A STUDY OF
ARYL HYDROCARBON HYDROXYLASE
IN CULTURED HUMAN LYMPHOCYTES

THESIS

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By

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Aryl hydrocarbon hydroxylase activity was studied in cultured human lymphocytes using 3-methylcholanthrene, 1,2-benzanthracene, and 4'-bromoflavone as inducers. The substrates used to run the 60 minute assay were benzo(a)pyrene and diphenyloxazole. At the optimum bromoflavone concentration for induction of aryl hydrocarbon hydroxylase, the induced enzymatic activity compared favorably with that of aryl hydrocarbon hydroxylase induced by 3MC in a 96 hour lymphocyte culture using BP as the assay substrate. The whole cell human lymphocyte system was found to have as much or more activity in 20 ml vials using Joklik's-Modified Minimum Essential Medium at a pH optimum of 7.5 with no co-factor added as did the Roswell Park assay system. The whole cell assay showed that levels of aryl hydrocarbon hydroxylase inducibility in lymphocytes from smokers and non-smokers varied without regard to the subjects' smoking habits. The assay system also indicated that intact lymphocytes generate a similar group of benzo(a)pyrene metabolites as that produced by a hepatic microsomal preparation from C57B1/6J mice.
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CHAPTER I

INTRODUCTION

Cancer is a class of diseases rather than a single disease (Greenstein, 1964; Clayson, 1962). The various types of cancer can be induced in a variety of tissues by a variety of agents. Many of these agents, such as insecticides, herbicides, dyes, food preservatives, and a number of other substances are found as environmental pollutants (Gelboin, 1971). The induction of cancer by xenobiotic agents is accompanied by phenotypic changes which include loss of cellular regulation and of specialized tissue or organ activity (Gelboin, 1971). The mechanisms by which carcinogenesis occurs have been postulated in three basic theories. The first theory is that of somatic mutation (Miller and Miller, 1971) in which a change occurs in the genetic material in the host cell accompanied by transformation of the cell line due to the presence of exogenous chemicals. This theory is illustrated by the malignant transformation of a cloned single cell line from C3H mouse prostrate by methylcholanganthrene (Heidelberger, 1970). A second theory involves the permissive growth of cancer cells in the presence of a defective immunologic system (Harnden, 1970; Burnet, 1964). The immunologic system of the host normally eliminates or suppresses the growth
of cancer cells that arise. Carcinogenic chemicals may cause a failure in the immunologic suppression followed by uncontrolled growth of abnormal cells (Gelboin, 1970; Burnet, 1964). The third concept suggests that viruses play an important role in the origin of cancer. Viral DNA associated with the host DNA may provide the route for alteration of host cell genetic mechanisms and growth regulation. Latent activation of this virion may occur through interactions with carcinogenic agents or radiation (Hubner, 1969; Gallo et al., 1970; Whitmire, 1973; Stitch, 1970).

These theories have specific differences, but all involve chemicals. Carcinogenic chemicals may effect genetic mutations, activate latent tumor viruses, or initiate a defect in the immune mechanism which ultimately alters the profile of gene expression (Commins, 1973; Gelboin, 1971a).

The class of chemical carcinogens considered in this paper is the polycyclic aromatic hydrocarbons (PAHs). The human population is exposed daily to low levels of benzpyrene (benzophenone, BP) and other polycyclic aromatic hydrocarbons (PAH) present in tobacco smoke (Commins et al., 1955; Cooper et al., 1954; Cantrell et al., 1973a) polluted city air (Falk, 1963), and smoked foods (Karatsune, 1960). Although it is difficult to prove a direct cause and effect line between PAHs and cancer, the high correlation between exposure to significant quantities of PAHs and the initiation of cancer strongly suggests that the PAHs are, in fact, associated with the production of cancer in man. Exposure to PAHs through
cigarette smoke increases the probability of lung cancer up to five fold (Tokuhata, 1964). Ninety percent of all lung cancer occurs in cigarette smokers (Progress Against Cancer, 1970). Recent studies have shown that the PAHs found in cigarette smoke markedly increase the activity of enzymes that hydroxylate BP in man (Alvares et al., 1972). PAHs are also found in automobile exhaust (Hoffman and Wynder, 1962) and in industrial plants (Dixon et al., 1970). In our environment we are, to a varying extent, almost continuously exposed to PAHs.

The enzymatic system induced by PAHs is one of the microsomal mixed function oxygenases. The cytochrome P450 associated enzyme system, aryl hydrocarbon hydroxylase (AHH), also called benzpyrene hydroxylase, metabolizes a variety of PAHs, including benzpyrene, to phenolic derivatives (Benedict, 1971; Nebert and Gelboin, 1969). This monooxygenase enzyme system requires NADPH and molecular oxygen during its metabolism of PAHs (Nebert et al., 1975), with ultimate reduction of the hydrophobic substrate being carried out by cytochrome P450. The incorporation of one atom of molecular oxygen into an aromatic substrate results in a reactive arene oxide (Epoxide) intermediate which can rearrange spontaneously to form a phenol and can be converted enzymatically to a trans-dihydriodiol or glutathione conjugate or become covalently bound to cellular nucleic acids or proteins (Wang, 1971; Swaisland, 1974).

In our environment we are exposed to many different PAH. Not all of these compounds are carcinogenic and not all affect
the AHH system in the same way. There are different types of monooxygenases induced by different classes of compounds (Lu, Strobel and Coon, 1969, 1970). Selected chemicals along with the enzymes they induce are the subjects of intense investigation.

The Mechanism of AHH
And its Metabolism of PAHs

Mixed function oxygenases are membrane bound, multicomponent enzyme systems (Mason, 1951) which require NADPH (Brodie et al., Estabrook et al., 1975) and molecular oxygen (Mason, 1957) for the oxidative metabolism of polycyclic hydrocarbons and related compounds. One of these mixed function enzyme systems is aryl hydrocarbon hydroxylase, also called benzpyrene hydroxylase (AHH). The nomenclature, aryl hydrocarbons hydroxylase, is preferred since the membrane bound hydroxylases convert a variety of polycyclic hydrocarbons to phenolic derivatives (Benedict et al., 1971). The substrate specificity of either the constitutive or the induced hydroxylase system from various mammalian tissues in culture and in vivo appears to be rather broad. Thus, many drugs and other exogenous substrates may be hydroxylated by this enzyme (Lee et al., 1969, 1970; Omura and Sato, 1964).

