THE EFFECT OF THE HOPS CONSTITUENT COLUPULONE ON HEPATIC CYTOCHROME P450 IN THE MALE RAT

By

Elizabeth Brownell Shipp

A Thesis

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

Master of Science

Department of Food Science and Human Nutrition

1992

c
c
r
c
a
i
p
i
m
t

ABSTRACT

The Effect of the Hops Constituent Colupulone on Hepatic Cytochrome P450 in the Male Rat

By

Elizabeth Brownell Shipp

The cytochrome P450 enzyme superfamily metabolizes a number of xenobiotics. Some of these are carcinogenic, either in their native state or after activation by cytochrome P450 isozymes. However, a number of these xenobiotics are detoxified by other cytochrome P450 isozymes. The possibility exists that cytochrome P450 metabolism could be manipulated to increasethe detoxification of food-borne carcinogens and procarcinogens.

To determine the ability of a hops constituent to alter cytochrome P450 metabolism, colupulone, one of the components of hops, was added to purified diet and fed to rats for a period of five days. Microsomal cytochrome P450 concentrations, metabolic activities, and steady-state RNA abundance for three cytochrome P450 isozymes were determined in control and treated animals. Colupulone treatment produced an increase in RNA abundance for two of the isozymes of interest. Total cytochrome P450 content and microsomal cytochrome P450 metabolism did not differ between treated and control animals.

This thesis is dedicated to Dr. Kathryn Grove Shipp and to Mrs. Emily McPherson, for all of their inspiration.

ACKNOWLEDGEMENTS

I would most of all like to thank my thesis advisor, Dr. Bill Helferich, for his help and understanding during my Master's degree program. I also want to thank the other members of my committee, Dr. Bursian and Dr. Gray, for their guidance and support. I owe a large debt to Dr. Christine S. Mehigh for her generous help with RNA isolation techniques and with the Northern blot hybridizations. My thanks also go to Dr. Denison for his willingness to share one of his figures for use in this manuscript. I would like to thank Kalsec Inc. for their generosity and support for this research. I appreciate the help and friendship I have received from the other students in Dr. Helferich's lab, and from the rest of the students in the department. I would also like to thank the Department of Food Science and Human Nutrition for their assistance during my program.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AFB_1 --- aflatoxin B_1
AFM_1 --- aflatoxin M_1
AFQ_1 --- aflatoxin Q_1
AIA --- aminoimidoazaarene
AIN 76 --- American Institute of Nutrition 1976 diet
ATCC --- American Type Culture Collection
B[*a*]P --- benzo[*a*]pyrene
BPDE --- benzo[*a*]pyrene diol epoxide
EDTA --- ethylenediaminetetraacetic acid
HEPES --- N[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid
3-MAB --- 3-methyl-4-monomethylaminobenzene
MAE --- MOPS, sodium acetate, EDTA
3-MC --- 3-methylcholanthrene
MOPS --- 3-[N-morpholino]propanesulfonic acid
NADP --- nicotinamide adenine dinucleotide phosphate
PB --- phenobarbital
PCB --- polychlorinated biphenyl
PCN --- pregnenolone-16α-carbonitrile
α-^{32}P-dCTP --- α-^{32}phosphorus-deoxycytidine triphosphate
PhIP --- 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine
SDS --- sodium dodecyl sulfate
SSC --- sodium chloride, sodium citrate
TCDD --- 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

INTRODUCTION

Environmental Factors Affecting Human Health

A number of factors in the environment are capable of impacting human health, either beneficially or detrimentally. Contaminants in the environment can have either acute or chronic effects. One class of these contaminants is the polychlorinated biphenyls (PCBs). These were once used in electrical transformers, paints, and in non-carbon copy paper. PCBs can enter the environment through improper disposal of contaminated oil or paint, from factory waste-water, and aerosolization of the PCBs themselves (Tatsukawa, 1976). PCBs cause chronic liver damage, skin pigmentation and irritation, and may be carcinogenic. Another example is methylmercury, which is used in a number of manufacturing industries, including paint and pharmaceutical formulation, as well as in dentistry. Methylmercury is released in wastes and in industrial accidents, as well as from mining of mercury itself. This neurotoxin can have both acute and long-lasting effects and can also produce birth defects after prenatal exposure.

The diet is also able to affect the status of human

f

f

t

e

Th

ca

da

Die

numb

arte

bloo

suff

itse

provi

maint

cells

health. Long-term overnutrition can lead to several chronic diseases, including heart disease, high blood pressure, and obesity. A chronic intake of excess calories may also be linked to the development of cancer. Many components of the food supply have been demonstrated to be rodent carcinogens. These include natural components of food, such as caffeine and many other compounds in coffee (Ames and Gold, 1990); cooking by-products such as benzo[*a*]pyrene (B[*a*]P) and the aminoimidoazaarenes (AIAs); and food contaminants such as the mycotoxin aflatoxin B_1 (AFB_1). Recent research has focused on the potential for dietary components to aid in the prevention of cancer (Ommen, *et al.*, 1988; Santamaria, *et al.*, 1988; von Hofe, *et al.*, 1991; DeWys, *et al.*, 1986)). These may either alter the metabolic activation of carcinogens or interfere with the ability of carcinogens to damage cells.

Diet

A high fat intake has been conclusively linked to a number of chronic health conditions. The development of arterial plaques during arteriosclerosis can lead to high blood pressure, weakening of the heart muscle, and lack of sufficient blood supply to major organs, including the heart itself. High calorie and high fat diets additionally provide more nutrition than is needed for energy maintenance. This extra energy is stored in the adipose cells, leading to significant weight gains over time. The

added body mass requires extra work by the heart and may be another cause of heart disease. However, nutrition in general has been linked to other chronic diseases as well. Laboratory animals fed high-calorie diets developed more tumors in response to one dose of diethylnitrosamine than did their counterparts whose caloric intake was restricted (Lagopoulos and Stalder, 1987). This would imply that general overnutrition can lead to the development of cancer. In similar experiments, animals maintained on an *ad libitum* (*ad lib*), high-fat diet developed more spontaneous tumors than animals fed an *ad lib* low-fat diet (Reddy, 1986).

Diet and Cancer

A number of specific or potential carcinogens have been identified in the human diet. Heterocyclic amines, especially common in cooked meats, have been shown to cause a wide variety of cancers in laboratory animals (Sugimura, 1985). Another food-borne carcinogen is AFB_1, a mycotoxin found in many grain crops and products (Lötter and Kröhm, 1988; Dvorácková, 1990).

Some dietary carcinogens are capable of damaging DNA in their native state. These direct-acting carcinogens need no metabolic activation after they are ingested. Vinyl chloride is one compound that falls into this category. Indirect-acting carcinogens, or procarcinogens, must be chemically altered before they are mutagenic. Two examples

of these chemicals are AFB_1 and B[a]P. Metabolic activation or detoxification of these and other compounds is often carried out by the hepatic cytochrome P450 enzyme system. A great deal has been discovered about this group of enzymes since its activity was first observed in 1954. The information developed since then has demonstrated the abundance and variety of isozymes in the cytochrome P450 superfamily. In humans, for example, twenty-four genes and three pseudogenes have been classified into ten enzyme families based on gene and protein sequence data (Nebert *et al.*, 1991). Both endogenous and exogenous xenobiotic substrates have been identified for a number of the isozymes characterized. Most xenobiotics metabolized by the cytochrome P450 system are detoxified by this metabolism. On the other hand, some substrates are activated to more reactive and mutagenic forms by cytochrome P450-mediated reactions. Most cytochrome P450 isozymes can be induced or inhibited by chemicals in the diet or in the environment, thereby altering the abundance and metabolic activity of specific isozymes, as well as the activation and detoxification of xenobiotics. This alteration of cytochrome P450 metabolism may be of benefit to human health, as modifying the human diet to include more of the compounds able to increase detoxification of xenobiotics may help to decrease the risk posed by food-borne or environmental procarcinogens. One group of compounds that may be useful in altering xenobiotic metabolism is the ß-

acids found in hops. These have been shown to induce certain cytochrome P450 isozymes in the mouse (Mannering *et al.*, 1992).

The objectives of this study were to evaluate the inductive effect of dietary colupulone, one of the hops ß-acids, on hepatic cytochrome P450 metabolism in the rat.

LITERATURE REVIEW

CYTOCHROME P450

Discovery and Isolation of Cytochrome P450

The hepatic xenobiotic-metabolizing enzyme system first received attention in the mid-1950s. Brown *et al.* (1954) indicated that the type of diet fed to experimental animals could affect the enzyme activity observed in liver homogenates. These studies were an outgrowth of a series of experiments on the metabolism of carcinogenic dyes. The N-demethylation of 3-methyl-4-monomethylaminoazobenzene (3-MAB), a carcinogenic aminoazo dye, was used as an indicator of xenobiotic metabolism in rats and mice. Altering the diet from a purified chemical diet to a commercially available chow produced striking increases in N-demethylase activity. The factor suggested as the cause of this increase was sterols formed in the commercial diet during storage. Since that time, a number of components naturally present in the diet (rather than processing by-products) have been shown to alter xenobiotic metabolism, including dietary protein (Butler and Dauterman, 1988), corn oil (Yoo *et al.*, 1990), carbohydrate, vitamins A and C, and some minerals (Yang *et al.*, 1992).

In another series of experiments, liver homogenates from rats administered 3-methylcholanthrene (3-MC, a polycyclic aromatic hydrocarbon) by intraperitoneal injection possessed increased aminoazo dye N-demethylase activity (Conney *et al.*, 1956). The specific enzyme responsible for this N-demethylation had not yet been identified. It was also not clear how 3-MC could produce the increase in demethylase activity. Combining heat-treated homogenates possessing no enzymatic activity with preparations from 3-MC treated rats did not change the demethylase activity seen in the 3-MC homogenates. This result ruled out the possibility that the liver homogenate itself contained a soluble effector of enzyme activity (Conney *et al.*, 1956, 1957).

It was then hypothesized that the inducer, such as 3-MC, might be increasing (inducing) N-demethylase activity by activating previously existing enzyme molecules. If this were the case, then both *in vitro* and *in vivo* addition of the inducer should produce the same effect. To test this, known N-demethylase inducers such as phenobarbital (PB), benzo[*a*]pyrene (B[*a*]P), and 3-MC were added to the homogenate. No inductive response was seen in these *in vitro* experiments (Conney and Burns, 1959; Conney *et al.*, 1956; Conney *et al.*, 1957; Conney and Burns, 1963; Conney, 1967), demonstrating that induction was an *in vivo* process.

Another avenue of research investigated the effect of endogenous hormones on induction of cytochrome P450 by xenobiotics. Adrenalectomy, hypophysectomy (Conney *et al.*, 1956), or ovariectomy (Conney, 1967) did not affect induction by 3-MC. Similarly, the response to PB treatment was not altered by adrenalectomy and castration, hypophysectomy, or thyroidectomy (Conney, 1967). Thus, enzyme induction by these xenobiotics did not depend on the hormonal status of the animals.

