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I am submitting herewith a dissertation written by Juarine Stewart Easley entitled "Evidence for the Existence of Cytochrome(s) P-450 Specifically Induced by the Antioxidants Butylated Hydroxyanisole, Butylated Hydroxytoluene and Ethoxyquin." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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EVIDENCE FOR THE EXISTENCE OF CYTOCHROME(S) P-450 SPECIFICALLY INDUCED BY THE ANTIOXIDANTS BUTYLATED HYDROXYANISOLE, BUTYLATED HYDROXYTOLUENE AND ETHOXYQUIN

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ABSTRACT

The purpose of this study was to determine whether or not the antioxidants butylated hydroxyanisole, butylated hydroxytoluene and ethoxyquin induce the same type cytochrome P-450 as phenobarbital or 3-methylcholanthrene in the mouse. It had previously been shown that treatment of mice with antioxidants protected them against the toxicity, mutagenicity and carcinogenicity of environmental pollutants such as the polycyclic hydrocarbons, while treatment of these animals with a polycyclic hydrocarbon enhanced the harmful effects of these compounds.

Though the cytochrome P-450 system (mixed function oxidases) is found in such tissues as the liver, lung, spleen, intestine and skin, the hepatic system was the one chosen for study since it contains the greatest amount of these enzymes.

Several different procedures were used to study the cytochrome P-450 obtained from phenobarbital-, methylcholanthrene-, and antioxidant-treated mice. Zoxazolamine paralysis times were used to determine the genetics of zoxazolamine hydroxylase induction after pretreatment with butylated hydroxyanisole, butylated hydroxytoluene, ethoxyquin, phenobarbital or methylcholanthrene. Methylcholanthrene induction has been shown to be strain-specific, while phenobarbital induction is not. Since cytochrome P-450 is

the substrate binding site of the mixed function oxidase system, various compounds were used in enzyme assays to determine if the P-450 from the different sources could use these substances as substrates to the same degree. Carbon monoxide binding studies were used to determine the relative amounts of P-450 in the liver of the variously-treated animals. Type I and Type II binding studies were used to show how tightly the cytochrome from each source bind different type substrates. Sodium dodecyl sulfate gel electrophoresis was the method chosen to show whether or not the cytochrome obtained from each pretreatment gave the same protein profile, thus demonstrating whether the proteins are the same structurally. High pressure liquid chromatography was done to separate the different metabolites of radioactive benzo(a)pyrene obtained when this compound is reacted with a P-450 preparation from each pretreatment group. The radioactivity was used to measure the relative amounts of each metabolite formed. It was thought that the antioxidants might alter the types and/or the amounts of metabolites formed.

The major findings of this research were that P-450 induction by the antioxidants is not strain-specific, thus showing that they are more like the phenobarbital-type inducer in that they induce zoxazolamine hydroxylase activity in all strains of mice tested; each pretreatment produced a

cytochrome with a substrate specificity different from that of the others; the gel profiles of the cytochrome from each source showed that a different combination of protein moieties (as determined by molecular weight) was produced by each pretreatment; and the metabolite profile of the reaction of benzo(a)pyrene with the cytochrome from the variously-treated mice suggest taht each type cytochrome metabolizes the benzo(a)pyrene molecule primarily at different positions.

The overall conclusion drawn from these studies is that the antioxidants are not like any of the inducers previously studied. These observed differences between the antioxidants and other inducers such as the polycyclic hydrocarbons could account for the marked differences between these two classes of compounds seen on the effect they have on the toxicity, mutagenicity and the carcinogenicity of environmental pollutants and other harmful compounds.

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GENERAL INTRODUCTION

For many years now it has been known that many environmental pollutants, and other chemicals such as benzo(a)-pyrene and 2-amino-acetylfluorene, could cause cancer, mutations, and toxic responses in man and laboratory animals (Miller and Miller, 1952; Miller and Miller, 1948; Mueller and Miller, 1948; Miller et al., 1945; and Stevenson et al., 1942). Most of these compounds have two things in common, they are highly lipid soluble and are seemingly harmless until they are metabolized in vivo. This metabolism is now known to be carried out by enzymes termed mixed function oxidases (Mason, 1957) embedded in the membrane of the endoplasmic reticulum of cells. These enzymes have been found in such tissues as lung, kidney, intestine and skin, but they are most prevalent in the liver.

These enzymes remained enigmatic for some time because of the difficulty of isolating them in an active form. Since they are embedded in the microsomal membrane, it was necessary to use detergents to remove them. The detergent solubilizes the membrane that holds the enzymes and thus releases them into the solubilizing medium. This process usually left an inactive protein since the protein's activity is fragile and detergent solubilization is a harsh method. In 1968, Lu and Coon isolated a partially purified,

active form of the enzyme by using detergent to partly dissolve the membrane and then applying this preparation to a diethylaminoehtyl (DEAE) cellulose chromatographic column. By using this method they obtained a reductase-containing fraction, a heat-stable fraction, and an enzyme fraction. All three of these fractions were shown to be necessary for complete enzyme activity.

The reductase-containing fraction was shown to be NADPH-cytochrome c reductase, an enzyme that had been isolated earlier by Horecker (1950), but its biological significance had been uncertain. Studies by Williams and Kamin (1962), Phillips and Langdon (1962) and Masters et al. (1973) have now shown that this reductase is an electron carrier in microsomal hydroxylations.

Studies by Strobel et al. (1970) revealed that electron transfer in the microsomal enzyme system was completely dependent upon the presence of microsomal lipid. The lipid seems to provide the physiologically important medium in which these enzymatic reactions can occur. By trying a variety of lipids in reconstituted mixed function oxidase preparations, it was determined that phosphotidylcholine was the heat-stable factor involved in these reactions.

The only part of the system left that was still a mystery was the actual enzyme, which has subsequently been shown to be cytochrome P-450.

While doing further spectral studies on cytochromes located in the microsomal membrane (Strittmatter and Ball, 1952), Klingenberg (1958) demonstrated the presence of a pigment which in the reduced form could combine with carbon monoxide to form a complex that gave a broad band with maximum absorption at 450 nm. Comparative studies by Garfinkel (1963) showed that this "CO-binding pigment" or "pigment (P)-450" was present in a number of tissues including liver, kidney, lung, intestine, testes, adrenal glands, spleen, pancreas and muscle. Keilin (1930) showed that the cytochromes consisted of three spectrally distinct components, which were called a, b, and c, in order of their absorption maxima: a absorbing at the longest wavelength and c at the shortest. Keilin also obtained evidence that cytochrome b was closest to the substrate side when the cytochrome functioning as a redox carrier, transferring electrons or reducing equivalents from the enzymes on one side to the terminal acceptors at the other. Omura and Sato (1964a) showed that cytochrome P-450 was a cytochrome of the b type and that it could be isolated from the liver microsomal membrane in an inactive form (Omura and Sato, 1964b).

Ryan and Engle (1957), in measuring the hydroxylation of corticosteroids at carbon-21, noticed similarities between their work with adrenal microsomes and earlier work (such as that done by Miller and Miller, 1952) with the mixed function

oxidases found in the microsomes of the liver. Both systems required atmospheric oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH). They also found that the steroid 21-hydroxylase was inhibited by carbon monoxide and that the inhibition was reversed by light. These observations indicated that cytochrome P-450 could be present in the enzyme system, but Ryan and Engel did not detect a pigment that combined with carbon monoxide in their preparations. It was not until the studies of Cooper et al. in 1965, who used photochemical studies, that cytochrome P-450 was found to be the terminal oxidase of the mixed function oxidase system. Carbon monoxide was shown to inhibit the oxidative demethylation of codeine. This inhibition could be completely reversed by monochromatic light at 450 nm. Cytochrome P-450 was also indentified as the oxygen-activating component of aerobic hydroxylase systems. Treatment of animals with inducers of aerobic hydroxylase activity of liver microsomes resulted in a marked increase in microsomal P-450, as measured by the degree of carbon monoxide binding. These observations together convinced Cooper et al. that P-450 is the terminal oxidase of this enzyme system. This cytochrome was also found to be the substrate binding site of the MFO system (Lu et al., 1973).

Characteristic spectral changes occur when a substrate is added to a microsomal hydroxylase preparation. Using the

carbon monoxide-difference spectrum, Imai and Sato (1966) found that aniline combined with a microsomal component. The shape of the aniline difference spectrum suggested the involvement of a hemoprotein. Several lines of evidence pointed to cytochrome P-450 being the substrate-binding factor, the main one being that the magnitude of spectral change induced by an excess of aniline was proportional to the content of P-450 in a number of different preparations of liver microsomes containing various amounts of P-450. Similar experiments led Schenkman et al. (1967) to reach the same conclusion.

This cytochrome is now known to be an enzymatically active protein whose principal function appears to be the hydroxylation of a variety of compounds including drugs, pesticides and carcinogens and such endogenous substrates as steroids and fatty acids (Mason et al., 1973); McIntosh et al., 1973; Gillette et al., 1972; Gunsalus et al., 1971; Peterson, 1971; Gillettee et al., 1969; Kuntzman, 1969; Estabrook et al., 1968; Gunsalus, 1968; Conney et al., 1967; Gillette, 1967; Omura et al., 1966; and Omura et al., 1965). These studies also reaffirmed the fact that cytochrome P-450 does not act alone but is dependent on other components of the MFO system. In the mammalian microsomal system these components, the flavoproteins, NADPH, and NADH, may act in

transferring the electrons needed to reduce the cytochrome according to the following equation:

$$RH + O_2 + \underset{(NADH)}{NADPH} \qquad ROH + H_2O + \underset{(NAD)}{NADP}$$

where RH represents the substrate. NADPH has been found to be the primary electron donor in the liver MFO system (Coon et al., 1973).

Now that all of the components of the system had been isolated, it was then possible to put together a more complete reaction scheme (Figure 1).

Because of the implication of this enzyme system in chemical carcinogenesis, numerous studies are being done on it. The low basal level of these enzymes in all of the mammalian species tested has made it necessary to use inducers to increase their activity and thereby facilitate measurements of the system. Past studies have revealed that various hormones, drugs, insecticides, and carcinogens are inducers of this system (Conney, 1967). This enhancement of enzyme activity is referred to as induction. The induction of liver microsomal enzymes is important pharmacologically because it leads to an accelerated biotransformation of drugs in vivo. This could alter the duration and intensity of drug action in animals and man. It may also enhance the hydroxylation of steroid hormones and other normal body constituents.

The stimulatory effect of foreign compounds on the liver MFO system was observed by Brown et al. (1954) while studying

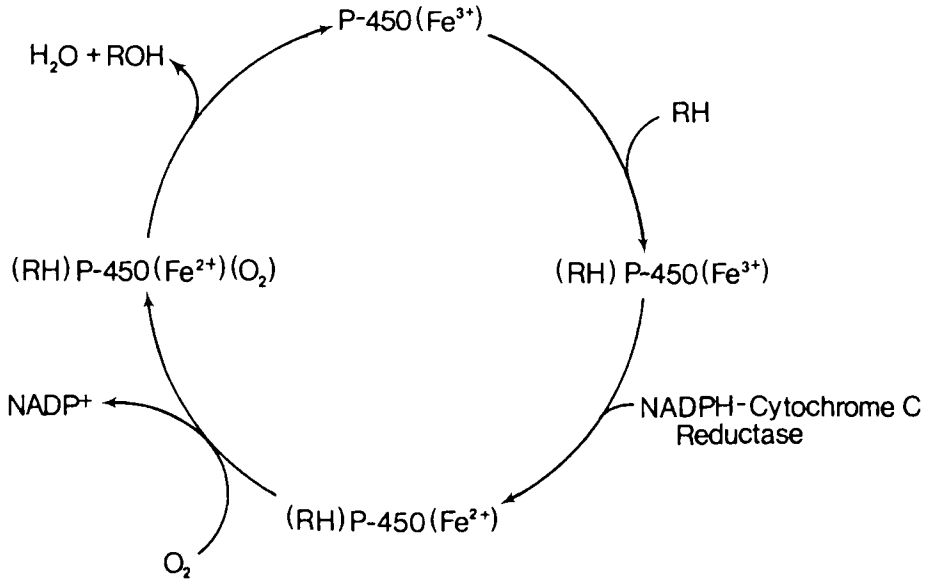

Figure 1. Overall scheme of a mixed function oxidase reaction.

the dietary factors that influence the activity of hepatic aminoazo N-dimethylase. Other studies (Burns, 1964; Gillette, 1963; Cramer et al., 1960; Conney et al., 1959; Reemer, 1959; Remmer, 1958; Gelboin et al., 1958; Conney et al., 1957; and Conney et al., 1956) have revealed that injection of small amounts of polycyclic hydrocarbons, barbituates and other drugs rapidly induce several-fold increases in the activities of these enzymes. It was also observed that the female of the species tested was always more inducible than the male of the species.

More than 500 drugs, insecticides, carcinogens and other compounds are now known to induce the mixed function oxidase system. "The characteristic pharmacological actions of these chemicals on the organism are extremely diverse and there is no apparent relationship between either their actions or structure and their ability to induce enzymes" (Conney, 1967). Most inducers are lipid-soluble at a physiological pH and the quantity of inducer necessary to have an appreciable effect on the MFO system varies by a factor of at least 30,000 (Niwa et al., 1975; Poland et al., 1974).

These inducers have been loosely classified into two categories: (1) those that induce the increased metabolism of a large number of chemicals and also induce a microsomal cytochrome that peaks at 450 nm when examined with carbon monoxide difference spectroscopy; and (2) those whose

inductive effects are directed toward a much smaller number of substrates and whose induced cytochrome peaks at 448 nm (Gillette et al., 1972; Hart and Fouts, 1965; and Conney, 1967). The first group is composed largely of drugs and insecticides. Sodium phenobarbital (PB) is a drug commonly used as an example of this group. The usual example of the second group which includes 3,4-benzo(a)pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin, is 3-methylcholanthrene (MC).

These inducers may cause their effect by increasing the rate of de novo protein synthesis, increasing the rate of activation of preexisting MFO components, or by decreasing the rate of degradation of these enzymes (Haugen et al., 1976). A combination of these events may also be at work in enzyme induction. Studies with MC (Gelboin, 1964; Gelboin and Sokoloff, 1964) and PB (Nebert and Gielen, 1971) have shown that there is a substantial increase in amino acid incorporation in vitro by rat liver microsomes and ribosomes. This induction also seems to occur in two phases, the first involving the synthesis of an induction-specific RNA and the second involving translation (Wiebel, 1974; Gelboin et al., 1972; and Nebert and Gelboin, 1970). Haugen et al., 1976 showed that these induction events are involved in de novo protein synthesis rather than any of the other above mentioned possibilities. These same induction events are involved in the experimental induction of mouse skin tumors (O'Brien et al., 1975) during the promotion stage, suggesting that the

in vivo metabolism (activation) of polycyclic hydrocarbons by the enzymes of the MFO system and the promotion of mouse skin tumors both involve gene activation.

Using the three components of the MFO system from several differently induced sources, it was demonstrated that the substrate specificity of the system changed only when a different cytochrome was used (Lu et al., 1972; Lu et al., 1971).

The substrate specificity of the MFO system differs in every species of animals tested and also according to the inducer used (Rasmussen and Wang, 1974; Conney et al., 1973). There are also differences between the strains and sex of animals of the same species. So by interchanging the reductase, lipid and cytochrome fractions from several sources, Lu et al., (1972 and 1974) showed that the substrate specificity of the MFO system resides in the cytochrome P-450 fraction.

Substantial evidence has accumulated to suggest that the difference observed with cytochrome P-450 could be due to multiple forms of this hemoprotein. Comai and Gaylor (1973) showed that rat liver microsomes contain three spectrally distinguishable forms of P-450. Each form is identified qualitatively and quantitatively by visible spectral changes that occur when the P-450's combine with various ligands, such as cyanide. The relative amounts of the three forms

were altered by treatment of the rats with 3-methylcholanthrene, phenobarbital, and dietary ethanol. Different rates of incorporation of (^{3}H)- or (^{14}C)- labelled ω-aminolevulinic acid into the heme moieties of the three forms also argue against their being the same cytochrome complexed with different tightly-bound endogenous substrates (Comai and Gaylor, 1973; Conney et al., 1973), but is compatible with there being a family of hemoproteins differing in the nature of the apoenzyme and their characteristics, yet very similar in spectral behavior.

Welton and Aust (1974) and Welton et al. (1975) used sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and antibody studies to show that there are three molecular weight classes of cytochrome P-450 present in rat liver microsomes and that the 45,000-dalton cytochrome induced by PB is immunologically distinct from the 53,000-dalton cytochrome which predominates in microsomes from untreated rats. Similar studies by Thomas et al. (1976) and diethylaminoethyl cellulose column chromatography by Ryan et al. (1975) support the hypothesis of at least three forms of cytochrome P-450. Alvares and Siekevitz (1973) used SDS gel electrophoresis to show that four forms of P-450 exist in the untreated animal.

Other compounds shown to induce a specific P-450 are ethanol (Villeneuve et al., 1976), 2,3,7,8-tetrachlorodibenzo-

p-dioxin TCDD (Johnson and Mueller-Eberhard, 1977), and β-naphthoflavone (Dean and Coon, 1977).