Reducing equivalents for AHH may be supplied by the co-factors NADPH (Brodie et al., 1955) and in some case NADH (Alvares et al., 1973) ultimately reducing a substrate via cytochrome P450 (Omura et al., 1965; Estabrook, 1975). A number of components of the hydroxylase system have been
reported, including a phospholipid moiety reported by Lee and Coon (1968), a non-heme iron protein (Omura et al., 1965), and two flavine moieties assumed to be NADH-cytochrome-c reductase (West et al., 1974) and NADH-cytochrome-b5 reductase.

One atom of molecular oxygen and the substrate are combined by AHH to form an epoxide intermediate (Jerina et al., 1968; Grover et al., 1972; Sims, 1970) which can react in one of four ways. It can rearrange spontaneously to form phenols (Heibeman & Ortez, 1968), be enzymatically converted to a trans-dihydriodiol by epoxide hydrase (Oesch and Daly, 1971), be conjugated with glutathione by glutathione s-epoxide-transferase (Boyland and Williams, 1965), or become covalently bound to cellular nucleic acids and proteins (Rogan and Cavalieri, 1974; Kinoshita and Gelboin, 1972; Wang et al., 1971). The epoxides, phenols, and dihydriodials may, in turn, be metabolized, explaining why dihydroxy and quinone derivatives of certain substrates are produced (Sims et al., 1975). The epoxides and phenols can also be further conjugated producing compounds considerably more polar than either the parent substrate, the epoxides, the phenols, or the various dihydriodials and which are more readily excreted (Oesch et al., 1971). The metabolites of interest in carcinogenesis research are the epoxides. The relationship between carcinogenicity of certain chemicals and the rate of reactive epoxide formation (Jerina and Daly, 1974; Oesch, 1972; Sims et al., 1975) are currently under investigation in numerous laboratories.
The metabolites of benzpyrene have been characterized more thoroughly than those of the other PAHs. Selkirk et al., (1974) characterized by mass spectrum fracture pattern, U.V. absorbance, and liquid chromatographic retention time, benzo(α)pyrene-4,5-oxide. Few other epoxides of polycyclic aromatic hydrocarbons have been identified. This may be due either to their extreme liability or to their rapid hydration to dihydrodiols by microsomal epoxide hydrases (Oesch et al., 1973). Trichloro-propeneoxide (TCPO), an epoxide hydrase inhibitor, blocks the formation of at least three BP dihydrodiols, a fact which suggests that the 7,8-diol, and the 9,10-diol, as well as the 4,5-diol are formed from epoxide intermediates. The 7,8 and 9,10 epoxides are very unstable under laboratory conditions and are thus difficult to synthesize and/or study. In order to clarify the pathways of polycyclic hydrocarbon metabolism, particularly the routes leading to detoxification and to carcinogen activation, it is important that all the intermediates and end products be characterized. The known BP metabolites include 9,10-dihydro-9,10-dihydroxybenzo(α)pyrene, 7,8-dihydro-7,8-dihydroxybenzo(α)pyrene, 4,5-dihydro-4,5-dihydroxybenzo(α)-pyrene, 3-hydroxybenzo(α)pyrene, 9-hydroxybenzo(α)pyrene, benzo(α)pyrene-1, 6-dione, benzo(α)pyrene-3,6-dione, and benzo-(α)pyrene-6,12-dione (Selkirk et al., 1975). Two additional metabolites have been isolated by Selkirk et al. (1976) using a new HPLC recycling technique. These metabolites, 1-hydroxybenzo(α)pyrene and 7-hydroxybenzo(α)pyrene, are chromatographically similar to 3-hydroxybenzo(α)pyrene.
Tissue Specificity

Aryl hydrocarbon hydroxylase activity is inducible in many mammalian tissues by polycyclic hydrocarbons (Conney, 1967; Gelboin, 1967; Gillette, 1963). The enzyme system may also be induced in cell culture by polycyclic hydrocarbons (Nebert and Gelboin, 1968). Nebert and Gelboin (1969) compared the in vivo AHH activity of liver, lung, gastrointestinal tract, brain, skeletal muscle and kidney in the monkey, hamster, and rat. Aryl hydrocarbon hydroxylase activity has been reported in a variety of cultured tissues. Primary cell cultures from human skin metabolize benzpyrene into phenols, quinones, and dihydriodols (Fox et al., 1975). Topical application of dimethylbenzantracene stimulates increased AHH activity in the skin of inducible mouse strains (Thomas et al., 1973).

AHH activity is found in human lymphocytes (Whitlock, 1972; Busbee et al., 1972; Selkirk et al., 1975), fibroblast cultures derived from fetal lung or skin (Pelkonen et al., 1975), pulmonary alveolar macrophages (Cantrell et al., 1973b), peritoneal macrophages (Ptashne et al., 1974), kidney (Wiebel et al., 1974), bowel (Gielen et al., 1972), and human foreskin (Levin et al., 1972; Alvares et al., 1973). The enzyme is inducible in fetal cells derived from whole hamster, mouse, rat and chick (Nebert and Gelboin, 1969). Further, hydroxylase activity is inducible in cell cultures derived from hamster fetal liver, lung, small intestine, and muscle tissue, and in mouse 3T3 cells, an established abnormal cell line (Nebert and Gelboin, 1969). The magnitude of aryl hydrocarbon hydroxylase
induction varies greatly with the tissue, from no induction to more than 100-fold increases in enzyme activity (Nebert and Gelboin, 1969).

**Induction of AHH**

Originally, the term enzyme induction (Jacob and Monod, 1961) described the biochemical mechanism in which a regulatory gene controlled the expression of a structural gene's production of a certain enzyme by the synthesis and release of repressor molecules. In the presence of an inducer, the repressor molecule could no longer exert an effect upon the DNA, allowing increased transcription of that structural gene with concomitant increased enzyme synthesis (Jacob and Monod, 1961). At present, the term "induction" is used in a general sense to describe an elevation in enzyme activity, without necessarily having any genetic connotation (Cutroneo et al., 1972). The process of induction denotes a relative increase in the rate of de novo synthesis or in the rate of activation of enzyme activity from pre-existing moieties, or in both, compared to the rate of breakdown of enzyme activity. Since the AHH system is a multicomponent membrane-bound system, there are technical difficulties in attempting to distinguish between de novo enzyme synthesis and increased activation of existing molecules (Gielen et al., 1971). Thus, in most cases, the rate of enzyme induction expresses the rate at which induced hydroxylase activity accumulates.