These experiments demonstrated that enzyme induction was not due to allosteric effects by soluble compounds, or to activation of pre-existing enzyme molecules. Hormonal status also did not have a role in enzyme induction by xenobiotics. It was therefore hypothesized that induction was the result of increased protein synthesis. This was first tested by using ethionine, an amino acid antagonist and protein synthesis inhibitor. Administration of ethionine *in vivo* before treatment with an inducer prevented any increase in enzyme activity (Conney and Burns, 1959; Conney *et al.*, 1957; Conney *et al.*, 1956, Conney and Burns, 1963). Adding ethionine to liver homogenates from 3-MC treated animals had no effect on enzyme activity (Conney *et al.*, 1956). This confirmed that the inducer acted before the homogenate was prepared. Animals treated with ethionine a short time after 3-MC administration demonstrated a higher N-demethylase activity than animals given ethionine and 3-MC

simultaneously. However, they possessed enzyme activities lower than did animals treated with 3-MC alone (Conney *et al.*, 1956). These results supported the hypothesis that inducers such as 3-MC and PB increased enzyme activity by increasing protein synthesis and thus enzyme abundance. Radiolabelling studies using ^{14}C-leucine given *in vivo* after treatment with PB confirmed this (Kato *et al.*, 1965). Incorporation of ^{14}C-leucine doubled in liver microsomes after PB treatment, but did not change in other subcellular fractions. *In vivo* treatment with PB also increased endoplasmic reticulum (ER) volume and total protein in liver cells (Remmer and Merker, 1965).

It was observed that bubbling carbon monoxide through a reduced microsomal suspension resulted in the formation of a spectral absorption peak at 450 nm (Klingenberg, 1958). This peak was not due to the cytochrome b_5 already identified in microsomal suspensions. Dissociation experiments with the carbon monoxide-complexed hemeprotein showed that it was not myoglobin or cytochrome b_5. This new carbon monoxide-binding pigment was a hemeprotein with a spectral absorption maximum at 450 nm (Omura and Sato, 1964). Like other cytochromes, the main function of the new hemeprotein was electron transport to oxygen from an NADPH-linked reductase, activating the oxygen to allow its addition to a substrate molecule. The protein was therefore labelled cytochrome P450.

Cytochrome P450 was originally found in the greatest concentration in liver microsomes, and has since been located in many tissues throughout the body in lower concentrations. Many of the xenobiotics that are metabolized by cytochrome P450 enter the body in the diet, either from plant foods or dietary contaminants. Localization of the primary cytochrome P450 activity in the liver would allow these compounds to be metabolized close to the site of first exposure. However, the presence of specific cytochrome P450 isozymes in the epithelial tissue of the lung and in the skin can also serve an important protective metabolic function by facilitating detoxification of toxicants inhaled or absorbed through the skin. The distribution of cytochrome P450 within a few different tissues and species is listed in Table 1. The identification of cytochrome P450 in adrenal cortex microsomes led to one of the earliest suggestions of a role for cytochrome P450, in the metabolism of endogenous compounds such as steroids. The in vitro steroid C-21 hydroxylase activity in these microsomes was inhibited by carbon monoxide. This inhibition could then be reversed by light at 450 nm (Omura and Sato, 1964). These results confirmed the involvement of cytochrome P450 in steroid metabolism.

In further experiments on the function and inducibility of cytochrome P450, Remmer and Merker (1965) observed that

Table 1. The occurrence of cytochrome P450 isozymes in a few different tissues and species.

Organism	Tissue	Reference
Cow	Adrenocortical Mitochondria	Tsubaki *et al.*, 1987
Herring Gull	Liver	Peakall *et al.*, 1986
Human	Granulocytes	Mungikar and Gothoskar, 1986
	Placental Mitochondria	Pasanes and Pelkonen, 1986
Pig	Kidney	Bergman and Postlind, 1990
Rabbit	Lung, Liver	Parandoosh *et al.*, 1987
Rat	Brain	Walther *et al.*, 1986
Avocado	Mesocarp	Bozak *et al.*, 1990
Soybean	Entire bean	Kochs and Griseback, 1989
Drosophila	Entire animal	Sundseth *et al.*, 1990
Streptomyces	Entire culture	O'Keefe *et al.*, 1988
Xanthobacter	Entire culture	Warburton *et al.*, 1990

in vivo administration of PB increased the magnitude of the 450 nm absorption peak in reduced and carbon monoxide-treated microsomes. This increase in the 450 nm peak was accompanied by an increase in both *in vitro* and *in vivo* barbiturate metabolism. Thus the elevated cytochrome P450 content corresponded to the induction of enzymes involved in drug metabolism. This was the first observation linking microsomal cytochrome P450 to drug oxidation and metabolism. The use of difference spectra and photodissociation of carbon monoxide complexes in microsomal preparations demonstrated that cytochrome P450 was the final oxidase in the drug/xenobiotic-metabolizing enzyme systems (Cooper *et al.*, 1965).

Because administration of an inducer produced an increased 450 nm peak and simultaneous increases in various enzyme activities, the cytochrome P450 system was originally thought to be one enzyme capable of several different reactions. However, the existence of more than one form of the N-demethylase enzyme was eventually hypothesized. Intraperitoneal administration of B[*a*]P increased some enzyme activities while having little or no effect on others (Conney *et al.*, 1959). N-Demethylation of 3-MAB was inhibited by SKF 525A, a very specific, very potent demethylase inhibitor, in microsomes from untreated and PB-treated rats. In microsomes from 3-MC treated rats, this same inhibitor had no effect on N-demethylase activity.

This suggested that the enzyme induced by 3-MC was different from that induced by PB (Sladek and Mannering, 1966). Similar inhibition was observed using 7,8-benzoflavone to inhibit metabolic activity in microsomes from untreated, 3-MC-treated, or PB-treated animals (Wiebel *et al.*, 1971), further suggesting the existence of at least two cytochrome P450 isozymes. Parke (1975) further verified the existence of multiple forms of cytochrome P450 when a spectral absorption profile for 3-MC microsomes had an absorption peak at 448 nm, rather than 450 nm. This 448 nm peak was not due to formation of a complex between 3-MC and the normal cytochrome P450 molecule (Fujita *et al.*, 1973). The second hemeprotein was labelled cytochrome P448. When the two proteins were purified and characterized, they were found to have different substrate specificities (Parke, 1975). This finding confirmed that cytochrome P448 and cytochrome P450 were two different proteins.

The question still remained of exactly how the two were different from each other. To investigate this, inducers could be used to selectively induce cytochromes P450 with differing substrate specificities and enzymatic activities. These purified forms could then be analyzed to determine their relationships to each other. Botelho *et al.* (1979) showed that at least three forms of cytochrome P450 were induced by Aroclor 1254 (a polycyclic aromatic hydrocarbon). This inducer is actually a mixture of many different

polychlorinated biphenyl congeners, each with a different pattern of chlorination on the parent molecule, but averaging 54% chlorine. The congeners have differing inductive abilities, explaining the induction by Aroclor 1254 of more than one form of cytochrome P450. These studies showed that the induced isoforms were different from each other on the basis of total amino acid composition and partial amino acid sequence data. The differences in amino acid sequence among the three proteins demonstrated that the proteins were the products of different genes rather than alternate splicing of the mRNA transcript or post-translational modification of the protein itself.

With the evidence from these and similar studies, Coon and Persson (1980) suggested that there were only five or six forms of cytochrome P450. They further hypothesized that inducers altered substrate specificity and regioselectivity of the enzymes toward the substrate. For example, one inducer would stimulate production of one group of metabolites from a given substrate, while another set of metabolites would be seen after treatment with another inducer. It was suggested that the inducer somehow changed the site of metabolism on the substrate, without altering the type of cytochrome P450 enzyme present. It has since been demonstrated that there are many different forms of cytochrome P450, and that inducers alter the type of metabolites produced from a given substrate by altering the

type of number of cytochrome P450 isozymes present. Research has focused on characterizing new forms of cytochrome P450 by means of their substrate specificity, their relationship to previously identified forms in amino acid sequence and composition, and their constitutive and induced levels of expression. The genes coding for a number of the cytochrome P450 isozymes have also been identified and mapped to specific locations of the chromosome. Using these new approaches, more than one hundred and fifty distinct forms of cytochrome P450 have been characterized in a number of species and tissues (Coon *et al.*, 1992). The isozymes identified to date have been organized within the cytochrome P450 superfamily on the basis of protein and gene sequence data. The organization developed for cytochrome P450 isozymes is listed in Table 2 (Nebert *et al.*, 1991). The nomenclature suggested in that review will be used for the remainder of this discussion.

Table 2. Nomenclature and organization of cytochrome P450 isozymes in higher animals. (Nebert *et al.*, 1991)

Organism	Family	Subfamily	Genes
Rat	I	A	1,2
	II	A	1,2,3
		B	1,2,3,8
		C	6,6P*,7,11,12, 13,22,23
		D	1,2,3,4,5
		E	1
		G	1
	III	A	1,2,9
	IV	A	1,2,3,8
		B	1
	VII		
	XI	A	1
		B	1,3
	XVII		
	XIX		
	XXVII		
Human	I	A	1,2
	II	A	6,7
		B	6,7P*
		C	8,9,10,17,18,19
		D	6,7P*,8P*
		E	1
		F	1
	III	A	3,4,5,7
	IV	A	9
		B	1
	VII		
	XI	A	1
		B	1,2
	XVII		
	XIX		
	XXI	A	1P*,2
	XXVII		
Rabbit	I	A	1,2
	II	B	4,4P*,5
		C	1,2,3,4,5,14, 15,16
		E	1,2
		G	1
	III	A	6

*P indicates a pseudogene present in the designated family and subfamily.

Table 2 (cont.)

Organism	Family	Subfamily	Genes
Rabbit (cont.)	IV	A	4,5,6,7
		B	1
	VII		
	XVII		
Trout	I	A	1
Dog	I	A	1,2
	II	B	11
		C	21
Monkey	I	A	1
	II	C	20
		E	1
	III	A	8
Mouse	I	A	1,2
	II	A	4,5
		B	9,10
		D	9,10,11,12,13
		E	1
	XVII		
	XXI	A	1,2
Hamster	I	A	2
	II	A	8,9
	III	A	10
Chicken	II	H	1,2
	XVII		
	XIX		
Cow	VII		
	XI	A	1
		B	1
	XVII		
	XXI	A	1
Pig	XI	A	1
	XVII		
	XXI	A	1

Induction and Repression of Cytochrome P450

Enzyme induction is defined (Gelboin, 1967) as a process that increases the rate of synthesis of new enzyme. Similarly, enzyme repression decreases the rate of synthesis below the usual rate. Several mechanisms exist that explain how inducers may affect cytochrome P450 synthesis (Conney, 1967). The inducer may bind to a receptor that then interacts with the DNA to increase transcription from the cytochrome P450 gene. It may interact antagonistically with repressors made by regulator genes, or may alter histone structure around the DNA. The former would prevent down-regulation of the gene, allowing increased transcription to occur, while the latter would make the DNA more accessible to the transcription complex. The inducer may act at the endoplasmic reticulum to increase protein translation from the mRNA on the ribosomes. One example of the processes involved in induction of a cytochrome P450 isozyme is the well-studied induction of cytochrome P450IA1 by the potent inducer 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The mechanisms and pathways of induction by TCDD are shown in Figure 1 (Denison, 1992). The polycyclic aromatic hydrocarbon ligand, such as TCDD, binds to the cytoplasmic receptor, causing receptor transformation and translocation into the nucleus. Here, the ligand-receptor complex binds to an enhancer sequence upstream of the cytochrome P450IA1 gene and increases mRNA transcription from the gene. The mRNA is exported to the cytoplasm, where the increased mRNA

