week or during the whole period of pregnancy induced kidney tumors in the offspring (Schmahl and Habs, 1980), but it was not found to be teratogenic (Alexandrov, 1968).

N-Nitrosopyrrolidine

N-Nitrosopyrrolidine (NPYR) has been reported to be carcinogenic in rodents (Preussmann and Stewart, 1984; Ishinishi et al., 1988) and mutagenic in bacteria (Guttenplan, 1987). Although NPYR is frequently found together with NDMA in food (usually at a higher concentration) and other environmental systems, its possible role in the development of human cancer is not known.

Distribution. N-Nitrosopyrrolidine is most commonly found in foods and beverages (Spiegelhalder et al., 1980; Sen and Seaman, 1981; Song and Hu, 1988), to a lesser extent in tobacco products and cigarette smoke (Brunnemann et al., 1980; Brunnemann et al., 1983) and occasionally in the rubber industry environment (Fajen et al., 1979). This compound has

been demonstrated as the main volatile N-nitrosamine in various types of cured meat products, especially fried bacon (Sen et al., 1988; Spiegelhalder et al., 1980; Ikins et al., 1986) at a range in concentration of 2.5 to 13.6 µg/kg (Ikins et al., 1986). Milk products occasionally contain NPYR at 0 to 0.8 µg/kg (Song and Hu, 1988). Sea foods, such as dried squid, have been reported to contain NPYR at concentrations of 2.4 to 12.9 µg/kg (Kawabata et al., 1980). Miscellaneous foods including pickled and salted vegetables, red chillies and lotus stems have been demonstrated to contain approximately 0.76 to 2.0 µg/kg of NPYR (Song and Hu, 1988; Siddiqi et al., 1988).

This N-nitrosamine has been detected at a concentration of 1.5 to 29 ng/cigarette in main stream smoke and 28 to 150 ng/cigarette in the side stream smoke of cigarettes (Brunnemann et al., 1980). Contamination with NPYR has also been reported in rubber and tire industry environments (Fajen et al., 1979). Urine has been found as the only human body fluid that contains detectible amounts of NPYR (Ohshima et al., 1982, 1987).

Chemistry and Formation. N-Nitrosopyrrolidine is a heterocyclic N-nitrosamine (Preussmann and Stewart, 1984), which is volatile and nonpolar (Eisenbrand et al., 1970; Fan et al., 1978). This compound is slightly less stable in acidic solution and is sensitive to light, especially ultraviolet light (International Agency for Research on Cancer, 1978).

N-Nitrosopyrrolidine is miscible with water and is soluble in organic solvents and lipids (International Agency for Research on Cancer, 1978).

Although the formation of NPYR in fried bacon has been studied extensively (Gray, 1981), its mechanism of formation in the environment is not fully understood. In people, this N-nitrosamine has been demonstrated in normal urine (Ohshima et al., 1982, 1987) and in urine from a bacterial infected urinary tract (Ohshima et al., 1987). In addition, NPYR can also be formed in the dog stomach from pyrrolidine and nitrite (Mysliwy et al., 1974). These studies suggest that NPYR has the potential to be formed endogenously.

Several possible precursors of NPYR in the environment have been reported, including collagen, the amino acids proline, ornithine, arginine, citrulline and the amines pyrrolidine, putrescine, agmatine, spermidine and spermine (Huxel et al., 1974; Gray and Dugan, 1975; Warthesen et al., 1975; Coleman, 1978). Nebelin et al. (1980) reported that formation of NPYR occurs upon reaction between the amino precursors and nitrites.

Proline appears to be the most likely precursor of NPYR (Spinelli-Gugger et al., 1981). The mode of exogenous formation of NPYR may be best represented by its mechanism of formation in bacon (Gray, 1981). Pork belly has been found to contain free proline (Lakritz et al., 1976), which in turn can be converted to NPYR during frying of bacon (Bharucha et al.,

1979). Formation of NPYR from proline can occur by two possible pathways (Bharucha et al., 1979). One pathway involves N-nitrosation of proline to N-nitrosoproline (NPRO), followed by decarboxylation to NPYR. The other pathway consists of initial decarboxylation of proline to pyrrolidine, then further N-nitrosation to yield NPYR. Bharucha et al. (1979) indicated that the pathway involving intermediacy of NPRO is the most likely route in the formation of NPYR because the conversion of NPRO to NPYR occurs at a lower temperature than the transformation of proline to pyrrolidine. Coleman (1978) suggested that free radicals may have important roles in the formation of NPYR. It is suspected that during frying of bacon, nitrous acid is transformed into N_2O_3 by continuous removal of water (Coleman et al., 1978; Bharucha et al., 1979). Then, N_2O_3 undergoes dissociation at temperature >100^{0}C to nitric oxide and $NO_2^{\cdot}$ radical (Coleman, 1978; Bharucha et al., 1979). The $NO_2^{\cdot}$ radical can act as the chain initiator and react with proline to produce a radical which combines with the $NO^{\cdot}$ radical to yield NPRO, and consequently NPYR (Coleman, 1978; Bharucha et al., 1979). Several factors may affect the formation of NPYR, including the method of cooking (Bharucha et al., 1979), nitrite concentration (Robach et al., 1980), N-nitrosamine inhibitors (Bharucha et al., 1980; Gray et al., 1982), preprocessing of pork bellies (Pensabene et al., 1980) and smoking (Bharucha et al., 1980).

Metabolism. Two possible pathways for the metabolism of NPYR have been described, including α- and β-hydroxylation (Hecht et al., 1981, 1982; Gilbert et al., 1982). Among these pathways, α-hydroxylation is considered to play an important role in the metabolic activation of NPYR to a mutagen (Gilbert et al., 1982, 1984). Although the cytochrome P450 dependent MFO has been demonstrated to catalyze NPYR activation, other pathways of metabolism may also be involved (Cottrell et al., 1983; Alldrick et al., 1985).

The in vitro metabolic activation of NPYR in rat liver fractions was summarized by several workers. The α-hydroxylation of NPYR is catalysed by cytochrome P450 in the presence of a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and O_2 (Chen et al., 1978; Hecht et al., 1978). This reaction results in the formation of an unstable intermediate 2-hydroxy-N-nitrosopyrrolidine (Hecht et al., 1978). Then, the intermediate compound undergoes spontaneous ring opening to diazohydroxide and after loss of nitrogen, it generates oxocarbonium ion (Chen et al., 1978). This ion, which could be the possible ultimate carcinogen derived from NPYR, is trapped by water resulting in the formation of 4-hydroxybutyraldehyde dinitrophenylhydrazone (Chen et al., 1978). This product exists predominantly as the cyclic hemiacetal 2-hydroxytetrahydrofuran, which is known as the major metabolite from α-hydroxylation of NPYR (Hecker et al., 1979). The latter product is in equilibrium with 4-hydroxy-

butanal (4-HB) and in the presence of postmicrosomal supernatant enzymes, it is rapidly converted to 1,4 butanediol or γ-hydroxybutyrate (Hecker et al., 1979). These compounds may be cycled into general cellular metabolism resulting in the production of CO_2 (Hecker et al., 1979), which is in agreement with the in vivo metabolism of NPYR to CO_2 by rats (Snyder et al., 1977). The first stable product of the α-hydroxylation pathway is 4-HB (Cottrell et al., 1983).

Metabolic activation of NPYR by β-hydroxylation has been demonstrated in vivo in rats and the 3-hydroxy-N-nitrosopyrrolidine metabolite has been identified in the urine (Cottrell et al., 1980).

Mechanism of Toxicity and Carcinogenicity. It has been reported that mutagenic and carcinogenic effects of NPYR are due to its metabolic products (Brambilla et al., 1981; Gilbert et al., 1984; Alldrick et al., 1985). Although the metabolic fates of NPYR are known, little is known about the identity of its mutagenic metabolites or the DNA binding capacity and DNA lesions produced by the metabolites (Alldrick et al., 1985). It is suggested that NPYR produces DNA lesions which exert their mutagenic effect through error-prone repair (Rao et al., 1981; Alldrick et al., 1985), base substitution (Rao et al., 1981) and damage to GC base pairs (Zielenska and Guttenplan, 1987). Several adducts from NPYR have been identified, including 7-methylguanine (Hunt and Shank, 1982), 7,8-cyclic guanine (Chung et al., 1989b) and cyclic

1,N^2-propanodeoxyguanosine (Chung et al., 1989a). Although these adducts may be associated with mutagenesis, their role in tumor formation has not been well characterized (Chung et al., 1989a, 1989b). In addition, the mechanism of the cytotoxic effects of NPYR following metabolic activation or upon binding to DNA and its organ specificity are also not well understood (Hecht et al., 1981; Alldrick et al., 1985).

Toxicity and Carcinogenicity in the Rat. Studies in laboratory animals, including rats, indicated that NPYR has the potential to induce tumors (Lijinsky and Reuber, 1981; McCoy et al., 1986; Ishinishi et al., 1988). In rats, the acute toxic LD_{50} for NPYR is 650 mg/kg of BW when given by the intraperitoneal route (Lee and Lijinsky, 1966). Although in vitro studies with rat hepatocytes indicated that NPYR is able to induce cytotoxic effects (Alldrick et al., 1985), acute lesions in rats due to exposure to this compound have not been well established.

The carcinogenic effects of NPYR in rats were mainly in the liver (Lijinsky and Taylor, 1976; Lijinsky and Reuber, 1981; Berger et al., 1987) in the form of carcinoma, sarcoma, cholangiocarcinoma (Lijinsky and Reuber, 1981; Berger et al., 1987), hemangioendothelioma and occasionally adenoma (Berger et al., 1987). Other locations that frequently develop tumors after administration of NPYR are the oral cavity and stomach (Berger et al., 1987) and to a lesser extent, esophagus (Lijinsky and Reuber, 1981), intestine, kidney, neural tissues

and in the hematopoietic and lymphatic systems (Berger et al., 1987). Formation of papillary mesothelioma in the tunica vaginalis testis (Greenblatt and Lijinsky, 1972) and olfactory carcinoma (Lijinsky and Taylor, 1976) has also been reported.

Polybrominated Biphenyls

Polybrominated biphenyls (PBBs) are a group of environmental contaminants (Carter, 1976; Kay, 1977) which are known to be toxic in several animal species and people (Sleight and Sanger, 1976; Allen et al., 1978; Anderson et al., 1978) and are carcinogenic in laboratory animals (Kimbrough et al., 1981; Gupta et al., 1983b).

Distribution. A complex mixture of PBBs, under the commercial name Firemaster BP-6 or FF-1, was used as a fire retardant (Gutenmann and Lisk, 1975; Rappe et al., 1979). The PBBs have been found to be an environmental pollutant after accidental addition of Firemaster into animal feed in Michigan in 1973 (Carter, 1976). This accident resulted in widespread contamination of farm animals (cattle, swine, sheep, chickens) and their products (Jackson and Halbert, 1974; Gutenmann and Lisk, 1975; Carter, 1976; Kay, 1977; Fries et al., 1978) as well as the soil, water (Jacobs et al., 1978), fish and water fowl (Jacobs et al., 1978; Zabik et al., 1978). Human exposure to PBBs can occur through the food chain (Lilis et al, 1978) or through occupational contact (Williams et al., 1984). Polybrominated biphenyls have been detected in blood (Lilis

et al., 1978; Landrigan et al., 1979), adipose tissue (Stross et al., 1979) and breast milk (Landrigan et al., 1979) of human beings.

Since the production of PBBs in the USA ended in 1974 (DiCarlo et al., 1978) and their use is strictly regulated, further widespread contamination is unlikely.

Chemistry and Formation. Polybrominated biphenyls are synthetic compounds, which are classified as polyhalogenated aromatic hydrocarbons (Rappe et al., 1979). The structure and biological properties of the PBBs are closely related to polychlorinated biphenyls (PCBs), dibenzodioxins (PCDDs), dibenzofurans (PCDFs) and naphthalenes (PCNs) (Rappe et al., 1979; McConnell and Moore, 1979).

Polybrominated biphenyls with three or more bromines are solid compounds (DeKok et al., 1977) which are very poorly soluble in water (Fries, 1985) but soluble in fat (Kay, 1977) and organic, nonpolar solvents (DiCarlo et al., 1978). These compounds are chemically unreactive (Kay, 1977), but they have been shown to be susceptible to photochemical debromination (Ruzo et al., 1976). Polybrominated biphenyls are highly resistant to degradation (Fries, 1985) and require a temperature above 300^{0}C before they begin to decompose (DiCarlo et al., 1978). Therefore, the PBBs are very stable in the environment and in animal tissues (Fries, 1985). In rats, the half life of FM in fat tissues is approximately 69 weeks (Miceli and Marks, 1981) and in people, the estimated

half life of 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB) is 6.5 years (Tuey and Matthews,1980).

Firemaster BP-6 (FM) is a complex mixture of about 12 to 14 major congeners (Moore et al., 1978; Moore and Aust, 1978; Dannan et al., 1982). Two major congeners that have been identified are 245-HBB, which represents approximately 50 to 70% of the entire FM mixture (Sundstrom et al., 1976; Aust et al., 1981; Robertson et al., 1984) and 2,3,4,2',4',5'-hexabromobiphenyl, which accounts for about 12% of the total mixture (Aust et al., 1981; Robertson et al., 1984).

Toxicity and Carcinogenicity in the Rat. The total LD_{50} value for daily oral doses of Firemaster FF-1 during a 90 day study was 3.28 g/kg of BW for male rats and 1.43 g/kg of BW for female rats (Gupta and Moore, 1979). Polybrominated biphenyls induce a delayed toxicity syndrome in rats and many other species of animals (Kimbrough et al., 1978a; Damstra et al., 1982; Fries, 1985). The toxic syndrome includes weight loss, porphyria, thymic and splenic atrophy, enlargement and fatty degeneration of the liver (Sleight and Sanger, 1976; Gupta and Moore, 1979; Render et al., 1982), hyperplastic goiter-like changes in the thyroid gland (Sleight et al., 1978) and depletion of hepatic vitamin A stores (McCormack et al., 1982). In addition, chronic progressive nephropathy, gastric ulcers and hyperplastic gastropathy were also observed in rats given PBBs (Gupta et al., 1983b).

In PBB-intoxicated rats, hepatic changes included

hepatocellular swelling, disruption of hepatic architecture, individual hepatocyte necrosis, fatty vacuolation of hepatocytes and bile duct proliferation (Sleight and Sanger, 1976; Gupta and Moore, 1979; Gupta et al., 1983a). The congener 245-HBB, which is relatively nontoxic, causes hepatocellular swelling and lipid vacuolation, whereas the highly toxic 3,3',4,4',5,5'-hexabromobiphenyl (345-HBB) causes bile ductule proliferation and more extensive lipid vacuolation (Render et al., 1982).

Since liver appears to be one of the target organs for PBBs (Kimbrough et al., 1978b), this organ might be expected to be the primary site for the carcinogenic process induced by this chemical mixture. Chronic oral administration of PBBs to rats produces hepatocellular carcinomas (Kimbrough et al., 1978a, 1981; Groce and Kimbrough, 1984) and cholangiocarcinomas (Gupta et al., 1983b).

Metabolism. Although some of the PBB congeners in the FM mixtures are metabolized by hepatic mixed function oxidases, their metabolic fates are not known (Dannan et al., 1978). In vitro studies with rat liver microsomes in the presence of NADPH and atmospheric O_2 indicated that some PBB congeners can be metabolized by cytochrome P450 monooxygenases (Aust et al., 1983; Mills et al., 1985). In vitro metabolism of several tetra-, penta- and hexabromobiphenyls has been demonstrated in rat liver microsomes following irradiation with ultraviolet light (Millis et al., 1985). The ability of tetra- and

pentabromobiphenyls to be metabolized in vivo has also been reported (Millis et al., 1985). Microsomes from rats pretreated with phenobarbital (PB) or 3-methylcholanthrene (3-MC) have been shown to metabolize some di-, tri- and tetrabromobiphenyls (Mills et al., 1985).