Antioxidants are also known to induce the cytochrome P-450 system (Branen, 1975; Botham et al., 1970; Creavan et al., 1969; Gilbert and Golberg, 1965). Several of these compounds, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and ethoxyquin, have been shown to decrease the toxic, mutagenic and carcinogenic effects of polycyclic hydrocarbons and other chemicals in laboratory animals.

Wattenberg (1972) demonstrated that BHA, BHT and ethoxyquin inhibit polycyclic hydrocarbon-induced carcinogenesis in the forestomach of the mouse and the mammary gland of the rat. BHA also reduces the formation of chemical carcinogen-induced pulmonary neoplasm in mice (Wattenberg, 1973).

Studies by Cumming and Walton (1973) showed that male mice are given substantial protection against mortality caused by toxic doses of ethyl methanesulfonate, n-propyl or isopropyl methanesulfonate, ethylene dibromide, diethylnitrosamine and cyclophosphamide when pretreated with BHA, BHT, or ethoxyquin. Cumming et al. (in preparation) also showed that the mutagenic effect of ethyl methanesulfonate (as determined by the dominant lethal assay) could be significantly reduced when the mice were fed BHT.

Wittig (1965) and Tappel (1965) suggested that antioxidants elicited their protective effects by stabilizing the lysosomal membranes, thus protecting them against injury by endogenous free radicals. However, Slaga and Bracken (1977) and Cumming and Walton (1973) have presented evidence that BHA and BHT do not act in their antioxidant capacity, that of free radical scavengers, when they protect cells against the harmful effects of some chemicals.

The exact mechanism(s) by which these antioxidants act has not yet been determined. Speier and Wattenberg (1975) offered two hypotheses: (1) some type of direct interaction may occur between the antioxidant and the reactive metabolites of polycyclic hydrocarbons; (2) the antioxidants may act indirectly to possibly alter enzyme activity (resulting in the alteration of the metabolism of polycyclic hydrocarbons). These hypotheses are broad enough to encompass any possible interaction between the antioxidants and any cellular constituent and therefore summarizes all the theories to date on this subject. Since it has already been shown that the antioxidants enhance the activity of the mixed function oxidase system, the second Wattenberg hypothesis was the one chosen to investigate for this thesis. An attempt is made in this study to determine the biochemical means by which the antioxidant inducers differ from other types of inducers (PB and MC), if at all, and to see whether these determinations help

explain how these compounds act to elicit their protective effects against carcinogens and mutagens. These studies have an additional importance in that these compounds, butylated hydroxyanisole, butylated hydroxytoluene and ethoxyquin, are widely used in fat-containing foods to retard spoilage.

In this thesis an in vivo study is made to determine whether or not the antioxidants induce zoxazolamine hydroxylase in all of mice (as does PB), or in just a few strains (as does MC). A measurement of the level of induction of several MFO enzymes, aminopyrine demethylase, hexobarbital demethylase, p-nitroanisole 0-demethylase, aniline demethylase, zoxazolamine hydroxylase and aryl hydrocarbon hydroxylase, caused by the antioxidant compounds is also made. These enzymes were chosen because they have been studied in earlier work with other inducers. A comparison between the effect of the antioxidant inducers and other inducers (such as PB and MC) would be helpful in determining if the antioxidants form a different class of inducer.

Sodium dodecyl sulfate polyacrylamide electrophoresis was done using microsomes from MC-, PB-, and antioxidant-treated animals to determine if the protein profiles are similar since a difference in the protein profiles would suggest the synthesis of a different protein by the antioxidant-treated animals. To test whether the antioxidant inducers altered the metabolic pathway of certain chemicals like the

polycyclic hydrocarbons, high pressure liquid chromatography was the procedure chosen since this method has been used successfully to separate many of the metabolites of the aromatic hydrocarbon benzo(a)pyrene.

CHAPTER 1

ZOXAZOLAMINE PARALYSIS TIMES

Introduction

Aryl hydrocarbon hydroxylase, AHH, one of the enzymes of the MFO system, is inducible in the tissues of many species of animals.

The basal and induced levels of AHH activity vary greatly among the various strains of mice, such as C57BL/6 and DBA. The level of the control enzyme as well as its capability for induction seems to be influenced by genetic factors (Nebert and Bausserman, 1971; Nebert and Gelboin, 1969). The first genetic experiments indicated that AHH induction by polycyclic hydrocarbons in mouse tissues was an all-or-none phenomenon. These experiments were done with C57BL (a highly inducible strain) and DBA (a noninducible strain). The genetics were expressed as a simple autosomal dominant trait (Nebert et al., 1972; Thomas et al., 1972). However, recent results with Af and AKR mice and offspring from genetic crosses between these inbred strains showed that this may not be the case (Burki et al., 1973a). Each mouse strain shows a particular pattern of susceptibility to enzyme inducers among hepatic and extrahepatic tissues (Wiebel et al., 1974; Burki et al., 1973b; Wiebel et al., 1973). The lack of inducibility in certain strains is specific to some tissues

such as liver, and may also be dependent upon the choice of inducers (Poland et al., 1974).

Studies have shown that the incidence of tumor in test animals is closely correlated with the genetics of AHH induction (Felton et al., 1976; Thorgeirrson et al., 1975; Thomas et al., 1973; Burki et al., 1973a; Nebert et al., 1973). These studies concluded that the more responsive the animals were to induction by the polycyclic hydrocarbons the higher the incidence of cancer in that strain.

Nebert et al. (1973) showed that this genetic specificity resides in the microsomal fraction containing cytochromes P-448 and P-450. AHH activity was examined in vitro with various combinations of the solubilized NADPH-cytochrome P-450 reductase, phospholipid, and cytochrome P-448 or P-450 fractions obtained from liver microsomes of B-naphthoflavone-treated C57BL/6J and DBA/2J strains of mice. Results showed that a strain difference toward the hydroxylation of benzo(a)pyrene was expressed only when the cytochrome was changed. Since there is a genetic relationship between AHH induction and carcinogenicity, mutagenicity, and cytotoxicity of certain chemicals, many studies are being conducted to determine the regulatory mechanism at work in controlling this induction.

Zoxazolamine hydroxylase has been reported to be associated with the same genetic locus (Ah) as aryl hydrocarbon hydroxylase (Robinson and Nebert, 1974). A simple

in vivo method of determining the activity at the Ah locus is to treat mice with zoxazolamine (a skeletal muscle relaxant) and measure the time required for them to regain their righting reflex. Zoxazolamine is mainly hydroxylated by the hepatic mixed function oxidase to 6-hydroxy-zoxazolamine a compound that has little or no muscle relaxant activity (Convey et al., 1960). An increase in the activity of the zoxazolamine metabolizing enzyme system has been shown to shorten the duration of action of this compound.

Materials

Zoxazolamine was purchased from McNeil Laboratories, Inc.

Mazola corn oil was obtained locally.

3-Methylcholanthrene was purchased from Eastman Kodak Co.

Sodium phenobarbital was obtained from Merck and Co., Inc.

Standard and treated diets were purchased from Tekland Laboratories. The diets were formulated by Dr. Ronald J. Rose, Director of Technical Services, Tekland Laboratories, Madison, Wisconsin. For complete description of diets see appendix. The special diets were used to eliminate the induction caused by the presence of BHT in regular animal feed.

Methods

The inbred strains of mice used were C57BL/10, C3Hf, C57BR, BALB/cs, A, SEC, SEA, CFCW, and 129. An outbred

strain, T, was also used. All mice were females, 10-14 weeks old at the time of experimentation. All strains are maintained in the Mammalian Genetics Section of the Biology Division of Oak Ridge National Laboratory.

Animals were maintained on a hardwood bedding (aspen) since studies by Vesell (1967) had shown that softwood bedding such as red cedar, white pine, or ponderosa pine contained substances that caused the induction of several mixed function oxidases. This induction was reversed when animals were placed on hardwood bedding. Preliminary studies conducted in this laboratory showed that animals kept on hardwood bedding had a much lower basal level of zoxazolamine hydroxylase and hexobarbital demethylase activity than those kept on softwood bedding. The bedding for all experiments was changed weekly. Animals had access to food and water ad libitum and were on a 12-hour light-dark cycle.

For control, MC-, and PB-treated groups, animals were on a standard high protein diet for at least two weeks. PB-pretreated animals were given a 1 mg/ml solution of sodium phenobarbital in the drinking water for seven days prior to the experiment. This procedure has been demonstrated to be more effective than the injection of PB (Marshall and McLean, 1969). MC-treated animals were injected intraperitoneally with 80 mg/kg body weight 3-methylcholanthrene dissolved in corn oil 48 hours prior to the experiment. For the other

pretreatments, animals were placed on diets containing 0.75% BHA, BHT, or ethoxyquin for two weeks prior to the experiment. This level of the antioxidants was used because it had been determined by Cumming and Walton in preliminary studies (personal communication) that it gave the maximum induction of the MFO system with the least incidence of death and harmful side effects to the animals tested.

Paralysis times were measured by the method of Robinson and Nebert (1974) with modification. Pretreated and control animals were injected with 200 mg/kg body weight zoxazolamine instead of the 225 mg/kg dose used by Robinson and Nebert since the nonspecific induction caused by softwood bedding and extraneous inducers in regular food pellets was reduced considerably by the use of hardwood bedding and the special diets. The higher dose killed many of the animals since they now had a lower tolerance for the drug. In a few minutes after the drug was administered, the animals became drowsy and were then placed on their backs. The paralysis time was measured from the time the animals could no longer stand to the time they regained their righting reflex.

Results

Table 1 shows the paralysis times determined with the ten strains of animals tested. Strains are classified as responsive (little or no paralysis time) or nonresponsive (paralysis time approaching control values), according to

Table 1. Zoxazolamine paralysis times (minutes).

Strains	Control	MC	PB	BHA	BHT	Ethoxyquin
C57BL/10	265.75 ± 29.24	0	22.50 ± 3.51	120.44 ± 13.92	41.75 ± 5.90	16.50 ± 2.59
C3Hf	309.50 ± 4.14	0	47.70 ± 10.07	162.20 ± 6.09	38.10 ± 2.77	39.60 ± 5.68
C57BR	243.83 ± 15.54	0	21.67 ± 9.29	100.33 ± 10.78	86.00 ± 10.39	---*
BALB/cs	254.20 ± 16.25	10.00 ± 12.70*	63.00 ± 15.22	120.75 ± 14.93	43.60 ± 3.91	25.20 ± 1.79
A	245.40 ± 1.67	36.27 ± 70.47*	28.33 ± 8.98	129.50 ± 21.60	50.20 ± 23.41	35.75 ± 0.96
SEC	228.33 ± 39.00	32.25 ± 18.19	21.50 ± 3.54	156.75 ± 32.88	33.25 ± 7.85	37.00 ± 3.46
SEA	284.67 ± 10.58	66.00 ± 19.80	28.00 ± 0.00	138.33 ± 0.58	57.67 ± 9.87	44.00 ± 2.82
CFCW	196.20 ± 36.61	95.75 ± 30.36	30.75 ± 2.63	94.40 ± 19.37	28.71 ± 3.15	19.60 ± 2.70
129	217.60 ± 48.51	153.80 ± 33.33	48.00 ± 18.49	109.60 ± 31.72	52.80 ± 9.34	---
T	283.40 ± 38.84	176.80 ± 18.68	36.89 ± 3.79	157.78 ± 28.50	48.00 ± 6.22	32.50 ± 4.93

--Each group contained 6-12 animals

*Animal did not survive regular or shortened pretreatment period of 1 week

the way mice respond to induction by polycyclic hydrocarbons (such as MC).

The data for MC-induced animals show that the C57BL/10, C3Hf, and C57BR strains are very responsive to this pretreatment (paralysis times of zero) while 129 and T are not (paralysis times of 153.80 and 176.80 minutes, respectively). The SEC, SEA, and CFCW strains form a middle group where the animals are not totally responsive to MC treatment but they are more responsive than the 129 and T strains. The paralysis times obtained for the BALB/cs and A strains are caused by an obvious dual response in these animals. Apparently some animals are paralyzed whereas others are not affected by the drug. This could be due to the genetic background of these strains, but the actual mechanism involved in this dual response is not known.

PB, BHT, and ethoxyquin cause a substantial lowering (approx. 65-94%) of the paralysis times of all strains as compared to the control values, though none of the animals are totally free from the effect of the zoxazolamine.

BHA lowers the paralysis times of all the strains by approximately 50%. This would tend to suggest that this compound is less effective as an inducer of zoxazolamine hydroxylase activity than either PB, BHT, or ethoxyquin, though they all reduce the effect of this drug in the mouse strains tested.

Discussion

The antioxidants seem to be good inducers of zoxazolamine hydroxylase activity in the strains tested. Unlike the polycyclic hydrocarbon inducer 3-methylcholanthrene, the antioxidants induce this hydroxylase activity in all strains. According to this test, BHA, BHT and ethoxyquin are more like the PB-type inducer than the MC-type since they cause a significant and consistent reduction in the test animals. There is not the genetic response with these pretreatments as there is with MC.

Ethoxyquin and BHT are as effective at reducing the paralysis times as PB, while BHA is significantly less so. This supports earlier work that suggested that BHA, while less toxic than BHT in rodents, is not as effective an inducer of the mixed function oxidase system (Gilbert and Golberg, 1967; Creavan et al., 1966). BHA also offered less protection against carcinogenic (Piekarski and Konkiewiez, 1975) and toxic substances (Cumming and Walton, 1973) than BHT. From the conclusions drawn from the earlier work and from preliminary studies with these antioxidants, it was expected that the antioxidants would decrease the zoxazolamine paralysis times of all the animals tested.

Determination of the zoxazolamine paralysis times is a short-cut method of testing the inducibility of the mixed function oxidase system in various strains or species of

animals. In this case it was used to determine whether the antioxidants induced the zoxazolamine metabolizing enzymes to the same extent in all strains (like PB) or only in certain strains (like MC). Most of the other inducers of the MFO system have fallen into the category of either the PB-type or MC-type inducer. At this point, it can be stated that the antioxidants are like the PB-type inducers.

The induction of drug metabolizing enzymes by the antioxidants BHA and BHT is of special importance since these antioxidants are used in a variety of foodstuffs used for human consumption. These compounds are known to cause an increase in the mixed function oxidase system of humans (Collings and Sharrat, 1970; Daniel et al., 1967). This increase of enzyme activity can cause significant modification in the level of therapeutic drugs prescribed for health reasons (Hunter and Chasseud, 1976). Knowledge of what biochemical changes the body undergoes from the constant onslaught of these inducers is critical. Studies such as the zoxazolamine paralysis times may be a step in the right direction.

CHAPTER 2

DETERMINATION OF THE SUBSTRATE SPECIFICITY OF THE ANTIOXIDANT-INDUCED ENZYME(S)

Introduction

Frequently used methods of increasing microsomal hydroxylase activities are by induction with phenobarbital and 3-methylcholanthrene (Gillette et al., 1972; Gram and Gillette, 1971; Gillette et al., 1969). However, any one of these treatments does not result in the simultaneous and equivalent increase of all the hydroxylation activities. In general, induction with PB increases the metabolism of many of the substrates of the microsomal enzymes while polycyclic hydrocarbons (such as MC) induce a smaller number. It has also been shown that PB increases the amount of NADPH-cytochrome c reductase in test animals, whereas MC does not (Gillette et al., 1972). These data suggest that the two inducers use different mechanisms to elicit their response. Another example of the difference between the inducers can be seen from the studies of Gnosspelius et al. (170). It was shown that PB and MC often increase the activities of the same enzymes but to different intensities. For example, induction of rat hepatic microsomes with PB increases aminopyrine demethylation while 3,4-benzo(a)pyrene hydroxylation is only slightly stimulated. MC, on the other hand,

causes significant induction of benzo(a)pyrene hydroxylase (AHH), with only a small increase in aminopyrine demethylase activity.

Previous studies have also shown that additive effects can be obtained by simultaneous induction with PB and MC (Gielen and Nebert, 1971; Bidleman and Mannering, 1970). If test animals are given doses of the inducers that are maximal for their characteristic enzyme inductions, and if then the enzyme activity observed is more than it would have been if either of the inducers were used separately, this would suggest that the inducers under investigation operated by different mechanisms. This was the theory considered when the antioxidant inducers were used in conjunction with either PB or MC in the multiple inducer study.

A study is made of the metabolism of aminopyrine, aniline, p-nitroanisole, hexobarbital, zoxazolamine and benzo(a)pyrene by the mixed function oxidase system of mouse liver. These compounds were chosen because detail procedures for the measurement of the enzymes responsible for their metabolism had already been developed and preliminary studies showed that with minor modifications these methods could be useful.

Materials

Aldrich Chemical Co., Inc., was the source for NADPH, aminopyrine, p-nitroanisole, aniline and benzo(a)pyrene. Hexobarbital was purchased from Merck and Co., Inc.

For source of zoxazolamine, diets and animals, see Chapter 1.

Methods

Animals and pretreatments: C57BL/10 female mice 10-14 weeks old at the time of sacrifice were used. The animals were treated with inducers as described in chapter 1, except for those studies in which more than one inducer was used.

For the studies with antioxidants plus PB, mice were on the special diets (which reduced extraneous induction) with regular water for a week. They then received a 1 mg/ml solution of PB in their drinking water for a week in addition to the special diets. For the combination of MC and antioxidants, mice were on the special diets for two weeks, with 80 mg/kg body weight MC being injected on the 12th day. The combination of PB and MC was accomplished by maintaining mice on the standard diet for one week, adding PB to the drinking water the second week and injecting MC 48 hours prior to the end of the two-week period.