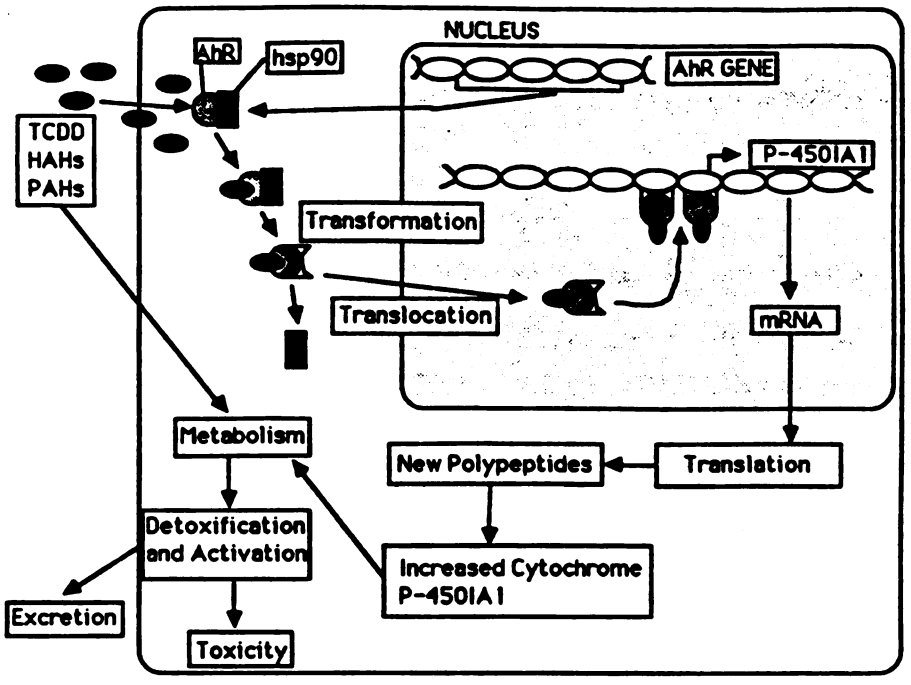


Figure 1. The induction of cytochrome P450 proteins by polycyclic aromatic hydrocarbons as mediated by the aromatic hydrocarbon receptor (Denison, 1992).

abundance produces increased translation of the message into cytochrome P450IA1 protein at the ribosomes on the endoplasmic reticulum. The mechanism of induction most frequently seen is an increase in transcription of the gene in question (Okey, 1989; Bock *et al.*, 1990). Cytochrome P450IIB induction in response to PB (Adesnik *et al.*, 1981) and methylenedioxybenzenes (Marcus *et al.*, 1990) is the result of both increased gene transcription rates and increased protein synthesis rates. 3-MC induces cytochrome P450IA by increasing the processing rate of the mRNA (Silver and Krauter, 1990). Pyridine induces the cytochrome P450IA subfamily by increasing transcription rates (Kim *et al.*, 1991). All these mechanisms of induction lead to an increase in at least one cytochrome P450 isozyme.

Induction of one cytochrome P450 isozyme may also be accompanied by a decrease or stabilization in the levels of other isozymes (Harada and Omura, 1981). This can be seen in the large increase of one isozyme, such as the induction of cytochrome P450IIB by PB, with a smaller increase in total cytochrome P450. This is also seen in treatment with aminoazotoluene, where cytochrome P450IA increases ten-fold and cytochrome P450IIB increases five-fold by twenty-four hours after treatment, while total cytochrome P450 concentration is only slightly increased (Tagashira *et al.*, 1985).

Several compounds that are inducers of cytochrome P450 are also substrates for the isozyme they induce. Some of the compounds in this group are toluene (Okey, 1989), B[*a*]P (Conney *et al.*, 1957), and naphthoflavones (Juchau, 1990). Increasing the metabolism of a toxic compound to less toxic products would reduce the toxicity of the chemical. Selective induction of the cytochrome P450 isozyme responsible for detoxifying a specific xenobiotic is an ideal method for increasing the excretion of the foreign compound. The oxidative reactions catalyzed by cytochrome P450 tend to detoxify foreign compounds and increase their excretion. However, they also have the potential to activate xenobiotics and make them more reactive towards DNA and proteins. The activation and detoxification of xenobiotics such as B[*a*]P by cytochrome P450 metabolism can often be simultaneous reactions whose outcomes depend on the induction state of the animal. Anything that changes the abundance of cytochrome P450 isozymes may alter the detoxification or activation of their substrates and thus have dramatic effects on their toxicity. In addition, inducing one cytochrome P450 isozyme to increase the detoxification of a specific xenobiotic may actually increase the activation of another foreign compound by the same isozyme. The decrease or stabilization in other cytochrome P450 isozymes that can accompany specific induction could also result in a lack of detoxification of some xenobiotics by the repressed or non-induced isozymes.

Role of Cytochrome P450 in Xenobiotic Detoxification and Activation

Three primary outcomes of cytochrome P450-mediated reactions have been suggested (Ryan and Levin, 1989). The first is the metabolism of endogenous substrates, such as the steroid hormones by cytochrome P450IIIA and the fatty acids by cytochrome P450IVA (Juchau, 1990). Another is the detoxification of lipophilic xenobiotics by hydroxylating them to more polar and less toxic metabolites. A closely related third result is the activation of some xenobiotics, such as B[*a*]P or AFB_1, to more toxic products by these same hydroxylation reactions.

Cytochrome P450 induction can be accompanied by the induction of several conjugating (Phase II) enzymes (Bock *et al.*, 1990). These enzymes add endogenous, highly polar compounds such as glutathione to reactive sites on xenobiotic substrates. This addition makes the xenobiotics more polar and more easily excreted. The ability of these Phase II enzymes to be induced by a foreign compound would be advantageous in reducing the toxicity of xenobiotics. Both cytochrome P450 (Phase I) and Phase II reactions are important in the metabolism of foreign compounds. However, further discussion of Phase II enzymes is beyond the scope of this discussion.

One well-studied xenobiotic metabolized by cytochrome

P450 is AFB_1. This mycotoxin is produced primarily by *Aspergillus flavus* on grain crops, either in the field or in storage (Palmgren and Ciegler, 1983). To exert its toxic or mutagenic effects, AFB_1 must undergo cytochrome P450 metabolism (Shimada *et al.*, 1987). The ultimate mutagenic potential of the metabolites is determined by the site of metabolism on the aflatoxin molecule. This in turn is determined by the relative abundance of specific cytochrome P450 isozymes. The most mutagenic product is AFB_1-2,3-oxide. Studies using human liver microsomes have shown that this metabolite is primarily produced by cytochrome P450IIIA4 (Shimada and Guengerich, 1989; Aoyama *et al.*, 1990; Guengerich *et al.*, 1991). However, antibodies to this isozyme only inhibited the activation of AFB_1 by 65% in human liver microsomes (Aoyama *et al.*, 1990). This indicates that other cytochrome P450 isozymes are involved in activating AFB_1. One of these forms has been identified as cytochrome P450IIB1 (Doehmer *et al.*, 1988).

Cytochrome P450 is also responsible for the detoxification of AFB_1. The 4-hydroxylation of AFB_1 to form AFM_1 has been assigned to cytochrome P450IA2 on the basis of cDNA expression and activation studies (Koser *et al.*, 1987; Faletto *et al.*, 1988; Koser *et al.*, 1988). AFM_1 is markedly less mutagenic in the Ames assay, possessing only about 3% of the mutagenicity of its parent compound, AFB_1 (Wong and Hsieh, 1976). In the rat, induction of the cytochrome

P450IIIA2 isozyme has been shown to increase the 9-hydroxylation of AFB_1 to AFQ_1 (Halvorson *et al.*, 1988), which has only 1% of the mutagenicity of the parent (Wong and Hsieh, 1976). Induction of either cytochrome P450IA2 or of cytochrome P450IIIA2 would thus shift the metabolism of AFB_1 in the direction of the less mutagenic hydroxylated products that can also be easily excreted. If the presence of AFB_1 in food were confirmed, then supplementation of the diet with some inducer of either of these two isozymes could potentially increase the detoxification of AFB_1, thereby reducing the risk of mutagenesis posed by the mycotoxin.

MUTAGENESIS AND CARCINOGENESIS

Chemically Mediated Mutation

AFB_1-2,3-oxide, produced by metabolic activity of cytochrome P450, binds covalently to macromolecules such as DNA or proteins. The DNA adduct formed most often by AFB_1-2,3-oxide is AFB_1-N^7-guanine. A number of other chemicals can also form DNA adducts after activation by cytochrome P450. One of these is B[*a*]P, which is activated to B[*a*]P-diol epoxide (BPDE).

DNA-xenobiotic adduct formation can destabilize the structure of the affected nucleosides (Moran and Ebisuzaki, 1991), and lead to their rearrangement or depurination. Neither the adduct formation nor the alteration in nucleoside structure is in itself a mutation. Mutation, a permanent change in DNA sequence, will only occur if the lesion is not repaired correctly before DNA replication occurs. If repair does not occur, the damaged area will be unable to serve as a template for replication. An incorrect base may be substituted at the lesion during replication, producing a permanent mutation.

Mutation in Carcinogenesis

If a lesion is left unrepaired in non-replicating DNA, the persistent damage will not produce a mutation. It has been demonstrated that unrepaired DNA damage cannot initiate carcinogenesis without cell proliferation, which is necessarily accompanied by DNA replication and mutation at the damaged sites (Farber, 1982).

A mutation may have no effect on the cell's physiology or function, or it may prove lethal. Between these two extremes, however, the mutation may give the cell a selection advantage when responding to a growth stimulus. This increased growth ability, initiation, is seen as the first step in chemical carcinogenesis (Farber, 1982). Environmental factors termed promoters allow clonal expansion of the initiated cell. This then increases the number of cells that can be initiated a second time (Farber, 1982). The cycle of initiation and promotion may be repeated more than once, each time allowing the cell to acquire new properties (Scherer, 1989). The number of mutational events required for the development of cancer from a progenitor cell is not currently known, although it has been suggested that more than one is necessary (Scherer, 1989). As the initiated cells respond to promotion and then continue to proliferate independently, the genome may undergo more drastic changes such as chromosomal rearrangement or deletion. This can lead to growth

deregulation and transformation to a neoplastic state. This stage of carcinogenesis is termed progression.

All of these steps are regulated to some extent by environmental factors. Initiation can be affected by the dose and type of carcinogen. If the xenobiotic is a promutagen requiring cytochrome P450-mediated activation, the environment can affect mutation rates by altering the abundance of specific cytochrome P450 isozymes responsible for this activation. This would then change the activation of the chemical to its DNA-binding form. Initiation by mutation may occur repeatedly due to continued exposure to the same carcinogen, or to others. After initiation, either exogenous or endogenous factors may act as promoters and select for clonal expansion of the initiated cells (Farber, 1982). This expansion can lead to further initiation by environmental compounds (Farber, 1982; Scherer, 1989). This series of changes can finally lead to the removal of the cell from normal growth controls, allowing its progression into neoplasia.