The ability of a PBB congener to be metabolized to any appreciable extent depends on the presence of adjacent nonhalogenated carbons in the ortho and meta or meta and para positions (Mills et al., 1985). In addition, a nonhalogenated para carbon is also required for metabolism (Dannan et al., 1978).

The rates of metabolism of PBB congeners can be associated with the degree of bromination and the positions of the bromines, the presence of other PBB congeners and the type of microsomal enzymes induced (Mills et al., 1985). A decrease in the rate of metabolism of a particular congener is generally related to increased bromination of the biphenyl molecule (Mills et al., 1985). Aust et al. (1983) indicated that some PBB congeners can induce enzymes required for their metabolism if they satisfy the structural requirements for toxicity and metabolism.

Mechanism of Toxicity and Carcinogenicity. The mechanism of toxicity for PBBs is a complicated process and has not been well established (Aust et al., 1983). Polybrominated biphenyl congeners with different structures will have different biochemical and toxicological effects (Mills et al., 1985).

It is suggested that the toxic and biochemical responses are mediated by the 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) receptor (Poland et al., 1979; Poland and Glover, 1980). The ability to serve as ligands for this receptor is correlated with the toxicity of the PBB congeners (Poland et al., 1979). Only the congeners which can exist in the planar configuration by relatively free rotation about the bridge can serve as ligands for the TCDD receptor and elicit a TCDD-like toxicity (Aust et al., 1983). The receptor will translocate the ligands to the nucleus, resulting in the induction of the synthesis of a number of enzymes (Aust et al., 1983). One of the enzymes which is coordinately expressed is a form of cytochrome P450 with aryl hydrocarbon hydroxylase (AHH) activity (Poland et al., 1976). It has been postulated that enzyme induction is an early event and that toxicity occurs later and requires persistent receptor occupation and gene expression (Poland and Knutson, 1982).

The individual PBB congeners are classified as PB (Dannan et al., 1982), 3-MC (Render et al., 1982; Robertson et al., 1982) or a combination of PB and 3-MC (mixed) type inducers (Robertson et al., 1980). The FM mixture can be classified as a mixed-type microsomal enzyme inducer (Dent et al., 1976).

A pure PB-type inducer, such as 245-HBB, induces cytochrome P450 isozymes, including NADPH-cytochrome P450 reductase, aminopyrine demethylase and epoxide hydratase (Moore et al., 1978). The PB-type induction is generally

associated with low toxicity of the congener and a structural pattern including bromination of one or both ortho carbons on each of the rings (Dannan et al., 1983). The congener 345-HBB, which is present in FM in very small amounts (Orti et al., 1983; Robertson et al., 1984) is a pure 3-MC type inducer (Render et al., 1982; Robertson et al., 1982, 1984). This congener induces cytochrome P448 isozymes such as aryl hydrocarbon hydroxylase (Robertson et al., 1982). This type of induction is generally related with high toxicity and a structural pattern which allows a coplanar configuration similar to TCDD (Robertson et al., 1982, 1984). The absence of ortho bromines and the presence of at least two adjacent lateral bromines (meta and para) appears to allow the coplanar configuration (Robertson et al., 1982, 1984). A known mixed-type inducer is the congener 2,4,5,3',4',5'-HBB (Dannan et al., 1978).

Although the PBBs have been known to induce hepatocellular carcinomas in rats (Kimbrough et al., 1978a, 1981), the mechanism or the role of this chemical mixture in the carcinogenic process is not entirely clear. The PBB mixture may contain congeners that have initiating and/or promoting effects and therefore can act as a complete carcinogen. On the other hand, the PBBs may have only a promoting effect and tumor formation can result from promotion of spontaneously or environmentally induced initiated cells (Pitot et al., 1980; Schulte-Hermann and Parzefall, 1981; Schulte-Hermann et al.,

1983).

Mechanism of Tumor Promotion. Polybrominated biphenyls have been shown to have a promoting effect in liver carcinogenesis (Jensen et al., 1983, 1984; Jensen and Sleight, 1986), but the mechanism of tumor promotion is not known. The FM mixture and some of the individual congeners, such as 245-HBB, 345-HBB and 3,4,3',4'-tetrabromobiphenyl (34-TBB) have been shown to promote hepatic enzyme altered foci (EAF) in initiated rats (Jensen et al., 1983; Jensen and Sleight, 1986; Dixon et al., 1988). The ability of the FM mixture to serve as a promotor may be related to the presence of very potent individual congeners in this chemical mixture (Jensen et al., 1982) or to a synergistic or additive effect of the mixture of congeners (Jensen et al., 1982; Jensen and Sleight, 1986; Evans and Sleight, 1989).

Jensen et al. (1983) suggested that the congener 345-HBB may act as liver tumor promoter by producing chronic toxicity and necrosis of noninitiated hepatocytes, resulting in a prolonged regenerative stimulus and proliferation of initiated cells. The "resistant hepatocyte" phenomenon (Schulte-Hermann, 1985; Farber and Sarma, 1987) may apply to this type of interaction, since the hepatocyte nodules found in the later stages of promotion with 345-HBB appear to be resistant to the lipid accumulation seen within normal hepatocytes (Jensen et al., 1983). In comparison to 345-HBB, the congener 245-HBB apparently has a different mechanism of

tumor promotion (Jensen et al., 1983; Jensen and Sleight, 1986) and is apparently an effective promoter at nontoxic doses (Jensen et al., 1982).

The promoting effect of some PBB congeners has also been suggested to be related to inhibition of intercellular communication, since Firemaster BP-6 and 245-HBB have been demonstrated in vitro to block metabolic cooperation at nontoxic doses (Trosko et al., 1981; Tsushimoto et al., 1982). Gap junctional intercellular communication is known to be important in the regulation of differentiation and proliferation of cells (Hooper, 1982; Trosko and Chang, 1984a). Blockage of intercellular communication by tumor promoters can cause the release of initiated cells from the growth control of surrounding normal cells (Trosko and Chang, 1984b), and consequently result in uncontrolled cell proliferation.

Chemical Hepatocarcinogenesis

A number of chemicals have been implicated by epidemiologic studies as contributing or causative factors for the development of human cancers (Farber, 1981). It is generally hypothesized that development of a majority of tumors in people and animals including experimental animals, is a multistage process (Farber and Cameron, 1980; Scherer, 1984). This process involves rare events as well as gene modulation in discrete cells and cell populations as they slowly evolve

toward cancer (Farber and Cameron, 1980). The multistage concept of chemical carcinogenesis is based on the assumption that cancer evolves from single normal cells whose progeny pass through a number of developmental processes ending with the advent of an invasive, metastasizing cell population (Farber and Sarma, 1987). Farber and Cameron (1980) proposed that carcinogenesis can be regarded as a chronic disease process which is not a continuum, but rather a "discontinuum" involving only a small number of altered cells at many steps.

Chemical hepatocarcinogenesis is characterized by sequential development of morphologically distinct lesions (Saeter and Seglen, 1990). The sequential lesions include formation of numerous clonal proliferations (foci) of phenotypically altered hepatocytes at the earliest stage, followed by development of a more limited number of neoplastic nodules (benign tumors) and eventually, formation of a few hepatocellular carcinomas.

Multistage hepatocarcinogenesis consists of at least 3 distinct stages; initiation, promotion and progression (Pitot et al., 1988). The identification of a compound with any of these properties does not imply that the compound is inactive in the other two respects. Thus, tumor initiators may have promoting activity and a promoting agent may also initiate carcinogenesis or induce tumor progression (Yamasaki, 1988).

Initiation. The initiation stage is an irreversible and inheritable cellular alteration induced by a carcinogen, creating a potential in the cell and its progeny for subsequent malignant transformation (Schulte-Hermann, 1985). The exact molecular events involved in the initiation step is unknown (Farber and Sarma, 1987). However, there is considerable circumstantial evidence that for many carcinogens, alterations in DNA, including DNA adduct formation, is probably a critical event in the initiation process (Miller and Miller, 1981b). The majority of chemical carcinogens either without ("direct acting" carcinogen) or after metabolic activation, interact with many cell constituents including DNA, RNA, proteins and various cell organelles (Miller, 1978; Miller and Miller, 1981a). Most known carcinogens are metabolically converted to highly reactive, and often electrophilic, metabolites (Miller and Miller, 1981b; Farber, 1981) by microsomal mixed function oxygenase (MFO) systems associated with the cytochromes P450 which are located predominantly in the microsomes (Farber, 1981; Becker and Stout, 1984). The electrophilic metabolites form covalent bonds with cellular nucleophiles, especially DNA, resulting in adduct formations (Miller and Miller, 1981b; Chambers, 1985). The interaction between electrophilic intermediates and cellular DNA may result in genomic alterations such as miscodings, recombinations and transpositions of DNA (Farber, 1981; Chambers, 1985).

Initiator-induced DNA lesions are usually efficiently repaired, unless a round of cell proliferation occurs before repair is complete (Cairns, 1975; Farber, 1984c). In the latter case the DNA-lesions may be "fixed" in the form of a mutation, and become permanent property of the affected cell and its progeny (Cairns, 1975; Farber, 1984c; Chambers, 1985). Since many initiators are able to interact with and damage DNA, they are commonly known as genotoxic agents (Pitot et al., 1981; Williams, 1983b). In the liver, one or more of these presumed changes in DNA must generate a new biochemical pattern that can be associated with certain conditions such as resistance to a wide variety of agents, potential for a new state of differentiation and a genetic program for redifferentiation (Farber and Sarma, 1987).

Cell proliferation in the liver can be induced by partial hepatectomy (Columbano et al., 1981) and administration of a primary mitogen, such as lead nitrate, or cyproterone (Columbano et al., 1987). The need for cell proliferation is fulfilled automatically in the neonate or very young animals, since the liver contains many dividing hepatocytes at this early time (Peraino et al., 1984).

Some carcinogenic chemicals induce tumors in the liver, but do not seem to interact biochemically, either directly or indirectly with DNA in this organ (Reddy and Lalwani, 1983). The biochemical basis for this type of initiation is still undetermined (Farber and Sarma, 1987). Several studies

emphasized the possible role of mutation in the activation of cellular proto-oncogenes (Bishop, 1987; Yamasaki, 1988; Yuspa and Poirier, 1988; Saeter and Seglen, 1990).

In summary, the initiation stage of carcinogenesis may be regarded as a two-step process involving the alteration of DNA and the perpetuation of this lesion by cell proliferation (Farber and Sarma, 1987). These "fixed" cellular alterations produce the initiated cells, which may undergo promotion to express a fully neoplastic phenotype (Farber, 1981; Hicks, 1983; Farber, 1988).

Promotion. The promotion stage is the process of proliferative expansion of the population of initiated cells (Schulte-Hermann, 1985) to express an altered phenotype (Farber, 1981; Hicks, 1983). The results of such clonal expansion of initiated cells in the liver are focal or nodular clusters of preneoplastic or benign neoplastic tissue (Farber and Sarma, 1987; Farber, 1988).

Promotion can be reversible or irreversible, depending on whether the clones of initiated cells are maintained or regress subsequent to removal of the promoting stimulus (Saeter and Seglen, 1990). In the liver, promoting effects would appear to be predominantly irreversible, since a few weeks of promoter treatment significantly increases the yield of tumors appearing many months later (Saeter et al., 1988a). In addition, at the focus stage, the majority of promoter induced lesions persist following cessation of promoter

treatment (Goldsworthy and Pitot, 1985; Ito et al., 1988).

On the other hand, promotion may be reversible and not heritable from one cell generation to the next (Pitot et al., 1988). A reversible alteration in the expression of genetic information within cells is the principal characteristic of the stage of promotion and the action of promoting agents (Pitot et al., 1988). For example, regulation of the expression of γ-glutamyl transpeptidase (GGT) and glutathione S-transferases (GST) genes is involved in xenobiotic metabolism by phenobarbital (Hendrich et al., 1987).

Most promoters apparently do not damage DNA, but rather induce epigenetic effects on the target cells, which can be associated with alterations in the expression of genetic information, contributing to development of tumors (Pitot and Sirica, 1980; Hicks, 1983; Williams, 1983a).

Several studies indicated that following the initiation stage, there are two or more additional "rare events" or "hits", analogous to genomic mutations (Emmelot and Scherer, 1980; Scherer, 1984; Yokoyama and Lombardi, 1985). The promoting agents may facilitate these events by increasing the susceptibility of the initiated cells, the error proneness of DNA replications or repair, and the effects of DNA adducts (Emmelot and Scherer, 1980; Scherer, 1984).

An increasing number of models of carcinogenesis have been developed in several organs in laboratory animals utilizing two or more agents as initiators and promoters

(Farber, 1988). In studies of hepatocarcinogenesis, approximately 9 models have been reported (Farber, 1984c). After initiation with a single dose of a genotoxic carcinogen, the initiated cell population may be increased by the addition of one of several different promoters (Farber, 1988). Several liver carcinogenesis promoters have been reported, including phenobarbital (Pitot et al., 1978; Lans et al., 1983), dichloro-diphenyl-trichloroethane (DDT) (Peraino et al., 1975), PCBs (Preston et al., 1981), PBBs (Jensen et al., 1983), TCDD (Pitot et al., 1980) and 2-acetylaminofluorene (2-AAF) (Saeter et al., 1988a).

Once the foci of altered hepatocytes enlarge to form persistent nodules, new options seem to appear. These include differentiation leading to remodeling and continued cell proliferation and evolution to cancer (Farber, 1984a, 1984c). This stage of the carcinogenic process is known as progression.

Progression. The progression stage is the transformation of initiated cells to the fully malignant phenotype as well as the acquisition of increasing degrees of anaplasia in malignant tumors (Schulte-Hermann, 1985). Two possible mechanisms of progression to cancer based on the "resistant hepatocyte" model are cell death and the production of growth factors and stimulation of oncogene expression (Farber and Sarma, 1987). It remains undetermined if the sudden appearance of cell death in the persistent nodule may act as a trigger

or as a consequence of the autonomous cell proliferation (Farber and Sarma, 1987). The production of growth factors during carcinogenesis is suspected as an autocrine mechanism (Sporn and Roberts, 1985). Since some oncogenes are related to some growth factors (Heldin and Westermark, 1984; Sporn and Roberts, 1985; Kaczmarek, 1986), it is possible that one or more oncogenes may become expressed or may show increased expression during the progression stage (Farber and Sarma, 1987). The continual proliferation of a small number of cells in the persistent nodules may proceed to cell evolution (Farber, 1984c) by mutation and selection (Cairns, 1975) or clonal evolution (Nowell, 1976). These properties may well become the major overall mechanisms for the progressive pressure toward more and more malignant behavior (Farber and Sarma, 1987). Therefore, progression can be associated with increased growth rate, increased invasiveness, the formation of metastases and alterations in the biochemical and morphological characteristics of the neoplasm.

Preneoplastic Lesions in Rat Hepatocarcinogenesis.

During hepatocarcinogenesis, new phenotypically altered clones of hepatocytes eventually appear (Emmelot and Scherer, 1980; Farber, 1984c). The first lesions to become evident in the liver after treatment of rats with hepatocarcinogens are microscopic foci or "islands" of altered cells, often called preneoplastic or altered hepatocellular foci (AHF) and enzyme-altered foci (EAF) (Pitot and Sirica, 1980; Emmelot and

Scherer, 1980; Williams, 1980; Farber and Sarma, 1987). The AHF appear to develop sequentially to nodules and hepatocellular adenomas and carcinomas (Farber and Sarma, 1987; Saeter and Seglen, 1990). This sequence of events has been interpreted as a feature of a developmental process in multistage hepatocarcinogenesis, where earlier lesions serve as precursors for more advanced alterations (Bannasch, 1986). The AHF and nodules are regarded as the clonal progeny of single initiated hepatocytes, and they are widely accepted as early indicators of neoplastic development (Farber and Cameron, 1980; Schulte-Hermann, 1985; Moore and Kitagawa, 1986; Hendrich and Pitot, 1987). However, only very few of the foci and nodules are able to progress to cancer and can be considered as preneoplastic lesions (Sell et al., 1987). Therefore, the final conclusion related to the carcinogenic potential of a compound (initiator or promoter) in the liver still requires the demonstration of a malignant neoplasm.