Preparation of microsomes: Animals were killed by cervical dislocation. Three livers from each group were pooled and 33% homogenates prepared using 0.05 M Tris-chloride buffer containing 1.15% KCL (pH 7.6). The numbers of livers pooled and the concentration of the homogenate was determined by preliminary studies to be sufficient for the amount of enzyme activity needed and the number of assays to be

performed. The 9000g supernatant was centrifuged at 105,000g for 60 minutes. The pellets were resuspended in 0.25 M sucrose and stored at -24°C.

Aminopyrine and hexobarbital demethylase assays were done by the method of Lu et al. (1969) with few exceptions. The complete reaction mixture contained 150 µmoles potassium phosphate (KPO_4) buffer (pH 7.5), 10 µmoles magnesium chloride (M Cl_2), 5 µmoles semicarbizide, 0.5 µmole NADPH, 6.0 µmoles aminopyrine or 4.0 µmoles hexobarbital, and microsomes in a final volume of 1.5 ml. The samples were incubated for 20 minutes at 37°C and the reaction was stopped with 1.5 ml acetone.

The formation of formaldehyde was measured by the method of Nash as modified by Cochin and Axelrod (1959). One ml each of 20% $ZnSO_4$ and a saturated solution of $Ba(OH)_2$ was added to the samples. They were then centrifuged to sediment the precipitate. An aliqout of the supernatant (2.5 ml) was treated with 1 ml of double-strength Nash reagent. The treated samples were then left at room temperature in the dark to incubate for 3 hours before the color intensity was determined at 415 nm.

p-Nitroanisole O-demethylase and aniline demethylase activities were determined by the procedure of Hansen and Hodgson (1971) with changes. The incubation mixture contained 2.0 µmoles p-nitroanisole or 100 µmoles aniline, 0.01 M NADPH,

7.5 mM $MgCl_2$, 0.05 M Tris buffer (pH 7.9) and microsomes in a total volume of 4 ml. The substrates were dissolved in methanol instead of buffer as directed by the Hansen and Hodgson method because it was determined that even with extraordinary procedures, enough of the substrates would not dissolve in the buffer to give the appropriate concentrations. The reaction tubes were then incubated at 37° for 30 min.

For determination of p-nitrophenol, the product of O-demethylation of p-nitroanisole, the reaction was stopped with 1 ml of 1 N HCl. The acidified contents were shaken with an equal volume of chloroform and centrifuged. The chloroform layer was then shaken with 5 ml of 0.5 N NaOH and centrifuged. The absorbance at 400 nm of the samples was then determined.

Aniline hydroxylation was determined by measuring the production of p-aminophenol after the method described by Schenkman et al. (1967). The reaction of the aniline samples was terminated by the addition of 1.5 ml of 20% trichloro-acetic acid and centrifuged at 18,000g for 5 min. To 1 ml of the supernatant was added 1 ml of 0.5 M NaOH containing 1% phenol and 1 ml of 1 M Na_2CO_3. This mixture was incubated for 20 minutes at room temperature before the color intensity was determined at 630 nm.

Zoxazolamine hydroxylase activity was determined by the method of Robinson and Nebert (1974) with modifications. The

reaction mixture contained 50 µmoles KPO_4 buffer (pH 7.0), NADPH, 120 nmoles zoxazolamine and microsomes. The substrate was dissolved in a minimal amount of 1N HCl and then diluted with water to the final concentration. The samples were incubated at 37° for 20 min. before the reaction was stopped by adding 1.0 ml of 0.5 N NaOH. This mixture was extracted with 2 ml of cold ethylene dichloride and centrifuged briefly. The ethylene dichloride extract was shaken with 1.0 ml 0.3 M sodium borate and the aqueous fraction was removed. The unmetabolized zoxazolamine was extracted from the ethylene dichloride into 2 ml of 1N HCl and was measured spectrophotometrically at 278 nm.

Aryl hydrocarbon hydroxylase assay was done by the method of Nebert and Gelboin (1968). The reaction mixture contained 50 µmole Tris buffer (pH 7.5), 0.36 µmole NADPH, 3 µmole $MgCl_2$, 0.80 nmole benzo(a)pyrene (added in 0.04 ml of methanol just prior to incubation) and microsomes in a total volume of 1 ml. The mixture was shaken at 37° for 30 min. before the reaction was stopped by the addition of 1.0 ml cold acetone. After the addition of acetone, 3.25 ml hexane was added and the sample was incubated with shaking for 10 min. at 37°. The organic phase was extracted with 3.0 ml of 1N NaOH and the concentration of hydroxylated benzo(a)pyrene in the alkali phase was determined spectrophotofluorometrically with activation at 396 nm and fluorescence at 522 nm.

Protein determinations for all of the microsomal preparations were done according to the procedure of Lowry et al. (1951).

All spectrophotometric readings were done on a Beckman Acta CIII spectrophotometer. Fluorimetric readings were made on an Aminco-Bowman Spectrophotofluorimeter.

Results

The values presented in Table 2 are the ratios of the specific activities of the induced enzymes. This table lists the types of activities assayed and the amount of increase over the control value for each type of treatment.

The MC-induced microsomes was very effective in demethylating p-nitroanisole and in hydroxylating benzo(a)-pyrene, as indicated by the ratios 8.20 and 6.02, respectively. The remainder of the substrates were not substantially affected by the MC enzyme. Phenobarbital induced enzymes that could use the compounds tested as substrates, though the p-nitroanisole O-demethylase activity seemed to be induced to the greatest intensity. p-Nitroanisole O-demethylase was the only enzyme activity increased by pretreating the animals with BHA. The activities obtained for the remaining substrates did not differ from the control values significantly except for the slight inhibitory response observed for aminopyrine demethylase. BHT also induced the O-demethylase enzyme to a significant level. There is also a small amount

Table 2. Ratio of specific activities of enzymes tested.

Type	Enzyme Assay	Pretreatments					
		None	3-MC	PB	BHA	BHT	Ethoxyquin
I	p-nitroanisole O-demethylase	1	8.20 ± 3.26*	7.14 ± 2.92	2.09 ± 0.28	2.75 ± 0.99	3.33 ± 1.05
II	Aniline demethylase	1	1.53 ± 0.06	3.13 ± 1.52	1.22 ± 0.19	1.56 ± 0.18	1.53 ± 0.31
I	Hexobarbital demethylase	1	1.27 ± 0.25	3.62 ± 0.53	1.04 ± 0.30	1.75 ± 0.50	2.61 ± 0.83
I	Aminopyrine demethylase	1	1.41 ± 0.32	3.35 ± 1.45	0.82 ± 0.26	1.37 ± 0.34	1.87 ± 0.62
	Aryl Hydro-carbon hydroxylase	1	6.02 ± 1.64	2.54 ± 0.80	1.15 ± 0.46	1.02 ± 0.39	1.21 ± 0.38
II	Zoxazolamine hydroxylase	1	1.44 ± 0.40	1.99 ± 0.52	1.48 ± 0.59	1.51 ± 0.46	2.11 ± 0.64

*Value is ratio of specific activities (induced value ÷ control value)

of hexobarbital demethylase activity induced by this pretreatment. Ethoxyquin increases the activities of more of the enzymes tested than either of the other antioxidants. p-Nitroanisole O-demethylase, hexobarbital demethylase, aminopyrine demethylase, and zoxazolamine hydroxylase were the enzymes induced by the ethoxyquin pretreatment. This data indicates that each of the treatments causes the induction of at least one of the enzyme activities tested. Except for the increase in p-nitroanisole O-demethylase activity by all the inducers, there is no correlation between the induction pattern of any of the compounds.

Table 3 gives the results of the effects of a combination of inducers on these same enzyme activities. In both studies the animals were given the amount of inducer necessary to obtain the maximal response in these enzyme assays. Even with the antioxidants which are given in the food pellets, it was determined by Cummin and Walton (1973) that the concentration of antioxidant given and the time allowed for the mouse to consume the food was sufficient to produce the maximum stimulation of the mixed function oxidase system for any group of animals tested. So it should be possible from this study to determine if any of the combinations of inducers used cause an additive effect, which would be indicative of the involvement of different mechanisms of action by those inducers.

Table 3. The effect of combined inducers.

Enzyme Activities	Pretreatments							
	Control	PB + MC	BHA + MC	BHT + MC	Ethoxyquin + MC	BHA + PB	BHT + PB	Ethoxyquin + PB
p-nitroanisole O-demethylase	1	3.43 ± 0.27*	4.40 ± 0.79	3.82 ± 0.41	3.70 ± 0.41	2.44 ± 0.21	2.04 ± 0.02	1.91 ± 0.14
Aniline demethylase	1	2.32 ± 0.19	1.87 ± 0.63	1.42 ± 0.03	1.74 ± 0.02	1.97 ± 0.50	2.11 ± 0.20	1.22 ± 0.04†
Hexobarbital demethylase	1	0.96 ± 0.03†	1.02 ± 0.03†	1.22 ± 0.03†	1.28 ± 0.06	1.05 ± 0.04†	1.21 ± 0.05	0.96 ± 0.16†
Aminopyrine demethylase	1	1.51 ± 0.11	1.21 ± 0.14†	1.61 ± 0.07	1.58 ± 0.12	1.64 ± 0.05	1.74 ± 0.04	1.58 ± 0.21
Aryl hydrocarbon hydroxylase	1	4.63 ± 0.32	5.22 ± 0.34	6.52 ± 1.76	4.94 ± 0.01	3.00 ± 0.70	1.80 ± 0.17	1.38 ± 0.48
Zoxazolamine hydroxylase	1	3.02 ± 0.62	3.38 ± 0.67	2.75 ± 0.36	2.99 ± 0.73	2.79 ± 0.19	2.73 ± 0.22	3.54 ± 0.53

*Values are ratios of specific activity

†Values not significantly different from 1 at 95% confidence level

From the data presented it seems that the combined effects of PB and MC gives additive results only in the zoxazolamine hydroxylase assay, when these values are compared to the values for the single inducers from Table 3. The other enzyme activities are inhibited by this combination of inducers. The same is true for the BHA+MC, BHT+MC, BHT+MC, and ethoxyquin+MC combinations.

The combinations of antioxidant and phenobarbital show inhibition of all the enzymes assayed, though for BHA+PB the aryl hydrocarbon and zoxazolamine hydroxylases are nearly additive. The same is true for the zoxazolamine hydroxylase activity obtained with ethoxyquin+PB.

Discussion

The results from Table 2, page 32, indicate that the pattern of enzyme activities increased by each of the inducers is different. As discussed earlier, MC treatment causes a significant increase in p-nitroanisole O-demethylase and aryl hydrocarbon hydroxylase activities. PB also causes a great increase in the p-nitroanisole metabolizing enzyme and at least a 2-fold increase in all of the other activities measured. This data supports the conclusiosn reached by previous studies which found that pretreatment of test animals with MC caused an increase in only a few of the mixed function oxidases while PB treatment increases the enzyme activity of many of these oxidases (Gillette et al.,

1972; Gram and Gillette, 1971; Gillette et al., 1969; Conney, 1967). The enzyme(s) induced by the antioxidants seem to be able to recognize p-nitroanisole as a substrate, but here the similarity stops. BHA gives a very small increase in the zoxazolamine hydroxylase activity when compared to the control. The only other enzyme activity enhanced by pretreatment with BHT is the hexobarbital hydroxylase. Ethoxyquin seems to be the more versatile of the antioxidant inducers in that it increases the activity of hexobarbital and zoxazolamine hydroxylases, aminopyrine demethylase, as well as the p-nitroanisole O-demethylase.

Each of the inducers tested also increased the activity of whatever enzyme they induced to a different intensity than any of the other compounds. For example, the p-nitroanisole O-demethylase activity is increased 8-fold in the microsomes obtained from MC-treated animals, 7-fold in PB-treated animals and from 2- to 3-fold in the antioxidant-treated animals. The induction ratio for hexobarbital demethylase activity in mice given PB is 3.62, it is only 1.75 and 2.61 in mice given BHT and ethoxyquin, respectively. It would seem then that each of these pretreatments uses a different mechanism of induction.

To further test this hypothesis, the effect of multiple inducers on these enzyme activities was tested. If additive effects are observed it would serve to strengthen the

argument for different induction mechanisms. Table 3, page 34, gives the results of that study. By comparing these data with the ones obtained for Table 2, page 32, for the single inducers it is possible to discern most of the combinations of inducers used resulted in the inhibition of the enzymes. The inducer combination of PB and MC gave additive results only for the zoxazolamine hydroxylase. The other activities measured in this group gave results significantly less than additive. The ratios for aniline demethylase and aryl hydrocarbon hydroxylase seems to be the average of the ratios for each of the inducers when used separately. In the BHA+MC study it appears that the BHA may inhibit the action of the MC in several instances. The p-nitroanisole O-demethylase activity and the aryl hydrocarbon hydroxylase activity are both reduced from the values obtained when MC is used alone when BHA is used in combination with the MC. The ratio for aniline demethylase in the combined inducer study is higher for BHA+MC than it is for either of the indicers used alone, but it is far from additive. The MC apparently cancels any inhibitory effect of BHA treatment on aminopyrine demethylase since no inhibition is observed when the inducers are used together. Only the ratio for zoxazolamine hydroxylase shows any additive response for this group.

The effect of MC treatment on p-nitroanisole O-demethylase is also reduced when this inducer is used in

combination with the antioxidant BHT. The slight stimulation of the hexobarbital demethylase activity by treatment of mice with BHT is cancelled when BHT+MC is used to induce the animals. The value for aryl hydrocarbon hydroxylase is the same for the combined inducers as for MC used alone. Again, only the zoxazolamine hydroxylase ratio shows an additive effect.

When used alone ethoxyquin causes the stimulation of hexobarbital demethylase, aminopyrine demethylase, zoxazolamine hydroxylase and p-nitroanisole O-demethylase. In combination with MC the hexobarbital and aminopyrine enzyme activitie are diminished, while the value obtained for the O-demethylase is significantly reduced from the 8-fold increase obtained when MC is used alone. Zoxazolamine hydroxylase is the only activity that gives additive results.

When the antioxidants were used in combination with PB, the results again, did not follow any pattern. BHA+PB treatment caused a cancellation of the inhibitory response observed for the aminopyrine demethylase activity when BHA was used alone. There was also near-additive results obtained for the aryl hydrocarbon hydroxylase and zoxazolamine hydroxylase activities. BHT+PB gave results for three of the enzyme activities that suggest that the effects of these two inducers is being averaged. The ratios for aniline demethylase and zoxazolamine and aryl hydrocarbon hydroxylases

show this averaging effect. There was a significant decrease in the value for the O-demethylase activity when ethoxyquin+PB was used as compared to the value obtained when either of the inducers was used alone. The same is true of the ratio for hexobarbital demethylase. There is a near-additive response for the zoxazolamine hydroxylase activity.

This inhibition of the enzyme activities is not easy to explain. It is possible that one inducer may increase the enzyme(s) responsible for the metabolism of the other and thereby decrease the number of total inducer molecules available to initiate the induction mechanism. It is also possible that some of the inducer molecules interact with each other leading to the same depletion of necessary molecules to start the induction process.

From Table 2, page 32, it is seen that the substrate specificity of the enzymes induced by each of the pretreatments differ considerably. It may be that the product measured in each assay is not the product being formed by each of the induced enzymes. Further studies on the possible alternate metabolites of these substrates need to be done. This may also aid in explaining the results obtained with the combined inducers. It could be possible that such inducers used in combination may alter the metabolic pathway of the substrate.

One theory that can be drawn from these studies is that the inducers used may operate by different mechanisms since the pattern of inhibition for each combination is different.

CHAPTER 3

DETERMINATION OF THE CYTOCHROME PRESENT IN ANTIOXIDANT INDUCED MICROSOMES

Introduction

The presence in liver microsomes of a carbon monoxide-binding pigment was reported by Garfinkel (1958) and Klingenberg (1958). This pigment was discovered by Garfinkel when he was doing further spectral work on the cytochromes previously studied by Strittmatter and Ball (1952). Klingenberg observed that the total hemin content of rat liver microsomes was twice as much as could be accounted for by cytochrome b_5 and when the microsomes were reduced and treated with carbon monoxide (CO), a broad absorption band at 450 nm appeared. Omura and Sato (1964a) showed that there was competition between CO and ethylisocyanide for the dithionite-reduced microsomes. It was then concluded that the microsomal CO- and ethylisocyanide-binding pigments were identical with each other. This, in turn, suggested that the pigment (P)-450 was a hemoprotein since the binding of reduced hemoproteins with ethylisocyanide is a characteristic of that class of compounds.

Carbon monoxide is known to inhibit the oxidative demethylation of codeine and monomethyl-4-aminopyrine and the hydroxylation of 17-hydroxyprogesterone at carbon-21

(Cooper et al., 1964). It was observed by these investigators that this inhibition could be reversed by bands of monochromatic light and that the maximum reversal was obtained at 450 nm, the light absorption maximum of cytochrome P-450. The agreement between the photochemical action spectrum and the spectrophotometric difference spectrum suggested that the CO-binding pigment was the terminal oxidase of mixed function oxidase systems of mammals. The method of establishing the nature of the terminal oxidase of a respiratory chain was demonstrated by the classic studies of Warburg (1949) on the influence of monochromatic light on the reversal of the CO inhibition of cellular respiration.