In the AHH system, the process of induction varies with the tissue type, but apparently requires continuous protein

**Type I and Type II Inducers**

More than 200 xenobiotic agents such as drugs, herbicides, and pesticides stimulate microsomal enzyme induction. The inducers are of at least two types (Conney et al., 1960; Gielen, 1971), exemplified by phenobarbital, a Type I inducer, and 3-methylcholanthrene (one of the polycyclic aromatic hydrocarbons), a Type II inducer. A variety of enzymatic pathways in liver microsomes are induced by compounds structurally related to phenobarbital. Phenobarbital stimulates the in vivo activity of microsomal enzymes to maximum levels after 3 days or more of repeated treatment, accompanied by marked proliferative changes in the smooth endoplasmic reticulum (Gielen, 1971a). Polycyclic hydrocarbons, administered as a single dose, generally induce the activity of several specific oxygenases to peak levels in about 24 hours with no marked proliferation of the microsomal membranes (Gielen, 1971; Conney et al., 1967). Using aryl hydrocarbon hydroxylase as an
enzyme inducible by either class of inducers, Nebert and Gelboin (1969) found that hydroxylase induction by BP occurs in vivo to a significant extent only in liver. In contrast, hydroxylase induction in vivo or by polycyclic hydrocarbons occurs rapidly in most mammalian tissue examined (Nebert and Gelboin, 1969). In fetal cell cultures derived from the entire hamster (Alfred and Gelboin, 1967; Nebert, 1970) or from mouse (Nebert, 1970), aryl hydrocarbon hydroxylase induction by polycyclic hydrocarbons in the growth medium occurs readily, whereas concentrations of phenobarbital as high as 10 mM did not induce the enzyme (Nebert, 1968). Gielen and Nebert (1971b) later found that when cell cultures derived from the entire rat were treated with 2 mM BP about a two-fold increase in hydroxylase activity was elicited. This finding is consistent with the possibility that in a heterogenous cell population the enzyme system in one of the types of cells present, such as hepatocytes, can be induced by BP.

**Cytochrome P450**

\(\Delta\)-amino-levulinate, is biosynthetically converted to cytochrome P450 by the enzymatic addition of side groups.

\[
\begin{align*}
2\text{HN-CH}_2 \\
\text{C}=\text{O} \\
\text{H-C-H} \\
\text{C-OOH} \\
\end{align*}
\]

\[
\begin{align*}
\text{HOOC-CH} \\
\text{(CH}_2\text{)_2-COOH} \\
\end{align*}
\]

\[
\begin{align*}
\text{2HN}_2\text{-HC} \\
\text{N} \\
\text{H} \\
\text{H-C-H} \\
\end{align*}
\]

\[
\begin{align*}
\rightarrow \\
\text{Porphyins} \\
\rightarrow \\
\text{P-450} \\
\end{align*}
\]

\(\Delta\)-aminolevulinate \hspace{1cm} \text{Porphobilinogen}
Cytochrome P450 functions in the oxidative metabolism of a wide variety of organic compounds (Omura and Sato, 1964; Gillette et al., 1969). This hemoprotein serves to activate molecular oxygen for the insertion of an atom of oxygen into the organic substrate molecule (Mason, 1957). In this way, non-polar lipophilic molecules become more polar and sites for conjugation with other compounds are generated. In general terms, this enzymatic process has long been recognized as a hydroxylation reaction or a mixed function oxidation reaction (Mason, 1957). Four general observations can be made about cytochrome P450 catalyzed reactions (Estabrook, 1975): (a) a substrate must have suitable lipophilicity and appropriate stereochemical configuration to permit binding at a site adjacent to the heme iron of cytochrome P450; (b) there must be a source of reducing equivalents in the form of reduced pyridine nucleotides; (c) there must be a suitable series of electron transfer components required to catalyze the reduction of cytochrome P450 by equivalents originating from reduced pyridine nucleotides; and (d) molecular oxygen must be bound to reduced cytochrome P450 to form a tertiary complex of oxygen, substrate, and the heme iron of cytochrome P450. In its reduced state, cytochrome P450, like many other ferrous hemoproteins, complexes with carbon monoxide (Griffin et al., 1975). However, the position of the Soret absorbance maximum of this complex at 450 nm is unique among nemo-proteins and serves to identify nemo-proteins belonging to this class (Omura and Sato, 1964). Further studies show that Type I inducers such as
phenobarbital induce cytochromes with a slightly different absorbance maximum than do the Type II inducers, such as 3-methylcholanthrene, which induce cytochromes with a maximum absorbance at 448 nm (Lu Levin, 1972). These results indicate at least two separate cytochromes, P450 and P448, that catalyze the same or similar hydroxylations in two separate systems. These two cytochromes have been partially purified (Lu and Levin, 1972), retaining catalytic activity.

The enzymatic reaction characteristic of cytochrome P450, in its most general form can be written as:

\[
\text{NAD(P)H} + \text{H}^+ + \text{O}_2 + \text{SH} \rightarrow \text{NAD(P)}^+ + \text{SOH} + \text{H}_2\text{O} \quad (\text{Griffin et al., 1975})
\]

There is a net transfer of two electrons from the reduced pyridine nucleotide and two electrons supplied by the substrate (SH) to molecular oxygen, resulting in the formation of a molecule of the hydroxylated substrate and a molecule of water (Estabrook et al., 1963), as shown in the diagram below.

Figure 1 --- The general enzymatic reaction characteristic of cytochrome P450.
The initial step is thought to consist of a substrate binding to the ferric molecule of the enzyme, which converts the heme protein to a high-spin state (Estabrook et al., 1972; Tyson et al., 1972). One electron is then transferred to the enzyme-substrate complex by a second protein component of the AHH system. Oxygen is subsequently bound, resulting in a distinct configuration of the enzyme which is presumed to be an enzyme-substrate-oxygen ternary complex. A one electron reduction of the ternary oxygenated enzyme species results in product formation and regeneration of the initial ferric state of the enzyme (Estabrook et al., 1969).

**DNA Binding**

The metabolism of polycyclic aromatic hydrocarbons proceeds via the oxidation of an intermediate arene oxide. These highly reactive epoxides may (a) interact with macromolecular tissue constituents, (b) be further metabolized or rearranged to dihydrodiols and phenols, or (c) conjugate with glutathione (Grover et al., 1971; Selkirk et al., 1971; Wang et al., 1972). Moreover, evidence suggests that K-region epoxides of polycyclic aromatic hydrocarbons are chemically and biologically reactive proximal or possibly ultimate carcinogens (Grover et al., 1971b; Ames et al., 1972). In contrast to these findings, Pietropaolo and Weinstein (1975) suggest that the nucleic acid binding of PAH through an epoxide intermediate was not the primary route because of several competing reactions (epoxide hydrase conversion to BP-diol, transferase conversion to conjugate with glutathione and the non-enzymatic conversion...
to a phenol) that utilize BP-epoxide and limit its availability.