Although the focal hepatocellular lesions are most clearly identified by their immunohistochemical staining patterns, they can also be seen with standard histologic staining, such as hematoxylin and eosin (Saeter and Seglen, 1990). After exposure to carcinogens, five distinctive types of AHF can be recognized in the liver. These include clear, acidophilic, basophilic, vacuolated and mixed cell foci (Squire and Levitt, 1975; Emmelot and Scherer, 1980; Williams, 1980; Institute of Laboratory Animal Resources, 1980; Harada

et al., 1989a). Further subclassification, particularly for basophilic and acidophilic cell foci has also been reported (Harada et al., 1989a). Clear and acidophilic foci stain as such due to increased amounts of glycogen and smooth endoplasmic reticulum, whereas basophilic foci have a low glycogen content and increased numbers of ribosomes (Bannasch, 1976; Ogawa et al., 1979) and vacuolated foci have a high fat content (Harada et al., 1989a). The nuclei in altered hepatocytes generally have a loose chromatin structure with prominent nucleoli (Bannasch, 1976; Ogawa et al., 1979). Evidence for sequential development of foci from clear and acidophilic types to mixed type, then to basophilic type has been reported (Enzmann and Bannasch, 1987).

Preneoplastic foci have increased proliferative activity when compared to the surrounding hepatocytes, as evident by an increased number of doublet cells (Cameron, 1989), changes in cellular and nuclear ploidy (Schwarze et al., 1984: Saeter et al., 1988b) and an increase in focus volume with the passage of time (Emmelot and Scherer, 1980). There is a considerable heterogeneity among individual foci within the same initiated liver (Saeter and Seglen, 1990). The heterogeneity includes the number and type of enzymatic alterations (Peraino et al., 1984; Tsuda et al., 1987), ploidy distribution (Sarafoff et al., 1986) and proliferative activity (Peraino et al., 1984).

Another common feature in the early stage of chemical

hepatocarcinogenesis is the proliferation of a group of cells known as biliary ductular epithelial cells or oval cells (Williams, 1980). It appears that these oval cells are a heterogenous population of epithelial cells which have the capability to transform or differentiate into hepatocytes (Evarts et al., 1987; Germain et al., 1988). Although the particular role of oval cells in the development of AHF is unknown (Williams, 1980), they may be precursors of hepatocytes or stem cells.

Foci of initiated cells may then regress, remodel or transform further to hepatocyte nodules (Farber and Sarma, 1987; Saeter et al., 1988a). These nodules have been variously designated as hyperplastic nodules, neoplastic nodules, adenomas (Institute of Laboratory Resources, 1980) or hepatocyte nodules. The term hepatocyte nodules is preferred because it is a neutral noninterpretative term (Farber and Sarma, 1987). Hepatocyte nodules are visible grossly as round, sometimes elevated, grayish-white spots in the liver (Farber, 1976; Institute of Laboratory Animal Resources, 1980). Histologically, the nodules have a cellular picture resembling that of foci, but often contain mixtures of clear, acidophilic, and basophilic cells (Bannasch, 1976). Hepatocyte nodules are sharply demarcated from and compress the surrounding normal liver tissue (Williams, 1980). The plates of hepatocytes comprising the nodule may be more than one cell thick, irregular or replaced by sheets of cells (Institute of

Laboratory Animal Resources, 1980).

Similar to preneoplastic foci, hepatocyte nodules have been shown to be monoclonal in origin (Tsuji et al., 1988). The nodules have elevated proliferative activity (Saeter et al., 1988b), increased cell turnover (Columbano et al., 1985) and alteration in enzyme activities as with AHF (Buchmann et al., 1985).

Hepatocyte nodules can be divided into reversible or persistent nodules (Farber and Sarma, 1987; Saeter and Seglen, 1990). Reversible nodules are those that remodel or differentiate into apparently normal hepatocytes after withdrawal of a carcinogen (Enomoto and Farber, 1982; Tatematsu et al., 1983). Persistent nodules include those which persist and have the capability to proliferate spontaneously upon withdrawal of the carcinogen or the promoter (Goldsworthy et al., 1984; Saeter et al., 1988c). About 2 to 5 % of the hepatocyte nodules remain as persistent nodules and may undergo further malignant progression into hepatocellular carcinomas (HCC) (Tatematsu et al., 1983; Farber, 1984b; Farber and Sarma, 1987). However, an occasional HCC may bypass the nodular stage and evolve directly from basophilic foci (Bannasch, 1976; Taper et al., 1983).

Hepatocellular carcinomas can be found in several forms, ranging from well differentiated trabecular carcinomas and adenocarcinomas to highly undifferentiated, anaplastic carcinomas (Squire and Levitt, 1975; Farber, 1976; Institute

of Laboratory Animal Resources, 1980). Several properties of HCC can be used to differentiate them from normal hepatocytes including hyperproliferation, high cell turnover, diploid or divisional growth as the predominant pattern, hyperresponsiveness toward growth stimuli and reduced protein degradation (Saeter and Seglen, 1990).

Immunohistochemical Markers in Preneoplastic Lesions. In chemical hepatocarcinogenesis in rats, AHF and hepatocyte nodules have been demonstrated as putative precursor populations (Farber and Cameron, 1980; Farber, 1984a; Scherer, 1984; Sell et al., 1987). They are characterized by a number of alterations in enzyme expression which have been used as immunohistochemical markers (Sato et al., 1983; Tatematsu et al., 1985; Moore and Kitagawa, 1986; Sato, 1988). These changes include increased activity of a large number of enzymes involved in drug detoxification (phase II enzymes), such as glutathione S-transferases (GSTs), γ-glutamyl transpeptidase (GGT) (Tatematsu et al., 1985; Sato, 1988), epoxide hydrolase (Enomoto et al., 1981) and uridine-diphosphate (UDP)-glucoronyl transferase (Bock et al., 1982). Parallel to these changes, there is commonly a decrease in the activity of enzymes involved in the activation of xenobiotics (phase I enzymes), such as cytochrome P450 (Tsuda et al., 1987), NADPH-cytochrome c reductase (Buchmann et al., 1985), aryl hydrocarbon hydroxylase (Cameron et al., 1976) and aminopyrine N-demethylase (Feo et al., 1978). The enzymatic

alterations may convey resistance for the altered cells against the toxic effects of a multitude of agents (Carr and Laishes, 1981; Carr, 1987) as well as protection against the genotoxicity of some carcinogens (Huitfeldt et al., 1988).

Hyperexpression of the multidrug resistance gene ("mdr") has been observed in hepatocellular nodules and carcinomas (Fairchild et al., 1987). Most likely it also contributes to the drug resistance of early foci (Saeter and Seglen, 1990).

The preneoplastic lesions may show a decrease in activity of glucose 6-phosphatase and an increase in glucose 6-phosphate dehydrogenase which are associated with increased glycogen storage and high pentose phosphate pathway activity, respectively (Klimek et al., 1984; Moore and Kitagawa, 1986). The pentose phosphate pathway may in turn generate NADPH and may contribute to an increased capacity for drug detoxification (Moore et al., 1986).

Other alterations described in preneoplastic foci include decreased activity of canalicular adenosine triphosphatase (ATPase) (Scherer, 1984), lower iron uptake (Williams, 1980) and reduced asialoglycoprotein receptor content and asialoglycoprotein uptake (Evarts et al., 1984). In addition, a decrease in fatty acid synthesis and lipid storage (Moore et al., 1986) and altered metabolism of individual amino acids (Schulte-Hermann, 1985; Moore and Kitagawa, 1986) have also been reported. Protein degradation has been found to be decreased in preneoplastic foci through down regulation of

protein autophagy (Schwarze and Seglen, 1985). Therefore, protein synthesis could be normal in preneoplastic lesions (Schwarze and Seglen, 1985).

Recent data suggest that at least some of the enzymatic alterations in foci may be the indirect results of mutational changes in target cell DNA induced by the initiating carcinogens (Saeter and Seglen, 1990). Moore et al. (1987) reported that 48 hours after carcinogen exposure, single GST placental form (GST-P) positive hepatocytes, the probable precursors of later appearing GST-P positive foci, can be identified in the liver. Furthermore, hyperexpression of GST-P and GGT can be induced in rat liver epithelial cell lines by transforming ras oncogenes (Sinha et al., 1986; Burt et al., 1988), suggesting that the presence of these markers in preneoplastic foci may reflect the mutational activation of ras genes or other oncogenes.

Although such enzymatic and biochemical alterations are used for the histochemical identification of preneoplastic cell populations, their relevance to hepatocarcinogenesis remains obscure (Saeter and Seglen, 1990). For example, expression of the "mdr" gene is increased (Fairchild et al., 1987) and the level of cytochrome P450 is decreased during liver regeneration (Carr and Laishes, 1981). Liver hyperplasia may also cause reduction in cytochrome P450 activities and elevation of GST-P (Roomi et al., 1986).

Glutathione S-Transferases in Preneoplastic Lesions. Among various immunohistochemical markers (Enomoto et al., 1981; Bock et al., 1982; Buchmann et al., 1985; Xu et al., 1990), rat GST-P has been identified as a reliable marker enzyme for preneoplastic lesions in rat hepatocarcinogenesis (Kitahara et al., 1984; Tatematsu et al., 1985; Satoh et al., 1985; Cameron, 1988; Sato, 1988). Glutathione S-transferases are a group of multifunctional dimeric proteins and are present in multimolecular forms in various organs of different species, including the liver of rats (Jakoby, 1978; Satoh et al., 1985; Mannervik et al., 1985; Sato, 1988). Although most of the glutathione S-transferases in the rat are localized in the cytosol, one microsomal form has been purified (Morgenstern et al., 1982; Mannervik et al., 1985; Morgenstern and DePiere, 1987).

Glutathione S-transferases catalyze the conjugation of electrophilic compounds with reduced glutathione (GSH), which is the first step of the mercapturic acid pathway in detoxification reactions (Chasseaud, 1979). Immunologically, GST-P does not cross react with other forms of GST, but GST-P from many species, such as the rat, mouse, hamster, dog, horse and human does cross react (Satoh et al., 1985; Roomi et al., 1985). The GST-P is present at very low levels in bile ductular cells of normal rat liver (Satoh et al., 1985) and can be detected immunohistochemically in the nuclei or cytoplasm of hepatocytes (Moore et al., 1987).

Glutathione S-transferase placental form is greatly elevated in putative AHF, nodules and carcinomas (Sato et al., 1984; Satoh et al., 1985; Tatematsu et al., 1985). This enzyme is an accurate marker for different stages in liver carcinogenesis, including the very early AHF or even single cells, since very few AHF or single GST-P positive cells may be detected in normal control animals (Moore et al., 1987). Single cells and AHF are predominantly located in the intermediate zone 2 and to a lesser extent in zone 1 of Rappaport in rat liver (Moore et al., 1987). Since the GST-P positive single cells may become the precursors of preneoplastic foci and nodules (Satoh et al., 1985), early detection of GST-P positive foci would appear to be important for screening of hepatocarcinogens.

Chemical Nasal Carcinogenesis

Epidemiologic studies indicated that the incidence of nasal tumors in human beings is higher in specific occupational groups, such as workers in the leather and wood industry (Buiatti et al., 1983) and in nickel refineries (Doll et al., 1977; Torjussen, 1983). In experimental animals, nasal tumors can be readily induced by various chemicals by various routes (Noronha and Goodall, 1972; Swenberg et al., 1980; Lee and Trochimowicz, 1982; Takano et al., 1982). However, animal models that have been used for studies of multistage carcinogenesis in nasal tissue are not available at present.

Metabolic Activation of Carcinogens in the Rat Nasal Cavity. Malignant conversion of cells by carcinogens is widely accepted as a multistage process (Farber and Sarma, 1987) with an initial interaction between the agent or its metabolites and the target cell's DNA, leading to permanent phenotypic lesions (Miller and Miller, 1981a).

The nasal cavity of rats is anatomically (Young, 1981; Popp and Martin, 1984; Monteiro-Riviere and Popp, 1984) as well as physiologically (Morgan et al., 1984) complex and has been demonstrated to play an important role in the metabolism of xenobiotics (Hadley and Dahl, 1982; Randall et al., 1987; Baron et al., 1988). The association of certain enzymes with particular cell types may reveal regional differences in metabolic activity which could subsequently account for regional susceptibility to xenobiotics (Randall et al., 1987). Xenobiotics which are biotransformed into active metabolites by particular enzymes usually exert relatively selective toxic effects within the different segments of the respiratory tract (Boyd, 1980; Haschek et al., 1983; Bermudez and Allen, 1984). The carcinogens frequently damage either a specific morphological cell type or groups of morphologically similar cells which are located within a selected area or region of the nasal cavity (Hecht et al., 1980; Kerns et al., 1983; Hurtt et al., 1988).

Nasal mucosa has been demonstrated to contain enzymes involved in drug detoxification reactions (Bond, 1983; Baron

et al., 1988) as well as enzymes involved in the activation of xenobiotics (Voigt et al., 1985; Baron et al., 1988). Cytochrome P450 isozymes can be found in respiratory epithelium, seromucous glands, Bowman's glands (Voigt et al., 1985; Baron et al., 1988) and olfactory epithelium (Voigt et al., 1985). Glutathione S-transferases B, C, and E and epoxide hydrolase have been demonstrated immunohistochemically in olfactory and respiratory mucosa and especially in Bowman's and seromucous glands (Baron et al., 1988). Other enzymes such as GGT, NADPH-cytochrome P450 reductase, alkaline phosphatase and glucose 6-phosphatase are also detected in the respiratory and olfactory regions of nasal mucosa (Randall et al., 1987). Although xenobiotics can be metabolized within olfactory and respiratory regions, the olfactory region appears to represent the major site for oxidative metabolism of xenobiotics (Baron et al., 1988).

Results of several in vivo studies in rats indicated that some carcinogens can damage DNA in epithelial cells of nasal mucosa after metabolic activation or by acting directly (Brittebo et al., 1983; Casanova-Schmitz and Heck, 1983; Bermudez and Allen, 1984). Evidences for covalent binding and crosslinking of DNA to proteins within cells of the nasal mucosa (Casanova-Schmitz and Heck, 1983) and the ability of nasal epithelium to repair some or all of the DNA damage (Bermudez and Allen, 1984) have been reported. Furthermore, formation of DNA adducts after exposure to cigarette smoke has

been demonstrated in the nasal cavity of rats (Gupta et al., 1989).

Based on the distribution and availability of some metabolic enzymes, evidence for DNA damage and adduct formation and further development of lesions to tumors, most likely the multistage concept of carcinogenesis also applies to the nasal cavity.

N-Nitrosamine Carcinogenesis in the Rat Nasal Cavity. Many N-nitrosamines have been known to induce tumors in the nasal cavity of various laboratory animals, including the rat (Cardesa et al., 1976; Reznik-Schuller, 1978; Lijinsky and Taylor, 1979; Green et al., 1980). Several studies indicated that nasal mucosa is able to metabolize some N-nitrosamines (Brittebo and Tjalve, 1981, 1982; Bermudez and Allen, 1984). This property can be associated with the presence of enzymes involved in the metabolic activation and detoxification of xenobiotics in the nasal tissues (Baron et al., 1988). Metabolites derived from activation of N-nitrosamines have been demonstrated to be capable of damaging DNA in the nasal mucosa (Loefberg et al., 1982; Castonguay et al., 1983; Bermudez and Allen, 1984). Therefore, formation of adducts leading to mutation might be possible. Further steps following metabolic activation to development of tumors in the nasal cavity are not well defined.

Tumors in the nasal cavity can arise from the anterior or posterior portion of this organ (Reznik-Schuller, 1983a).