Because the environment can have such a large impact on chemical carcinogenesis, a major area of research is focused on modifying the environment to decrease cancer risks. One avenue of risk reduction is through dietary modification. The goals of altering the diet would be to both reduce the intake of carcinogens and to increase the consumption of

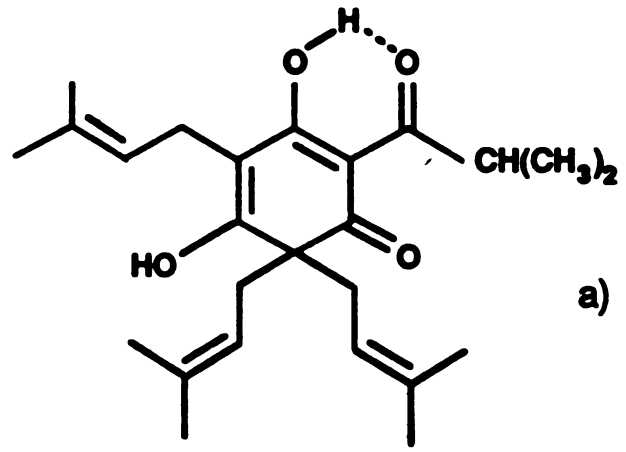
compounds able to favorably alter xenobiotic metabolism. These alterations could include either increasing detoxification of activated carcinogens or decreasing activation of procarcinogens.

HOPS CONSTITUENTS

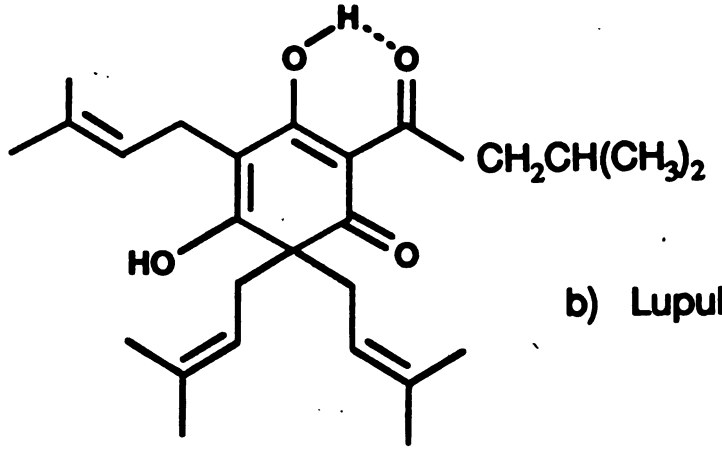
One group of compounds that may have potential for altering cytochrome P450-mediated activation and detoxification of xenobiotics are the ß-acids found in hops. The structures of some of these compounds are shown in Figure 2.

Hops constituents have been studied previously (Mannering and Deloria, 1988; Mannering *et al.*, 1992). Interest in hops compounds was initiated by the finding that hops ß-acids adsorbed onto brewer's yeast could induce cytochrome P450 metabolism in the mouse (Mannering and Deloria, 1988). Further research showed that colupulone was the primary ß-acid responsible for this induction (Mannering *et al.*, 1992). This inductive ability was demonstrated by measuring total cytochrome P450 concentration, by using antibodies to several cytochrome P450 isozymes, and by measuring cytochrome P450 metabolic activity using B[*a*]P, aniline, and ethylmorphine as substrates for cytochromes P450IA, P450IIE1, and P450IIIA4 respectively.

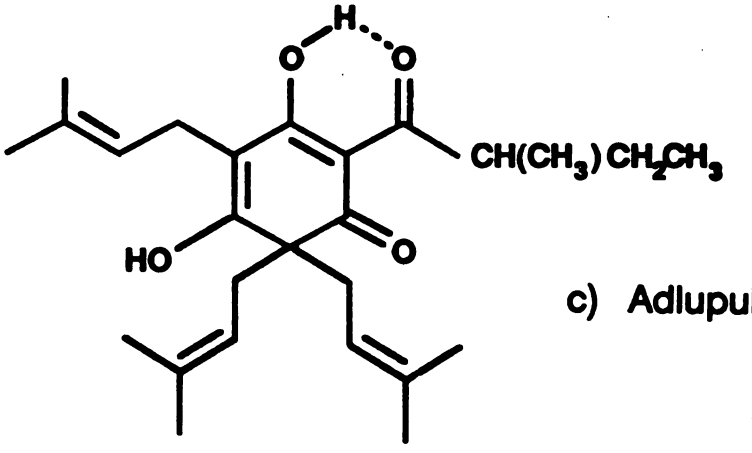
ß-acids are present in both alcoholic and non-alcoholic



a) Colupulone



b) Lupulone



c) Adlupulone

Figure 2. The structures of three hops ß-acids: a) colupulone; b) lupulone; c) adlupulone (Mannering *et al.*, 1992).

hopped beverages. Because these beverages can be ingested in high quantities (several liters per day), it is essential to define the effect of hops ß-acids on cytochrome P450 abundance and metabolic activity.

The isozymes examined in the current experiment were selected because of their involvement in the activation and detoxification of a number of important procarcinogens. For example, cytochromes P450IA1 and P450IA2 activate the heterocyclic amine 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Wallin *et al.*, 1990), and the carcinogenic dye 3-methoxy-4-aminoazobenzene (Yamazaki *et al.*, 1991). Cytochrome P450IA2 is known to metabolize AFB_1 to the less mutagenic AFM_1, and also deactivates the carcinogen 1,3-dinitropyrene (Shimada and Guengerich, 1990). Cytochrome P450IIB1 is involved to some extent in the activation of AFB_1 to the mutagenic, reactive AFB_1-2,3-oxide. This reaction is also carried out by cytochrome P450IIIA4, as is some of the activation of 3-methoxy-4-aminoazobenzene (Yamazaki *et al.*, 1991). This isozyme also deactivates 1,6-dinitropyrene to a non-carcinogenic metabolite (Shimada and Guengerich, 1990). Examining the ability of colupulone to induce these forms of cytochrome P450 may produce a clearer understanding of the potential chemopreventive effects of dietary colupulone.

METHODS AND MATERIALS

Chemicals

Colupulone (Kalsec Inc., Kalamazoo, MI) was provided as 95% pure by high-pressure liquid chromatography. Pregnenolone-16α-carbonitrile was a gift of UpJohn Inc. (Kalamazoo, MI). Grade I glucose-6-phosphate dehydrogenase was obtained from Boehringer Mannheim (Indianapolis, IN) as a 5 mg/ml solution in ammonium phosphate and stored at 4°C in a desiccator between uses. Glucose-6-phosphate, monosodium salt (98% purity) was also from Boehringer Mannheim Biochemicals. Grade II, 97% pure NADP from Sigma Chemical Co. (St. Louis, MO) was stored at -20°C. Carbon monoxide was obtained from AGA Gas Co. (Lansing, MI) at 99% purity. All other chemicals were of the highest grade obtainable.

Animals

Male Sprague Dawley rats (Harlan Sprague Dawley Co., Indianapolis, IN) weighing 200-225 g were used for this experiment. Animals were housed in polycarbonate boxes on hardwood chip bedding. The room was temperature- and humidity-controlled with a 12-hour light/dark cycle. Animals were allowed free access to food and water

throughout the study.

Diets. Animals were acclimatized to a modified American Institute of Nutrition (AIN) 76 diet for six days prior to treatment. The original composition of the AIN 76 diet was: cellulose, 5%; cornstarch, 15%; casein, 20%; sucrose, 50%; DL-methionine, 0.3%; AIN vitamin mixture, 1%; AIN mineral mix, 3.5%; choline, 0.2%; corn oil, 5% (AIN, 1977). In the modified diet, cornstarch was increased to 49.2%, while sucrose was decreased to 10%. This new formulation provided the same nutrient density as the original composition. Purified diet was mixed and stored in 1-kg lots at 4°C. All feedings and treatments took place at 10:30 each morning.

Treatments. Colupulone and pregnenolone-16α-carbonitrile (PCN) were each given to a separate group of four animals. PCN, a fairly specific inducer for cytochrome P450IIIA4, was suspended in corn oil to give a concentration of 93.75 mg/ml (Wrighton *et al.*, 1985) This suspension was administered daily by oral gavage to provide 300 mg PCN/kg body weight per day for four days. Colupulone was mixed into modified AIN 76 diet at a level of 0.36% (w/w) (Mannering *et al.*, 1992). Colupulone-supplemented diet that was not used immediately was stored at -20°C. This diet was fed *ad lib* for five days. A third group of four animals were untreated and allowed *ad lib* access to modified AIN 76 diet and water.

Tissue Collection. Animals were euthanized by CO_2 asphyxiation and cervical dislocation. Livers were quickly removed, rinsed in ice-cold 150 mM KCl, and quickly frozen in liquid nitrogen. The tissues were then stored in liquid nitrogen until use.

Microsomal Assays

Microsome Preparation. Two to three grams frozen liver tissue were allowed to thaw on ice in 6 ml of 19.8 mM Tris/1.5% KCl and homogenized on speed setting 5 of a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) for 20 to 30 seconds. Homogenates were centrifuged for 20 minutes at 10,000x g in a Sorvall RC-5B centrifuge. The supernatant was filtered through two layers of cheesecloth and centrifuged at 105,000x g for 60 minutes in a Beckman L7-65 ultracentrifuge. The resulting pellet was resuspended in 4 ml of 0.39 M sucrose/0.77 mM pyrophosphate buffer, pH 7.6, and homogenized with a Polytron on speed setting 3 for 10 seconds. The suspension was then centrifuged at 105,000x g for 60 minutes in the Beckman L7-65. The second pellet was resuspended in 1.5 ml of 150 mM KCl and homogenized on speed 3 of the Polytron for ten seconds. All steps in the preparation were carried out on ice.

Protein and Cytochrome P450 Determination. Microsomal protein concentrations were determined by the Biuret protein

concentration method (Gornall *et al.*, 1949). Microsomal suspension (100 µl) was diluted in 1.9 ml of distilled water and 2 ml of 6% sodium hydroxide. Biuret color reagent (200 µl) (Biuret reagent: 1.19 M sodium bicarbonate, 69 mM copper sulfate) were added. The reaction mixture was allowed to incubate at room temperature for 10 minutes. The tubes were centrifuged in a Beckman clinical centrifuge for five minutes to sediment any precipitate. The absorbance of the supernatant was then measured against a blank of distilled water. Absorbance values were then compared to a standard curve of bovine serum albumin in 150 mM KCl, prepared along with each protein concentration determination. Microsomal protein concentrations were calculated by linear regression.

Microsomes were diluted to 1 mg protein/ml in 100 mM Tris, pH 7.4, and analyzed for cytochrome P450 concentration by the method of Omura and Sato (1964). Approximately 5 mg sodium dithionite were added to the suspension and mixed well. The absorption of this reduced suspension was then recorded from 500 to 400 nm, against a blank of the same reduced suspension. Carbon monoxide was bubbled through the sample solution for 20 seconds, and absorbance was again measured between 500 and 400 nm, with the same reduced blank as before. Peak height at 450 nm was used to calculate cytochrome P450 concentration, using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ (Omura and Sato, 1964).