The somatic mutation theory (Miller and Miller, 1971) implies that cancer is a consequence of DNA modification. There is current evidence to indicate that the attachment of intermediate PAH epoxides to DNA could cause such modifications (Brooks, 1975) which are reported to be mutagenic in selected bacterial strains (Ames et al., 1972). Burki et al. (1974) found increased binding of 3-methylcholanthrene -11,12-oxide to calf thymus DNA in the presence of trichloro-2-propeneoxide (TCPO), an inhibitor of epoxide hydrase. Wang et al. (1972) reported that a PAH K-region arene oxide, the 4,5-epoxide of BP, was much more active in binding to DNA in vitro than the other metabolites tested. The K-region epoxides of polycyclic hydrocarbons are considered to be proximal carcinogens if they have a higher carcinogenic potential than do the corresponding parent compounds in animal test systems and/or if it can be shown that the metabolic formation of arene oxides in the whole animal is related to the carcinogenic process (Burki et al., 1974). Conflicting evidence on reactivity of arene oxides is reported in preliminary comparative studies of the carcinogenic activity of MCA-11,12-oxide, and of the parent compound, where experimental tumorigenesis on mouse skin, either by single or repeated topical application or in initiation-promotion experiments with phorbol ester as a promoter, revealed a low carcinogenic potential of the epoxide compared to that of the parent compound (Burki et al., manuscript in preparation). Similar characteristics of epoxides of other polycyclic hydrocarbons (Van Duren,
suggest that in situ formation of the epoxide at a target site is vital for carcinogenesis to occur. Although epoxides do react with DNA, the products are not identical to those resulting from in vivo binding of the parent carcinogen (Baird et al., 1973).

Recently, Borgen et al. (1974) reported that BP 7,8-dihydrodiol, in the presence of liver microsomes and NADPH, binds to DNA to a ten fold greater extent than does BP. Sims et al. (1974) reported that BP 7,8-dihydrodiol was in favorable stereochemical state to effect the binding of DNA. Wisloki et al. (1976) found that BP 7,8-oxide may be carcinogenic per se, or it may undergo further metabolism to BP-7,8-diol-9,10-epoxide which is highly reactive. This high mutagenicity suggests that the diol epoxide may be the ultimate carcinogen of BP.

**Statement of Problem**

In this thesis, many aspects of the AHH system are examined using cultured human lymphocytes. 4'-bromoflavone, a non-carcinogenic polycyclic hydrocarbon is studied as an inducer of the AHH system. This PAH is tested for its characteristic effects on lymphocytes, including (a) AHH inducibility, (b) optimum AHH induction time, and (c) optimum inducer concentration.

The assay for AHH in lymphocytes is examined for pH, cofactor, and buffer requirements. This AHH assay system can later be used to study the difference in inducibility of AHH in lymphocytes between smokers and non-smokers. High pressure liquid chromatography was used to study the distribution of BP meta-
bolites in human lymphocytes as compared to the BP metabolites in C57Bl/6J mice liver microsomes.
CHAPTER II
MATERIALS AND METHODS

Experimental Animals

C57Bl/6J mice were obtained from Jackson Laboratories, Bar Harbor, Maine, and maintained on Purina Rat Chow and water ad libitum in the vivarium of the Biology Department of North Texas State University.

Lymphocyte Isolation and Culture

Human lymphocytes were isolated for cell culture by drawing venous blood and diluting it with one half volume of sterile, heparinized, normal saline (0.9%). This mixture was carefully layered onto 8 ml of Ficoll-Hypaque (180 ml H₂O, 50 ml 50% sodium diatrizoate (Hypaque, Winthrop Laboratories) containing 6% Ficoll and centrifuged at 800 x g for 15 min. The erythrocytes agglutinated in the presence of Hypaque and sedimented to the bottom of the centrifuge tube along with polymorphonuclear leukocytes. Lymphocytes and monocytes formed a discrete layer at the interface between the plasma and the Ficoll Hypaque and were removed using a sterile Pasteur pipette. The lymphocyte/monocyte mixture was washed by centrifugation from sterile heparinized saline, and the cell pellet obtained was resuspended to approximately 2 x 10^6 cells per 10 ml in Joklik's Modified Minimum Essential Medium containing phytohemagglutinin, pokeweed mitogen,
50 units of heparin/ml, and 15% fetal calf serum (Gibco). The cells were induced by the immediate addition of 10 μl of either 5 mM 1,3-benzanthracene, or 1 mM 3-methylcholanthrene or of specified concentrations of 4'-bromoflavone dissolved in acetone. Cultures were incubated in a 5% CO₂, humidified environment at 37°C for 96 h.

**Cell Harvest and Assay**

Cells cultured for 96 hours were transferred to 15 ml conical centrifuge tubes and centrifuged for 3 min. at 100 x g. The growth medium was removed by aspiration and cells were resuspended to 10⁶ cells/ml in EMSAD buffer at pH 8.0. EMSAD buffer contains 3 mM MgCl₂, 125 mM sodium chloride, 0.1% bovine serum albumin, 10 mM dextrose, and 25 mM 4-(2-hydroxyethyl)-1-1-piperazine propane sulfonic acid (EPPS, pK 7.95). Two μg of benzo(a)pyrene in a volume of 10 μl of methanol were added, and the mixture was incubated in the dark for 60 min. at 37°C. The reaction was stopped by adding 0.5 ml of neutral 20% formalin with gentle shaking. After waiting at least 5 min., 0.5 ml of 1.0 N NaOH was added, and the mixture was vigorously agitated to suspend the cells. The fluorescence of the suspension was immediately determined in an Aminco-Bowman spectrophotofluorometer, with excitation at 465 nm and emission at 522 nm. Earlier phases of this study used the fluorometric analysis described by Busbee et al. (1972) in which a Tris buffered assay system was utilized and excitation for fluorometry was accomplished at 396 nm. The distribution of BP
metabolites was also determined using $^3$H-BP as a substrate. The reaction was stopped by adding 10 ml of ethyl acetate to a 5ml sample and vortex mixing for 20 sec. The organic phase was removed, dried under $N_2$, and frozen at $-80^\circ C$ until metabolite separation was accomplished by high pressure liquid chromatography.