The anterior portion consists of squamous, basal, mucous, or ciliated cells (Young, 1981) and these cells have the potential to become precursors of tumors in this area. The benign tumors found in this region are papillomas, papillary adenomas and occasionally adenomas (Reznik-Schuller, 1983a), whereas the malignant tumors include squamous cell carcinomas and adenocarcinomas (Feron et al., 1990). Papillomas and papillary adenomas are usually accompanied by multifocal hyperplasia and/or squamous metaplasia, although these lesions do not always develop into neoplasms (Reznik-Schuller, 1983a). Multiple proliferations of basal cells in the respiratory epithelium leading to squamous metaplasia are considered as precursor lesions of squamous cell carcinomas induced by treatment with some N-nitrosamines (Reznik-Schuller, 1983a). The reaction of basal cells to treatment with N-nitrosamines may vary at different locations in the nasal cavity (Reznik-Schuller, 1983a). Hyperplasia of Bowman's gland epithelium with cytoplasmic basophilia and large hyperchromatic atypical nuclei are always associated with neoplastic transformation (Brown, 1990).

The posterior portion of the nasal cavity consists of olfactory sensory cells, neuroblasts, sustentacular cells, basal cells and epithelial cells of Bowman's gland (Young, 1981). It can be assumed that neoplasms in this region may arise from these types of cells. Neoplasms in this region consist of adenomas, well- and poorly differentiated

adenocarcinomas, neurogenic tumors (Hecht et al., 1980), and squamous cell carcinomas (Reznik-Schuller, 1983b).

Result of studies with N-nitrosomethylpiperazine (Reznik-Schuller, 1983b) indicated sequential changes starting as severe toxic degeneration of sensory cells, followed by proliferation of basal and neuroendocrine cells, and finally progression to adenocarcinomas.

Potential Immunohistochemical Markers in Nasal Lesions. After treatment with some N-nitrosamines, there is hyperplasia of basal cells and/or neuroendocrine cells in the nasal mucosa (Reznik-Schuller, 1983a). Since most of these lesions have been found to progress to tumors (Reznik-Schuller, 1983a), they may be considered as "preneoplastic" lesions in the nasal cavity. Brown (1990) reported that other potentially preneoplastic changes can be found in the Bowman's gland.

Some studies indicated that various enzymes (including glutathione S-transferases) in the nasal cavity can be detected with immunohistochemical staining (Voigt et al., 1985; Randall et al., 1987; Baron et al., 1988). Furthermore, the presence of glutathione S-transferases B, C, and E has been demonstrated in both respiratory and olfactory regions of nasal cavity (Baron et al., 1988). Since GST-P is assumed to be present in various normal tissues at low concentration (Sato, 1988), the immmunohistochemical method using GST-P as a marker enzyme may be a useful technique in detecting preneoplastic lesions in the nasal cavity.

CHAPTER 1

SEQUENTIAL STUDY IN RATS OF NASAL AND HEPATIC LESIONS INDUCED BY SINGLE OR DUAL EXPOSURE TO N-NITROSODIMETHYLAMINE AND N-NITROSOPYRROLIDINE

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SUMMARY

N-Nitrosodimethylamine (NDMA) and N-nitrosopyrrolidine (NPYR) are primary hepatocarcinogens and can be metabolized in the liver and nasal mucosa of rats. To characterize the development of acute lesions in the liver and nasal cavity, female Sprague-Dawley rats were given NDMA and NPYR, singly or in combination. Doses used were 10, 30 and 60 mg/kg of body weight (BW) of NDMA and 10, 30 and 100 mg/kg of BW of NPYR. Rats were killed at 6 and 12 hours and 1, 3, 10 and 30 days. With combination exposure, doses used were 15 and 30 mg/kg of BW of each chemical and the rats were killed at 3 and 30 days. Liver and nasal tissues were stained with hematoxylin and eosin and immunohistochemically for glutathione S-transferase placental form (GST-P). Necrosis of olfactory epithelium and Bowman's glands was seen as early as 6 hours with NDMA or NPYR and was most severe by 3 days. Regeneration was incomplete by 30 days after exposure to high doses of either chemical. With

combination exposure, necrosis of olfactory epithelium and Bowman's glands were evident at 3 days with NDMA or NPYR and their combination and at 30 days with combined exposure to NDMA and NPYR. Liver necrosis was seen with NDMA or with a combination of NDMA and NPYR. With either chemical, there were single GST-P positive hepatocytes as early as 1 day and small altered hepatocellular foci (AHF) at 30 days. With dual exposure, larger AHF were seen at 30 days. Results indicate that a single dose of NDMA or NPYR can have prolonged toxic effects on nasal tissues. Immunohistochemical staining for GST-P is useful in determining single positive cells and very early foci in the liver. Combination exposure appeared to cause additive effects in the liver and nasal cavity. Preneoplastic lesions were absent from nasal tissues.

Introduction

N-Nitrosamines are a group of environmental chemicals which are commonly found in foods and beverages (Spiegelhalder et al., 1980), tobacco products (Hoffmann et al., 1979), industrial products (Fan et al., 1977; Spiegelhalder and Preussmann, 1983), and industrially polluted environments (Spiegelhalder and Preussmann, 1987). These compounds are known for their ability to induce malignant tumors in a wide variety of target organs, including liver and nasal cavity (Schmahl and Habs, 1980; Lijinsky and Reuber, 1981).

Most N-nitrosamines require metabolic activation for

conversion into ultimate carcinogens (Miller and Miller, 1981a) which is mediated by cytochrome P450 (Chan et al., 1978; Lai and Arcos, 1980). The activation of N-nitrosamines results in the formation of electrophilic intermediates (Michejda et al., 1982) which may form covalent bonds with DNA or react with other macromolecules (Harris, 1987). This interaction may result in adduct formation (Miller and Miller, 1981b) and consequently lead to point mutations (Singer, 1984). Barbacid (1986) indicated that mutation of cellular proto-oncogenes may be an essential step in the multistage process of carcinogenesis. It can be assumed that metabolic intermediates may act additively to produce toxic, metabolic and carcinogenic effects (Harris et al., 1982).

The organ-specific carcinogenesis of N-nitrosamines may be associated with the distribution of the parent carcinogen, tissue-specific bioactivation, DNA repair, cell division and inherent tissue susceptibility because of the presence of proto-oncogenes (Kleihues et al., 1987). Many carcinogens, including N-nitrosamines have been demonstrated to damage either a specific morphological cell type or groups of morphologically similar cells which are located within a selected region of the nasal cavity (Hecht et al., 1980; Kerns et al., 1983; Hurtt et al., 1988).

N-Nitrosodimethylamine (NDMA) and N-nitrosopyrrolidine (NPYR) are the most commonly detected N-nitrosamines in food systems (Spiegelhalder et al., 1980; Song and Hu, 1988).

N-Nitrosodimethylamine has been known to induce acute cellular injuries and tumors in many laboratory animals, including rats (Schmahl and Habs, 1980; Bogovski and Bogovski, 1981; Hard et al., 1984; Ishinishi et al., 1988). In rats, acute toxic effects of NDMA occur mainly in the form of cellular necrosis and can be found in the liver (Shank, 1975) and kidney (Hard et al., 1984). Montesano et al. (1980) described the repair capability of liver of rats after exposure to low doses of NDMA.

Studies in laboratory animals, including rats, indicated that NPYR can produce mainly tumors in the liver (Lijinsky and Reuber, 1981; McCoy et al., 1986; Berger et al., 1987; Ishinishi et al., 1988). Although in vitro studies with rat hepatocytes indicated that NPYR is able to induce cytotoxic effects (Alldrick et al., 1985), acute lesions in rats due to exposure to this compound have not been well described.

Since N-nitrosamines are commonly present in various foods and environments, simultaneous exposure to several carcinogens, including NDMA and NPYR is likely to be possible. Schmahl and Habs (1980) indicated that these carcinogens may act additively, synergistically or inhibitorily. Although the precise mechanisms of such actions are not well understood, they may relate to enzyme-inducing or enzyme-inhibiting effects, competitive or noncompetitive inhibition of substrates on the enzymes, alterations of the rates of cell division and cell specific protein synthesis (Schmahl and

Habs, 1980). Syncarcinogenic studies in laboratory animals indicated there are additive effects of very low doses of N-nitrosodiethylamine (NDEA), N-nitrosodiethanolamine (NDELA) and NPYR on the formation of liver tumors (Berger et al., 1987). Comparable amounts of N-nitrosamines may be found in various foods or in the environment and these amounts would be assumed not to be carcinogenic if present individually.

Previous studies indicated that NDMA and NPYR can be metabolized in the liver (Brambilla et al., 1981; Streeter et al., 1990) and nasal mucosa of rats (Brittebo et al, 1981; Bermudez, 1986) and their injurious effects have been suggested to be related to their metabolites (Brittebo and Tjalve, 1983; Alldrick et al., 1985). It was hypothesized that a single dose of NDMA or NPYR can produce acute lesions in the nasal cavity and NPYR can acutely damage the liver. Acute cellular lesions in both organs may partially or completely regenerate by a given time. Lesions in the DNA of highly targeted cells may persist and these cells may become precursor cells early in the carcinogenic process, which can be determined by immunohistochemical staining. Dual exposure to NDMA and NPYR may act additively in producing acute lesions in the liver and nasal cavity since both of them target the same organs.

The specific objectives of this experiment were to characterize the development and fate of morphologic and preneoplastic lesions associated with acute exposure to NDMA

or NPYR and to determine the possible additive or synergistic effects of dual exposure to NDMA and NPYR.

Materials and Methods

Animals and Experimental Protocol. Female Sprague-Dawley rats weighing 150 to 180 g were purchased from Charles River Corporation, Portage, Michigan. Rats were housed in clear propylene cages and were randomly assigned into groups of three per cage. The room temperature was maintained at 22^0C with a 12 hour light/dark cycle. The animals were given commercial feed (Rodent Laboratory Chow #5001, Purina Mills, Inc., St. Louis, Missouri) and water ad libitum. Rats were acclimated for 7 days before treatment. The animals were randomly assigned to groups of three animals each.

Each rat in treated groups was given a single intraperitoneal dose of 10, 30, or 60 mg of NDMA or 10, 30, or 100 mg of NPYR/kg of BW. Since NDMA is known as a potent hepatotoxin (Shank, 1975), the high dose of NDMA was less than that for NPYR to avoid severe hepatic toxicity and early death. Rats were killed at 6 or 12 hours and at 1, 3, 10 or 30 days after treatment.

In the combination study, each rat was given an intraperitoneal dose of NDMA or NPYR. Doses used were 15 or 30 mg/kg of BW of either chemical, a combination of 15 mg/kg of NDMA and 15 mg/kg of NPYR, or a combination of 30 mg/kg of NDMA and NPYR. Rats were killed at 3 and 30 days after

treatment. A control group without treatment was also included in these studies.

Chemicals. N-Nitrosodimethylamine and N-nitrosopyrrolidine were purchased from Sigma Chemical Company, St. Louis, Missouri.

Necropsy and Collection of Tissue Samples. At the prescribed intervals, rats were killed with CO_2, decapitated and necropsied. Nasal cavities were infused with 10% buffered formalin. Sections of liver and nasal tissues were placed in the same fixative.

Histologic Preparation. Nasal tissues were decalcified by the formic acid-sodium citrate method and were sectioned according to standard procedures (Young, 1981). Formalin-fixed liver and nasal tissues were processed, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. Serial sections of nasal tissues were also stained with alcian blue-periodic acid-Schiff (AB/PAS).

Immunohistochemical Staining. Immunohistochemical staining of formalin-fixed liver and nasal tissues for GST-P was according to the procedure described by Bourne (1983).

Results

Development of Lesions in the Liver. Histologic changes related to treatment were seen in the liver of rats given NDMA, and consisted of multifocal centrolobular necrosis and vacuolation of hepatocytes. A photomicrograph of a histologic

section of liver from an untreated rat is shown in Figure 1.1. By 1 day rats given 30 mg/kg of NDMA had moderate, multifocal necrosis and vacuolation, mainly in the centrolobular regions. At 1 day rats treated with NDMA (60 mg/kg) had moderate to severe, multifocal centrolobular necrosis and vacuolation (see Figure 1.2.). Three days after exposure, rats given NDMA (30 mg/kg) had mild, multifocal centrolobular necrosis and vacuolation. Moderate necrotic changes were seen in groups of rat treated with NDMA (60 mg/kg). By 10 and 30 days after treatment, the livers appeared to have regenerated completely. Rats treated with NPYR or a low dose (10 mg/kg) of NDMA had no significant hepatic changes throughout the experiment.

In the combination study, histologic lesions related to treatment were seen only at 3 days after treatment. At this time, rats given NDMA (30 mg/kg) had moderate, multifocal centrolobular necrosis and vacuolation. Severe, multifocal necrosis with hemorrhages were found in the liver of rats treated with a combination of NDMA and NPYR (30 mg/kg of each) (see Figure 1.3.).

Development of Lesions in the Nasal Cavity. Histologic lesions related to treatment occurred in the nasal cavity of rats treated with NDMA or NPYR (see Table 1.1.). The lesions consisted of necrosis and vacuolation of olfactory epithelium and Bowman's glands. Areas most severely and consistently affected were between the nasal septum and dorsal meatus. The respiratory epithelium and related glands seemed to be spared.

A photomicrograph of a section of nasal cavity from an untreated rat is shown in Figure 1.4. and AB/PAS staining of the same section is shown in Figure 1.5. Necrotic changes in nasal tissues were seen in rats treated with 30 mg/kg of BW (medium dose) and 60 or 100 mg/kg of BW (high dose) of each chemical.

At 6 hours post treatment, rats given NDMA (30 mg/kg) had mild, multifocal necrosis and vacuolation of olfactory epithelium and Bowman's glands (see Figure 1.6.). The same rats had a decrease in AB/PAS positive material in Bowman's glands (see Figure 1.7.).

Lesions in the nasal cavity of rats treated with NPYR were evident as early as 6 hours at a dose of 100 mg/kg of BW. By 12 hours after exposure, necrosis of olfactory epithelium and Bowman's glands was seen with either chemical. At this period, NDMA produced more severe lesions in the olfactory epithelium, whereas NPYR caused more severe effects in Bowman's gland epithelium (see Figures 1.8.). This difference was most apparent in rats given the medium dose of each compound. Severe, diffuse necrosis of olfactory epithelium and Bowman's glands with disorganization and desquamation of olfactory epithelium was found after 1 day of treatment with high doses of NDMA or NPYR (see Figure 1.9.).

Both chemicals induced the most severe lesions at 3 days after treatment, especially in rats given high doses of either chemical. The lesions consisted mainly of atrophy of Bowman's

Table 1.1. Histopathologic Evaluation of the Nasal Cavity of Rats Exposed to NDMA or NPYR[a].

Intervals	Lesions[b]	Dose (mg/kg of BW)			
		NDMA		NPYR	
		30	60	30	100
6 hours	Necrosis of OE[c]	Mi	Md	-	Mi
	Necrosis of BE[d]	Mi	Mi	-	Md
	AB/PAS[e]	Mi	Md	-	Md
12 hours	Necrosis of OE	Md	Se	Mi	Se
	Necrosis of BE	Mi	Md	Md	Se
	AB/PAS	Md	Se	Md	Se
1 day	Necrosis of OE	Se	Se	Md	Se
	Necrosis of BE	Md	Se	Md	Se
	AB/PAS	Se	Se	Se	Se
3 days	Necrosis of OE	Md	Se	Se	Se
	Atrophy of BE	Md	Se	Se	Se
	Hyperplasia of ED[f]	Mi	-	Mi	-
	Hyperplasia of basal cells	-	Md	-	Md
	Atrophy of ON[g]	-	Se	-	Se
	AB/PAS	Md	Se	Se	Se
10 days	Necrosis of OE	Md	Se	Md	Se
	Atrophy of BE	Md	Se	Md	Se
	Hyperplasia of ED	Se	-	Se	-
	Hyperplasia of basal cells	Mi	Se	Md	Se
	Squamous metaplasia	-	Se	-	Se
	Atrophy of ON	-	Se	-	Se
	AB/PAS	Md	Td	Md	Td
30 days	Necrosis of OE	-	Se	-	Se
	Atrophy of BE	-	Se	-	Se
	Hyperplasia of ED	Md	-	Md	-
	Hyperplasia of basal cells	-	Se	-	Se
	Squamous metaplasia	-	Se	-	Se
	AB/PAS	-	Td	-	Td

[a]Each treated group comprised of 3 rats
[b]Lesions from level 3 of the nasal cavity of rats given medium and high doses of NDMA or NPYR
[c]Olfactory epithelium
[d]Bowman's gland epithelium
[e]Decrease in AB/PAS positive material in Bowman's glands
[f]Cells of excretory ducts of the Bowman's glands
[g]Olfactory nerve bundles
Mi = Mild; Md = Moderate; Se = Severe; Td = Total depletion

Table 1.2. Histopathologic Evaluation of the Nasal Cavity of Rats Exposed to Combination of NDMA and NPYR[a].