For the first time a function could be assigned to the CO-binding pigment of microsomes.

The carbon monoxide difference spectrum can be used to detect different forms of this cytochrome present in hepatic microsomes from variously-treated animals by showing a shift in the absorption maximum. MC is known to induce a cytochrome that peaks at 448 nm instead of 450 nm, while the PB-induced carbon monoxide difference spectrum has the same maximum as that of the untreated microsomes. This technique is used here to determine if the BHA, BHT, and ethoxyquin-treated microsomal preparations contain a cytochrome like any of the ones previously studied. The intensity of the CO peak can also be used to measure the relative amounts of cytochrome induced by each treatment.

Materials

Carbon monoxide was purchased from Matheson Gas Co., and the sodium dithionite was purchased from Fisher Scientific Co.

Methods

C57BL/10 female mice 10-14 weeks old at time of sacrifice were used. Treatment of animals with inducers and preparation of microsomes were done as described in Chapter 2, except that samples were recentrifuged after the first resuspension to get rid of any hemoglobin that might interfere with spectra.

Carbon monoxide difference spectra were done according to the procedure of Omura and Sato (1964a), using a Beckman Acta CIII spectrophotometer equipped with a turbid sample attachment. An equal volume of the microsomal suspension was delivered to both sample and reference cuvettes. A baseline was obtained by scanning the difference between the two cuvettes from 500 nm to 350 nm. Sodium dithionite was then added to both cuvettes and CO was bubbled through the sample cuvette for one minute. The scan was repeated between the above-mentioned wavelengths.

Results

Figure 2 is a composite of the carbon monoxide difference spectra obtained when each of the microsomal preparations were reduced with dithionite. All of the treatments induced a cytochrome with a maximum peak at 450 nm except MC, which peaked at 448 nm.

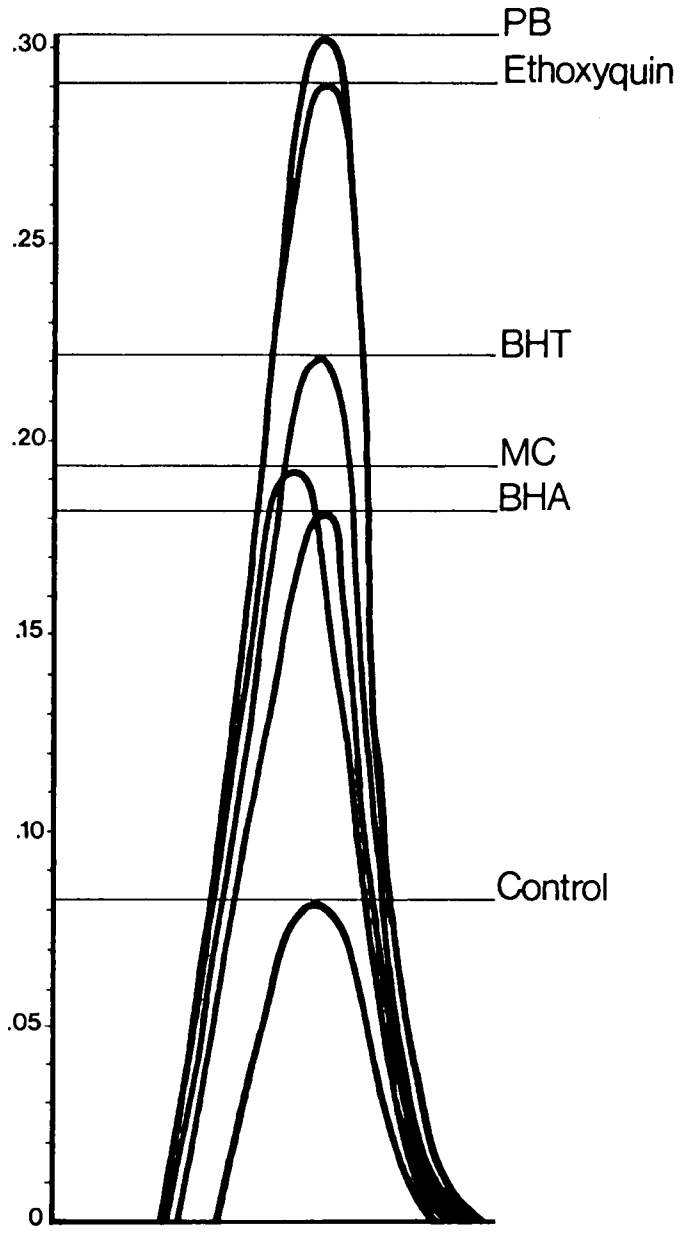

Figure 2. Composite of cytochrome P-450 and P-448 difference spectra.

Since the same amount of protein was used for each spectrum, it is possible to estimate the relative amounts of P-450 induced by each pretreatment from the heights of the peaks. All the pretreatments cause a substantial increase in the amount of cytochrome P-450 in mouse liver microsomes. BHT and BHA increase P-450 levels approximately 2-fold. Ethoxyquin and PB both cause a greater than 3-fold induction of P-450 over the control value. The amount of P-448, induced by MC, is twice the amount of P-450 present in the control animals. The values for PB and MC are in good agreement with previously published data (Remmer et al., 1967; Mannering et al., 1968).

Discussion

Since BHA-, BHT-, and ethoxyquin-induced microsomal preparations all caused a peak at 450 nm in the carbon monoxide difference spectra, one would assume that the cytochrome present in these samples is the same spectrally as the ones in the control and PB-induced preparations.

Recently, it has been shown that although the PB-induced microsomes yield one peak at 450 nm when treated with dithionite and carbon monoxide, there may actually be more than one cytochrome present in these preparations. Ingelman-Sundberg and Gustafsson (1977) used isoelectric focusing to show that PB-induced rabbit liver microsomal cytochrome P-450 can be resolved into four heme containing forms with

isoelectric points between pH 7 and 9. Immunochemical studies by Thomas et al. (1976) demonstrated that multiple forms of cytochrome were present in the P-450 fraction from PB-treated rats. Other studies have shown at least two forms of P-450 from this source (Haugen et al., 1975; van der Hoeven and Coon, 1974; van der Hoeven et al., 1974). In all cases it was reported that each of these forms was responsible for the metabolism of a particular substrate from the "pool" of substrates PB-induced animals are known to metabolize, suggesting that the substrate specificity of the PB-induced system is dependent upon the presence of all of these different forms of the cytochrome. The same situation may exist for each of the other cytochromes (those induced by BHA, BHT, MC and ethoxyquin).

The discovery of multiple forms of the cytochrome(s) induced by antioxidant treatment could account for the differences in substrate specificity reported in Chapter 2. Some of the inducers may not enhance the activities of the form of cytochrome responsible for the metabolism of certain substrates.

It has already been shown that the mixed function oxidase system is involved in the detoxification of foreign compounds that enter the body but it is also involved in the activation of certain compounds to more harmful intermediates (Gillette, 1963). If, then, the cytochromes are present in multiple

forms in induced animals it is possible that one or more of the forms could be involved in activation, while other forms are active in the detoxification of toxins, mutagens, and carcinogens. The observed effects of the exposure of induced animals to harmful substances would then be the resulting balance of these two processes.

Additional studies on the possible involvement of multiple forms of the cytochrome induced by antioxidant treatment needs to be done before it is decided if these cytochromes are the same as that induced by phenobarbital. A later chapter of this report deals with this problem.

CHAPTER 4

TYPE I AND TYPE II BINDING

Introduction

When substrates, inhibitors or other compounds bind to the various species of cytochrome P-450, they cause characteristic shifts in the oxidized difference spectra (Schenkman et al., 1967). Those compounds that produce a shift to lower wavelengths (maximum at 390 nm; minimum at 420 nm) are referred to as type I, whereas substances causing a shift to higher wavelengths (maximum, 420 nm; minimum, 390 nm) are type II compounds. Type I spectra are caused by many compounds including drugs, pestidices, and steroid hormones, while type II are caused by aromatic and aliphatic amines, isocyanides, and a modified type II from certain steroids and alcohols (Hodgson, 1976). Schenkman et al. (1967) suggested that type II spectra were due to the formation of a ferrihemochrome from a basic amine and a ferrihemoprotein. Mailman et al. (1973) concluded from their studies that type II spectra are caused by ligands with a nitrogen atom in which sp^2 or sp^3 nonbonded electrons are sterically accessible. Type I spectra, on the other hand, are believed to be due to interaction with a lipophilic site close to but not at the heme iron. If either of these spectra were a reflection of the formation of an enzyme-substrate complex,

a correlation between spectral binding and the rate of metabolism might be expected, but as of yet this possibility has not been investigated.

Materials

Benzphetamine was purchased from Upjohn Laboratories and pyridine was purchased from Eastman Kodak Co.

Methods

The binding spectra were done by the procedure of Schenkman et al. (1967) using benzphetamine as the type I substrate and pyridine as the type II substrate. Both the sample and reference cuvettes contained an equal volume of the microsomal suspension. Readings were taken from 500 nm to 350 nm to get a baseline. Benzphetamine or pyridine was then added to the sample cuvette and the scan repeated. These measurements were made using a Beckman Acta CIII spectrophotometer equipped with a turbid sample attachment.

The microsomes were prepared as described in Chapter 3.

C57BL/10 female mice were used. They were 10-14 weeks old at the time of sacrifice. Treatments with inducers were as described in Chapter 2.

Results

Figure 3 shows that type I and type II substrates tend to bind to microsomes from variously-treated mice to different degrees. In increasing order of binding (as determined by

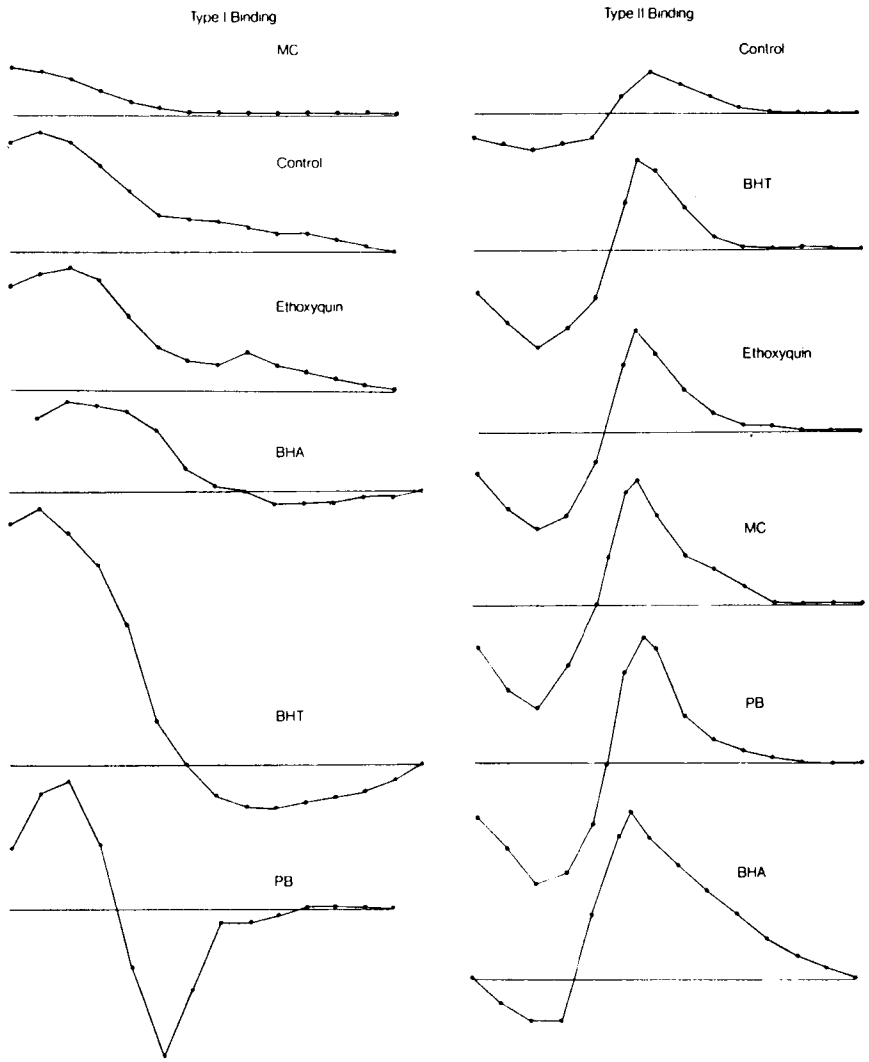

Figure 3. Type I and type II binding spectra of enzyme from variously-pretreated animals.

peak height) benzphetamine (type I) binds microsomes from MC-, control, ethoxyquin-, BHT-, BHT-, and PB-treated mice. Pyridine binds control, BHT-, ethoxyquin-, MC-, PB-, and BHA-treated microsomes to an increasing degree.

Discussion

There seems to be no established pattern to the binding of the type I and type II substrates to the microsomes of the induced animals. If this binding is a reflection of the formation of an enzyme-substrate complex, which is necessary for enzyme activity, it should be possible to correlate the extent of binding to the rate of metabolism of type I and type II substrates. One might predict then that PB, which shows the greatest tendency to bind type I substrates (Figure 3), would be better able to use p-nitroanisole (type I substrate) than MC. Yet, checking Table 2, page 32, one sees that this is not the case. p-Nitroanisole is a very good substrate for the MC-induced enzyme, though the type I binding study showed that microsomes from MC-induced animals do not bind these type substrates. The same is true for the metabolism of benzo(a)pyrene.

The type I binding spectra of methylcholanthrene could be obscured if methylcholanthrene itself is bound to the enzyme. This phenomenon would interfere with the binding of the benzphetamine used in the experiment. Other studies have shown that the microsomes isolated from MC-treated animals

contain only negligible amounts of the compound (Shoeman et al., 1973; Fujita and Mannering, 1971). Using radioactive MC, it was determined that very little, if any, radioactivity was present in the microsomal preparations. It may be possible that benzphetamine is not a good substrate for the MC-induced enzyme, whereas p-nitroanisole has a stronger tendency to bind to this enzyme.

The degree of type I and type II binding also does not compare to the amount of P-450 induced by each of the pretreatments, except phenobarbital. An increase in the amount of cytochrome P-450 is indicative of an increase in the binding of substrates to the enzyme since P-450 was determined to be the substrate-binding site of the mixed function oxidase system (Lu et al., 1973). According to Figure 2, page 44, all of the inducers cause a substantial increase in P-450 or P-448 over the control level. Looking at the type I binding, one can see that the level of substrate binding does not correlate with this data. Type II binding also does not agree with this data.

The significance of this binding is not known. The discrepancies observed between the data from this study and the substrate specificity and carbon monoxide-binding studies may be caused by factors as yet undetermined.

CHAPTER 5

DETERMINATION OF BAND PROFILES OF INDUCED ENZYMES BY SODIUM DODECYL SULFATE GEL ELECTROPHORESIS

Introduction

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis is used to separate polypeptide chains according to their molecular weights. The size of the polypeptide chains of a given protein can be determined by comparing their electrophoretic mobilities on SDS gels to the mobilities on SDS gels to the mobilities of marker proteins with well-characterized polypeptide chain molecular weights.

It has previously been shown that solubilization of partially purified cytochrome P-450 by SDS results in polypeptide(s) with a molecular weight close to 50,000 (Levin et al., 1972; Dellinger and Schimke, 1972). Welton and Aust (1974) demonstrated that the pattern of bands obtained with microsomes from PB- and MC-treated mice clearly showed substantial differences in the distribution of the protein constituents having apparent molecular weights of approximately 50,000. MC-treatment of rats induced a protein of apparent molecular weight 53,000 while PB-treatment induces two proteins having molecular weights of 47,000 and

44,000. It would seem then that the two inducers stimulate the synthesis of different cytochromes. It has not been shown whether the two molecular weight species in the PB preparation is actually two different proteins or subunits of the same protein. Using partially purified microsomal preparations, Alvares and Siekevitz (1973) obtained similar results.

Since it has already been shown that the antioxidants induce a cytochrome that peaks at 450 nm like that from control and PB-treated animals (Chapter 3) it is important to determine if the gel profiles of these inducers are the same. This would determine if these compounds are triggering the synthesis of the same or different proteins, and would aid in the understanding of the catalytic differences observed for these inducers in Chapter 2.

Materials

Sodium dodecyl sulfate and coomassie brilliant blue were purchased from Fisher Scientific Co. Acrylamide, N,N,N',n'-tetramethylethylenediamine, N, N'-methylenebisacrylamide, and ammonium persulfate were purchased from Bio-Rad Laboratories. The protein standard was purchased from BDH Chemical Ltd. It has a molecular weight range of 14,300 to 71,500.

Methods

The gels were done according to the method of Weber et al. (1972). All the stock solutions were prepared as described.

The resultant gels contained 0.1% SDS and were 12 centimeters long. The samples and standard were treated with SDS and 2-mercaptoethanol before they were placed on the gels. Since it is possible that some nonenzymatic hydrolysis of peptide bonds can occur during the preparation, generating low molecular weight bands, and these bands could become more prominent if the sample is heated, it was decided to omit the treatment. Gels run at 2 milliamps per gel.

The microsomes for this study were prepared as described in Chapter 2.