Substrate Metabolism Assays. Two specific substrates were used. Each was incubated with the microsomal fraction and an NADPH-generating system. Aminopyrine (cytochrome P450IIB1) and erythromycin (cytochrome P450IIIA4) N-demethylase activities were determined by the formation of formaldehyde, which was quantified spectrophotometrically (Nash, 1953). The incubation mixture consisted of 250 µl 183 mM HEPES buffer, pH 7.6; 10 µl 197 mM $MgCl_2$; 20 µl 354 mM glucose-6-phosphate, pH 7.0; and 1 unit glucose-6-phosphate dehydrogenase. NADP (0.5 mg) was added to the sample incubations but omitted from the blanks. Microsomal protein (0.8 mg), diluted in 150 mM KCl, was added to the incubation mixture on ice. The mixtures were pre-incubated for three minutes at 37°C in a shaking water bath. The reaction was initiated by the addition of the substrate (aminopyrine, 20 µmole; erythromycin, 0.5 µmole), followed by vortexing. The reaction mixtures were then allowed to incubate for 20 minutes at 37°C in a shaking water bath. The reactions were stopped by adding 1.0 ml of 20% zinc sulfate and 1.0 ml saturated barium hydroxide, vortexing, and replacing the tubes on ice. The tubes were centrifuged in a Beckman clinical centrifuge for five minutes at 1000x g. 1.0 ml of the supernatant was combined with 1.0 ml of Nash reagent (1.95 M ammonium acetate, 37.2 mM acetylacetone, 50 mM acetic acid; Nash, 1953) and incubated for 10 minutes at 60°C. The tubes were centrifuged as before and allowed to cool. Color development was measured

at 412 nm on a Varian Cary-3E dual beam spectrophotometer. A new formaldehyde standard curve was prepared each time the assays were performed. Absorbance values for blank and sample incubations were compared to the standard curve, and nanomoles of formaldehyde produced were calculated by linear regression.

RNA Isolation and Analysis

RNA Isolation. All glassware used was baked overnight at 300°C. All plasticware was handled with latex gloves before autoclaving and was autoclaved for 25 minutes at 250°C, or was used from pre-wrapped, sterile individual packages. Solutions were filter-sterilized through a 0.2 μm filter (Nalgene Inc., Rochester, NY) into sterile glass bottles. The homogenizer probe was autoclaved before use, and was rinsed with approximately 5 ml chloroform between tissues to remove debris without introducing RNAse contamination. RNA was isolated using a modification of the Chirgwin procedure (Chirgwin *et al.*, 1978), as previously described (Helferich *et al.*, 1990). Approximately 0.25 g liver tissue was completely homogenized in 4 M guanidinium isothiocyanate buffer, pH 7.0 (4 M guanidinium isothiocyanate,1 M sodium citrate, 0.1 M ß-mercaptoethanol, 0.5% N-laurylsarcosine). The homogenate was centrifuged at 10,000 rpm for 10 minutes in a Sorvall SS-34 rotor. Four ml of the supernatant were layered over 1 ml of 5.7 M cesium chloride (5.7 M cesium chloride, 0.1 M EDTA, pH 8.0) and centrifuged for 12 hours

at 150,000x g at 20°C in a Beckman L7-65 ultracentrifuge and SW 50.1 rotor. After centrifugation, the cesium chloride and guanidinium isothiocyanate were decanted and the tube walls were wiped with a Kimwipe^{R}. The pellet was resuspended in 1 ml of 7 M guanidine hydrochloride buffer, pH 7.0 (7 M guanidine hydrochloride, 19.8 mM sodium acetate, 0.97 M dithiothrietol, 1 mM EDTA) and transferred to microcentrifuge tubes. RNA was precipitated by the addition of 0.5 volume absolute ethanol and 0.05 volume 0.3 M sodium acetate at -20°C for 60 minutes. RNA pellets were then resuspended in 3 M sodium acetate and precipitated as before. The resulting pellet was washed with 66% ethanol and 33 mM sodium acetate at 4°C, and washed again with absolute ethanol at -20°C. The final pellet was resuspended in 1 ml 10 mM Tris, 1 mM EDTA, pH 8.0. The RNA suspensions were scanned with a Varian Cary-3E dual beam spectrophotometer from 220 to 320 nm, and the concentrations were determined from the absorbance at 260 nm using an extinction coefficient of 40 μg/ml, as described in Maniatis *et al.* (1986). The solutions were stored at -80°C.

RNA Gel Electrophoresis. RNA solutions were allowed to thaw on ice and diluted with 10 mM Tris, 1 mM EDTA, pH 8.0, to 1 μg/μl. The RNA dilutions (20 μl of each) were mixed with 60 μl of RNA denaturing buffer (10x MOPS (*N*-morpholino-propane-sulfonic acid) buffer: 0.4 M MOPS, pH 7.0, 100 mM sodium acetate, 10 mM EDTA; RNA denaturing buffer: 13% 10x MOPS,

23% formaldehyde, 64% formamide) and heat-denatured at 50°C for 15 minutes. Twenty μl of RNA loading buffer (50% glycerol, 1 mM EDTA, 0.4% xylene cyanol, 0.4% bromphenol blue) were added and the solutions were mixed by pipeting repeatedly. Five μg of each RNA sample were loaded onto one of three 1.2% agarose-10% formaldehyde denaturing gels made up with 1x MAE buffer (10x MOPS, 100 mM sodium acetate, 10 mM EDTA) in running buffer of 1x MAE buffer. The gels were electrophoresed at a constant voltage of 20 volts for 16 hours. The gels were then stained by the addition of 0.02 μg ethidium bromide per ml of running buffer and placing the gels on a rotating shaker for 60 minutes. Destaining was accomplished by replacing the staining buffer with 1x MAE buffer for three hours on a rotating shaker. The gels were prepared for Northern blotting by denaturation in 50 mM sodium hydroxide and 10 mM sodium chloride for 45 minutes followed by neutralization in 0.1 M Tris, pH 7.5, for 45 minutes.

Northern Blotting. The gels were soaked in 20x sodium chloride-sodium citrate (20x SSC: 3 M sodium chloride, 0.3 M sodium citrate) for 10 minutes, then the RNA was transferred to Hybond N nylon membrane (Amersham Inc., Arlington Heights, IL) for 24 hours with 10x SSC. The transfer apparatus was assembled as described in Maniatis *et al.* (1986). After transfer, the blots were rinsed briefly in 20x SSC, then allowed to air dry for two hours. The RNA was

cross-linked to the membrane by two minutes of ultraviolet radiation.

Northern Blot Hybridization. The blots were hybridized with cDNAs of mouse cytochrome P450IA2 from ATCC (Kimura *et al.*, 1984), rat cytochrome P450IIB1 (Doehmer *et al.*, 1988), or human cytochrome P450IIIA4 (Bork *et al.*, 1989). The probes were labelled with a Dupont-NEN (Boston, MA) random-prime labelling kit. Fifty microcuries of α-^{32}P-dCTP from Dupont NEN were used per probe, with a specific activity of 3097 curies/mmole. Labelled probe was purified from the unincorporated nucleotides by centrifuging through Sephadex G-50 in 1 M Tris, 1 mM EDTA, 100 mM sodium chloride, pH 8.0. The radiolabelled probe was then stored at -20°C until use. All prehybridizations, hybridizations, and washes were carried out in a Robbins hybridization oven. Blots for cytochromes P450IA2 and P450IIIA4 were prehybridized at 36.5°C for four hours in 15 ml 45% formamide prehybridization solution (45% formamide, 3x SSC, 5x Denhardt's solution, 50 mM phosphate buffer). 5 mg herring sperm DNA was heat-denatured at 95°C for five minutes before addition to the pre-hybridization solution. The prehybridization solution on each blot was replaced with 7 ml of 45% formamide hybridization solution (45% formamide, 3x SSC, 5% dextran sulfate, 50 mM phosphate buffer, 5x Denhardt's solution, 0.1% SDS), 2.5 mg heat-denatured herring sperm DNA, and heat-denatured probe. Hybridization

hours. The blots were washed with 100 ml of 2x SSC, 0.1% sodium dodecyl sulfate (SDS) at 45°C for 30 minutes, followed by 100 ml of 2x SSC, 0.1% SDS at 45°C for 60 minutes. The cytochrome P450IIB1 blot was prehybridized in 15 ml of 50% formamide prehybridization solution (50% formamide, 3x SSC, 5x Denhardt's solution, 50 mM phosphate buffer) and 5 mg heat-denatured herring sperm DNA at 42°C for four hours. The prehybridization solution was then replaced with 7 ml 50% formamide hybridization solution (50% formamide, 3x SSC, 5% dextran sulfate, 50 mM phosphate buffer, 5x Denhardt's solution, and 0.1% SDS), 2.5 mg heat-denatured herring sperm DNA, and heat-denatured probe. Hybridization was carried out at 42°C for sixteen hours. Washing was conducted in 100 ml of 2x SSC, 0.1% SDS at 65°C for sixty minutes, followed by a twenty-five minute wash at 65°C in 100 ml 0.3x SSC, 0.1% SDS. All blots were allowed to air-dry for two hours, and exposed to X-Omat autoradiography film (Kodak Inc., Rochester, NY) at -80°C. Autoradiographs were developed according to Kodak directions for X-Omat film.

Statistical Analysis

Means and standard errors were calculated for: feed eaten per animal; body weight and liver weight; microsomal protein and cytochrome P450 isolated; and aminopyrine and erythromycin N-demethylase activities. Differences between the means of control and treated groups were determined by

the one-tailed Student's *t* test (Bhattacharyya and Johnson, 1977). The criterion for significance was set at 0.05.

RESULTS AND DISCUSSION

Feed Intake and Daily Body Weight

Daily feed intake for control, colupulone-treated, and PCN-treated animals is presented in Figure 2. Days -3 to -1 are the acclimatization period, with treatment starting at 10:30 AM on day 0. Before treatment, the amount of feed consumed by animals in all three groups was similar. After treatment began, feed intake by the colupulone treatment group dropped from an average of 25.8 g per animal before treatment to 20.1 g per animal during treatment. This decrease was significant at $P < 0.05$. This level of feed consumption was also significantly less than the 26.8 g per animal eaten by the control animals on days 1-5. Feed consumption by the PCN treatment group showed no real change during treatment.

Average daily body weight in grams for each treatment group is shown in Figure 3. Neither treatment group was significantly different from the controls either before or during the course of the experiment.

Final Body Weight and Liver Weight

Neither the initial nor the final body weights were significantly different between the control and the treated animals, at a significance level of $P < 0.05$. Liver weights also were not different between groups. However, liver weight expressed as a percentage of body weight was significantly increased at $P < 0.05$ in the PCN-treated group (6.4% for PCN compared to 5.6% for the control group). These data are presented in Table 3.

Microsomal Protein and Cytochrome P450 Isolation

For the control group, 18.3 mg microsomal protein was isolated per gram of liver used. As presented in Table 4, this was slightly but not significantly higher than the yield in the colupulone-treated group (17.8 mg/g) and in the PCN-treated group of animals (17.9 mg/g). Microsomes from control animal livers also had a higher cytochrome P450 concentration (0.5 ng/g protein) than microsomes from either colupulone-treated or PCN-treated animals (0.3 ng/g protein for both). Using a significance level of $P < 0.05$, however, this difference was not significant.