**AHH Induction in Animals**

C57Bl/6J mice were induced *in vivo* by intraperitoneal (IP) injection of 3-methylcholanthrene (20 mg/kg body weight) in corn oil. Experimental animals were denied food and $H_2O$ during the final 24 h. of induction. The animals were sacrificed by cervical dislocation 72 h. after injection.

**Animal Assay**

Experimental animals were sacrificed, and their livers were removed. Livers were diced, washed, and homogenized in cold TMS buffer ($0.050 \text{ M Tris-HCl pH 7.5}, 0.200 \text{ M sucrose}, \text{ and } 0.003 \text{ M } MgCl$). Centrifugation of the homogenate at $10,000 \times g$ for 15 min. removed mitochondria, nuclei, and other cellular debris. The supernatant was removed and centrifuged at $105,000 \times g$ for 60 min. in a Beckman Model L Preparative Ultracentrifuge equipped with a 50 Ti rotor. The pellet was resuspended in cold TMS at a final concentration of 10% microsomal protein and was frozen at $-80^\circ C$ until used.

The microsomal mixture was assayed for aryl hydrocarbon hydroxylase by the addition of 1 mg NADPH and 50 $\mu$g $^3$H-benzo-(α)pyrene (Amersham-Searle 79.3 mCi/mg) to 5 ml of a 1% micro-
somal preparation followed by incubation in the dark for 30 min. at $37^\circ C$. The reaction was stopped by adding 10 ml of ethyl acetate and vortex mixing for 20 sec. The organic phase was removed by Pasteur pipette, and the aqueous phase was re-extracted with ethyl acetate. The two organic phases were pooled, dried under $N_2$, and stored at $-80^\circ C$ until metabolite assay was accomplished.

**Metabolite Separation by High Pressure Liquid Chromatograph**

A Waters model 1401 high-pressure liquid chromatograph fitted with a C$^{18}$ Bondapak column was used to separate the benzo($\alpha$)pyrene metabolites. A reverse phase gradient system was utilized for benzo($\alpha$)pyrene metabolite separation, and consisted of a 60:40 methanol in $H_2O$ solvent front increasing to 100% methanol at termination. A pressure of 1,000 pounds per square inch produced a flow rate of 0.5 ml/min. Fractions were collected at one minute time intervals. Eluate was constantly monitored using a Waters model 440 U.V. absorbance detector (254 nm) and a Fisher Recordall series 5000 strip chart record.

**Radiometric Analysis**

A 10 $\mu$l aliquot was taken from each sample collected from the HPLC and placed into a 20 ml scintillation vial to which 10 ml of Aquasol-2 (New England Nuclear) was added. Each vial was then counted for 1 min. in a Beckman LS - 250 liquid scintillation counter. The tritium counting efficiency was 37.7%.
CHAPTER III

RESULTS

Preface

A study of aryl hydrocarbon hydroxylase (AHH) in cultured human lymphocytes was undertaken to examine several aspects of the enzyme system. 4'-bromoflavone was tested as an inducer of AHH. The optimum dosage of bromoflavone and optimum time for induction of AHH in cultured lymphocytes were determined. The optimum pH, co-factor, and buffer requirements of the assay for AHH in cultured human lymphocytes were also examined. AHH was assayed to determine the difference in inducibility in smokers vs. non-smokers and to examine the distribution of BP metabolites produced by cultured human lymphocytes. Enzymatic activity of AHH was determined by fluorometrically measuring the phenolic derivatives of benzo(a)pyrene (BP). This technique indirectly measures the amount of enzyme activity. In order to determine the distribution of the BP metabolites, $^3$H-BP was used as substrate for the enzymatic reaction and the mixture of metabolites generated was extracted, separated by high pressure liquid chromatography, and collected for quantitation. The total levels of BP metabolites were radiometrically determined.

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**4'-Bromoflavone as an Inducer of the AHH System**

An induction dose response curve of 4'-bromoflavone (BrF) in cultured human lymphocytes using BP as a substrate is shown in figure 2. The experiment indicates an increase in AHH activity at BrF concentrations of 3 µM and 10 µM, with activity decreasing at higher BrF concentrations. Because of the wide range between the concentrations, further experimentation was needed to determine the optimum dosage of inducer. A narrow range of BrF concentrations with maximal activity at doses from 6 to 10 µM is seen in figure 3. The BrF concentrations were compared with optimum doses of 1,2-benzanthracene (BA) and 3-methylcholanthrene (3MC). Of the Type II inducers, 1,2-benzanthracene elicited almost twice as much activity as either 3MC or BrF, the inducer in question.

A BrF time course of induction experiment using 5 µM BA and 10 µM BrF as inducers of the AHH system in cultured human lymphocytes is presented in figure 4. The activity for each inducer increases with time up to 96 hours, decreasing to a lower level at 120 hrs. The AHH activity induced by BrF at 96 hrs., decreasing to a lower level at 120 hrs. The AHH activity induced by BrF at 96 hrs. is one-half that induced by BA.

Bromoflavone was compared to BA and 3MC as an inducer, and diphenyloxazole (PPO) (10 µg) was compared to BP (25 µg) as a substrate (Figure 5). Each inducer and substrate was paired to find the optimum combination. BrF showed the lowest AHH induction of three inducing agents tested using both BP and PPO as substrates. Induced enzyme levels were highest using BA as
an inducer and BP as a substrate. The data show relatively high levels of activity using PPO as the substrate with each inducer (Figure 5). Although AHH activity is low using BrF as an inducer, in BA and 3MC induced cells the activity of PPO is comparable to that of BP.

**An Assay for AHH in Cultured Human Lymphocytes**

Results of a co-factor study using varying concentrations of NADH is presented in figure 6. The induced enzyme activity was highest in intact cells and found to be independent of the concentration of either NADH or NADPH. This whole-cell independence may reflect the ability of intact cells to produce their own co-factors. Sonicated cells, however, required NADH and NADPH in the AHH system. The concentrations of both co-factors used were 0.3 mg/ml and 3 mg/ml. The co-factor concentrations producing the highest AHH activity were 0.3 mg/ml of NADH and 0.3 mg/ml of NADPH with a 30 min. incubation of the sonicated cells. The 120 min. sonicated cell assay showed its highest activity using 3 mg/ml of both NADPH and NADH.