C57BL/10 female mice, 10-14 weeks old at the time of sacrifice were used. Treatment with inducers was a described in Chapter 2.

Reflectance scans were done on a Gilford Gel Scanner. The destained gels were placed in a cuvette and placed before the light sources of the scanning apparatus. Wherever a band occurred on the gel a peak appeared on the scan.

Results

Microsomes prepared from animals from each of the experimental groups were applied to gel and Figure 4 shows the results after staining and destaining. As can be seen, there are a number of bands on each gel but the most prominent ones are in the 50,000 molecular weight region. One can readily see from the gels that there is a definite difference in the distribution of the bands on each gel. The

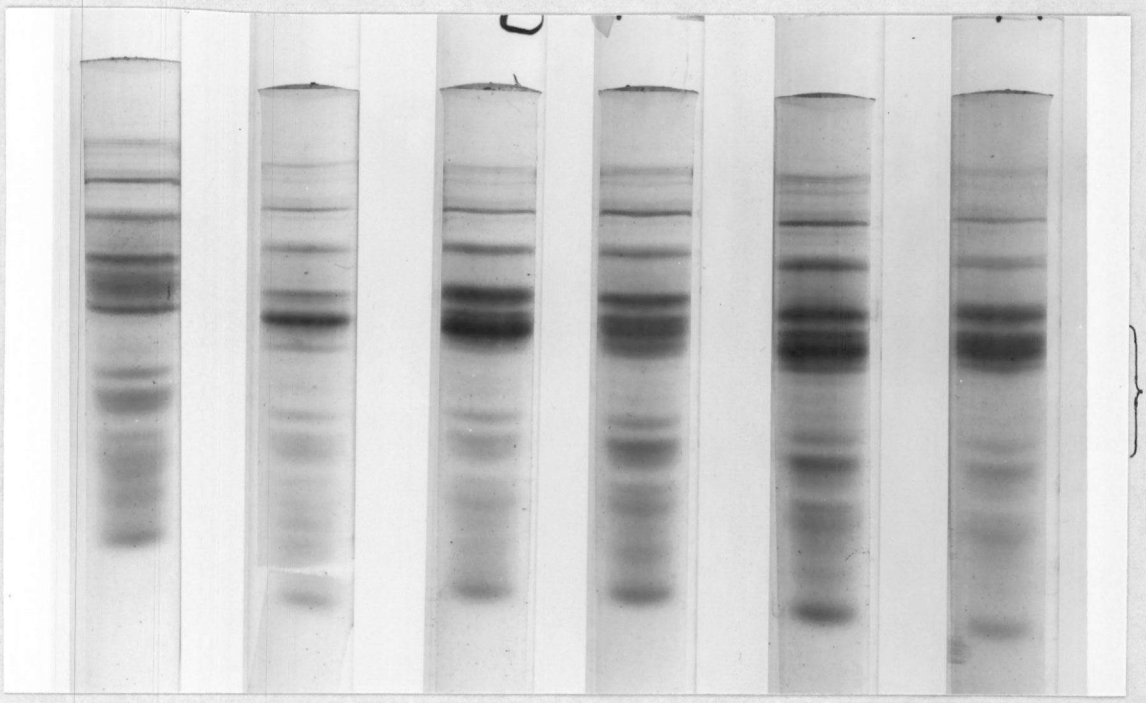

Figure 4. Protein profiles of the enzymes from variously pretreated mice.

intensity of the color in each band is indicative of the amount of protein in that band. As can be seen there are differences in the intensity of the bands for each gel. A better view of the 50,000 molecular weight region is given by the results of the reflectance scans (Figure 5).

There are give peaks (representing the bands) in the control gel. Calculations from the marker gels give the following values for these bands: (a) 49,000; (b) 53,000; (c) 55,000; (d) 57,000; and (3) 60,000.

All the treatments yield some 49,000 and 60,000 molecular weight proteins, so the distinction between the profiles lie in the differences in peaks b, c, and d.

Microsomes from MC-treated animals show a significant amount of peak c at the expense of peak b. This peak actually seems to be a combination of peaks c and d.

The gel profile of the PB-induced microsomes shows a great increase in peak b over the control with peak c lost in the shoulder. There is a small peak at position d.

A scan of the gel of BHA-induced microsomes gives no peak at position b and moderate amounts of peaks c and d. A substantial increase in peak b is shown on the BHT gel and the scan of the ethoxyquin gel shows increased amounts of peaks b and c.

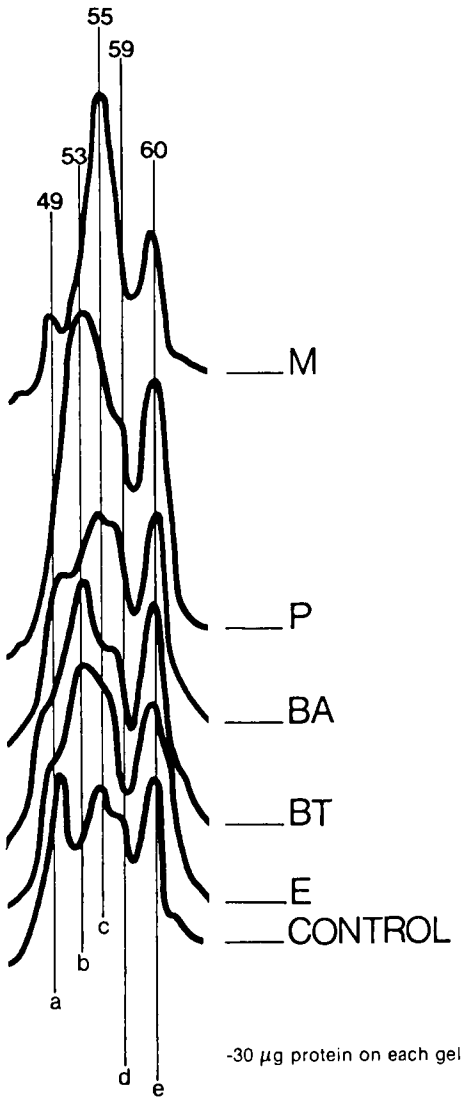

Figure 5. Reflectance scans of SDS gels.

Discussion

The relative amount of protein in each band in the 50,000 molecular weight region was determined by scanning the destained gels. As previously stated, the control gel contains five protein moieties with the molecular weights 49,000, 53,000, 55,000, 57,000, and 60,000. Treatment of the animals with 3-methylcholanthrene causes an increase in the 55,000 molecular weight band, indicating that this protein predominates in MC-treated microsomes. Phenobarbital-treatment causes an increase in the 53,000 and 57,000 molecular weight bands. Using different techniques, other studies have shown that the cytochrome P-450 from PB-induced animals could have multiple components (Ingelman-Sundberg and Gustafsson, 1977; Thomas et al., 1976).

Other SDS gel studies with PB and MC have shown comparable results. Alvares and Siekevitz (1973) using rat liver microsomes, obtained four bands on the control gel with colecular weights 47,000, 49,000, 51,000, and 52,000. MC caused an increase in the 52,000 band while PB-treated microsomes showed an increase in the 47,000 and 49,000 molecular weight bands. Only two bands showed on the control gel of Levin et al. (1976). These bands had molecular weights of 50,000 and 59,000. MC treatment increased the 50,000 molecular weight band. PB treatment increased both. Another experiment with rat liver microsomes by Welton and Aust (1974)

showed bands of 44,000, 50,000, and 53,000 on the control gel. The band increased on the gel from MC-treated animals was the 53,000-dalton band, while PB treatment caused an increase in only the 44,000 molecular weight region.

In this study the gel profiles obtained from the microsomes of the antioxidant-induced animals suggest that they do not contain the same protein moieites (or combination of protein) as either the MC- or PB-treated microsomes. The BHA gel profiles show an increase in the 55,000 and 57,000 molecular weight bands. The BHT-induced enzyme gives an increase in the 53,000 molecular weight region while the ethoxyquin profile shows an increase in the 53,000 and 55,000 molecular weight bands.

The enhancement of different enzyme activities by different inducers may be caused by the size and shape of the inducer molecule. As can be seen from Appendix A the inducers used in these studies are of various sizes and shapes. The work in 1961 by Arcos et al., showed that there is a requirement for an optimal molecular size range and coplanarity for maximal enzyme induction. They found that polycyclic hydrocarbons of an optimal molecular size of 85 to 150 Å induce the synthesis of liver microsomal enzymes. However, it must be mentioned that certain compounds that fell within this optimal range were inactive as enzyme inducers. Although optimal molecular size was not a unique

requirement for inducer activity, it appeared to be a steric factor limiting the inducer activity of the hydrocarbon. Other factors such as the shape, coplanarity, polarizability, and reactivity for metabolic transformation may also affect the ability of a hydrocarbon to react with cellular constituents. The same principle might apply to the inducers used in this study. The type or amount of protein might depend upon the steric arrangement of the inducer molecule. This would correlate with the receptor work done by Poland et al. (1976). It had been previously hypothesized (Poland et al., 1974), that the genetic trait of aromatic hydrocarbon nonresponsiveness (the failure of polycyclic hydrocarbons to induce aryl hydrocarbon hydroxylase activity in certain inbred strains) was due to mutation which results in an induction receptor with a diminished affinity for the inducing compound. These strains also showed a diminished affinity for the more potent inducer 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Using labeled TCDD in <u>in vitro</u> studies, Poland et al. showed that the hepatic accumulation of the label was greatest in C57BL/6J mice, intermediate in the hybrid B6D2F_1/J mice, and least in DBA/2J mice, a pattern which coincided with the strain sensitivity to aryl hydrocarbon hydroxylase induction by TCDD. This data was compatible with the mutation theory and suggested that the hepatic uptake of TCDD was determined by the affinity of the receptor.

In vitro studies on the binding of (^{3}H)-TCDD to hepatic cytosol from C57BL/6J mice revealed a small pool of high affinity sites which could stereospecifically and reversibly bind TCDD. The binding affinity of 23 halogenated dibenzo-p-dioxins and dibenzofurans for this hepatic cytosol-binding species closely correlated with the potencies of these compounds as inducers of hepatic aryl hydrocarbon hydroxylase activity. This data suggested that the hepatic cytosol species which could bind TCDD was the receptor for the induction of the hepatic hydroxylase activity.

It is possible that there exists in animals a pool of receptors for each type inducer used. An interaction between the inducer and its receptor would form a "unique" complex responsible for the activation of specific genes, thus increasing the synthesis of particular enzyme activities. This may explain the different protein profiles obtained with SDS gel electrophoresis. These different treatments could result in the formation of isoenzymes of a given enzyme. The different proteins that make up this set of isoenzymes would have similar but not necessarily identical enzyme properties. Each of the isoenzymes may be responsible for the metabolism of a specific substrate, thus accounting for the broad substrate specificity observed for the mixed function oxidase system.

The implication(s) of multiple forms (different molecular weight classes) of P-450 in carcinogenesis and metagenesis is

not yet fully understood. It is known that the antioxidant inducers protect against cancer and mutation in rodents (Cumming and Walton, 1973; Wattenberg, 1972), and that they do not induce the activity of aryl hydrocarbon hydroxylase, the enzyme responsible for the metabolism of the carcinogenic polycyclic hydrocarbons. The forms of P-450 present in the antioxidant-induced animals could be those responsible for the detoxification of foreign compounds. Phenobarbital has been shown to be effective in the reduction of mutations by ethyl methanesulfonate (Cumming et al., data in press) but the effect of this inducer on the carcinogenicity of certain compounds has not been tested. Methylcholanthrene is the only one of these inducers known to enhance the promotion of cancer in test animals. Therefore, it would seem that the form of cytochrome P-450 present in the MC-induced animal is the form responsible for the activation of carcinogenic compounds.

Though the differences between the different protein profiles are obvious, the results need to be interpreted with care since the samples used to obtain these results were whole microsomes and not purified enzyme preparations. Further work on the isolation of these forms needs to be done so that more biochemical tests can be done to determine the extent of the difference between these multiple forms.

CHAPTER 6

EFFECTS OF INDUCERS ON THE METABOLISM OF BENZO(A)PYRENE—ANALYSIS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

Introduction

We are exposed daily to a constant onslaught of chemical carcinogens. These compounds are present in the air we breathe, the food we eat, and the water we drink. Industry pollutes our waterways by its uncontrolled waste disposal (United States Department of Labor, 1973), emissions from automobiles and industrial smoke stacks fill the atmosphere with cancer-causing pollutants (Committee on the Biologic Effects of Atmospheric Pollytants, 1972), even our personal habits (such as cigarette smoking) contribute to our exposure to carcinogens.

These chemicals (mostly polycyclic hydrocarbons) are the major cuase of cancer in humans, and from the above-mentioned sources it would seem that people living in cities have the highest incidence of exposure. It has been found that virtually 100% of all urban dwellers autopsied showed the presence of asbestos in lung tissue (Langer et al., 1971). Individuals living in rural areas are also exposed to some carcinogens. Emissions from farm machinery and the spraying of arsenical insecticides have contributed to the increased

rate of lung cancer in people from rural areas (Roth, 1957; Occupational Safety and Health Administration, 1975).

The polycyclic hydrocarbons are not harmful in an unmetabolized state. It is only after they are "activated" by the mixed function oxidase system do they exert their damaging effect (Gelboin et al., 1972; Gelboin et al., 1969). This enzyme system is most prevalent in hepatic tissue, but it can also be found in skin, the gastrointestinal tract, lung and other tissues.

When studying the carcinogenic effect(s) of the hydrocarbons on test animals (such as mice), it was found that some strains did not develop tumors or skin ulcerations (like DBA) while other strains did (like C57BL). After further investigations, it was found that the presence of the enzyme aryl hydrocarbon hydroxylase (AHH), which metabolizes polycyclic hydrocarbons, was a determining factor. Data presented by Thomas et al. (1973) and Burki et al. (1973) indicated that susceptibility to cancer produced by these hydrocarbons is associated with higher inducible levels of AHH activity. They also showed that the C57BL strain had this higher level of AHH when induced while the DBA strain did not.

One of the most potent of the polycyclic hydrocarbons tested was benzo(a)pyrene (BP). Its use in most experiments as a cancer-producing agent is in line with its prevalence

in atmospheric pollution (Committee on the Biologic Effects of Atmospheric Pollutants, 1972). The number of studies being done on the metabolism of benzo(a)pyrene by hepatic AHH testifies to the importance of the elucidation of this pathway to the understanding of chemical carcinogenesis.

To date, the metabolites of BP, as analyzed by high pressure liquid chromatography (HPLC), have been shown to consist of phenols (Selkirk et al., 1976; Falk et al., 1963), dihydrodiols, quinones and epoxides (Yang et al., 1975; Selkirk et al., 1974; Holder et al., 1974; Kinoshita et al., 1973), as shown in Appendix D.

Several studies have presented evidence that suggests that benzo(a)pyrene and other polycyclic hydrocarbons undergo metabolism via arene oxides that are precursors for dihydrodiols and phenols (Yant et al., 1976; Huberman et al., 1976; Holder et al., 1974; Daly et al., 1972). Some of these epoxides and dihydrodiols have been found to be active in carcinogenicity. The phenols, while found to be toxic, do not seem to be involved in carcinogenicity.

Grover et al. (1971) found that the epoxides and cisdihydrodiols derived from the K-region double bond (the phenenthrenoid bond) of benz(a)anthracene and dibenz(a,h)-anthracene are active in the production of malignant transformation in hamster embryo cells.

The compound derived from the trans-7,8-diol of BP in Chinese hamster cells, 7α,8β-dihydroxy-9β, 10β-epoxy-7,8,9,10-

tetrahydrobenzo(a)pyrene, is the major intermediate and possibly the ultimate carcinogen in BP carcinogenesis in these cells (Huberman et al., 1976).

Since it has already been shown that different forms of cytochrome P-450 obtained from the same source hydroxylate BP in different positions, it would be interesting and informative to determine if the different forms of the cytochrome induced by various treatments also causes changes in the products of BP metabolism. It may be that these enzyme inducers shift the metabolism of BP toward noncarcinogenic or less carcinogenic products.

Materials

(^{3}H)BP was purchased from New England Nuclear.

Methods

C57BL/10 female mice 10-14 weeks old at the time of sacrifice were used.

Treatment of animals with inducers was the same as described in Chapter 1, and the isolation of microsomes was the same as described in Chapter 2.

Incubation and high pressure liquid chromatography was done by the procedures of Selkirk et al. (1975) with few exceptions. The metabolites were formed by incubating mouse liver microsomes with (^{3}H)benzo(a)pyrene as follows. Each tube contained 100 µg of microsomal protein, 0.36 µmole of

NADPH, 3 μmole of $MgCl_2$, 50 μmole of Tris-HCl buffer (pH 7.5) and 100 nmole of BP dissolved in 0.040 ml of methanol in a total volume of 1.0 ml. The tubes were incubated for 30 min. at 37°C under subdued lights, and the reaction was stopped by the addition of 1.0 ml of acetone. The mixture was then extracted with 2.0 ml of ethyl acetate; 10 extracts were pooled and dried over anhydrous magnesium sulfate, the solvent was evaporated under vacuum, and the residues (metabolites) was dissolved in 0.1 ml of methanol. Isolation and characterization of the labeled metabolites was performed on a high pressure liquid chromatograph. The chromatographs were done by Dr. James Selkirk and coworkers using a Dupont model 830 fitted with a permaphase column (1-m ODS). The column was eluted with a reverse phase gradient system, with methanol and water (30:70 initially, and 70:30 at the end). The gradient rate of change was 3% per minute; the column temperature was 50°C; the pressure was 350 pounds per square inch; flow rate was 0.6 ml/min. The elute was monitored by ultraviolet absorption at 254 nm. Fractions were collected at 20-second intervals, and the radioactivity was determined in a Beckman 350 scintillation counter with Aquasol (New Englang Nucler) as the counting medium.