Aminopyrine and Erythromycin N-Demethylase Activities

Measurements of the metabolic activities of two cytochrome P450 isozymes, presented in Table 5, did not show significant differences between untreated and treated animals. For aminopyrine N-demethylation, there was a

nonsignificant trend towards increased activity in both colupulone-treated (5.60 nanomoles formaldehyde/mg protein/minute) and in PCN-treated animals (5.63 nanomoles/mg protein/minute) when compared to control animals (4.57 nanomoles/mg protein/minute).

The same trend was apparent for erythromycin N-demethylase activity, although the increase was not as large. Erythromycin N-demethylase activity in control microsomes was 1.68 nanomoles formaldehyde/mg protein/minute. Enzyme activity in microsomes from colupulone-treated animals was 2.06 nanomoles/mg protein/minute, slightly higher than the activity in microsomes from PCN-treated animals, 2.02 nanomoles/mg protein/minute.

Northern Blot Hybridization

Figure 4 is a photograph of an autoradiogram of cytochrome P450IA2 hybridization to hepatic RNA isolated from untreated, colupulone-treated, and PCN-treated animals. The signal produced by hybridization in the colupulone-treated lanes is somewhat more intense than that seen in the control lanes. This indicates that colupulone may have effected a slight increase in the level of hepatic cytochrome P450IA2 RNA. On the other hand, some reduction in signal strength can be seen in the PCN-treated lanes, suggesting that PCN somehow decreased message abundance for

cytochrome P450IA2.

A similar figure for the hybridization of cytochrome P450IIB1 cDNA is presented in Figure 5. Here, however, the difference between signal strength in untreated and colupulone-treated lanes is more intense. This increased hybridization by the labelled cDNA probe to RNA from colupulone-treated animals indicates that the RNA level for this isozyme is increased by colupulone administration. The hybridization signal is also more intense in the PCN-treated lanes than in the control lanes, indicating increased message levels in response to PCN treatment.

Hybridization of the cytochrome P450IIIA4 cDNA is shown in Figure 6. While the hybridization of the probe in the control lanes is more evident than in Figures 4 and 5, there is also a noticeable increase in signal intensity in the colupulone-treated lanes. This indicates an increase in message level for cytochrome P450IIIA4 in response to colupulone treatment. An even more intense is evident in the PCN-treated lanes, demonstrating that PCN is able to increase the message level of cytochrome P450IIIA4.

The results of the current study do not correspond to the results described by Mannering *et al.* (1992). In that study, treatment of Swiss-Webster mice with 0.36% colupulone in purified diet resulted in an 11-fold induction of

cytochrome P450IIIA4 metabolic activity, as well as doubling cytochrome P450IIE1 metabolic activity and inducing that of cytochrome P450IA1 by three-fold. An increase in the content of hepatic cytochrome P450 and of the cytochrome P450IIIA4 isozyme itself, as indicated by Western blot analysis, were also observed.

There are several possible explanations for the differences between these studies. Cytochrome P450IIIA4 may be less sensitive to a given chemical, such as colupulone, in the rat than it is in the mouse. This would therefore require more of the chemical to produce the same amount of induction in the rat. Alternatively, the time required for translation of the cytochrome P450IIIA4 message into protein may be longer in the rat than in the mouse. Colupulone did produce an increase in cytochrome P450IIIA4 message in the rat. RNA levels for the isozymes in question were not measured in the previous study (Mannering *et al.*, 1992). It is thus possible that the increase in message abundance would indeed have been translated into increased cytochrome P450IIIA4 if treatment time in the rat had been extended.

Erythromycin is the diagnostic substrate used to measure cytochrome P450IIIA4 activity in the current experiment, whereas Mannering *et al.* (1992) used ethylmorphine as the cytochrome P450IIIA4 substrate. It is

possible that after N-demethylation of the erythromycin molecule, the reaction product complexes with the active site of the cytochrome P450 protein and inhibits further reactions (Pershing and Franklin, 1982). If this is the case, formation of this complex could obscure any increase in the metabolic activity of the cytochrome P450IIIA4 isozyme. It is therefore possible that using ethylmorphine would produce results demonstrating an induction of cytochrome P450IIIA4 metabolic activity in response to dietary colupulone supplementation. This would agree with the cytochrome P450IIIA4 RNA data obtained in the current experiment.

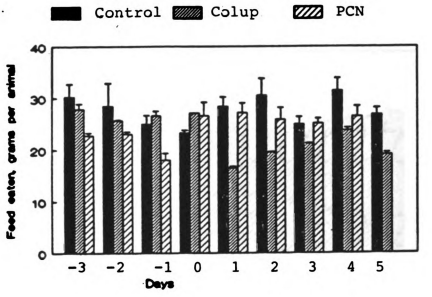


Figure 3. Average grams of feed eaten per animal per day prior to and during colupulone and PCN administration (error bars represent standard error of the mean) with treatment beginning at 10:30 AM on Day 0.

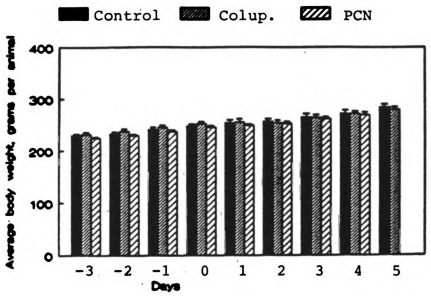


Figure 4. Average body weight in grams of each animal each day prior to and during colupulone and PCN administration (error bars represent standard error of the mean) with treatment starting at 10:30 AM on Day 0.

Table 3. Initial body weights, final body weights, liver weights, and relative liver weights in rats treated for five days with colupulone or for four days with PCN.

Treatment	Initial Body Weight (g)	Final Body Weight (g)	Liver Weight (g)	Relative Liver Weight (%)
Control	229.5 ± 3.1[1](4)	284.2 ± 5.8 (4)	16.0 ± 0.8 (4)	5.6 ± 0.2 (4)
Colupulone	231.9 ± 4.2 (4)	279.7 ± 4.9 (4)	15.6 ± 0.4 (4)	5.5 ± 0.1 (4)
PCN[2]	224.5 ± 2.0 (4)	269.1 ± 4.8 (4)	17.1 ± 0.4 (4)	6.4 ± 0.1[3](4)

[1]Data are expressed as mean ± standard error; numbers in parentheses refer to sample size.
[2]PCN is pregnenolone-16 alpha-carbonitrile treatment group.
[3]Values are significantly different from controls, at $P < 0.05$ (one-tailed Student's t test; Bhattacharyya and Johnson, 1977).

Table 4. The effect in the rat of administration of colupulone for five days or PCN for four days on hepatic microsomal protein and cytochrome P450 concentration.

Treatment	mg protein/g liver	ng cytochrome P450/mg protein
Control	18.27 ± 1.45[1](4)	0.52 ± 0.16 (4)
Colupulone	17.82 ± 1.29 (4)	0.30 ± 0.10 (4)
PCN[2]	17.86 ± 1.75 (4)	0.30 ± 0.08 (4)

[1]Data are expressed as mean ± standard error; numbers in parentheses refer to sample size.
[2]PCN is pregnenolone-16 alpha-carbonitrile treatment group.

Table 5. The effect in the rat of administration of colupulone for five days or PCN for four days on hepatic microsomal aminopyrine N-demethylase and erythromycin N-demethylase activities.

Treatment	Aminopyrine N-demethylase, nm formaldehyde/mg protein/minute	Erythromycin N-demethylase, nm formaldehyde/mg protein/minute
Control	4.57 ± 0.43[1](4)	1.68 ± 0.30[1](4)
Colupulone	5.60 ± 0.95 (4)	2.06 ± 0.26 (4)
PCN[2]	5.63 ± 1.04 (4)	2.03 ± 0.39 (4)

[1]Data are expressed as mean ± standard error; numbers in parentheses refer to sample size.
[2]PCN is pregnenolone-16 alpha-carbonitrile treatment group.

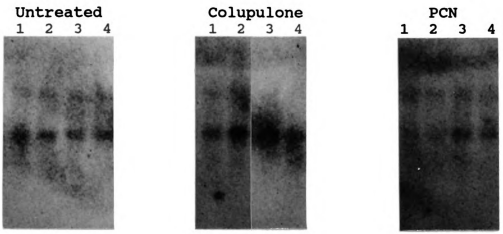


Figure 5. Northern blot hybridization of cytochrome P450IA2 mouse cDNA to RNA from livers of untreated, colupulone- and pregnenolone-16α-carbonitrile-treated rats.

Approximately 5 μg RNA was loaded into each lane. After transfer and hybridization as described in Methods and Materials, the blot was washed with a low-stringency wash of 100 ml 2x SSC, 0.1% SDS SSC, 0.1% SDS at 45°C for 30 minutes, followed by a low-stringency wash in 100 ml 2x SSC, 0.1% SDS at 45°C for 60 minutes. The washed blot was exposed to X-Omat autoradiography film for 11 days at -80°C. The increase in signal in the colupulone-treated lanes compared to the signal compared to the signal in the control lanes indicates an increase in cytochrome P450IA2 mRNA abundance in response to colupulone treatment.

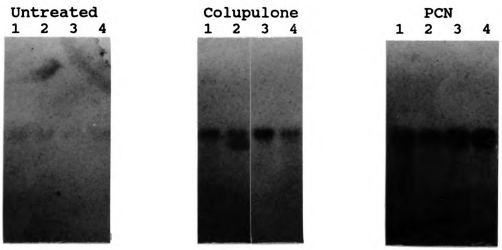


Figure 6. Northern blot hybridization of cytochrome P450IIB1 rat cDNA to RNA from livers of untreated, colupulone- and pregnenolone-16α-carbonitrile-treated rats.

Approximately 5 μg RNA was loaded into each lane. After blotting and hybridization as described in Methods and Materials, the blot was washed in a high stringency wash of 2x SSC, 0.1% SDS at 65°C for 60 minutes, followed by a 25 minute wash in 0.3x SSC, 0.1% SDS at 45°C. The washed blot was exposed to X-Omat autoradiography film at -80°C for 11 days. There is an increase in signal intensity visible in the colupulone-treated lanes, indicating that colupulone induces an increase in cytochrome P450IIB1 message abundance. There is also an increase in signal intensity in the PCN-treated lanes, demonstrating that PCN is able to increase mRNA abundance for cytochrome P450IIB1.

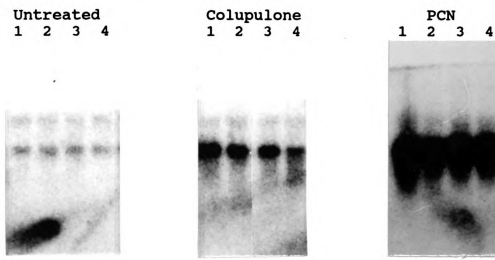


Figure 7. Northern blot hybridization of cytochrome P450IIIA4 human cDNA to RNA from livers of untreated, colupulone- and pregnenolone-16α-carbonitrile-treated rats.