Intervals	Lesions[b]	Dose (mg/kg of BW)					
		NDMA		NPYR		NDMA and NPYR	
		15	30	15	30	15	30
3 days	Necrosis of OE[c]	-	Md	-	Mi	-	Se
	Necrosis of BE[d]	-	Mi	Mi	Md	Md	Se
	Hyperplasia of BC[e]	-	-	-	-	-	Md
	Atrophy of ON[f]	-	-	-	-	-	Se
	AB/PAS[g]	-	Mi	Mi	Td	Se	Td
30 days	Necrosis of OE	-	-	-	-	-	Se
	Necrosis of BE	-	-	-	-	-	Se
	Hyperplasia of BC	-	-	-	-	-	Se
	Squamous metaplasia	-	-	-	-	-	Se
	AB/PAS	-	-	-	-	-	Se

[a]Each treated group comprised of 3 rats
[b]Lesions from level 3 of the nasal cavity of rats given NDMA or NPYR (single or combination)
[c]Olfactory epithelium
[d]Bowman's glands epithelium
[e]Basal cells
[f]Olfactory nerve bundles
[g]Decrease in AB/PAS positive material in Bowman's glands
Mi = Mild; Md = Moderate; Se = Severe; Td = Total depletion; NDMA and NPYR = Dual exposure to 15 or 30 mg/kg of BW of each compound.

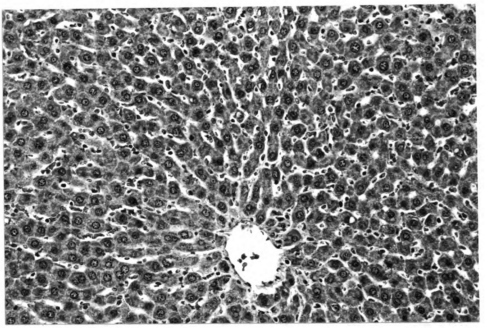

Figure 1.1. Photomicrograph of a section of liver from a rat in the untreated control group. Notice the normal hepatic architecture with cords of hepatocytes radiating from the central vein and the prominent sinusoidal spaces between the cords. (H & E stain, 224 X).

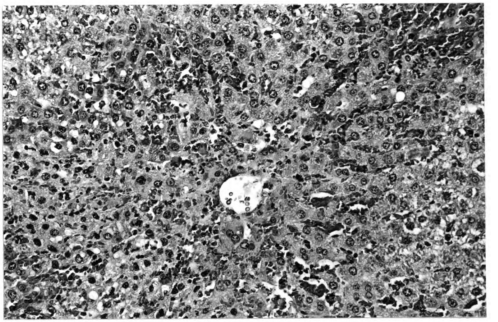

Figure 1.2. Photomicrograph of a section of liver from a rat given NDMA (30 mg/kg) at 1 day after treatment. Notice the necrosis with some vacuolation of the hepatocytes in the centrolobular area. Mild inflammatory reaction is evident. (H & E stain, 224 X).

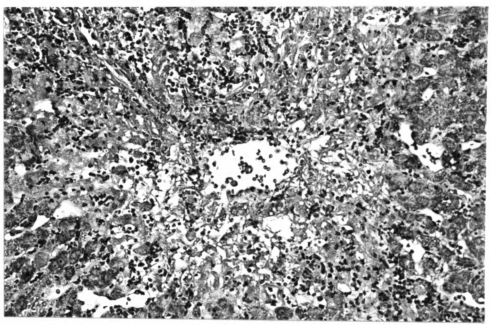

Figure 1.3. Photomicrograph of a section of liver from a rat given combination of NDMA and NPYR (30 mg/kg of each) after 3 days of exposure. Notice severe necrosis with vacuolation of hepatocytes in the centrolobular area. Some hemorrhages with mild inflammatory reaction are evident. (H & E stain, 224 X).

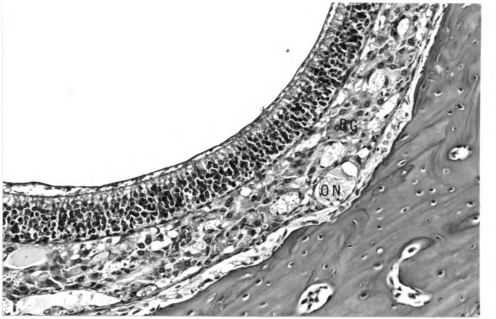

Figure 1.4. Photomicrograph of a section of nasal mucosa from a rat in the untreated control group. Notice the normal arrangement of olfactory epithelium which is covered by a mucus layer. The olfactory epithelium (OE) is comprised of sustentacular, sensory, basal cells and basement membrane. The lamina propria consists of Bowman's glands (BG), olfactory nerve bundles (ON), blood and lymph vessels. The Bowman's glands have indistinct epithelial cells with excretory ducts in the olfactory epithelium area. (H & E stain, 224 X).

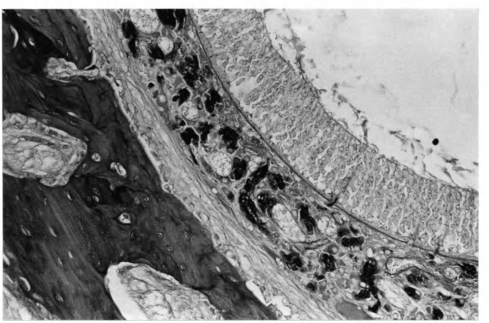

Figure 1.5. Photomicrograph of a section of nasal mucosa from a rat in the untreated control group. Notice the dark AB/PAS positive granules in the Bowman's glands. (AB/PAS stain, 224 X).

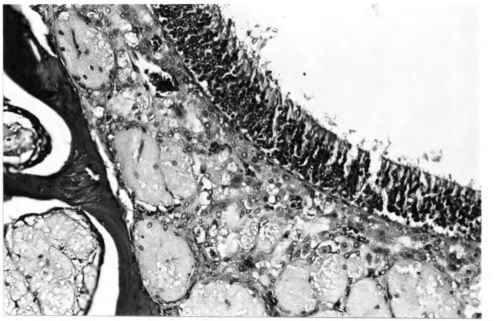

Figure 1.6. Photomicrograph of a section of nasal mucosa from a rat 6 hours after being given NDMA (30 mg/kg). Notice mild necrosis with pyknotic and rounded nuclei and vacuolation in the olfactory epithelium and Bowman's glands. (H & E stain, 224 X).

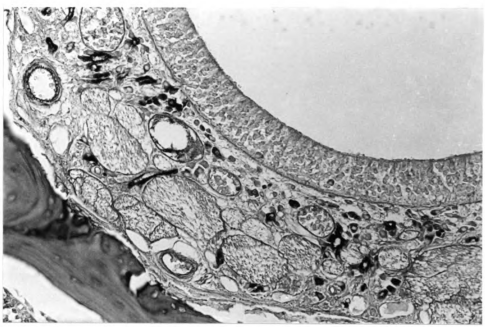

Figure 1.7. Photomicrograph of a section of nasal mucosa from a rat 6 hours after being given NDMA (30 mg/kg). Notice a decrease in the amount of AB/PAS positive material in the Bowman's glands. (AB/PAS stain, 224 X).

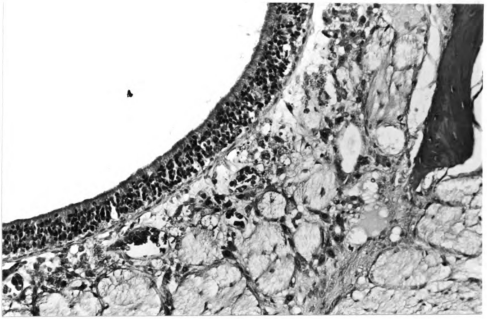

Figure 1.8. Photomicrograph of a section of nasal mucosa from a rat 12 hours after being given NPYR (30 mg/kg). Notice moderate necrosis of the Bowman's gland epithelium and mild changes in the olfactory epithelium. (H & E stain, 224 X).

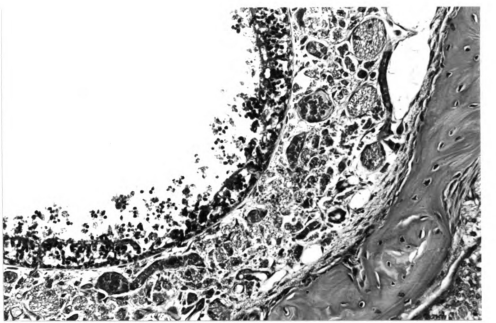

Figure 1.9. Photomicrograph of a section of nasal mucosa from a rat 1 day after being given NPYR (100 mg/kg). Notice extensive necrosis of olfactory epithelium and Bowman's glands. Focal desquamation of olfactory epithelium with only basal cells remaining is evident.
(H & E stain , 224 X).

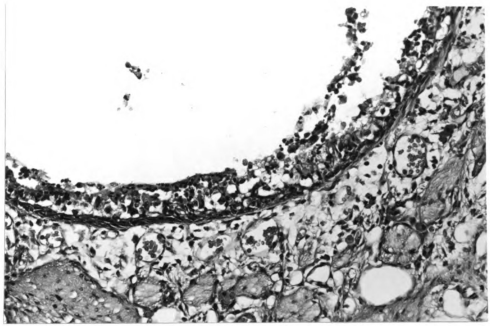

Figure 1.10. Photomicrograph of a section of nasal mucosa from a rat 3 days after being given NPYR (100 mg/kg). Notice extensive necrosis of olfactory epithelium with evidence of hyperplasia of basal cells. Bowman's glands epithelium and olfactory nerve bundles are severely atrophic. (H & E stain, 224 X).

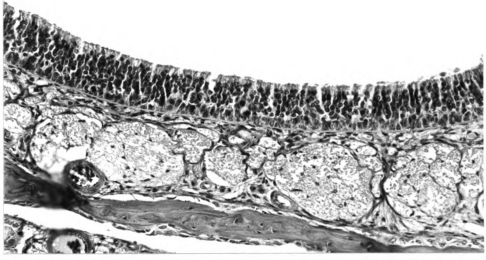

Figure 1.11. Photomicrograph of a section of nasal mucosa from a rat 10 days after being given NPYR (30 mg/kg). Notice moderate necrosis of olfactory epithelium with hyperplasia of basal cells and severe atrophy of Bowman's glands epithelium. Some hyperplasia of epithelium of ducts of the Bowman's glands is evident in the olfactory epithelium area. (H & E stain, 224 X).

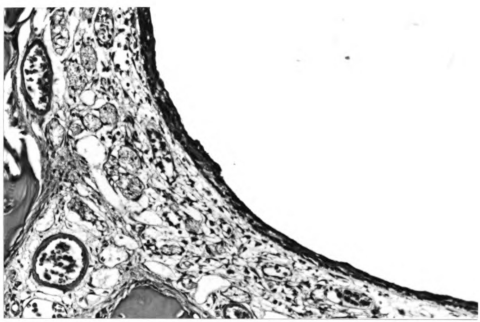

Figure 1.12. Photomicrograph of a section of nasal mucosa from a rat 10 days after being given NDMA (60 mg/kg). Notice desquamation of the olfactory epithelium with only hyperplastic basal cells remaining intermixed with undifferentiated epithelial cells as an indication of early stage of squamous metaplasia. Atrophy of olfactory nerve bundles and mild infiltration of inflammatory cells are evident. (H & E stain, 224 X).

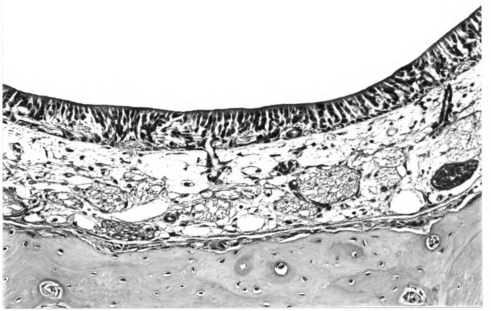

Figure 1.13. Photomicrograph of a section of nasal mucosa from a rat 30 days after being given NDMA (60 mg/kg). Notice squamous metaplasia with hyperplasia of basal cells and formation of axon bundles in the regenerating epithelium. Hyperplasia is evident in remnants of excretory ducts of Bowman's glands. (H & E stain, 224 X).

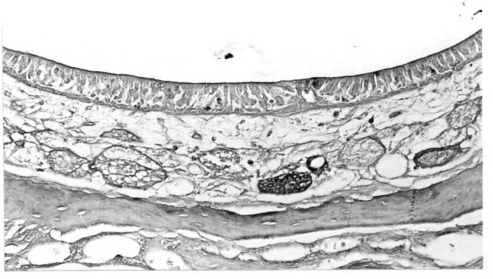

Figure 1.14. Photomicrograph of a section of nasal mucosa from a rat 30 days after being given NDMA (60 mg/kg). Notice the absence of AB/PAS positive material in the Bowman's glands. (AB/PAS stain, 224 X).

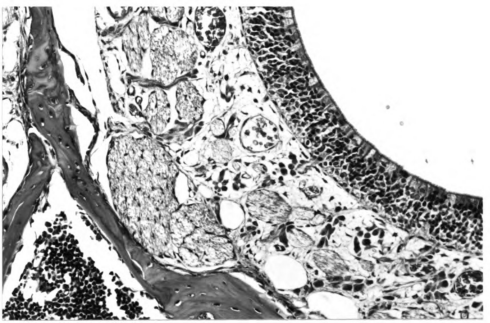

Figure 1.15. Photomicrograph of a section of nasal mucosa from a rat 3 days after being given a combination of NDMA and NPYR (15 mg/kg of each). Notice moderate necrosis of Bowman's glands and mild changes in the olfactory epithelium. Hyperplasia of epithelium of ducts of Bowman's glands is evident. (H & E stain, 224 X).

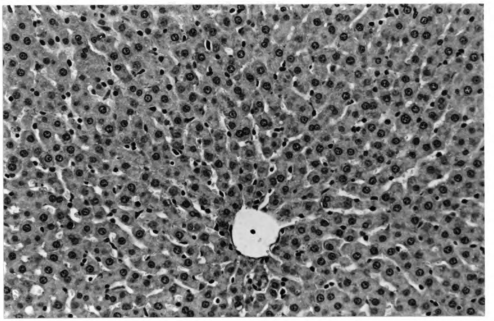

Figure 1.16. Photomicrograph of a histochemically-stained section of liver from a rat in the untreated control group. Notice the absence of GST-P positive hepatocytes. (GST-P stain, 224 X).

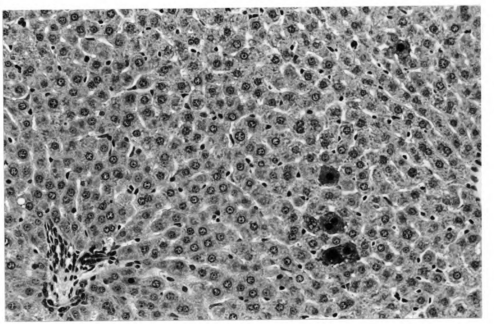

Figure 1.17. Photomicrograph of a histochemically-stained section of liver from a rat 3 days after being given NDMA (30 mg/kg). Notice the presence of single and doublet GST-P positive hepatocytes. (GST-P stain, 224 X).