Results

Figure 6, page 69, shows the graphs obtained by plotting the radioactivity versus the sample number. The numbers on

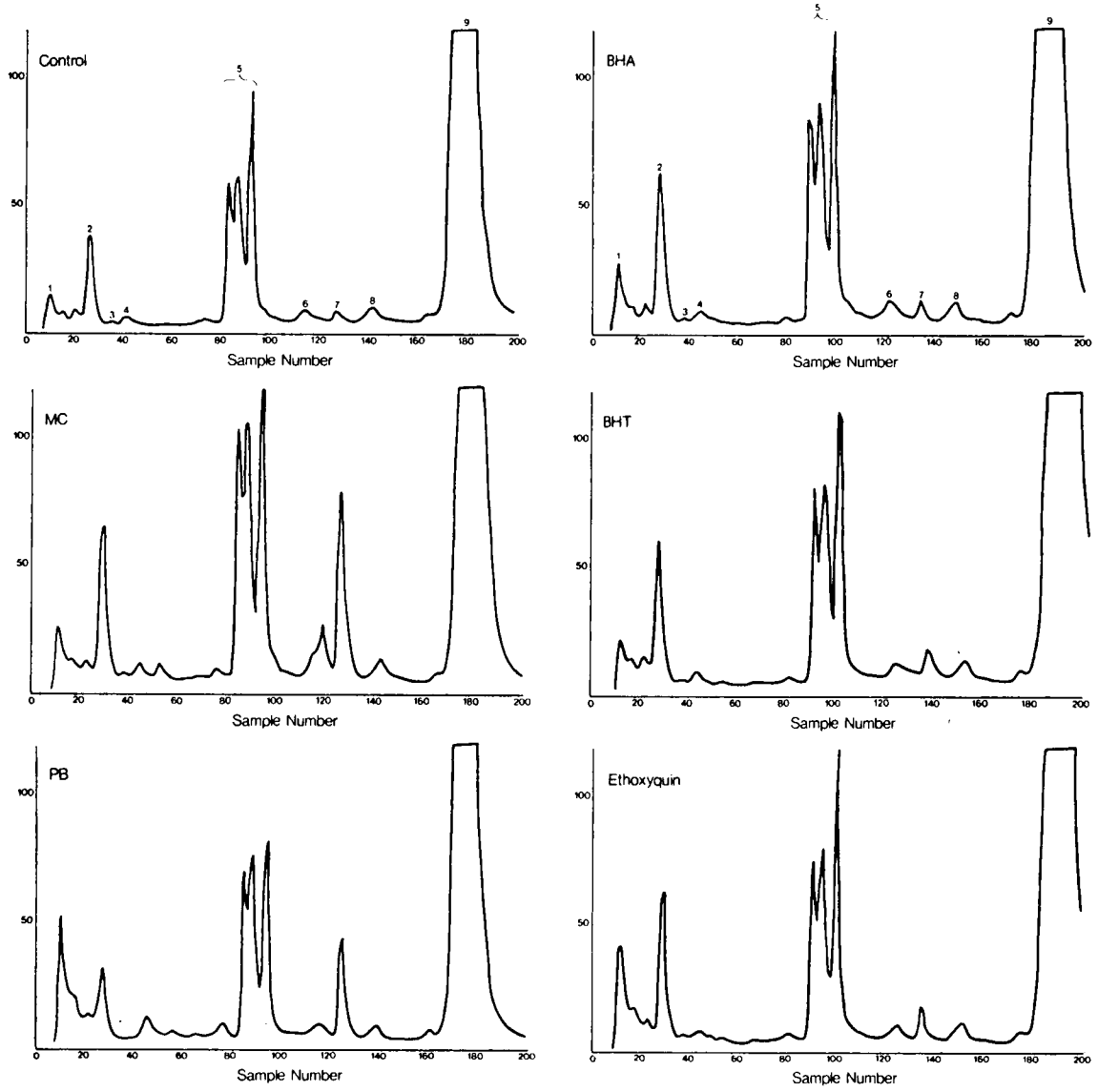

Figure 6. Profile of products formed by the metabolism of (^{3}H)benzo(a)pyrene.

the graphs denote the different metabolites: (1) an unknown substance; (2) 9,10-diol; (3) 4,5-diol; (4) 7,8-diol; (5) the quinones,1,6-, 3,6-, 6,12-; (6) 9-OH; (7) 3-OH; (8) unknown; (9) unmetabolized BP. Samples do not differ from control.

Table 4 shows the ratios of the values for the metabolites from each treatment divided by the value for the metabolites from the control. The numbers used for these measurements were obtained by determining the amount of radioactivity under each peak. For peak 1 an increase is seen for each treatment used, especially for PB, BHA, and ethoxyquin. For peak 2 PB shows a slight reduction when compared to the control value. The other treatments show a small increase. The 4,5-diol (peak 3) does not have enough radioactivity over the baseline to be measured for the PB, BHT, BHA and ethoxyquin treatments, and the MC treatment yields the same amount as the control. Peak 4 (the 7,8-diol) is increased in the PB and BHA samples, but not in the MC, BHT, or ethoxyquin samples. The BHT treatment even shows a reduction in the 7,8-diol. In general, the quinones (peak 5) are increased slightly by each treatment. MC is the only treatment which gives a significant increase in peak 6, the 9-OH metabolite, BHA and BHT both give a small increase in this metabolite. The 3-OH metabolite is increased to a very large extent with the MC and PB treatments, while the other treatments yield significant but smaller increases. The

Table 4. Ratios of peak intensities of Benzo(a)pyrene Metabolites produced by enzyme from different sources.

Metabolites	MC	PB	BHA	BHT	Ethoxyguin
Unknown I.	1.86	3.33	2.00	1.61	2.93
9,10-diol	1.11	0.84	1.63	1.58	1.63
4,5-diol	1.00	-	-	-	-
7,8-diol	1.00	1.70	1.43	0.71	-
1,6-quinone	1.67	1.13	1.37	1.30	1.03
3,6-quinone	1.69	1.16	1.45	1.29	1.10
6,12-quinone	1.21	0.84	1.21	1.16	1.26
9-OH	2.80	1.00	1.50	1.50	1.00
3-OH	10.00	5.00	1.88	2.50	2.50
Unknown II	1.25	0.83	1.25	1.25	1.00

second unidentified compound is only slightly increased by the MC, BHA, and BHT treatment, while the PB treatment shows a slight reduction of this compound when it was compared to the control value.

These data indicate that each of the treatments used in this study causes a different amount of each of the metabolites of benzo(a)pyrene to be produced, thus further suggesting that they each induce different enzymes and that these enzymes may metabolize the benzo(a)pyrene molecule at different sites.

Discussion

The initial oxidation in the oxidative metabolism of polycyclic hydrocarbons, catalyzed by the enzyme aryl hydrocarbon hydroxylase, results in the formation of reactive epoxides, which are subsequently converted to dihydrodiols, quinones, and phenolic metabolites (Jerina et al., 1970; Oesch, 1973).

Epoxides are three-membered cyclic ethers. Most of them are highly strained molecules and are extremely reactive because of the ease with which the ring can be opened. In 1950, Boyland suggested that epoxides might be formed during the metabolism of polycyclic hydrocarbons by animals. The evidence for the biological conversion of olefinic compounds to epoxides was first demonstrated for the insecticide heptachlor (Davidow and Radomski, 1953). They found that

dogs which were fed this insecticide had high levels of the epoxide in their body fat. It was suggested then that not only might olefinic bonds be attacked to form epoxides, but that bonds with aromatic character might also be epoxidized. Further studies showed that rats were also able to convert heptachlor to its epoxide. The concentration of the epoxide was highest in the liver, suggesting that epoxide formation occurred in this organ. A sex difference was found in this conversion, female rats producing more than males (Radomski and Davidow, 1953).

Hydroxylation of aromatic rings is a general biotransformation process which increases the polarity of the compound and therefore both aids its excretion and provides a focus for conjugation. As stated earlier, hydroxylation usually proceeds through formation of an intermediate epoxide which might rearrange at physiological conditions to a phenol, be hydrated to a trans dihydrodiol or conjugate with glutathione (Daly et al., 1972). Since the polycyclic hydrocarbons are aromatic compounds, they can be hydroxylated at any of several double bond positions in the rings. Studies with these compounds have mainly concerned the metabolism at the K-region (the phenanthrene bond), this being the most reactive position, and epoxides at the K-region seem to be easier to prepare than at other positions (Keysell et al., 1973).

Benzo(a)pyrene, the polycyclic hydrocarbon used in this study, is metabolized at the 1,2; 2,3; 4,5; 7,8 and 9,10 double bonds. The epoxide at the 4,5 position has been identified as a metabolite formed during the metabolism by rat liver microsomes (Grover and Sims, 1972). The non-K-region epoxides at the 7,8 and 9,10 positions have been synthesized and have been shown to easily rearrange to the respective phenols and are converted by rat liver homogenates to the trans dihydrodiol (Waterfall and Sims, 1972). Borgen et al. (1973) showed that the amount of 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene bound to DNA is more than ten times as great as the amount of BP bound, indicating that this metabolite is more active than the parent compound. Huberman et al. (1976) also showed that the 7,8-epoxide (precursor for 7,8-diol) is metabolically and stereoselectively formed from BP. The metabolic formation of this diol-epoxide and its extraordinarily high mutagenicity indicate that this metabolite may be the major ultimate carcinogenic form of BP. The amount of this diol-epoxide relative to other metabolites may be important in the determination of the carcinogenicity of BP. The amount of the 7,8- and other epoxides produced during the metabolism of polycyclic hydrocarbons is dependent on the concentration of the parent compound reaching the epoxidase enzymes (in the mixed function oxidase system). The activity of these enzymes can be increased by pretreating

animals with inducers such as methylcholanthrene and phenobarbital. There is a positive correlation between the inducibility of polycyclic hydrocarbon metabolism and the susceptibility to hydrocarbon carcinogenesis in mice (Kouri et al., 1973) and possibly in humans (Kellerman et al., 1973). The use of a particular enzyme inducer might shift the metabolism of polycyclic hydrocarbons away from the production of the 7,8-epoxide and toward the other metabolites, quinones and phenols. This might be accomplished by an increase in the enzymes responsible for the rearrangement of these epoxides to phenols, by a decrease in the enzymes that attack the benzo(a)pyrene molecule at the 7,8-position, or a combination of these events. Since the antioxidants have been reported to decrease the carcinogenic effects of benzo(a)pyrene (Wattenberg, 1973; Wattenberg, 1972), it seemed necessary to determine whether or not they decreased the formation of 7,8-epoxides and/or increased the formation of the other metabolites. The results from the high pressure liquid chromatographic study show that of the antioxidants, only BHT and ethoxyquin treatment reduced the amount of 7,8-epoxide formed (as measured by the presence of 7,8-diol). They all showed an increased amount of the quinones and phenols over the control values. A decrease in the amount of 4,5-diol is seen for each antioxidant. The significance of this result is not known. It should also be noted that there are

increases in the unidentified products (unknown I and II). Additional studies on these peaks need to be done to determine whether they are important products of BP metabolism. They could be important in the understanding of the effects of inducers on BP metabolism.

Since methylcholanthrene treatment has been shown to increase the carcinogenicity of benzpyrene (Kouri et al., 1973), it is important to compare the results of MC treatment in this study to that of antioxidant treatment since they cause opposite responses. Table 4, page 71, shows that the differences observed between these two classes of compounds is in the amounts of unknown I, 4,5-diol, 7,8-diol (variable for the antioxidants), 9-OH and 3-OH metabolites. Whether these differences can be correlated to the differences in the effect of these compounds on the carcinogenicity of BP has not yet been determined.

Before definite conclusions can be drawn from this study, it should be noted that the use of tritium to calculate the amount of metabolites formed requires that the tritium be retained on the benzpyrene molecule during metabolism. Since each position of the molecule may not be tritiated to the same extent and because the formation of the different BP metabolites necessitates the loss of tritium to varying degrees, it may not be appropriate to establish a relationship between the amount of radioactivity collected in a metabolite fraction

and the concentration of metabolites. With this in mind, it is cautiously concluded that the antioxidants do cause a slight alteration in the BP metabolite profile. This alteration may be one of the causes of the protective effect of the antioxidants BHA, BHT, and ethoxyquin against chemical carcinogenesis.

CHAPTER 7

SUMMARY, CONCLUSIONS AND IMPLICATIONS

Summary

This study was concerned with the characterization of the antioxidants butylated hydroxyanisole, butylated hydroxytoluene, and ethoxyquin as related to the induction of the mixed function oxidase system of hepatic tissue. Interest in these compounds was strengthened when it was determined that they could induce some of the enzymes of this system. As stated previously, the inducers of this system tend to fall loosely into two categories, one exemplified by the anticonvulsant sodium phenobarbital and the other by the carcinogenic polycyclic hydrocarbon 3-methylcholanthrene.

There are few similarities between any of the inducers of the microsomal enzymes, but certain general physiochemical properties of a compound may render them particularly potent as inducers. Such characteristics include lipophilicity, long biological half-life, and tight binding to some biologic entity that is responsible for the increase in enzyme activity (possibly a receptor site). These characteristics originate directly from the biology of the microsomal enzymes, which, embedded in the lipid envelope of the smooth endoplasmic reticulum, require for adequate function the integrity of this lipid envelope, as well as access to

molecular oxygen and NADPH. Chemicals that induce hepatic microsomal enzymes must be lipid soluble to penetrate the lipid envelope of the hepatic smooth endoplasmic reticulum. To enhance enzyme activity inducers must generally have a sufficiently long biological half-life and be administered in a large enough dose to attain a high concentration at the site of microsomal enzyme. Once at the activation site, the inducer must possess strong enough affinity for the enzyme to remain in their proximity for the time required to stimulate protein synthesis. Each of the inducers used in this study seem to possess these characteristics.

Drugs and other foreign compounds (such as polycyclic hydrocarbons) enter the body through gastrointestinal, respiratory or cutaneous routes by a process of passive diffusion of which an important determinant is the degree of lipid solubility (Hunter and Chasseaud, 1976). These polycyclic hydrocarbons are known to interact with macromolecules either through weak physical interactions (Ts'O and Lu, 1964) or through covalent linkage (Brookes and Lawley, 1964). The formation of these covalent linkages seemed to require the conversion of the relatively inert hydrocarbons to more reactive species. An enzymatic basis for this conversion was found to exist. Since these hydrocarbons are primarily metabolized by the microsomal oxidases, Gelboin et al. (1972) studied the effect of enzymatically active microsomal

preparations on the formation of DNA-hydrocarbon complexes and found that it was dependent on a microsome catalyzed reaction. These same enzymes also catalyze the binding of polycyclic hydrocarbons to proteins (Grover and Sims, 1968). Since it has not yet been determined whether or not this binding to DNA or protein has any effect on the carcinogenicity of the compounds tested, it would be beneficial to understand the effect of these inducers. It has been determined that an increase in the microsomal oxidases (especially aryl hydrocarbon hydroxylase) is associated with the increase of cancer in laboratory animals (Kouri et al., 1973). The enzyme inducers then must play an important role in the research on any carcinogen.

The study of the antioxidants as microsomal enzyme inducers is especially important since they are used widely as food additives. These compounds help to maintain the quality of many food products by preventing oxidation of labile lipid components. Most antioxidant formulations contain BHA and BHT, while ethoxyquin is used mainly in animal feeds.

This study was designed to further explain the results of many studies which showed that these antioxidants were effective in reducing the carcinogenic, mutagenic, and toxic effects of a number of chemicals. The effects of the antioxidants was compared with those of a drug (phenobarbital)

and a carcinogenic polycyclic hydrocarbon (methylcholanthrene) since these compounds have long been known to induce the mixed function oxidase system of test animals. Several approaches were used: (1) the determination of the genetics of antioxidant induction; (2) the determination of the substrate specificity of the antioxidant-induced enzyme(s); (3) the determination of the type and amount of cytochrome P-450 induced by each treatment; (4) the measurement of the amount of binding of type I and type II substrates to induced enzymes; (5) the determination of the electrophoretic profile of each induced enzyme; (6) the measurement of the amount of each metabolite of a polycyclic hydrocarbon produced by each treatment. These steps were important since each shed a different kind of light on the antioxidant induction story.