The increase with time in accumulated phenolic derivatives of BP in sonicated cells is demonstrated in figure 7. In the absence of either NADH or NADPH added to the sonicated preparation, essentially no activity was found, indicating dependency of the AHH system on NADH and NADPH as external co-factors. A pH curve of AHH activity in intact lymphocytes is seen in figure 8. The activity increases in BA induced cells to pH 7.5 and plateaus, remaining high through pH 9.
The difference in AHH activity assayed in a variety of buffers at a pH of 8.5 is shown in figure 9. The buffers include Tris, Bicine (pka 8.4), EPPS (pka 7.95), HEPES (pka 7.5), TES (pka 7.5), Tricine (pka 8.1), along with phosphate-carbonate buffered medium. Each of the buffers was presented in the growth medium at a concentration of 25 mM. The varying activity was probably a reflection of the different pkas of each of the buffers and their ability to stabilize the growth environment at a pH of 7.5. The data from this experiment indicate that lymphocytes may be induced to a higher AHH activity in Jokliks-Modified MEM medium than in any of six other buffered media.

A comparison between the BA-induced activity of AHH in human lymphocytes grown in Jokliks-Modified Minimal Essential Medium (GIBCO F-13), and the activity found in identical cell preparations cultured in Roswell Park Memorial Institute (RPMI-1640) medium which were also induced with BA is seen in figure 10. When the cells were grown in 20 ml vials, AHH activity was highest in Jokliks MEM. The opposite results were obtained when cells were cultured in 25 cm\(^2\) (30 ml) T-flasks, with RPMI cultured cells showing an induced AHH activity twice that of the MEM cells.

The inducibility of AHH in human lymphocytes between smokers and non-smokers was compared in figure 11. Two substrates were used, BP (25 µg) and PPO (10 µg), to assay in lymphocyte samples from each individual. The level of induction varied without regard to the subject's smoking habits.
A comparison was made between the distribution of BP metabolites produced by cultured human lymphocytes and by C57B1/6J mouse hepatic microsomes treated with the inducer, 3MC (Figure 12). The mouse microsomes showed a high production of 9,10-diol as compared to almost no 9,10-diol metabolites produced in the human lymphocytes. The production of large quantities of the phenolic compounds in both lymphocytes and microsomes indicates high levels of enzymatic conversion of intermediate epoxide(s) by epoxide hydrase. There is also an unusually high level of the 6,12-quinone produced by mouse hepatic microsomes.
Figure 2 --- A dose response curve of BrF as an inducer of the AHH system. The concentrations used were 0.3 μM, 1 M, 3 μM, 10 μM, and 30 μM with a 96 hour culture period. Assays were incubated over 120 minutes using BP (25 μg) as a substrate. Fluorescence was measured using an Aminco-Bowman spectrophotofluorometer at excitation 396 nm and emission 522 nm with fluorescence values converted to picomoles of 3-hydroxybenzpyrene.
Figure 3 --- AHH activity is compared using 1 μM 3-MC, 5 μM BA, and varying concentrations BrF for induction. The lymphocytes were cultured in 25 cm² (30 ml) T-flasks over 96 hours.
Figure 4 --- AHH time course of induction over 120 hours in cultured human lymphocytes induced with 5 μM BA and 10 μM BrF. Samples were assayed every 24 hours using BP (25 μg) as a substrate. Fluorescence values are shown converted to picomoles of 3-OHBP.
Figure 5 --- AHH activity measured using 5 μM BA, 1 μM 3-MC, and 10 μM BrF as inducers in a 96 hour culture. BP (25 μg) and PPO (10 μg) were used as substrates for the 60 minute assay.
Figure 6 --- Intact and sonicated cells were used to study co-factor requirements. BA (5 μM) was used as an inducer with BP (25 μg) as a substrate. NADPH and NADH were added to the assay mixture of concentrations of 0, .3, and 3 mg/ml. Each lymphocyte culture had approximately 1.8 x 10^6 cells. One F (fluorescent unit) corresponds to one picomole of 3-OHBP.
A 30 MIN. SONICATED

• 120 MIN. SONICATED

\[
\begin{align*}
\text{F/18x10^6} & \\
(15) & \\
(10) & \\
(5) & \\
0 & 0.3 & 3.3 & 0.3 & 3.3 & 0.3 & 3.3
\end{align*}
\]

NADH +
0 NADPH
0.3 NADPH
3. NADPH
Figure 7 --- A co-factor study using 0.3 mg/ml and 3 mg/ml of both NADH and NADPH. The cells were sonicated for 15 seconds. They were then assayed for 30 minutes and 120 minutes using BP (25 μg) as a substrate. One F (fluorescent unit) corresponds to one picomole of 3-OHBP.
[NADPH] + [NADH]

$F/18 \times 10^6$

MINUTES

30  60  120

0.3

3.0

0
Figure 8 --- AHH activity levels at different pH values in BA induced human lymphocytes. The pH was adjusted by the addition of either 1N HCl or 1N NaOH. The cells were assayed for 120 minutes using BP (25 μg) as a substrate.
Figure 9 --- The effect of different culture medium buffers on AHH activity levels. Six buffers, Bicine (pka 8.4), EPPS (pka 7.95), HEPES (pka 7.5), TES (pka 7.5), Tricine (pka 8.1), or Tris were added individually to the culture medium at a concentration of 25 mM. Approximately $3 \times 10^6$ cells were added to each culture and incubated for 96 hours using BA as an inducer. BP (25 µg) was added for the last 60 minutes. Metabolites of BP were then extracted and quantified. One F (fluorescent unit) corresponds to one picomole of 3-OHBP.
Figure 10 --- A comparison of culture media. AHH activity levels were compared in Gibco's Joklik-Modified minimal essential medium (Gibco F-13) and Roswell Park Memorial Institute medium (RPMI 1640). The BA induced cells were cultured in 20 ml vials and 25 cm$^2$ (30 ml) T-flasks.
Figure 11 --- AHH activity levels in non-smokers and smokers. BA was used to induce the lymphocyte cultures with BP (25 μg) and PPO (10 μg) as substrate. Fresh medium was added at 72 hours and the cells were assayed at 96 hours.
Figure 12 --- The distribution of BP metabolites in human lymphocytes was compared to the BP metabolism in C57B1/6J mice liver microsomes, A. mixture of standard compounds, B. mice liver, C. lymphocytes. The microsomes were assayed using 200 μCi of 3H-benzo(a)pyrene along with a 30 minute incubation. Each sample was separated by high pressure liquid chromatography and collected in 0.5 ml fractions. Each fraction was then counted using a scintillation counter. The separation of standards was monitored at 254 nm with U.V. detector.
CHAPTER IV