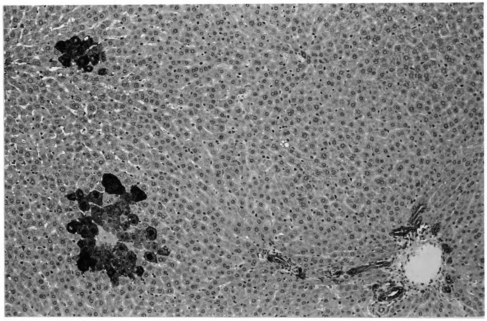

Figure 1.18. Photomicrograph of a histochemically-stained section of liver from a rat 30 days after being given a combination of NDMA and NPYR (30 mg/kg of each). Notice the presence of GST-P positive medium and large foci. (GST-P stain, 112 X).

gland epithelium, necrosis and decreases in the number of layers of olfactory epithelium, and hyperplasia of duct epithelium of Bowman's glands and basal cells (see Figure 1.10.). Inflammation and atrophy of olfactory bundles were seen at 3 and 10 days after exposure in rats given high doses of NDMA or NPYR.

Regeneration of olfactory epithelium and Bowman's glands in rats given NDMA or NPYR (30 mg/kg) was evident by 10 days (see Figure 1.11.) and was nearly complete by 30 days after exposure. By 10 days after treatment with high doses of NDMA or NPYR, squamous metaplasia was evident (see Figure 1.12.). Severe atrophy of Bowman's glands, hyperplasia of basal cells and duct epithelium of Bowman's glands were also present. Disappearance of Bowman's glands, multifocal squamous metaplasia with hyperplasia of basal cells and duct epithelial cells of Bowman's gland, and formation of glandular structures and axon bundles in the regenerating epithelium were seen at 30 days after exposure to high doses of either compound (see Figure 1.13.).

Decreased AB/PAS positive material in Bowman's glands was seen in rats given medium or high doses of NDMA or NPYR (see Table 1.1.). By 6 hours, the AB/PAS positive material was decreased in rats given a medium dose of NDMA or a high dose of NPYR. By 10 days after treatment with medium doses of either chemical, AB/PAS positive material was only slightly decreased and by 30 days the content of positive material was

nearly normal. At high doses of NDMA or NPYR, the AB/PAS positive material was totally depleted at 30 days after exposure (see Figure 1.14.). Rats given a low dose of either chemical had no detectible lesions in the nasal cavity throughout the experiment.

The groups of rats in the combination study had a similar pattern of lesions to those seen with single exposure to either chemical (Table 1.2.). At 3 days after treatment, rats given NDMA (30 mg/kg) had mild changes in the Bowman's glands and moderate alterations in the olfactory epithelium. On the contrary, lesions in the nasal cavity induced by NPYR appeared to be more prominent in the Bowman's glands compared to olfactory epithelium.

The combination exposure to NDMA and NPYR (30 mg/kg of each) caused multifocal necrosis and atrophy of Bowman's glands epithelium, necrosis and disorganization of olfactory epithelium with hyperplasia of basal cells. Rats exposed to NPYR (15 mg/kg) had mild atrophy of Bowman's epithelium, whereas rats treated with NDMA (15 mg/kg) did not. Simultaneous exposure to NDMA and NPYR (15 mg/kg of each) induced mainly necrosis of Bowman's gland epithelium (see Figure 1.15.).

At 30 days, rats treated with a combination of NDMA and NPYR (30 mg/kg of each) had lesions that were similar to rats treated with a single high dose of NDMA or NPYR in the previous experiment. All other groups of rat at this period

did not have any apparent lesions in the nasal cavity.

Decreases in AB/PAS positive material was seen at 3 days in groups of rats given NDMA (30 mg/kg), NPYR (15 or 30 mg/kg), or a combination of both compounds (15 or 30 mg/kg of each) (see Table 1.2.). By 30 days, decreases in AB/PAS positive material was only seen with combination exposure to 30 mg/kg of each chemical.

Immunohistochemical Examination. Glutathione S-transferase placental form (GST-P) expression was seen in the liver of rats exposed to medium and high doses of NDMA or NPYR. An immunohistochemical stained section of liver from an untreated control rat is presented in Figure 1.16. Single and doublet GST-P positive hepatocytes were detected as early as 1 day in rats given a medium dose of either chemical (see Figure 1.17.) and at 3, 10 and 30 days in rats treated with medium and high doses of NDMA or NPYR. At 30 days, there were a few small altered hepatocellular foci (AHF) consisting of approximately 3 to 10 cells in rats treated with a medium dose of NDMA or NPYR.

In the combination exposure study, development of GST-P positive hepatocytes was seen in all treated groups at 3 and 30 days after exposure. By 3 days, all treated rats had single and doublet GST-P positive hepatocytes. In addition, small AHF were found in livers of rats given a combination of 15 mg/kg of NDMA and NPYR. At 30 days, small, medium (approximately 11 to 20 cells) and large AHF (more than 20 cells) were present

in rats in all treated groups (see Figure 1.18.). Immunohistochemical evaluation of GST-P in the nasal cavity did not show any consistent pattern of specific staining.

Discussion

The severity of the lesions in the liver due to treatment with NDMA is dose dependent, as evident from the difference in the response to medium and high doses as well as in the response in the combination exposure study. Simultaneous exposure to NDMA and NPYR had additive effects on lesions in the liver as evident in rats given NDMA and NPYR (30 mg/kg of each) after 3 days of treatment. Such findings have not been reported, although additive effects of NPYR, NDEA and NDELA in producing liver tumors have been demonstrated by Berger et al. (1987).

The ability of NDMA to induce liver necrosis in a relatively short period can be associated with the high level of cytochrome P450 in this organ (Hadley and Dahl, 1982), which is required for metabolic activation (Druckrey, 1973) as well as deactivation of NDMA (Rowland, 1988). A high concentration of cytochrome P450 in the liver will result in the rapid metabolism of NDMA (Amelizad et al., 1989) and consequently a fast excretion of the toxic metabolites from the body. Therefore, the acute toxic effects may not be persistent after withdrawal of this chemical.

Histologic examination of the nasal cavity indicated that

a single dose of NDMA or NPYR produced necrosis in the olfactory epithelium and Bowman's gland. Several researchers have described damage by carcinogens to specific cell types or groups of morphologically similar cells which are located within a region of the nasal cavity (Hecht et al., 1980; Kerns et al., 1983; Hurtt et al., 1988). The susceptibility of certain cells to carcinogens can be related to the presence of enzymes that are involved in detoxification and activation reactions (Voigt et al., 1985; Baron et al., 1988). The sensitivity of olfactory epithelium and Bowman's glands to the toxic effects of NDMA and NPYR may be related to the presence of metabolizing enzymes in these cells. In this study, NDMA appeared to produce more damage to the olfactory epithelium whereas NPYR seemed to cause more severe effects in the Bowman's gland epithelium. Although the reason for the differences are not known, the variations in sensitivity were most clearly evident at the medium doses. The areas in the nasal cavity that most severely affected by NDMA and NPYR are between the nasal septum and dorsal meatus. Similar findings have been reported after exposure to some compounds, such as dimethylamine (Buckley et al., 1985), dibasic esters (Keenan et al., 1990) and dichlobenil (Brandt et al., 1990). The susceptibility of these sites may be related to the difference in the levels of metabolizing enzymes (Brandt et al., 1990). In addition, the most heavily targeted areas may have the most active blood flow in the nasal cavity (Brandt et al., 1990),

resulting in their being exposed to a higher level of NDMA and NPYR.

In the present experiment, development of lesions in the nasal cavity was similar to that described for other chemicals, such as dimethylamine (Buckley et al., 1985), dibasic esters (Keenan et al., 1990) and dichlobenil (Brandt et al., 1990). Initially the olfactory epithelium and Bowman's glands undergo degeneration or necrosis and later on most of these cells will appear to be atrophic. Necrosis or complete destruction of sensory cells results in the atrophy of the olfactory bundles in the lamina propria. However, the nerve bundles are completely repaired by 30 days after exposure.

Damage to the olfactory epithelium may completely resolve, regenerate in a disorganized fashion or the epithelium may be replaced by squamous or respiratory epithelium as a metaplastic reaction (Hurtt et al., 1988; Gaskell, 1990). A similar pattern of regeneration of olfactory epithelium was evident in this experiment. The factors that control the type of differentiation during the recovery stages are not known. In this study, sequential regeneration of the olfactory epithelium closely resembles that seen following exposure to methyl isocyanate (Bucher et al., 1987) and methyl bromide (Hurtt et al., 1988). This type of regeneration proceeds through periods of basal cell proliferation, epithelial repopulation and gradual reorganization to reform the original olfactory epithelium with varying degrees of

residual damage.

The basal cells (Moulton, 1974; Hurtt et al., 1988) and cells originating from Bowman's glands (Uraih et al., 1987) have been suggested as the progenitor cells of the olfactory epithelium. In this experiment, a similar regenerative process may have occurred in rats given medium and high doses of NDMA or NPYR. On histopathologic evaluation of the nasal cavity, proliferation of basal cells occurred quickly following acute injury and these cells continued to multiply to reform the damaged olfactory epithelium. Epithelium of the ducts of Bowman's glands became more prominent and underwent hyperplasia, which may contribute to the regeneration of the olfactory epithelium. In this study, squamous metaplasia appeared to develop mostly from basal cells.

Regeneration of Bowman's glands appeared to be associated with the presence of intact excretory ducts cells in the olfactory region. If the duct cells were completely destroyed, there would be permanent loss of Bowman's glands. Decreases in AB/PAS positive material in the Bowman's gland coincided with the development of lesions in this gland. Destruction of the Bowman's gland will result in total depletion of the AB/PAS positive material. Therefore, the presence of this material can be used as an indicator of the function of the Bowman's gland.

In the combination study, it was evident that NDMA and NPYR have additive effects in producing lesions in the nasal

cavity. In this experiment, rats given a combination of NDMA and NPYR (15 mg/kg of each) had more severe lesions than those exposed to a single dose of either chemical. Additive effects were also apparent in nasal tissues of rats given a combination of NDMA and NPYR at a dose of 30 mg/kg of each chemical.

Immunohistochemical evaluation indicated that a single exposure to NDMA or NPYR induced single GST-P positive hepatocytes as well as small AHF. These single cells have been postulated as precursors of preneoplastic foci and nodules (Satoh et al., 1985; Moore and Kitagawa, 1986) and their appearance can be assumed as a result of permanent cellular DNA damage in the liver (Chambers, 1985). In the combination study, single GST-P positive hepatocytes were found to progress to small and large AHF after 30 days of single or dual exposure to NDMA or NPYR. This further indicated that NDMA or NPYR are able to induce preneoplastic lesions in the liver.

In summary, the results indicate that NDMA produced necrosis in the liver, whereas NPYR appeared not to cause any detectable changes in this organ. Either compound appeared to induce prolonged toxic effects on nasal tissues. Combination exposure to NDMA and NPYR resulted in additive effects on the induction of lesions in the liver and nasal cavity. Single or dual exposure to NDMA or NPYR induced preneoplastic lesions in the liver, which may indicate a permanent DNA damage in the

hepatocytes. Immunohistochemical staining for GST-P indicated that this procedure is able to detect single positive cells and very early foci in the liver. Neither chemical appeared to be able to induce preneoplastic lesions in the nasal tissues during the 30 day study.

CHAPTER 2

DEVELOPMENT OF PRENEOPLASTIC LESIONS IN THE LIVER AND NASAL CAVITY OF RATS INITIATED WITH N-NITROSODIMETHYLAMINE OR N-NITROSOPYRROLIDINE AND PROMOTED WITH POLYBROMINATED BIPHENLS

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SUMMARY

N-Nitrosodimethylamine (NDMA) and N-nitrosopyrrolidine (NPYR) induce primary liver and to a lesser extent nasal tumors in rats. Polybrominated biphenyls (PBBs) are liver tumor promoters and are highly lipophilic and persist in tissues of rats. To characterize the development of preneoplastic lesions in the liver and nasal cavity, female Sprague-Dawley rats were initiated at day 0 with 30 mg of NDMA or NPYR/kg of body weight (BW) and at day 3 with 10 mg/kg of BW. Promotion was begun at day 10 with 100 mg of PBB/kg of BW. Rats were killed after 30, 120 or 180 days of promotion. Liver and nasal tissues were stained with hematoxylin and eosin and immunohistochemically for glutathione S-transferase placental form (GST-P). Significantly more altered hepatocellular foci (AHF) were evident in rats initiated with NDMA or NPYR and promoted with FM compared to nonpromoted

groups or rats given only FM. Appreciable numbers of AHF were seen at 120 and 180 days in livers of rats in all other treatment groups, whereas the untreated controls rats had no AHF. Volume of the liver (%) occupied by AHF was significantly higher in rats given NDMA with promotion compared to rats given only NDMA or FM. Lesions related to toxicity were absent in the liver and nasal cavity of rats initiated with NDMA or NPYR without promotion. These results indicated that a single oral dose of FM significantly enhanced development of AHF in rats initiated with NDMA or NPYR. Short term exposure to either compound appeared to have carcinogenic potential as evidenced by an increase in AHF. Neither chemical was able to induce preneoplastic lesions in the nasal tissues.

Introduction

Carcinogenesis in people and animals, including experimental animals, is generally considered as a multistage process (Farber and Cameron, 1980; Scherer, 1984) consisting of initiation, promotion and progression (Farber, 1984b; Pitot et al., 1988). This multistage concept has been demonstrated to be valid in experimental hepatocarcinogenesis (Scherer, 1984; Schulte-Hermann, 1985). Although nasal tumors can be induced experimentally in rats (Lee and Trochimowicz, 1982; Kerns et al., 1983), clear distinctions of each step in nasal carcinogenesis has not been established. In rats, experimental models for hepatocarcinogenesis usually include initiation and

promotion (Goldsworthy et al., 1986). The initiation stage is irreversible, involving alteration of DNA and perpetuation of this lesion by cell proliferation (Farber and Sarma, 1987). The initiated cells appear phenotypically normal and may not progress to malignancy, but they are able to respond to tumor promotion by clonal proliferation and formation of a focal population of cells (Schulte-Hermann, 1985). The promotion stage is characterized by the proliferative expansion of the population of initiated cells (Schulte-Hermann, 1985) to express an altered phenotype (Hicks, 1983). Promotion can be reversible or irreversible, depending on whether the clones of initiated cells are maintained or regress subsequent to removal of the promoting stimulus (Saeter and Seglen, 1990). Most promoters are known to induce epigenetic effects on the target cells, which can be associated with alteration in the expression of genetic information, contributing to development of tumors (Hicks, 1983; Williams, 1983a). Promotion requires multiple treatments with a promoting agent (Miller and Miller, 1981b) and is a slow process that may take weeks, months or years to be effective (Goldsworthy et al., 1986). Two-stage hepatocarcinogenesis models in rats have been developed by several workers (Solt and Farber, 1976; Pitot et al., 1978; Herren et al., 1982) and may be used to test the ability of a chemical to act as an initiator, promoter or a complete carcinogen (Goldsworthy et al., 1986). In rats, the first morphological indication of chemically induced carcinogenic

changes in the liver are the appearance of preneoplastic or altered hepatocellular foci (AHF) (Emmelot and Scherer, 1980; Schulte-Hermann, 1985; Farber and Sarma, 1987). Histologically, preneoplastic lesions consist of phenotypically altered cells which have no obvious neoplastic nature, but have a high probability of progressing to a benign or malignant neoplasm (Bannasch, 1986). The preneoplastic foci, which are regarded as the clonal progeny of single initiated hepatocytes (Moore and Kitagawa, 1986; Hendrich and Pitot, 1987), appear to develop sequentially to nodules and hepatocellular adenomas and carcinomas (Farber and Sarma, 1987). The foci and nodules are characterized by alterations in enzyme expression which have been used as immunohistochemical markers (Moore and Kitagawa, 1986; Sato, 1988). In rats, glutathione S-transferase placental form (GST-P) has been used as a reliable marker enzyme for preneoplastic lesions (Cameron, 1988; Sato, 1988) at different stages of liver carcinogenesis, including early appearing foci or even single cells (Moore et al., 1987). However, development of preneoplastic lesions following exposure to a carcinogen has not been demonstrated in the nasal cavity of rats.