The genetics of antioxidant induction was determined by the study of zoxazolamine paralysis times. Robinson and Nebert (1974) have shown that the enzyme responsible for the metabolism of zoxazolamine has the same genetic locus as aryl hydrocarbon hydroxylase, the enzyme that metabolizes the polycyclic hydrocarbons. Since zoxazolamine is a drug with visible effects, the paralysis of the test animals, it was possible to indirectly measure the amount of its hydroxylase by measuring the time necessary for animals to regain their righting reflexes after the onset of paralysis. Enzyme inducers increase the amount of the zoxazolamine hydroxylase

and should therefore decrease the paralysis times. Methylcholanthrene, the polycyclic hydrocarbon inducer, increases the amount of hydroxylase in some strains (responsive) but not in others (nonresponsive), while phenobarbital increases the hydroxylase concentration in all strains. Since the antioxidants had not been in this type study before, it was not known which course they would follow. This study in Chapter 1 showed that though the antioxidants in most cases, are not as effective as PB in inducing zoxazolamine hydroxylase activity, they still elicit a response in all strains tested; unlike MC, which only induces the hydroxylase in certain strains. This implies that the antioxidant inducers are able to operate the complete mechanism of zoxazolamine hydroxylase induction in all these strains of mice. It has been shown that another inducer of the MC-category (2,3,7,8-tetrachlorodibenzo-p-dioxin) with 30,000 times the potency as an inducer of the P-448 activity as MC (Niwa et al., 1975; Poland et al., 1974) was capable of inducing this activity in the strains that do not respond to MC treatment. This suggested that MC might not be a strong enough inducer to fully operate the mechanism of induction of some strains.

The carbon monoxide difference spectra was another study which suggested that the antioxidant inducers are of the PB-type. In these spectra the cytochrome present in microsomes

obtained from antioxidant-treated animals peaked at 450 nm, like the PB and control samples. It has been shown that this does not necessarily mean that the cytochromes in each of these samples is the same. Haugen et al. (1975) and van der Hoeven (1974) showed that the PB-induced enzyme itself is present in microsomes in multiple forms. Another indication of the multiplicity of the PB-induced enzyme was given by Wiebel et al. (1975) when they determined that the different forms of P-450 isolated from PB-induced microsomes specifically oxygenated the benzo(a)pyrene molecule in different positions.

To determine whether or not the cytochrome P-450 from antioxidant-treated animals was present in more than one form, gel electrophoresis was done on microsomal preparations from these animals. In Chapter 5 it was found that there are differences in the molecular weights of the protein(s) produced by each antioxidant. BHA treatment yields moderate amounts of 55,000 and 57,000 molecular weight protein whereas induction with BHT gives a protein of 53,000 molecular weight and ethoxyquin treated microsomes show a predominance of 53,000 and 55,000 molecular weight protein. The data for the other treatment used show that a molecular weight protein of 55,000 is present in MC-induced microsomes while PB-treated animals give proteins of 53,000 and 57,000 molecular weight. Whether these data suggest that some of the pretreatments yield two cytochromes of multiple forms or

that the cytochrome contains more than one subunit has not yet been determined. However, it is clear that each treatment gives a protein profile distinctly different from the other treatments.

It is clear now that even though the antioxidants are more like the PB-type inducer than the MC-type, they are different from either inducer and they are different from each other.

Another study which shows their uniqueness is the determination of their substrate specificity (Chapter 2). It was found that the enzymes from the antioxidant-treated animals do not catalyze every substrate tested (like PB), nor do they react with benzo(a)pyrene substantially (which is a characteristic of the MC-induced enzyme). These data can be explained on the basis of results from Chapter 5. Each of the treatments used in these studies could be inducing different enzymes, each responsible for the metabolism of a different substrate. This would also explain the fact that the substrates catalyzed and the degree of conversion of each substrate to product varied from antioxidant to antioxidant.

Studies in Chapter 4 on the binding of substrate to the microsomes from variously-treated mice again showed that the antioxidants may form a different class of inducers. The binding of type I and type II substrates to antioxidant-

induced microsomal preparations give no characteristic spectra like that of the PB-induced spectra (see Figure 2, page 44), but rather is scattered over the entire range of binding. There is no correlation between the binding of the type I and type II and the extent to which they are catalyzed by the microsomes from the pretreated animals.

The determination of the differences between the inducers under investigation here may lead to an understanding of their effect on the carcinogenicity of certain chemicals. It has been shown that methylcholanthrene enhances the carcinogenicity of polycyclic hydrocarbons by inducing aryl hydrocarbon hydroxylase (AHH) activity which activates these compounds via arene oxides. These oxides are very reactive and interact with many of the macromolecular constituents of the cell, which could trigger a carcinogenic response in these cells. Phenobarbital is also known to increase AHH activity (Chapter 2), but its effectiveness in enhancing cancer production has not been studied. It has been shown, however, that the antioxidants BHA, BHT, and ethoxyquin significantly reduce the carcinogenicity of polycyclic hydrocarbons, and the mutagenicity and toxicity of ethyl methanesulfonate. The different results obtained for MC and the antioxidants could be due to the fact that the antioxidants do not increase the activity of AHH (Chapter 2), but as can be seen from the results in Chapter 6 the antioxidants do metabolize polycyclic

hydrocarbons. Here again the data fits the theory that each inducer increases the activity of several enzymes, each reacting with a different substrate or at a different position on the same substrate, thus giving rise to different products than those detected by the enzyme assay used.

All of these studies were based on the assumption that only the protein moiety of the cytochrome induced by MC, PB, BHA, BHT, or ethoxyquin is different. It has been shown by earlier studies that the synthesis of the cytochromes P-450 and P-448 requires the synthesis of apoprotein and heme (Correia and Meyer, 1975; Rajamanickam et al., 1975; Siekevitz, 1973; Black and Bresnick, 1972). Maine and Anders (1973) characterized the heme moiety of these cytochromes using gas chromatography and mass spectrometry. They determined that the heme of both cytochromes are identical and are the same as that of hemoglobin, namely protoporphyrin IX. So the differences between these two cytochromes was concluded to be caused by differences in the protein moiety. The same could be true for the antioxidant-induced cytochrome(s), though the heme has not yet been characterized.

The inhibition observed in the combined inducer studies in Chapter 2 is not yet understood, but it serves as the basis for the following discussion on the hypothesis used to explain all of the results obtained in this study.

If each of the inducers used in the combined inducer study increased the activity of a different cytochrome or a

combination of cytochromes, it could be possible that they are competing for substrate and the product of one of the enzymic reactions is not the product measured by the assay used. An alternate possibility is that one of the inducers increases the level of the enzyme that metabolizes the other. This can be more easily understood by the schematic representation of the data obtained in Chapter 5 (shown in Figure 7).

When PB and MC are used simultaneously as inducers, there is an increase in the C_2 and C_3 cytochromes. These cytochromes are known to be involved in the metabolsim of a variety of substrates, not just the one being assayed. So it is possible that the C_2 from PB-induced animals can recognize MC as a substrate and thus eliminate some of the potential inducer molecules before they reach their target. The same is true for the C_3 cytochrome from MC-induced animals. Some of the PB could be metabolized before it can induce the mixed function oxidase system. In this study, what one sees then is the net effect of these actions.

When MC and BHA are used simultaneously to induce the MFO system, there is an increase in the zoxazolamine hydroxylation. This too can be explained by Figure 7. The C_3 cytochrome is increased by both of these inducers. This cytochrome could be responsible for the zoxazolamine hydroxylase activity. As can be seen there is an additive

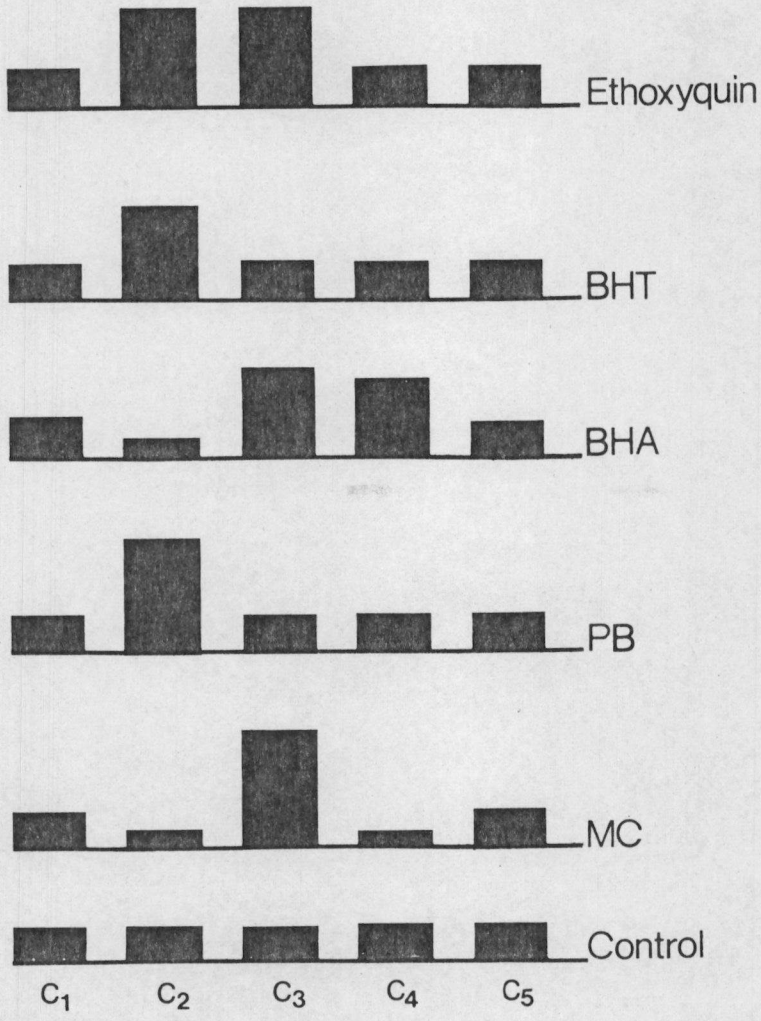

Figure 7. Schematic representation of the induction profiles of cytochromes.

effect for these inducers. As stated earlier (Chapter 2), this additive effect could be indicative of different mechanisms of induction used by these compounds. The other activities assayed in Chapter 2 were found to be inhibited by this combination of inducers. It is possible that one or more of the inducers is interacting with the substrate to metabolize it to a product not measured by the assay. All of the studies reported here indicate that the antioxidants are not like any of the other inducers previously studied.

Conclusions

There are several conclusions that can be drawn from the results obtained in this study. The first is that the induction by the antioxidants butylated hydroxyanisole, butylated hydroxytoluene, and ethoxyquin is not strain specific. The antioxidants induce the activity of the mixed function oxidase system in every strain of mouse tested. The results of the zoxazolamine paralysis times in Chapter 1 demonstrates this. The second conclusion drawn from the data is that methylcholanthrene and BHA use different mechanisms for the induction of the MFO system. This was found to be true when the two inducers were used simultaneously in the combined inducer study in Chapter 2. Both inducers were used in amounts that gave maximal responses when used singly. When an additive response was observed upon using the

inducers together, this indicated the use of different mechanism of action for these compounds.

The results of the substrate specificity study (Chapter 2) and the electrophoretic study (Chapter 5) show that the antioxidants induce a cytochrome different from that of PB and MC and the cytochrome induced by each antioxidant is different from the cytochrome induced by each of the other antioxidants.

The metabolite profile of the reaction of benzo(a)pyrene with the enzyme obtained from variously-treated mice show that each of these enzymes primarily attack benzpyrene at different positions. The high pressure liquid chromatography data from Chapter 6 shows this.

The one general conclusion drawn from this study is that the antioxidants are not like any of the previously studied inducers.

Implications

The intake of BHT by man has been estimated to be 2 mg/person/day in America (Food and Drug Administration, 1964). Most of this ingestion is the result of BHT and BHA being used as food additives in some fat-containing foods. Given a dose of BHT, 75% of it is excreted by man in the urine and feces during the first 24 hours, but the remainder is excreted more slowly (Collings and Sharrat, 1970). The five-day half-life of this second phase of excretion is attributed to

the slow release of BHT or its metabolites from the tissues (Daniel et al., 1967). At this level of intake and rate of excretion, BHT could accumulate until the body contains 4 mg. If this is evenly distributed through the total body fat it can produce a concentration of 0.4 ppm BHT (Collings and Sharrat, 1970). The same could be true for BHA consumption but not for ethoxyquin since it is used mainly in livestock feeds.

The cyclic metabolic pathway for BHT, as proposed by Chen and Shaw (1974), may aid in the explanation of the lengthy elimination time of this compound. Because of this slow rate of excretion and the daily ingestion of these antioxidants by man, the biochemical effects of these compounds must be determined.

The antioxidants are known to be inducers of the microsomal mixed function oxidase system. Since hepatic microsomal enzymes are responsible for the metabolism of many different drugs and endogenous substances, hepatic enzyme induction is potentially of value in therapeutics by modifying the extent and duration of action of these compounds (Hunter and Chasseud, 1976).

Unconjugated hyperbilirubinaemia is the only condition for which the deliberate induction of hepatic enzymes has become standard clinical practice but with more work being done on the antimutagenic, anticarcinogenic and antitoxic

properties of the antioxidant inducers, it may only be a matter of time before they are used routinely in the treatment of disease. However, before administering enzyme inducers indiscriminately for their possible beneficial effects, it must be remembered that they may produce some considerably physiological changes. Some of these changes cause an increased rate of drug metabolism, and others stem from the effect of increased enzyme activity on endogenous compounds. So additional work needs to be done before any clinical application of the antioxidants is possible.

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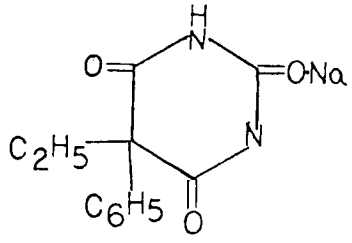
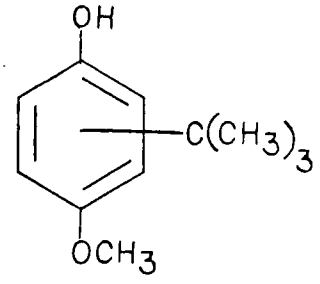
APPENDIXES

APPENDIX A

STRUCTURES OF INDUCERS USED



Sodium Phenobarbital



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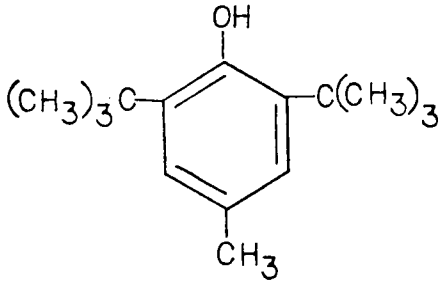

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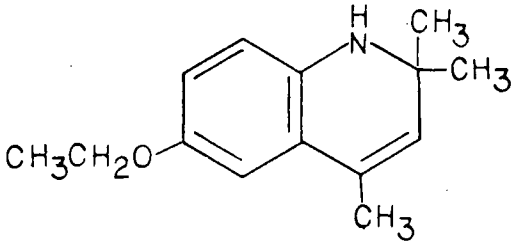
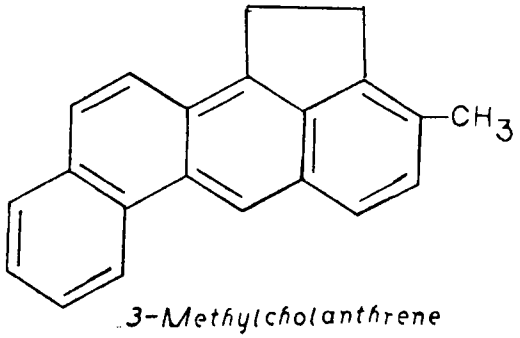


Ethoxyquin



3-Methylcholanthrene

APPENDIX B

COMPLETE DESCRIPTION OF THE TEST DIETS USED

50% Standard Protein Diet (TD 77204)

	g/kg
Casein, High protein	574.713
Corn starch	150.0
Sucrose	180.287
Corn oil	50.0
Mineral mix, William-Briggs Modified	35.0
Vitamin mix, Teklad	10.0

50% Protein Diet with 0.75% Antioxidant Added (Mod. TD 77204)

Same formula as for TD 77204, except:

(1) Antioxidant (BHA, BHT, or ethoxyquin) was added at the rate of 7.5 g/kg of diet.
(2) Sucrose content was adjusted to 172.787 g/kg.