DISCUSSION

Chemical carcinogenesis associated with enzymatic activation and breakdown of polycyclic aromatic hydrocarbons is the subject of investigation in numerous laboratories. In vivo studies of AHH metabolism of PAH carcinogens may be complicated by absorption, distribution, and metabolism of the inducer, nutritional and hormonal variables, and systemic toxicity of the PAH in the whole animal (Cutroneo et al., 1972). These interacting factors contribute to ambiguities confusing the determination of actual levels of AHH activity existing in tissues. In vitro studies lack these variables and allow more consistency in examinations of the AHH enzymatic system. The AHH system has been well characterized (Kouri et al., 1973; Nebert and Gelboin, 1969; Cantrell and Bresnick, 1972; Burki et al., 1972; Fisher and Spencer, 1972; Gelboin and Blackburn, 1964) using liver homogenates and hepatic microsomes. These experiments provided the foundation for extensive research using several AHH assays and correlating tumorigenesis in experimental animal tissues with AHH activity. Increased AHH activity levels were shown to be directly linked to tumorigenesis. Unfortunately, this information was not directly applicable to human studies. With the development by Busbee et al., (1972) and Whitlock et al. (1972) of a technique for assaying AHH in cultured human lym-
lymphocytes, the direct association between AHH activity and chemical carcinogenesis in man could be more thoroughly investigated. The cultured lymphocyte assay is relatively inexpensive, thus, with further development for large scale application, it has potential for use as a screening test to predict the genetic susceptibility of an individual to chemically initiated cancer.

One of the characteristics of the AHH enzyme system in mammalian tissues is its response to the administration of various inducing agents (Conney, 1967). The inducibility of this enzyme by PAHs may be important in protecting against ingested and inhaled environmental pollutants. A class of hydrocarbons, the flavones, induce an AHH system with the same characteristics as that induced by 3MC, yet lack the carcinogenic potential of 3MC (Wattenberg et al., 1968a). Potentially, these compounds are very important in carcinogenesis research, but have been incompletely characterized, and, consequently, further studies are needed using this class of compounds as an inducer of the AHH system.

Wattenberg et al. (1968b) found that the flavones, when substituted at the para position on the phenyl ring, exert a marked enhancement on microsomal enzyme induction in liver and lung tissues. If this chemical phenomenon were due simply to the blocking of the para position, one might expect similar results when both the halogens and the methoxyl group are used as blocking agents. This is not the case. The halogen-substituted flavones show greater inducing capacities than do those compounds containing the methoxyl group, with bromine substituted
compounds having the highest inducing capacity. The chemical changes in flavones after halogen substitution may be due to the electrostatic effect, to increasing the compound's polarity, or to altered resonance of the molecule. Cutroneo et al., (1971) found that when 4'-bromoflavone (BrF) was added to fetal liver explants, it elicited induced levels of AHH twice that observed with 8-napthoflavone (BNF), a potent in vivo inducer among the flavones. Those results stimulated the current study of BrF as an inducer of the AHH system in cultured human lymphocytes.

At the optimum BrF concentration for induction of AHH, the induced enzymatic activity compared favorably with that of AHH induced by 3MC in a 96 hour lymphocyte culture using BP as the assay substrate. However, 1,2-benzanthracene (BA), a more effective inducer of AHH in human lymphocytes, elicited a much higher response than either 3MC or BrF. BrF and 3MC probably act by a similar mechanism in stimulating the induction of AHH. BrF, at its optimum concentration, was paired with diphenyloxazole (PPO) in an inducer-substrate system for studying AHH activity in cultured human lymphocytes so as to reduce added risk of handling carcinogens. The AHH activity level using BrF as an inducer and PPO as a substrate was very low compared to those activity levels in BA and 3MC induced cells using BP as a substrate. Additional experimentation and characterization of BrF as an inducer of AHH in human lymphocytes is justified, due to its potential as a non-carcinogenic inducing agent of AHH.

A recent report by Gurtoo et al. (1975) indicates that increased concentrations of NADPH and NADH in the reaction mix-
tures enhance the sensitivity of the AHH assay in homogenized cells. Our laboratory has modified the AHH assay using an intact cell system rather than the disrupted cell system reported by Grutoo et al. (1975). This change reduces two major sources of variation in the AHH assay system. First, the enzyme is not destroyed by increased temperatures produced during sonication or homogenization. Secondly, exogenous NADPH or NADH are not required in intact cells, as reported by Cantrell et al. (1976). Upon comparing the AHH levels in intact and sonicated cells, intact cells were seen to exhibit more than twice the activity of sonicated cells. These considerations with the added advantage of decreasing the assay cost by deleting NADPH, suggest the intact cell assay is more appropriate.

The optimum pH for the AHH assay has been reported for many different systems to be 7.5 (Nebert and Gelboin, 1968; Nebert et al., 1975; Alvares et al., 1973; Busbee et al., 1972) also reported this pH in a study of human leukocyte homogenates. With the development of the whole cell assay, a new pH optimum for intact cells was reported (Cantrell et al., 1976; Coomes et al., 1976), corroborating the report by Gurtoo et al. (1975) of a pH optimum of 8.6 for the homogenized cell assay. The present study indicates a broad range in the AHH pH optimum with very little activity difference from 7.5 to 9.0 for the whole cell assay method.

In an effort to improve the whole cell assay for AHH activity, a buffer study was done in our culture medium (Joklik's-Minimum Essential medium) using six buffers with pKa ranging
from 7.5 to 8.4 (Bicine, pka 8.4; EPPS, pka 7.95; Hepes, pka 7.5; Tes, pka 7.5; Tricine, pka 8.1; Tris). Previously, the cultures were set up in MEM (buffered with phosphate-sodium bicarbonate) at a pH of 7.5. The cultures became more acidic with time, and this decrease in pH caused cells to clump together in many cultures. The current experiment shows higher AHH activity in the cultures to which no extra buffer was added. We also observed the same amount of clumping regardless of the buffer content. These results indicate that during culture the lymphocytes' pH requirement is at optimum in MEM along. The added buffers lower growth potential along with AHH activity. The cell clumping phenomenon is apparently due to certain inherent lymphocyte characteristics that vary between individuals.