The carcinogenicity of NDMA and NPYR has been demonstrated in rats by different routes. N-Nitrosodimethylamine can induce carcinomas, hemangioendothelial sarcomas (Lijinsky and Reuber, 1984), and cholangiocellular tumors in the liver (Terracini et al., 1967), kidney tumors

(Hard and Butler, 1971), and occasionally adenocarcinomas and squamous cell carcinomas in the lung (Lijinsky and Reuber, 1984). In the nasal cavity, NDMA causes squamous cell carcinomas (Noronha and Goodal, 1972). In rats, NPYR induces carcinomas, sarcomas, cholangiocarcinomas, hemangioendotheliomas and occasionally adenomas in the liver (Berger et al., 1987). This compound may also cause tumors in the oral cavity and stomach (Berger et al., 1987) and to a lesser extent, esophagus (Lijinsky and Reuber, 1981), intestine, kidney, neural tissue, hematopoietic and lymphatic systems (Berger et al., 1987), testes and nasal cavity (Lijinsky and Taylor, 1976).

Polybrominated biphenyls (PBBs) are a group of environmental contaminants (Carter, 1976), which were used as fire retardants under the commercial name Firemaster (FM) BP-6 or FF-1 (Gutenmann and Lisk, 1975; Rappe et al., 1979). The PBBs accidentally contaminated farm animals and their products in 1973 in Michigan and consequently entered the human food chain (Carter, 1976; Kay, 1977; Fries et al., 1978). Polybrominated biphenyls are very stable in the environment and are highly lipophilic (Fries, 1985) with structural and functional similarities to polychlorinated biphenyls, chlorinated dibenzofurans and dibenzodioxins (McConnell and Moore, 1979; Rappe et al., 1979). Firemaster is composed of 12 to 14 major PBB congeners (Moore and Aust, 1978; Dannan et al., 1982).

Although PBBs have been shown to act as hepatic tumor promoters in initiated rats (Jensen et al., 1982; Jensen and Sleight, 1986), the mechanisms of action are not fully understood. The ability of FM to act as a promoter may be related to the presence of potent individual congeners in this chemical mixture (Jensen et al., 1982) or to a synergistic or additive effect of the mixture of congeners (Jensen and Sleight, 1986; Evans and Sleight, 1989). The FM mixture and some of the individual congeners, such as 2,4,5,2',4',5'-hexabromobiphenyl (245-HBB), 3,4,5,3',4',5'-hexabromobiphenyl (345-HBB) and 3,4,3',4'-tetrabromobiphenyl (34-TBB) have been shown to promote hepatic enzyme altered foci in rats initiated with N-nitrosodiethylamine (Jensen et al., 1982, 1983; Jensen and Sleight, 1986; Dixon et al., 1988). The PBB congener, 245-HBB has been suggested to have a different mechanism of hepatic promotion than another PBB congener, 345-HBB (Jensen et al., 1983; Jensen and Sleight, 1986). The congener 345-HBB may act as a tumor promoter by producing chronic toxicity and necrosis of noninitiated cells (Jensen et al., 1983). On the contrary, the congener 245-HBB is apparently an effective promoter at nontoxic doses (Jensen et al., 1982). The congener 245-HBB comprises about 50 to 70% of the FM mixture (Robertson et al., 1984) and is a phenobarbital-type inducer of hepatic microsomal enzymes (Moore et al., 1978). The toxic congener, 345-HBB is found in very small quantities in the FM mixture and is known as a 3-methylcholanthrene-type inducer of

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microsomal enzymes (Aust et al., 1981).

Since PBBs are highly persistent in animal tissues (Fires, 1985) and since most PBB congeners are poorly metabolized (Dannan et al., 1978) it was hypothesized that a single oral dose of PBBs would have tumor promoting effects on the liver and nasal cavity after initiation with NDMA or NPYR as indicated by the increase in the number of GST-P positive foci in both organs.

The major objective of this experiment was to determine the promoting effect of PBBs on the expression of GST-P positive foci in the liver and nasal cavity following initiation with NDMA or NPYR. A further objective was to determine if there were long term toxic and carcinogenic effects of NDMA or NPYR on the liver and nasal cavity after short term exposure to either compound.

Materials and Methods

Animals and Experimental Protocol. Female Sprague-Dawley rats weighing 50 to 75 g were purchased from Charles River Laboratory, Portage, Michigan. Rats were housed in clear propylene cages and were randomly assigned into groups of three per cage. The room temperature was maintained at 22^{0}C with a 12 hour light/dark cycle. The animals were given commercial feed (Rodent Laboratory Chow #5001, Purina Mills, Inc., St. Louis, Missouri) and water ad libitum. Rats were acclimated for 7 days before subjected to treatment.

The rats used in the initiation-promotion protocol were randomly assigned to groups of 5 rats each, whereas FM treated or untreated control groups consisted of 3 animals each. Rats were initiated on day 0 with an intraperitoneal administration of 30 mg of NDMA or NPYR/kg of BW and on day 3 with 10 mg of NDMA or NPYR/kg of BW. The promotion stage was begun on day 10 by using Firemaster BP-6 as a promoter. The Firemaster was dissolved in corn oil vehicle and was given to the rats by gavage as a single dose of 100 mg/kg of BW. The rats were killed on day 30, 120 or 180 after administration of FM.

Chemicals. N-Nitrosodimethylamine and NPYR were purchased from Sigma Chemical Company, St. Louis, Missouri. The source of Firemaster BP-6 was from Michigan Chemical Company, St. Louis, Michigan.

Necropsy and Collection of Tissue Samples. At the prescribed intervals, rats were killed with CO_2, decapitated and necropsied. Nasal cavities were infused with 10% buffered formalin and nasal tissues along with portions of liver were placed in the same fixative.

Histologic Preparation. Nasal tissues were decalcified by the formic acid-sodium citrate method and were sectioned according to the procedure of Young (1981). Formalin-fixed liver and nasal tissue samples were processed, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. Serial sections of nasal cavity were also stained with alcian blue-periodic acid-Schiff (AB/PAS).

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Immunohistochemical Staining. Formalin fixed liver and nasal tissues were stained for GST-P according to the procedure described by Bourne (1983). Five representative samples from each liver were stained for GST-P and the diameter and area of each focus and area of each field were measured using a laser scanning confocal microscope (Carl Zeiss, Inc., West Germany). The number of GST-P positive foci of 100 μm (approximately 10 to 12 cell sized) or larger in diameter and the number of fields were counted. The number of altered hepatocellular foci (AHF) per cm^3, mean diameter of AHF, volume of the liver (%) occupied by AHF and mean volume of AHF (mm^3) were calculated according to the procedure of Campbell et al. (1982).

Statistical Analysis. The number of AHF and mean volume of AHF were square root transformed, whereas volume of the liver (%) occupied by AHF was arc sin square root transformed. Data were subjected to a 2 x 5 factorial analysis of variance (ANOVA) (completely random), then tested for multiple comparisons with Student Newman Keuls (SNK) test (Steel and Torrie, 1980) at a significance level of $p \leq 0.05$.

Results

Histopathologic Evaluation of the Liver. By 30 days after promotion, the liver of rats in all treated groups appeared normal. By 120 days of promotion, liver from rats given NDMA or NPYR and promoted with FM and rats given only FM had mild

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hepatocellular hypertrophy and microvacuolation mainly in the centrolobular areas. Moderate hypertrophy and microvacuolation of hepatocytes were seen in rats in promoted groups and rats given FM by 180 days of promotion (see Figure 2.1.). Rats given only NDMA or NPYR did not have these lesions in the liver by 120 and 180 days of promotion.

Foci of altered hepatocytes were seen in the liver of rats given NDMA or NPYR (with/without promotion) and in rats treated with FM. Foci were primarily of the acidophilic type. Other types seen occasionally included basophilic, vacuolated cell, or mixed type foci. At 30 days of promotion, a few small acidophilic cell foci were seen in liver of rats in promoted groups, whereas rats in nonpromoted groups and rats given FM did not have AHF. By 120 days of promotion, rats in promoted groups had mostly acidophilic cell foci (see Figure 2.2.) and occasionally a few basophilic, vacuolated or mixed cell foci. At the same time, a few acidophilic cell foci were seen in liver of rats given only FM. Rats in the nonpromoted groups had a few acidophilic cell foci and an occasional vacuolated or basophilic cell foci by 120 days of promotion. Large acidophilic cell foci involving several adjacent lobules were seen in liver of rats in promoted groups by 180 days of promotion. In addition, a few basophilic (see Figure 2.3.), vacuolated (see Figure 2.4.) or mixed cell foci were also present. By 180 days of promotion, rats given FM had a few acidophilic cell foci and rats in the nonpromoted groups (NDMA

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or NPYR) had mostly acidophilic cell and a few basophilic or vacuolated cell foci. At 180 days of promotion, one of the rats given NDMA and promoted with FM had a hepatic nodule, comprised of mainly cells arranged in glandular pattern (see Figure 2.5.). In addition, cells with acidophilic cytoplasm, enlarged nuclei and prominent nucleoli were also present within the nodule. Lesions were absent from rats in the untreated control groups throughout this experiment.

Histopathologic Evaluation of the Nasal Cavity. Histologic lesions related to treatment were seen only in rats given NPYR at 30 days of promotion. Changes in rats given NPYR (with/without promotion) consisted of moderate hyperplasia of epithelium of the ducts of Bowman's glands. Depletion of AB/PAS positive material was absent throughout this experiment.

Immunohistochemical Evaluation of the Liver. Foci of altered hepatocytes positive for GST-P were seen as early as 30 days of promotion in rats given NDMA or NPYR (with/without promotion) and in rats given FM. At this time, small (approximately 3 to 10 cells), medium (approximately 11 to 20 cells) and large (more than 20 cells) sized foci were seen in rats given NDMA or NPYR (with/without promotion). Rats treated with FM had small sized foci. At 120 and 180 days of promotion, many foci in the promoted groups were equivalent to several adjacent lobules in size (see Figure 2.6.).

Data for the number of AHF per cm^3 of liver are presented

Fig

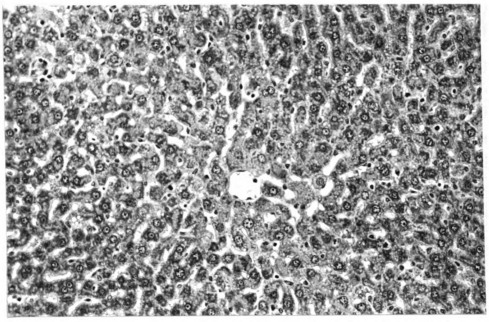

Figure 2.1. Photomicrograph of a section of liver from a rat given FM (100 mg/kg) at 180 days. Notice the moderate hypertrophy and microvacuolation of hepatocytes with subsequent narrowing of sinusoidal spaces and disruption of the cellular architecture. (H & E stain, 224 X).

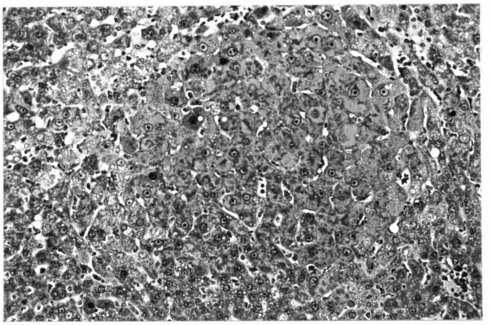

Figure 2.2. Photomicrograph of a section of liver from a rat initiated with NDMA (30 and 10 mg/kg) and promoted with FM (100 mg/kg) after 120 days of promotion. Notice the roughly circular area of an acidophilic cell focus. These proliferated hepatocytes are variable in size with enlarged nuclei and prominent nucleoli and are not sharply demarcated from the surrounding normal hepatocytes. On H&E staining, the cytoplasm was diffusely pale acidophilic. (H & E stain, 224 X).

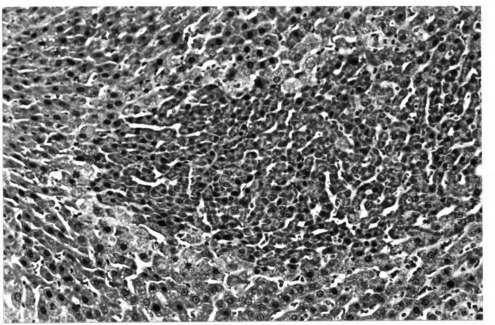

Figure 2.3. Photomicrograph of a section of liver from a rat initiated with NPYR (30 and 10 mg/kg) and promoted with FM (100 mg/kg) after 180 days of promotion. Notice a basophilic cell focus, consisting of small sized cells with hyperchromatic centrally located nuclei which are slightly demarcated from the surrounding normal hepatocytes. On H&E staining, the cytoplasm was diffusely basophilic. (H & E stain, 224 X).

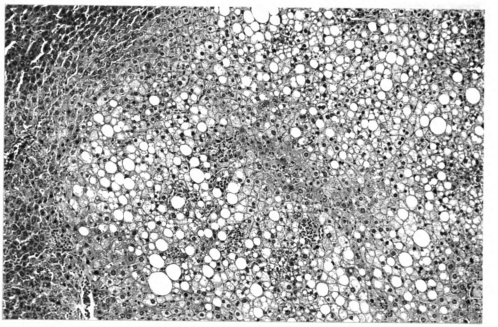

Figure 2.4. Photomicrograph of a section of liver from a rat initiated with NPYR (30 and 10 mg/kg) and promoted with FM (100 mg/kg) after 180 days of promotion. Notice a vacuolated cell focus, consisting of variable sized cells with macro and microvacuolated cytoplasm. The cells within the focus appear to be slightly compressing the surrounding hepatocytes. (H & E stain, 224 X).

Fig

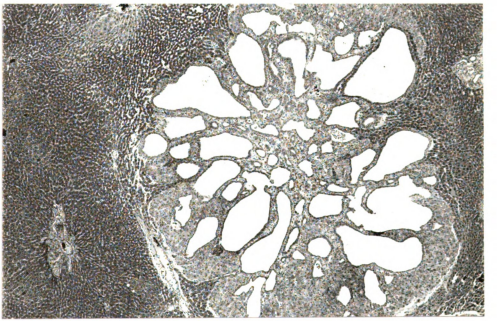

Figure 2.5. Photomicrograph of a section of liver from a rat initiated with NDMA (30 and 10 mg/kg) and promoted with FM (100 mg/kg) after 180 days of promotion. Notice a hepatic nodule which is sharply demarcated from the surrounding hepatocytes and is comprised mainly of cells arranged in glandular pattern. Notice the nodule has compressed the surrounding hepatocytes.
(H & E stain, 44 X).

Figure 2.6. Photomicrograph of a histochemically-stained section of liver from a rat initiated with NPYR (30 and 10 mg/kg) and promoted with FM (100 mg/kg) after 180 days of promotion. Notice a large focus involving several adjacent lobules. Another smaller focus is evident near the central vein. (GST-P stain, 47 X)

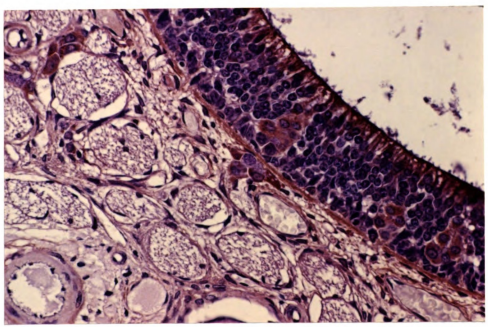

Figure 2.7. Photomicrograph of a histochemically-stained section of nasal mucosa from a rat initiated with NPYR (30 and 10 mg/kg) and promoted with FM (100 mg/kg) after 30 days of promotion. Notice the GST-P positive staining in the epithelium and ducts of Bowman's glands. (GST-P stain, 403 X).