Approximately 5 mg RNA was loaded into each lane. The blotting and hybridization techniques used are described in Methods and Materials. After hybridization, the blot was washed by low-stringency washing in 2x SSC, 0.1% SDS at 45°C for 30 minutes, followed by a 60 minute wash in 2x SSC, 0.1% SDS at 45°C. The blot was then allowed to air dry for two hours and exposed to X-Omat autoradiography film at -80°C for 11 days. Colupulone produced an increase in cytochrome P450IIIA4 mRNA level, as demonstrated by the greater signal in the colupulone-treated lanes when compared to the control lanes. The very intense signal in the PCN-treated lanes indicates that PCN increases the abundance of cytochrome P450IIIA4 message.

SUMMARY AND CONCLUSIONS

In this experiment, administration of colupulone in modified AIN 76 diet to rats had no significant effect ($P < 0.05$) on body weight, liver weight, or cytochrome P450-mediated metabolism of aminopyrine and erythromcyin. These data do not agree with earlier results demonstrating an inductive effect of colupulone on cytochrome P450 in the mouse (Mannering *et al.*, 1992). This difference in the response to colupulone may be due to a number of factors, such as species differences or the difference in cytochrome P450 substrates used (erythromycin instead of ethylmorphine). However, Northern hybridization in the current study did reveal an increase in the abundance of cytochrome P450IIIA4 mRNA after treatment with colupulone, as well as a slight increase in the message level of cytochromes P450IA2 and P450IIB1. This message level-induction, of cytochrome P450IIIA4 especially, was not observed at the protein level, as indicated by the lack of increase in total cytochrome P450 concentration in response to colupulone treatment.

Neither study examined the effect of colupulone

administration on metabolism of substrates which may be activated or detoxified by cytochrome P450IIIA4. Further research is required to determine whether inductive effects of colupulone, as seen at the mRNA level, may have an impact on human health.

The use of any agent to alter cytochrome P450-mediated promutagen or procarcinogen metabolism needs to be evaluated with reference to other xenobiotics entering the body, as well as to endogenous cytochrome P450 substrates. Because a compound able to induce one cytochrome P450 isozyme may either induce or repress other isozymes at the same time, directed induction to increase detoxification of one xenobiotic may actually increase activation or decrease detoxification of another xenobiotic. The possibility also exists that induction of one isozyme to detoxify a given promutagen may lead to the increased activation of other xenobiotics which are substrates for the same isozyme. In addition, altering xenobiotic metabolism may alter cytochrome P450 metabolism of medications, anesthetics, and endogenous compounds such as steroid hormones and cholesterol. For these reasons, the effect of any potential chemopreventive agent on overall cytochrome P450 metabolism needs to be thoroughly defined before that agent is added to the diet in pharmaceutical quantities.

LIST OF REFERENCES

LIST OF REFERENCES

Abbott, V., Deloria, L., Guenthner, T., Jeffery, E., Kotake, A., Nerland, D., Mannering, G. (1976) Comparison of hepatic microsomal drug-metabolizing systems from rats fed crude and purified diets. *Drug Met. Dispos.* 4:215-222.

Adesnik, M., Bar-Nun, S., Maschio, F., Zunich, M., Lippman, A., Bard, E. (1981) Mechanism of induction of cytochrome P450 by phenobarbital. *J. Biol. Chem.* 256:10340-10345.

American Institute of Nutrition. (1977) Report of the American Institute of Nutrition ad hoc committee on standards for nutritional studies. *J. Nutr.* 107:1340-1348.

Ames, B.N., Gold, L.S. (1990) Too many rodent carcinogens: mitogenesis increases mutagenesis. *Science* 249:970-971.

Aoyama, T., Yamano, S., Guzelian, P.S., Gelboin, H.V., Gonzalez, F.J. (1990) Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically activate aflatoxin B_1. *Proc. Natl. Acad. Sci.* 87:4790-4793.

Bergman, T., Postlind, H. (1990) Characterization of pig kidney microsomal cytochrome P450 catalysing 25-hydroxylation of Vitamin D_3 and C_{27} steroids. *Biochem. J.* 270:345-350.

Bhattacharyya, G.K., Johnson, R.A. (1977) *Statistical Concepts and Methods*. Wiley and Sons, New York.

Bock, K.W., Lipp, H-P., Bock-Hennig, B.S. (1990) Induction of drug-metabolizing enzymes by xenobiotics. *Xenobiotica*. 20:1101-1111.

Bork, R.W., Muto, T., Beaune, P.H., Srivastava, P.K., Lloyd, R.S., Guengerich, F.P. (1989) Characterization of mRNA species related to human liver cytochrome P450 nifedipine oxidase and the regulation of catalytic activity. *J. Biol. Chem.* 264:910-919.

Botelho, L.H., Ryan, D.E., Levin, W. (1979) Amino acid composition and partial amino acid sequence of three highly

purified forms of liver microsomal cytochrome P450 from rats treated with polychlorinated biphenyls, phenobarbital, or 3-methylcholanthrene. *J. Biol. Chem.* 254:5365-5640.

Bozak, K.R., Hong, Y., Sirevaeg, R., Christoffersen, R.E. (1990) Sequence analysis of ripening-related cytochrome P450 cDNAs from avocado fruit. *Proc. Natl. Acad. Sci.* 87:3904-3908.

Brown, R.R., Miller, J.A., Miller, E.C. (1954) The metabolism of methylated aminoazo dyes. IV. Dietary factors enhancing demethylation in vitro. *J. Biol. Chem.* 209:211-222.

Butler, L.E., Dauterman, W.C. (1988) The effect of dietary protein levels on xenobiotic biotransformations in F344 male rats. *Tox. Appl. Pharm.* 95:301-310.

Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J., Rutter, W.J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18:5294-5300.

Conney, A.H. (1967) Pharmacological implications of microsomal enzyme induction. *Pharmacol. Rev.* 19:317-366.

Conney, A.H., Burns, J.J. (1959) Stimulatory effect of foreign compounds on ascorbic acid biosynthesis and on drug-metabolizing enzymes. *Nature.* 184:363-364.

Conney, A.H., Burns, J.J. (1963) Induced synthesis of oxidative enzymes in liver microsomes by polycyclic hydrocarbons and drugs. in: *Advances in Enzyme Regulation, Vol. I.* ed.: George Weber. pp. 189-223. Macmillan Co., New York.

Conney, A.H., Gillette, J.R., Ensloe, J.K., Trams, E.R., Posner, H.S. (1959) Induced synthesis of liver microsomal enzymes which metabolize foreign compounds. *Science.* 130:1478-1479.

Conney, A.H., Miller, E.C., Miller, J.A. (1956) The metabolism of methylated aminoazo dyes. V. Evidence for induction of enzyme synthesis in the rat by 3-methylcholanthrene. *Cancer Res.* 16:450-459.

Conney, A.H., Miller, E.C., Miller, J.A. (1957) Substrate-induced synthesis and other properties of benzopyrene hydroxylase in rat liver. *J. Biol. Chem.* 228:753-766.

Coon, M.J., Ding, X., Pernecky, S.J., Vaz, A.D.N. (1992) Cytochrome P450: Progress and predictions. *FASEB J.* 6:669-673.

Coon, M.J., Persson, A.V. (1980) Microsomal cytochrome P450: A central catalyst in detoxication reactions. in: *Enzymatic Basis of Detoxication, Vol. I.* ed.: W.B. Jakoby. pp. 117-134. Academic Press, New York.

Cooper, D.Y., Levin, S., Narasimhulu, S., Rosenthal, O., Estabrook, R.W. (1965) Photochemical action spectrum of the terminal oxidase of mixed function oxidase systems. *Science.* 147:400-402.

Denison, M.S. (1992) personal communication.

DeWys, W.D., Costlow, R.D., Malone, W.F. (1986) The National Cancer Institute's Cancer Prevention Research Program. *J. Occup. Med.* 28:902-905.

Doehmer, J., Dogra, S., Friedberg, T., Monier, S., Adesnik, M., Glatt, H., Oesch, F. (1988) Stable expression of rat cytochrome P450IIB1 cDNA in Chinese hamster cells (V79) and metabolic activation of aflatoxin B_1. *Proc. Natl. Acad. Sci.* 85:5769-5773.

Dvorácková, I. (1990) *Aflatoxins and human health.* CRC Press, Boca Raton.

Faletto, M.B., Koser, P.L., Battula, N., Townsend, G.K., Maccubbin, A.E., Gelboin, H.V., Gurtoo, H.L. (1988) Cytochrome P_3-450 cDNA encodes aflatoxin B_1-4-hydroxylase. *J. Biol. Chem.* 263:12187-12189.

Farber, E. (1982) Sequential events in chemical carcinogenesis. in: *Cancer. Vol. 1: Etiology: Chemical and Physical Carcinogenesis.* ed.: F.F. Becker. pp. 485-506. Plenum Press, New York.

Fujita, T., Shoeman, D.W., Mannering, G.J. (1973) Differences in P450 cytochromes from livers of rats treated with phenobarbital and with 3-methylcholanthrene. *J. Biol. Chem.* 248:2192-2201.

Gelboin, H.V. (1967) Carcinogens, enzyme induction, and gene action. in: *Advances in Cancer Research.* eds.: A. Haddow and S. Weinhouse. pp. 1-81. Academic Press, New York.

Gornall, A.C., Baldwill, A.J., David, M.M. (1949) The determination of serum protein by means of a Biuret reaction. *J. Biol. Chem.* 177:751-761.

Halvorson, M.R., Safe, S.H., Parkinson, A., Phillips, T.D. (1988) Aflatoxin B_1 hydroxylation by the pregnenolone-16α-carbonitrile-inducible form of rat liver microsomal cytochrome P450. *Carcinogenesis.* 9:2103-2108.

Harada, N., Omura, T. (1981) Selective induction of two different molecular species of cytochrome P450 by phenobarbital and 3-methylcholanthrene. *J. Biochem.* 89:237-248.

Helferich, W.G., Jump, D.B., Anderson, D.B., Skjaerlund, D.M., Merkel, R.A., Bergen, W.G. (1990) Skeletal muscle α-actin synthesis is increased pretranslationally in pigs fed the phenethanolamine ractopamine. *Endocrinology.* 126:3096-3100.

Juchau, M.R. (1990) Substrate specificities and functions of the P450 cytochromes. *Life Sciences.* 47: 2385-2394.

Kato, R., Loeb, L., Gelboin, H.V. (1965) Microsome-specific stimulation by phenobarbital of amino acid incorporation *in vivo*. *Biochem. Pharmacol.* 14:1164-1166.

Kim, S.G., Reddy, S.L., States, J.C., Novak, R.F. (1991) Pyridine effects on expression and molecular regulation of the cytochrome P450IA gene subfamily. *Mol. Pharmacol.* 40:52-57.

Kimura, S., Gonzalez, F.J., Nebert, D.W. (1984) The murine *Ah* locus. *J. Biol. Chem.* 259:10705-10713.

Klingenberg, M. (1958) Pigments of rat liver microsomes. *Arch. Biochem. Biophys.* 75:376-386.

Kochs, G., Grisebach, H. (1989) Phytoalexin synthesis in soybean: purification and reconstitution of cytochrome P450 3,9-dehyroxy-pterocarpan 6α-hydroxylase and separation from cytochrome P450 cinnamate 4-hydroxylase. *Arch. Biochem. Biophys.* 273:543-553.