Reports showing development of a more sensitive technique for assaying AHH activity appear frequently in the literature. Gurtoo et al. (1975) reported a 17-fold increase in sensitivity attained by using Roswell Park Memorial Institute Medium 1640 (RPMI 1640) and providing a larger surface area during culture. Gurtoo also reported a 72 hour induction maximum with considerable decay of activity after continued incubation to 96 hours. The method of AHH analysis was reproduced in our laboratory and compared to our whole cell assay system. Cultures were run simultaneously using 20 ml vials and 25 cm² (30 ml) T-flasks. AHH activity in cells grown in 20 ml vials using Gibco's Joklik-Modified Minimum Essential Medium (MEM) was higher than that found in cells grown in RPMI 1640 in 20 ml vials. However, by providing a larger surface area during
culture in the 30 ml T-flask, the enzyme activity in lymphocytes grown in RPMI 1640 exceeded that of the MEM cultured cells. The overall evaluation of this experiment suggests that equally high or higher levels of AHH activity may be attained using MEM in 20 ml vials as compared with cells cultured in T-flasks in RPMI 1640 medium. The one-step AHH assay in human lymphocytes (Cantrell et al., 1976) also shortens experimental time by simplification of the laborious multiple extraction assay methods previously reported (Nebert and Gelboin, 1968).

Studied by Welch et al. (1971) have demonstrated that AHH can be induced in lungs of rat following exposure of the animals to an atmosphere containing cigarette smoke. Cantrell et al. (1973c) also showed the induction in vivo in human pulmonary alveolar macrophages. The level of AHH activity in cultured human lymphocytes was measured in our laboratory using the one step whole cell assay in smokers and non-smokers. Two substrates were used, BP (25 g) and PPO (10 g), to determine the level of AHH induction in lymphocyte samples from each individual.

The level of induction varied without regard to the subjects' smoking habits, indicating a factor(s) other than cigarette smoke inhalation directly affecting enzyme induction in the cultured cells.

The use of high pressure liquid chromatography provides a rapid, efficient, and reproducible method for the separation of metabolites produced during metabolism of benzo( )pyrene by cellular monooxygenase enzymes (Selkirk et al., 1974). The system was first used in related studies to separate products.
formed during the incubation of $^3$H - or $^{14}$C - benzo(a)pyrene with liver microsomes from male Sprague-Dawley rats that had been treated with methylcholanthrene. The BP metabolites soluble in organic solvent were extracted, concentrated by evaporation, and applied to the liquid chromatograph. Discrete metabolite peaks contained in the eluate fractions obtained from the liquid chromatograph were isolated and characterized by ultraviolet absorption spectra and mass spectra (Selkirk, 1974), and by comparison with metabolites isolated by thin layer chromatography (Kinoshita et al., 1973). The HPLC technique showed four classes of compounds present as BP metabolites, including phenols, quinones, dihydrodiols, and unidentified conjugated water soluble derivatives. Included in the four broad classes were 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene, 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene, 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene, 3-hydroxybenzo(a)pyrene, 9-hydroxybenzo(a)pyrene, benzo-(a)pyrene-1, 6-dione, benzo(a)pyrene-3,6-dione, and benzo(a)pyrene-6,12-dione. Selkirk et al. (1976) reported a modification of this system to allow repeated recycling of the unresolved peaks. Using this new technique, and comparing the metabolites to authentic standards, Selkirk isolated and identified 1-hydroxybenzo(a)pyrene and 7-hydroxybenzo(a)pyrene as metabolites of the rat liver microsomal preparation which had previously chromatographed with 3-hydroxybenzo(a)pyrene. The high pressure liquid chromatograph has also been used to study alterations in the patterns of benzo(a)pyrene metabolism (Freudenthal et al., 1975) and for monitoring the benzo(a)pyrene content of
cigarette smoke condensate fractions (Walters et al., 1974). High pressure liquid chromatographic techniques for the separation of BP metabolites were standardized in our laboratory using authentic standards obtained from the National Institutes of Health. The HPLC column eluate was monitored using ultraviolet absorption at 254 nm. After the system was standardized, we compared the distribution of BP metabolites produced by C57B1/6J mouse liver microsomes with those produced by human lymphocytes. The presence of increased levels of AHH in cultured human lymphocytes induced with BA and used to generate BP metabolites had been previously determined using the one-step assay system reported by Cantrell et al. (1976).

Further modifications of this in vitro system are needed to provide insight into the mechanism of PAH metabolism by the aryl hydrocarbon hydroxylase system. There are many variables in this system that remain to be characterized including: (a) the mode of enzyme induction by PAHs including BrF, (b) the actual effect of the induced AHH system with respect to the mechanisms of carcinogenesis (whether activation increases or reduces carcinogenesis), and (c) enzymatic formation of the ultimate carcinogen and its mode of action.

In spite of these shortcomings, the in vitro system for studying the correlation between AHH activity and chemical carcinogenesis is promising. The use of 4'-bromoflavone as a non-carcinogenic inducer of the enzyme system should prove favorable in the development of an inexpensive clinical screening test to predict genetic susceptibility of an individual.
to chemically initiated lung cancer. The use of the intact human lymphocyte cell system with the current modifications should also be applicable to the development of a system for characterizing the mechanism of AHH metabolism of PAHs.
There were multiple findings as a result of this study. First, 4'-bromoflavone, although not as potent as 1,2-benzanthracene, was comparable to 3-methylcholanthrene as an inducer of the aryl hydrocarbon hydroxylase system. The similarity of induction by BrF and 3MC may be due to their stereochemical arrangement, similar molecular dimensions, or to the fact that they both have similar regions of electron availability and charge distributions for inducer activity. Secondly, the assay for AHH in cultured human lymphocytes in the whole cell system showed higher activity than sonicated cells. This whole cell system was found to have as much or more activity in 20 ml vials using Joklik's-Modified Minimum Essential Medium (GIBCO) as in 25 cm² (30ml) T-flasks cultured with Roswell Park Memorial Institute Medium 1640. We also found that the MEM needs no additional buffer for increased sensitivity. The sonicated cell system required NADPH and NADH at a concentration of 0.3 mg/ml for maximal activity (in a 60 minute assay). The pH optimum was found to be 7.5, with activity remaining high through a pH of 9.0. This whole cell assay was used to measure levels of inducibility in smokers and non-smokers. The findings showed varied levels of induction of AHH without regard to the subjects' smoking habits. This assay was also used to compare
the distribution of BP metabolites in human lymphocytes with those of C57Bl/6J mice hepatic microsomes. The high pressure liquid chromatograph studies indicate similar distributions of metabolites in the two tissues.
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