Table 2.1. The Number of Altered Hepatocellular Foci[a] per Cm^3 of Liver of Experimental Rats[b].

Treatments	N	Intervals[c]	
		120 days	180 days
NDMA	5	335 ± 95	501 ± 70
NDMA + FM	5	1489 ± 212[e,f]	2184 ± 585[g,h]
NPYR	5	267 ± 98	377 ± 84
NPYR + FM	5	731 ± 162[i]	1279 ± 339[j,k]
FM	3	52 ± 16	244 ± 173
Control[d]	3	0	0

[a]Data expressed as mean ± S.E.
[b]Rats were initiated with NDMA or NPYR and promoted with FM
[c]Number of days of promotion
[d]Untreated control group
[e]Significantly different from rats given only NDMA ($P \leq 0.01$)
[f]Significantly different from rats given only FM ($P \leq 0.01$)
[g]Significantly different from rats given only NDMA ($P \leq 0.01$)
[h]Significantly different from rats given only FM ($P \leq 0.05$)
[i]Significantly different from rats given only FM ($P \leq 0.05$)
[j]Significantly different from rats given only NPYR ($P \leq 0.05$)
[k]Significantly different from rats given only FM ($P \leq 0.05$)

Table 2.2. Mean Diameter (μm) of Altered Hepatocellular Foci[a] in Experimental Rats[b].

Treatments	N	Intervals[c]	
		120 days	180 days
NDMA	5	260 ± 12	290 ± 16
NDMA + FM	5	327 ± 16	311 ± 25
NPYR	5	284 ± 35	254 ± 10
NPYR + FM	5	292 ± 17	300 ± 24
FM	3	337 ± 49	508 ± 220
Control[d]	3	0	0

[a]Data expressed as mean ± S.E.
[b]Rats were initiated with NDMA or NPYR and promoted with FM
[c]Number of days after promotion
[d]Untreated control group

Table 2.3. Volume of the Liver (%) Occupied by Altered Hepatocellular Foci[a] in Experimental Rats[b].

Treatments	N	Intervals[c]	
		120 days	180 days
NDMA	5	0.24 ± 0.09	0.80 ± 0.22
NDMA + FM	5	2.86 ± 0.85[e,f]	3.28 ± 1.39[g]
NPYR	5	0.54 ± 0.33	0.30 ± 0.08
NPYR + FM	5	0.95 ± 0.34	2.51 ± 0.79
FM	3	0.07 ± 0.02	0.32 ± 0.08
Control[d]	3	0	0

[a]Data expressed as mean ± S.E.
[b]Rats were initiated with NDMA or NPYR and promoted with FM
[c]Number of days of promotion
[d]Untreated control group
[e]Significantly different from rats given only NDMA ($P \leq 0.05$)
[f]Significantly different from rats given only FM ($P \leq 0.01$)
[g]Significantly different from rats given only FM ($P \leq 0.05$)

Table 2.4. Mean Volume (mm^3) of Altered Hepatocellular Foci[a] in the Liver of Experimental Rats[b].

Treatments	N	Intervals[c]	
		120 days	180 days
NDMA	5	0.007 ± 0.001	0.016 ± 0.003
NDMA + FM	5	0.018 ± 0.003	0.024 ± 0.008
NPYR	5	0.013 ± 0.005	0.008 ± 0.001
NPYR + FM	5	0.012 ± 0.002	0.017 ± 0.004
FM	3	0.017 ± 0.006	0.099 ± 0.086
Control[d]	3	0	0

[a]Data expressed as mean ± S.E.
[b]Rats were initiated with NDMA or NPYR and promoted with FM
[c]Number of days of promotion
[d]Untreated control group.

in Table 2.1. A significant increase in the number of AHF per cm^3 was evident in rats initiated with NDMA and promoted with FM at 120 and 180 days. Promotion with FM for 180 days resulted in a significant increase in the number of AHF in rats initiated with NPYR. Rats initiated with either NDMA or NPYR and promoted with FM had significantly more AHF than those given only FM. Appreciable numbers of AHF were seen at 120 and 180 days of promotion in livers of rats in all other treatment groups, whereas untreated controls had no AHF. There were no significant differences in the mean diameter of AHF among rats in the various treatment groups (see Table 2.2.). Volume of the liver (%) occupied by AHF was significantly greater in promoted rats given NDMA than in the nonpromoted group at 120 days (see Table 2.3.). At 120 and 180 days, promoted rats given NDMA had a significantly greater volume of liver (%) occupied by AHF than rats given only FM. All other promoted groups initiated with either NDMA or NPYR had a numerical increase in the volume of liver (%) occupied by foci. In general, the mean volume of AHF (mm^3) in the liver of promoted groups was greater than in the nonpromoted groups. However, these differences were not statistically significant (see Table 2.4.). Rats in the untreated control groups had a lack of GST-P positive foci throughout this experiment.

Immunohistochemical Evaluation of the Nasal Cavity. Evaluation of GST-P staining in the nasal cavity did not show any indication of development of preneoplastic lesions. The

only indication of GST-P positive staining was seen in the Bowman's glands and a few cells in the olfactory epithelium region of few rats given NPYR or NDMA (with/without promotion) by 30 days of promotion (see Figure 2.7.). Results of GST-P staining were negative in nasal tissues of rats at 120 and 180 days.

Discussion

Promotion with a single oral dose of FM significantly increased the number of AHF in rats initiated with either NDMA or NPYR. Lack of significant statistical difference in the mean diameter of foci may be due to the lack of a sequential development of foci in the promoted groups, resulting in their diverse size. Heterogeneity in the size of foci will further affect the calculated values for the volume of the liver occupied by AHF and mean volume of AHF. The volume of liver (%) occupied by AHF in rats given NDMA and promoted with FM was increased significantly.

Results in the current study indicated that FM may have an enhancing effect on the growth of AHF in rats initiated with NDMA or NPYR. This is in agreement with the suggestion that most of the potent hepatocarcinogens cause an increase in the number and, in some cases, the size of foci prior to the appearance of liver tumors (Popp and Goldsworthy, 1989). Enhancement of AHF in this initiation-promotion protocol could be considered as a tumor promoting activity (Emmelot and

Scherer, 1980; Goldsworthy and Pitot, 1985; Williams, 1989) since hepatic tumor promoters have been shown to accelerate the appearance of AHF (Goldsworthy et al., 1986), and are able to increase the number and size of AHF at a given interval (Goldsworthy and Pitot, 1985; Pitot et al., 1989a; Xu et al., 1990).

Although tumor promotion is considered as a slow process (Goldsworthy et al., 1986) that requires multiple treatments with a promoting agent to be effective (Miller and Miller, 1981b), two oral doses of FM 24 hours apart (total 130 mg/kg of body weight) have been shown to promote the development of AHF (Rezabek et al., 1987). In this study, a single oral dose (100 mg/kg of body weight) of FM was also effective in promoting the development of AHF in initiated rats. Such findings can be related to the nature of PBBs which are very poorly metabolized and slowly excreted (Miceli and Marks, 1981). The major congeners of FM have been shown to persist in the tissues of rats (Rezabek et al., 1987), which could stimulate induction of microsomal enzymes, leading to the promotion of AHF. Therefore, a single dose or a short term treatment will result in persistent internal exposure to this chemical mixture.

An appreciable number of AHF was evident in rats initiated with low doses of either NDMA or NPYR without promotion with FM. Such findings may be related to factors such as diet or environment which can modify initiation or act

as a promoting stimulus to the development of AHF. Dietary composition has been shown to affect initiation and promotion in liver carcinogenesis (Hendrich et al., 1989; Pitot et al., 1989b; Flodstrom et al., 1991). Crude, cereal-based diets are suggested to contain unidentified promoting or carcinogenic agents, which apparently affect the development of AHF (Hendrich et al., 1989; Pitot et al., 1989b; Flodstrom et al., 1991). In the current study, dietary factors may have had a role in the development of AHF in rats initiated with NDMA or NPYR, including the promoted groups. However, the fact that both compounds are complete carcinogens (Lijinsky and Reuber, 1984; Berger et al., 1987) with initiating and promoting ability must be taken into consideration.

In this initiation-promotion protocol, rats treated only with FM had a low number of AHF at 120 and 180 days after promotion. Similar findings were reported in PBB treated rats (Jensen et al., 1982, 1984), and with other liver tumor promoters (Pereira et al., 1982; Kitagawa et al., 1984; Xu et al., 1990). Tennant et al. (1987) indicated that PBBs are some of the most potent nongenotoxic carcinogens in rodents. Therefore, it is possible that FM has some potential to act as a complete carcinogen with initiating and promoting activity. Results of studies with 3,4,3',4'-tetrabromobiphenyl, which is a minor congener in FM that can be metabolized, indicated that this congener has weak initiating activity (Dixon et al., 1988). Perhaps there are other

congeners or constituents in FM which also have initiating activity. On the contrary, the PBBs may have only promoting effects and formation of AHF may result from promotion of spontaneously or environmentally induced initiated cells (Pitot et al., 1980; Schulte-Hermann et al., 1983).

Since NDMA and NPYR have been demonstrated to be present at low concentrations in various food products (Spiegelhalder et al., 1980; Song and Hu, 1988) and in the environment (Fajen et al., 1979; Rounbehler et al., 1979; Spiegelhalder and Preussmann, 1987), daily exposure of human beings throughout their lifetime is very likely. Results of epidemiologic studies have indicated a correlation between consumption of certain N-nitrosamine-containing foods and the incidence of nasopharyngeal cancers (Yu and Henderson, 1987; Sarkar et al., 1989). Development of tumors may be enhanced because people may also be exposed to promoters, such as PBBs, polychlorinated biphenyls (PCBs), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) or other promoting agents in the diets, insecticides, drugs or sex steroids. In the current study, short term exposure of rats to a low dose of either NDMA or NPYR was found to induce preneoplastic lesions, indicating a possible long term carcinogenic effect resulting from such exposure. Similarly, short term exposure of people or animals to relatively low doses of NDMA or NPYR may result in the initiation of sensitive target cells and these cells may be exposed to environmental chemicals or other factors which

may act as tumor promoters and thus lead to the development of cancer.

Immunohistochemical evaluation of the nasal cavity did not show any indication of development of preneoplastic lesions after 120 and 180 days of promotion. Although evidence of GST-P positive staining was seen in a few rats at 30 days of promotion, these effects were not persistent through 120 and 180 days of promotion. The reason is not known. It may be related to the ability of the nasal cavity to repair the DNA damage caused by either compound and the lack of continuous stimulation of microsomal enzymes in the nasal cavity. The ability of nasal cavity to repair DNA damage has been reported previously (Bermudez and Allen, 1984). In chapter 1, it was found that the regeneration of the nasal cavity following treatment with NDMA or NPYR (30 mg/kg) was nearly complete after 30 days of exposure. In addition, the negative results may also be associated with the lack of sensitivity of GST-P as a marker for preneoplastic lesions in the nasal cavity. This is based on the observation that respiratory and olfactory regions of nasal cavity only contain glutathione S-transferases B, C, and E (Baron et al., 1988), whereas GST-P does not cross react with any of these glutathione S-transferases (Satoh et al., 1985; Roomi et al., 1985).

On histopathologic examination of the liver, hepatic abnormalities found in rats treated with NDMA or NPYR (with promotion) and rats given FM were similar to the changes

described previously with PBBs (Sleight and Sanger, 1976; Gupta et al., 1983a). In this study, absence of histopathologic lesions in liver or nasal cavity of rats initiated with NDMA or NPYR without promotion indicated the lack of long term toxic effects induced by short term exposure to either compound. In contrast to the the liver, histologic lesions related to treatment with FM were absent from nasal cavity. This may imply a low probability of promoting activity of FM on nasal cavity, since most tumor promoters are known to be toxic to the target organ in which they exert their promoting activity (Ward et al., 1989).

On H&E-stained sections, 4 types of AHF (Squire and Levitt, 1975; Institute of Laboratory Animal Resources, 1980; Harada et al., 1989a) were observed. Acidophilic foci were the most common with basophilic, vacuolated and mixed types occurring less frequently in rats given NDMA, NPYR or FM. These types of foci have been demonstrated previously in rats treated with chemicals, such as N-nitrosomorpholine, 1-amino-2,4-dibromoanthraquinone (ADBAQ), methyl carbamate or 4-hydroxyacetanilide (Harada et al., 1989b; Bannasch et al., 1989). Although only very few of AHF are able to progress to hepatic neoplasms (Sell et al., 1987), their appearance is often a useful predictor of hepatocarcinogenic potential of a chemical (Harada et al., 1989b). Development of a hepatic nodule in one of the rats initiated with NDMA and promoted with FM at 180 days may be regarded as an indication of

progression of the AHF toward formation of tumors. This sequential development has been reported previously (Saeter et al., 1988a; Bannasch et al., 1989).

In summary, the current study found that a single oral dose of FM significantly enhanced development of AHF in rats initiated with NDMA or NPYR. Short term exposure to NDMA or NPYR may have a long term carcinogenic effect on the liver, which appears to be modified by factors in the diet. Toxic effects due to short exposure to either compound are not likely to persist in the liver or nasal cavity. In this experiment, neither compound induced detectable preneoplastic lesions in the nasal cavity after 180 days of promotion as determined by GST-P staining.

CONCLUSIONS

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The major conclusions based on findings from the first experiment are as follows:

1) A single intraperitoneal dose of either NDMA or NPYR caused extensive degeneration and necrosis of the olfactory epithelium and Bowman's glands. The earliest changes were seen as early as 6 hours, and were most severe by 3 days. The targeted nasal tissues in rats given the medium dose of either chemical was regenerated by day 30, whereas regeneration was incomplete after exposure to the highest dose. The results indicate that a single exposure to a high dose of NDMA or NPYR can adversely affect these tissues for at least several weeks.

2) Simultaneous intraperitoneal exposure to NDMA and NPYR had additive effects on the severity of lesions in the nasal tissues and the liver.

3) At the dose used, NPYR was non-necrogenic to the liver whereas NDMA caused severe acute hepatic necrosis. Repair of the hepatic lesions occurred within 10 days.

4) Immunohistochemical staining for GST-P was successful in identifying what were considered to be initiated hepatocytes as early as 1 day after rats were given a single dose of either NDMA or NPYR. Small foci of GST-P positive

cells were seen at 30 days. With combination exposure to NDMA and NPYR, the AHF appeared larger than those with single exposure to either compound.

The most significant conclusions from the second experiment are:

5) A single oral dose of PBB significantly enhanced the development of AHF in rats initiated with NDMA or NPYR.

6) Even without promotion, appreciable numbers of AHF developed in rats exposed to two non-necrogenic doses of NDMA or NPYR.

7) These results provide evidence that even short term exposure to these N-nitrosamines can have long term carcinogenic potential even when there is no known exposure to tumor promoting agents.

8) Under conditions of these experiments, we could not demonstrate preneoplastic lesions in the nasal cavity.

Since immunohistochemical staining for GST-P is a sensitive procedure for detection of very early preneoplastic lesions, this technique may be useful in screening for carcinogenic potential of N-nitrosamines or other carcinogens in food products so as to prevent or minimize further exposure of human beings.

Recommendations for future research include:

1) Ultrastructural characterization of the early lesions in the liver and nasal cavity.

2) Induction of preneoplastic lesions in the nasal cavity

by application of multiple doses of NDMA or NPYR and the use of more potent promoters such as TCDD. More sensitive marker enzymes may be needed for detection of these lesions.

3) Better characterization of preneoplastic lesions by identifying cell proliferation activity using bromodeoxyuridine labelling procedure and correlating this activity with the expression of marker enzymes.

4) The use of potential cancer inhibitors including naturally occurring compounds which may be present in food systems to prevent development of preneoplastic lesions or to inhibit further progression of lesions to the formation of tumors.

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