Salt Mix (Williams and Briggs modified)

Formula:	Percent Composition:
Calcium carbonate	20.714
Calcium phosphate dibasic	32.284
Cupric sulfate	0.037
Ferric citrate	0.431
Magnesium sulfate	6.571
Manganese sulfate	0.440
Potassium chloride	20.857
Potassium iodate	0.002
Sodium phosphate dibasic	18.600
Zinc carbonate	0.060

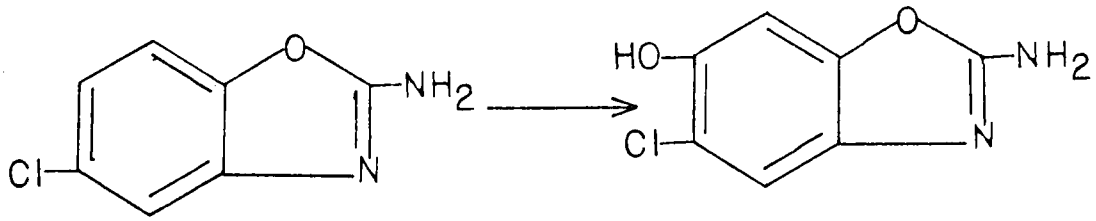
Vitamin Mix (Teklad)

Formula:	g/kg
p-Aminobenzoic acid	11.0132
Ascorbic acid, Coated (97.5%)	101.6604

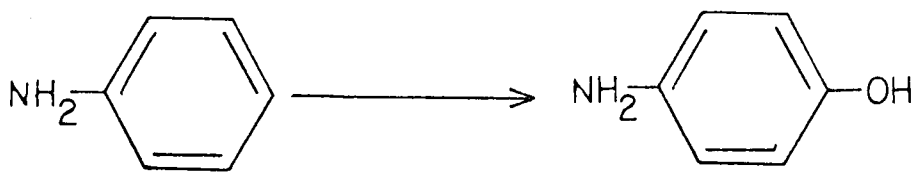
Biotin	0.0441
Vitamin B_{12} (0.1% trituration in mannitol)	2.9736
Calcium pantothenate	6.6079
Choline dihydrogen citrate	349.6916
Folic acid	0.1982
Inositol	11.0132
Menadione (Vitamin K_3)	4.9559
Niacin	9.9119
Pyridoxine HCl	2.2026
Riboflavin	2.2026
Thiamine HCl	2.2026
Dry vitamin A palmitate (500,000 U/g)	3.9648
Dry vitamin D_2 (500,000 U/g)	0.4405
Dry vitamine E acetate (500 U/g)	24.2291
Corn starch	466.6878

APPENDIX C

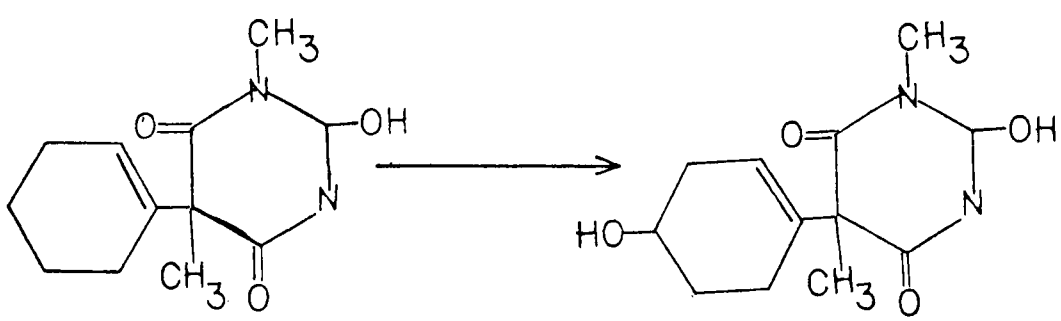
REACTIONS STUDIED IN CHAPTER 2



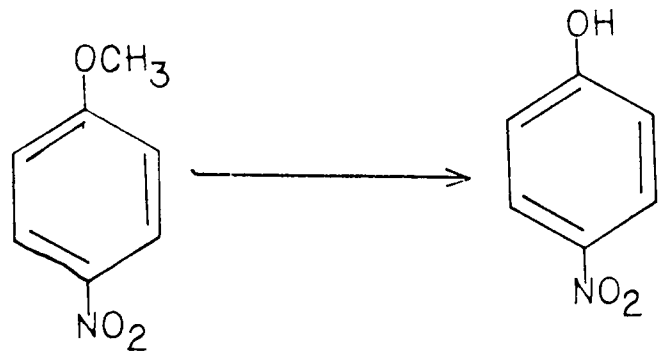
Zoxazolamine — 6-Hydroxyzoxazole



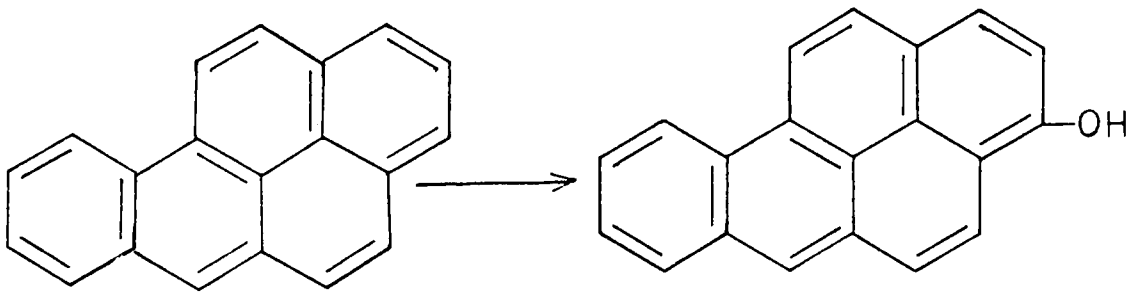
Aniline — p-Hydroxyaniline



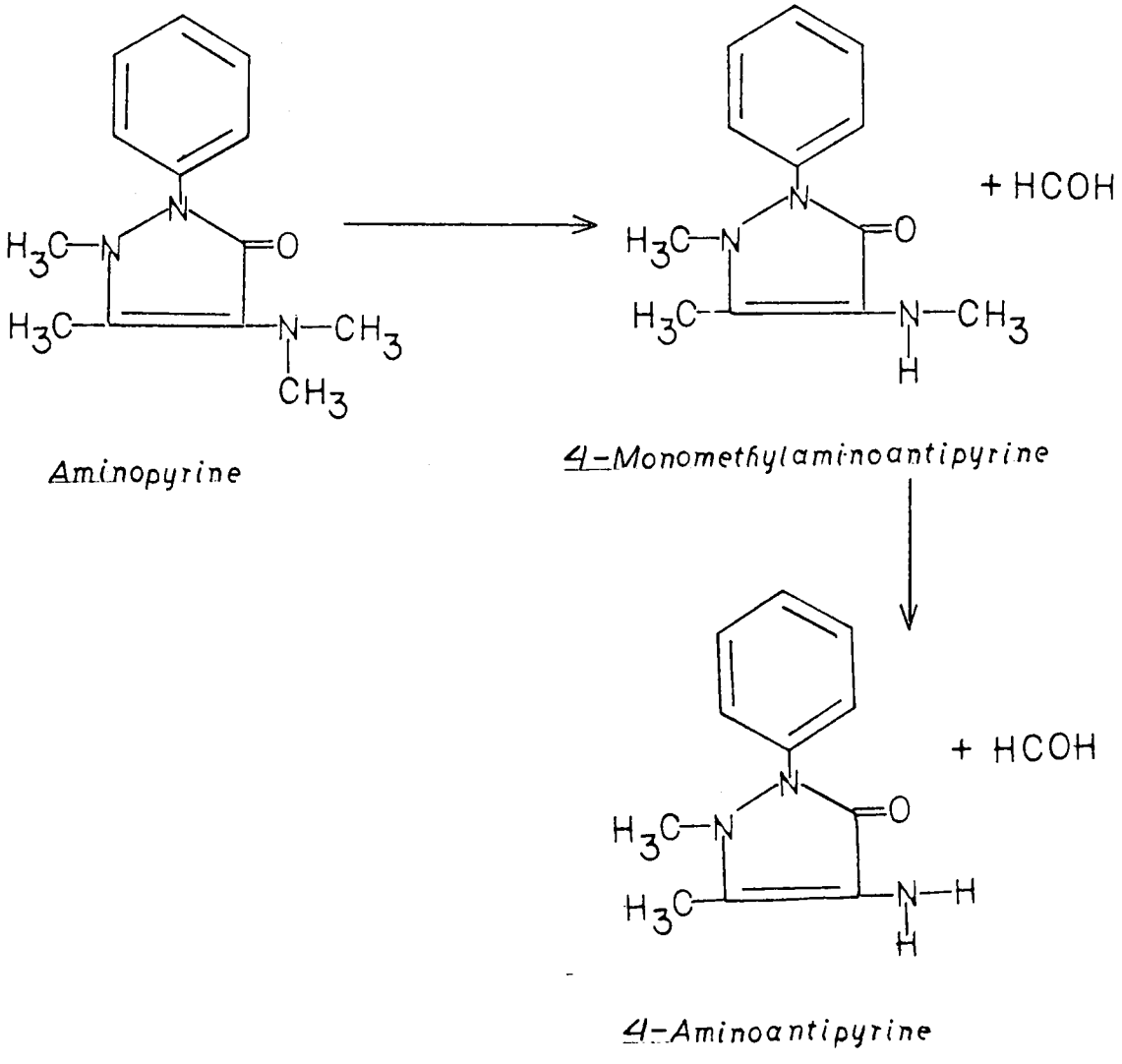
Hexobarbital — Hydroxyhexobarbital



p-Nitroanisole *p-Nitrophenol*



Benzo(a)pyrene *3-Hydroxybenzo(a)pyrene*



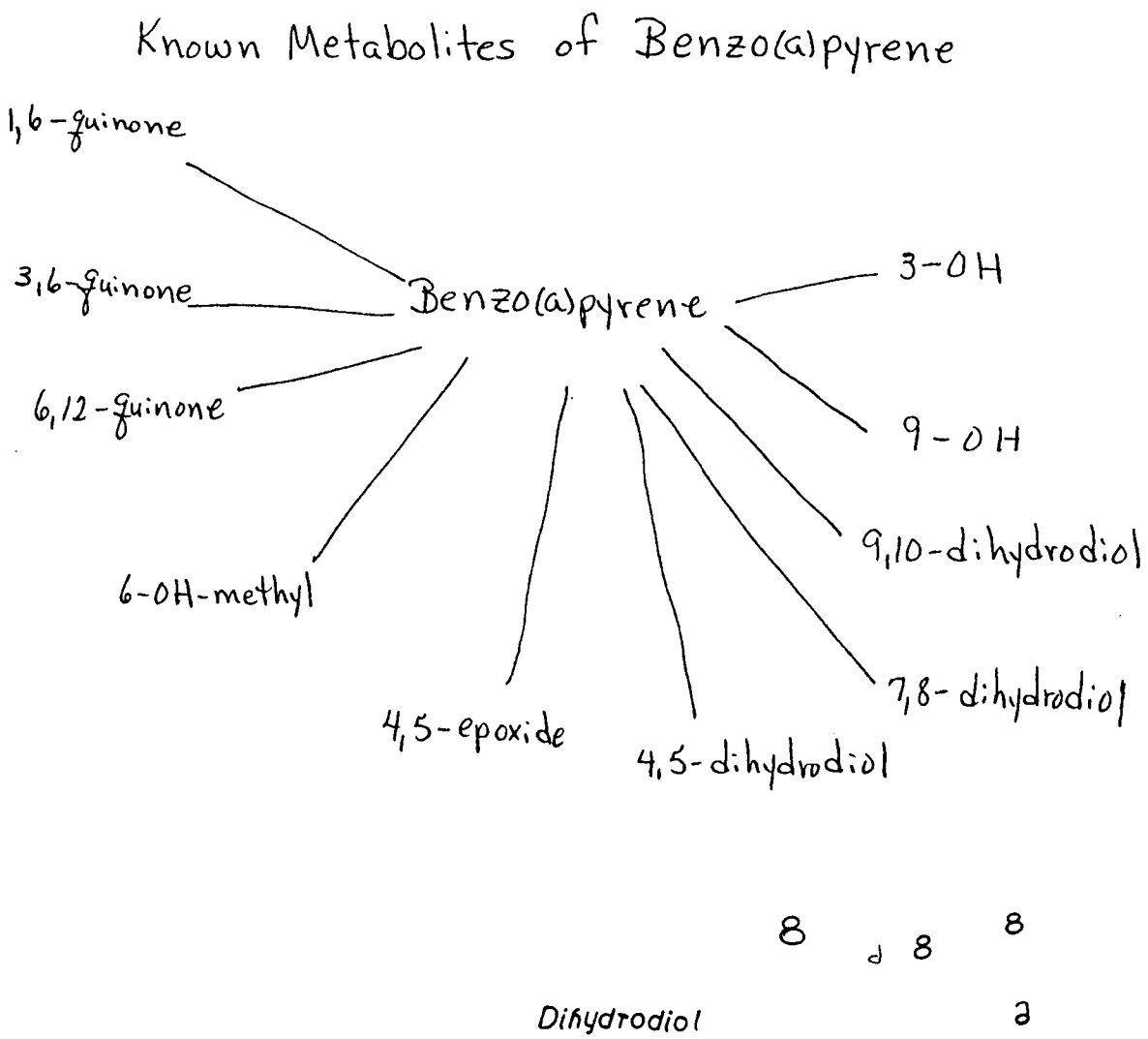
Aminopyrine

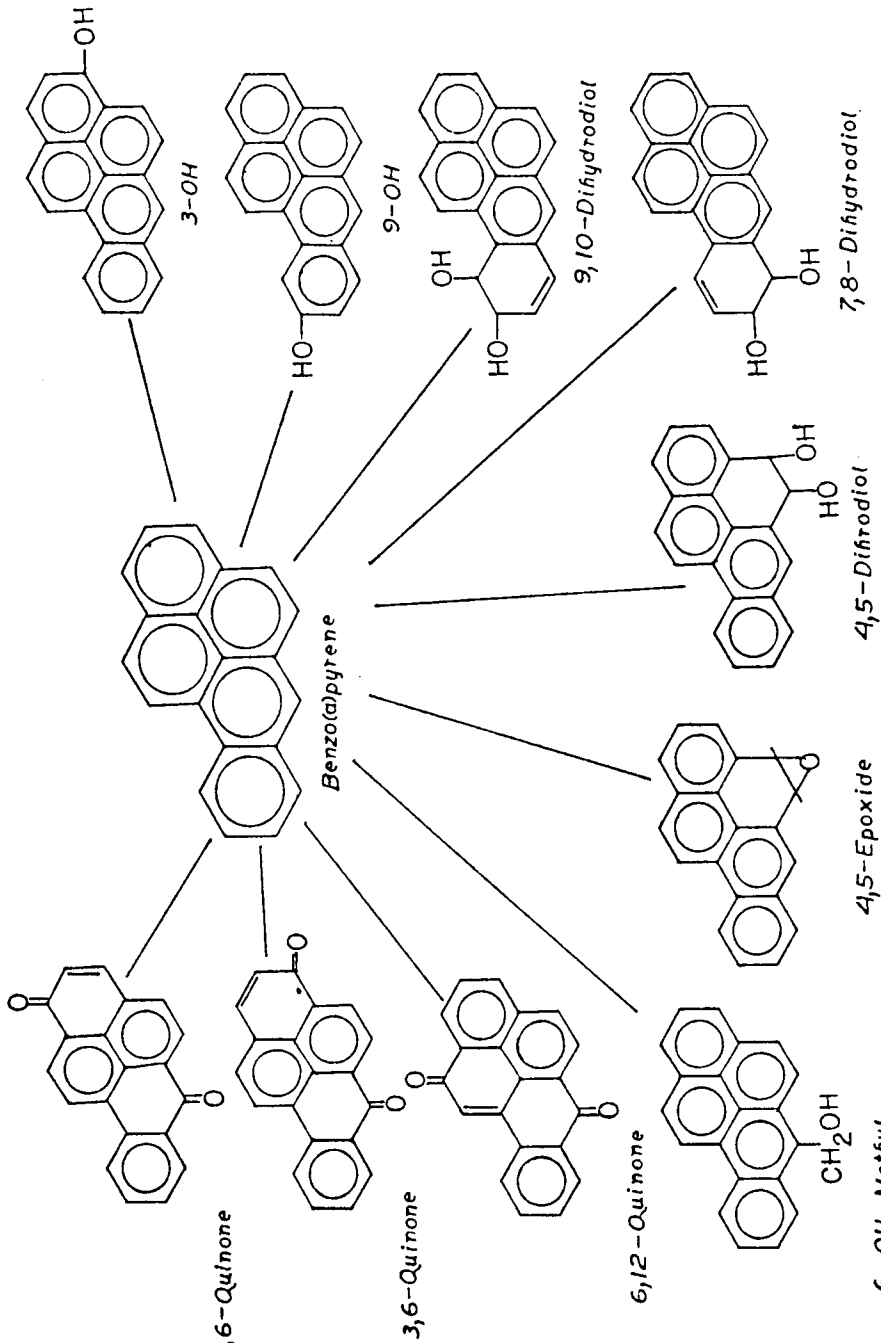
4-Monomethylaminoantipyrine

4-Aminoantipyrine

APPENDIX D

KNOWN METABOLITES OF BENZO(A)PYRENE




Benzo(a)pyrene
3-OH
9-OH
9,10-Difydrodiol
7,8-Difydrodiol
4,5-Difrodiol
4,5-Epoxide
6,12-Quinone
3,6-Quinone
1,6-Quinone
6-OH-Metfyl
OH
HO
CH2OH

VITA

Juarine Stewart Easley was born in Selma, Alabama, on October 20, 1951. She received her primary education in that city and was graduated from the former Tipton High School in May 1969. The following September she entered Knoxville College in Knoxville, Tennessee, and in June 1973 she received a Bachelor of Science degree in Chemistry. In the fall of 1973 she entered the University of Tennessee, Oak Ridge Graduate School of Biomedical Sciences on a fellowship granted by the Carnegie Corporation of New York and began study toward a Doctor of Philosophy degree. This degree was awarded in August 1978.

During the summer before her degree was awarded, Mrs. Easley served as assistant director and instructor in mathematics of the Premedical Enrichment Program for minority and disadvantaged students at the University of Tennessee. She will continue to work with this program while deciding on a postdoctoral position in her field.

The author is a member of the Beta Kappa Chi Scientific Honor Society, the Alpha Kappa Mu Honor Society and the Alpha Kappa Alpha Sorority.

She is married to Stenson Easley, a native of Knoxville, Tennessee.