Koser, P.L., Faletto, M.B., Bansal, S.K., Caballes, L., Bresnick, E., Hines, R.N., Gurtoo, H.L. (1987) Aflatoxin B_1-4-hydroxylase is associated with cytochrome P_3-450 in C57BL/6 mouse liver. *Biochem. Biophys. Res. Comm.* 142:872-878.

Koser, P.L., Faletto, M.B., Maccubbin, A.E., Gurtoo, H.L. (1988) The genetics of aflatoxin B_1 metabolism. *J. Biol. Chem.* 263:12584-12595.

Lagopoulos, L., and Stalder, R. (1987) The influence of food intake on the development of diethylnitrosamine-induced liver tumors in mice. *Carcinogenesis.* 8:33-37.

Lötter, L.H., Kröhm, H.J. (1988) Occurrence of aflatoxins in human foodstuffs in South Africa. *Bull. Environ. Contam. Toxicol.* 40:240-243.

Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory. pp. 383-385, 466-468. Cold Spring Harbor, New York.

Mannering, G.J., Deloria, L.B. (1988) Hops and lupulon, an antibiotic component of hops, are potent inducers of cytochrome P450 systems. *FASEB J.* 2:1793.

Mannering, G.J., Shoeman, J.A., Deloria, L.B. (1992) Identification of the antibiotic hops component, colupulone, as an inducer of hepatic cytochrome P4503A in the mouse. *Drug Metab. Disp.* 20:142-147.

Marcus, C.B., Wilson, N.M., Kieth, I.M., Jefcoate, G.R., Omiecinski, C.J. (1990) Selective expression of cytochrome P450 isozymes by 4-*n*-alkyl-methylenedioxybenzenes in rat lung cells. *Arch. Biochem. Biophys.* 277:17-25.

Moran, M.F., Ebisuzaki, K. (1991) In vivo benzo[*a*]pyrene diol epoxide-induced alkali-labile sites are not apurinic sites. *Mut. Res.* 262:79-84.

Mungikar, A.M., Gothoskar, B.P. (1986) Partial purification of cytochrome P450 from human normal granulocytes. *Res. Commun. Chem. Pathol. Pharmacol.* 51:281-284.

Nash, T. (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* 55:416-421.

Nebert, D.W., Nelson, D.R., Coon, M.J., Estabrook, R.W., Feyereisen, R., Fujii-Kuriyama, Y., Gonzalez, F.J., Guengerich, F.P., Gunsalus, I.C., Johnson, E.F., Loper, J.C., Sato, R., Waterman, M.R., Waxman, D.J. (1991) The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. *DNA.* 10:1-14.

Okey, A.B. (1989) Enzyme induction in the cytochrome P450 system. *Pharmacol. Ther.* 45:241-298.

O'Keefe, D.P., Romesser, J.A., Leto, K.J. (1990) Identification of constitutive and herbicide-inducible cytochromes P450 in *Streptomyces griseolus*. *Arch. Microbiol.* 149:406-412.

Omenn, G.B., Goodman, G.E., Kleinman, G.D., Rosenstock, L., Barnhart, S., Feigl, P., Thomas, D.B., Kalman, D., Lund, B., Prentice, R.L., Henderson, M.M. (1988) The role of intervention studies in ascertaining the contribution of dietary factors in lung cancer. *Annals N.Y. Acad. Sci.* 534:575-583.

Omura, T., and Sato, R. (1964) The carbon-monoxide-binding pigment of liver microsomes. I. Evidence for its hemeprotein nature. *J. Biol. Chem.* 239:2370-2378.

Palmgren, M.S., Ciegler, A. (1983) Aflatoxins. in: *Handbook of Natural Toxins. Vol. 1: Plant and Fungal Toxins.* eds.: R.F. Keeler and A.T. Tu. pp. 299-323. Marcel Dekker, New York.

Parandoosh, Z., Fujita, V.S., Coon, M.J., Philpot, R.M. (1987) Cytochrome P450 isozymes 2 and 5 in rabbit lung and liver. Comparisons of structure and inducibility. *Drug Metab. Disp.* 15:59-67.

Parke, D.V. (1975) Induction of the drug-metabolizing enzymes. in: *Basic Life Sciences, Vol. 6, Enzyme Induction.* ed: D.V. Parke. pp. 207-271. Plenum Press, New York.

Pasanes, M., Pelkonen, O. (1986) Purification and immunological characterization of human placental mitochondrial cytochrome P450. *J. Steroid Biochem.* 24:669-675.

Peakall, D.B., Norstrom, R.J., Rahimtula, A.D., Butler, R.D. (1986) Characterization of mixed-function oxidase systems of the nestling herring gull and its implications for bioeffects monitoring. *Environ. Toxicol. Chem.* 5:379-386.

Pershing, L.K., Franklin, M.R. (1982) Cytochrome P450 metabolic-intermediate complex formation and induction by macrolide antibiotics; a new class of agents. *Xenobiotica.* 12:687-699.

Reddy, B.S. (1986) Dietary fat and cancer: specific action or caloric effect. *J. Nutr.* 116:1132-1135.

Remmer, H., Merker, H.J. (1965) Effect of drugs on the formation of smooth endoplasmic reticulum and drug-metabolizing enzymes. *Annals N.Y. Acad. Sci.* 123:79-97.

Ryan, D.E., Levin, W. (1989) Purification and characterization of hepatic microsomal cytochrome P450. *Pharmacol. Ther.* 45:153-239.

Santamaria, L., Bianchi, A., Arnaboldi, A., Ravetto, C., Bianchi, L., Pizzala, R., Andreoni, L., Santagati, G., Bermond, P. (1988) Chemoprevention of indirect and direct chemical carcinogenesis by carotenoids as oxygen radical quenchers. *Annals N.Y. Acad. Sci.* 534:584-596.

Scherer, E. (1989) Neoplastic cell stages and progression in experimental hepatocarcinogenesis. in: *Cancer Growth and Progression. Vol. 2: Mechanisms of Carcinogenesis.* ed.:

E.K. Weisburger. pp. 128-144. Kluwer Academic Publishers, Dortrecht, Netherlands.

Shimada, T., Guengerich, F.P. (1989) Evidence for cytochrome $P450_{NF}$, the nifedipine oxidase, being the principal enzyme involved in the bioactivation of aflatoxins in human liver. *Proc. Natl. Acad. Sci.* 86:462-465.

Shimada, T., Guengerich, F.P. (1990) Inactivation of 1,3-, 1,6-, and 1,8-dinitropyrene by cytochrome P450 enzymes in human and rat liver microsomes. *Cancer Res.* 50:2036-2043.

Shimada, T., Nakamura, S.I., Imaoka, S., Fuane, Y. (1987) Genotoxic and mutagenic activation of aflatoxin B_1 by constitutive forms of cytochrome P450 in rat liver microsomes. *Tox. Appl. Pharm.* 91:13-21.

Silver, G., Krauter, K.S. (1990) Aryl hydrocarbon induction of rat cytochrome P450d results from increased precursor RNA processing. *Mol. Cell. Biol.* 10:6765-6768.

Sladek, N.F., Mannering, G.J. (1966) Comparison of phenobarbital and methylcholanthrene induction of microsomal enzyme systems which N-demethylate ethylmorphine and 3-methyl-4-mono-methylaminoazobenzene. *Fed. Proc.* 25:418.

Sugimura, T. (1985) Carcinogenecity of mutagenic heterocyclic amines formed during the cooking process. *Mut. Res.* 150:33-41.

Sundseth, S.S., Nix, C.E., Waters, L.C. (1990) Isolation of insecticide-related forms of cytochrome P450 from *Drosophila melanogaster*. *Biochem. J.* 265:213-217.

Tagashira, Y., Yonekawa, H., Watanabe, J., Hara, E., Hayashi, J.I., Gotoh, O., Kawajiri, K. (1985) Metabolic activation of chemical carcinogens by two molecular species of cytochrome P450. in: *P450 and Chemical Carcinogenesis*. eds.:Y. Tagashira and T. Omura. pp. 69-79. Plenum Press, New York.

Tatsukawa, R. (1976) PCB Pollution of the Japanese environment. in: *PCB Poisoning and Pollution*. ed.: K. Higuchi. pp. 147-179. Academic Press, New York.

Tsubaki, M., Ohkubo, H., Tsuneoka, Y., Tomita, S., Hiwatashi, A., Ichikawa, Y. (1987) Existence of multiple forms of cytochrome $P450_{SCC}$ purified from bovine adrenocortical mitochondria. *Biochim. Biophys. Acta.* 914:246-258.

von Hofe, E., Newberne, P.M., Kennedy, A.R. (1991) Inhibition of *N*-nitrosomethylbenzylamine-induced esophageal

neoplasms by the Bowman-Birk protease inhibitor. *Carcinogenesis*. 12:2147-2150.

Wallin, H., Mikalsen, A., Guengerich, F.P., Ingelman-Sundberg, M., Solberg, K.E., Rossland, O.J., Alexander, J. (1990) Differential rates of metabolic activation and detoxication of the food mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by different cytochrome P450 enzymes. *Carcinogenesis*. 11:489-492.

Walther, B., Ghersi-Egea, J.F., Minn, A., Siest, G. (1986) Subcellular distribution of cytochrome P450 in the brain. *Brain Res*. 375:338-344.

Warburton, E.J., Magor, A.M., Trower, M.K., Griffin, M. (1990) Characterization of cyclohexane hydroxylase; involvement of a cytochrome P450 system from a cyclohexane grown *Xanthobacter* sp. *FEMS Microbiol. Lett*. 66:5-10.

Wiebel, F.J., Leutz, J.C., Diamond, L., Gelboin, H.V. (1971) Aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase in microsomes from rat tissues: differential inhibition and stimulation by benzoflavones and other organic solvents. *Arch. Biochem. Biophys*. 144:78-86.

Wong, J.J., Hsieh, D.P.H. (1976) Mutagenicity of aflatoxins related to their metabolism and carcinogenic potential. *Proc. Natl. Acad. Sci*. 73:2241-2244.

Wrighton, S.A., Scheutz, E.C., Watkins, P.B., Maurel, P., Barwick, J., Bailey, B.S., Hartle, H.T., Young, B., Guzelian, P. (1985) Demonstration in multiple species of inducible hepatic cytochromes P450 and their mRNAs related to the glucocorticoid-inducible cytochrome P450 of the rat. *Mol. Pharmacol*. 28:312-321.

Yamazaki, H., Degawa, M., Funae, Y., Imaoka, S., Inui, Y., Guengerich, F.P., Shimada, T. (1991) Roles of different cytochrome P450 enzymes in bioactivation of the potent hepatocarcinogen 3-methoxy-4-aminoazobenzene by rat and human liver microsomes. *Carcinogenesis*. 12:133-139.

Yang, C.S., Brady, J.F., Hong, J-Y. (1992) Dietary effects on cytochromes P450, xenobiotic metabolism, and toxicity. *FASEB J*. 6:737-744.

Yoo, J-S.H., Hong, J-Y., Ning, S.M., Yang, C.S. (1990) Roles of dietary corn oil in the regulation of cytochromes P450 and glutathione S-transferases in rat liver. *J. Nutr*. 120:1718-1726.