XENOBIOTIC METABOLISM AND MUTATION IN DIPLOID HUMAN LYMPHOBLASTS

BY

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Submitted to the Department of Nutrition and Food Science on May 18, 1982 in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Toxicology

ABSTRACT

A gene-locus mutation assay at the hypoxanthine guanine phosphoribosyl transferase locus has been developed in a line of human lymphoblasts competent for oxidative xenobiotic metabolism. This human lymphoblasts cell line, which has been designated AHH-1, was sensitive to the mutagens ethyl methanesulfonate, ICR-191, benzo(a)pyrene, 1-methylphenanthrene, lasiocarpine, dimethyl nitrosamine, 2-acetoaminofluorene, cyclopenteno(c,d)pyrene, and aflatoxin B_1. AHH-1 cells were not mutated by either perylene or fluoranthene.

AHH-1 cells had high basal levels and inducible benzo(a)pyrene hydroxylase (AHH) activity and 7-ethoxyresorufin (ER) deethylase activity. These activities were induced by pretreatment with polycyclic aromatic hydrocarbons, such as benzo(a)pyrene or beta-naphthoflavone, but not induced by pretreatment with phenobarbitol or arochlor 1254. These activities were inhibited by carbon monoxide, alpha-naphthoflavone, and SKF-525 A. The basal and induced AHH activity and ER deethylase activity had different kinetic parameters, indicating they were not the same enzyme.

Benzo(a)pyrene (BP) biotransformation was examined in detail. AHH-1 cells produced primarily phenolic metabolites of BP with minor amounts of quinones and dihydrodiols. AHH-1 cells did not produce any known trans-dihydrodiol-BP metabolites. AHH-1 cells did produce a metabolite which cochromatographed with the cis-4,5-dihydrodiol BP.

The BP-DNA adducts have been partially characterized. BP-4,5-oxide accounts for approximately 7% of the DNA adducts and 9-hydroxy-4,5-oxide-BP accounts for approximately 2% of the adducts. The bay region trans-diolepoxide-BP metabolites were not found to be bound to cellular DNA.

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ABBREVIATIONS

2AAF	Acetoaminofluorene
AFB_1	Aflatoxin-B_1
AHH	Aryl hydrocarbon hydroxylase
ANF	Alpha-naphthoflavone (7,8-benzoflavone)
B(a)A	Benzo(a)anthracene
BNF	Beta-naphthoflavone (5,6-benzoflavone)
BP	Benzo(a)pyrene
CEPE	Cyclopenteno(c,d)pyrene
DAB	3'-Methyl-4'-dimethyl-aminoazobenzene
DB(a,c)A	Dibenz(a,c)anthracene
DB(a,h)A	Dibenz(a,h)anthracene
DMN	Dimethylnitrosamine
EMS	Ethyl methanesulfonate
ER	7-Ethoxyresorufin
Fla	Fluoranthene
HGPRT	Hypoxanthine-guanine-phosphoribosyltransferase
HPLC	High performance liquid chromatography
ICR-191	6-Chloro-9-((3-(2-chloroethylamino)-propyl)amino)-2-methoxyacridine dihydrochloride
LSCP	Lasiocarpine
3MC	3-Methylcholanthrene
MFO	Mixed function oxidase
MNNG	N-Methyl-N'-nitrro-N-nitrosoguanidine
1MP	1-Methylphenanthrene

NP	Nitrosopyrrolidine
PAH	Polycyclic aromatic hydrocarbon
Per	Perylene
PMS	Post mitochondrial supernatant
SKF-525 A	2-Diethyl-2,2-diphenyl valerate
TCDD	2,3,7,8-Tetrachlorodibenzo-p-dioxin
TCPO	1,2-Epoxy-3,3,3-trichloropropane
TFT	Trifluorothymidine
TG	6-Thioguanine
TK	Thymidine kinase

I. Introduction

Genetic toxicology is the study of the interaction of physical and chemical agents with the process of heredity. Human genetic diseases arise from at least three different modes of failure to accurately transmit genetic information: aneuploidization, clastogenesis and gene-locus mutations. Aneuploidization is the loss of gain of whole chromosomes. Clastogenesis is the production of microscopic breaks, gaps and rearrangements in chromosomes. And gene-locus mutations are changes in the DNA sequence altering the function of a gene. The discipline of genetic toxicology has a responsibility to participate in the evaluation of sources of genetic risk and to ultimately reduce the incidence of human genetic disease.

This a project about humans in general, and in particular, about human cells. Human cells were chosen because they are the closest thing to real people you can grow in a laboratory, expose to deadly chemicals and expect to avoid incarceration. The goal of this project was to develop an improved system for the study of the induction of gene-locus mutations which more closely approximates humans. The improvement over existing systems was the use of human target cells which were competent for xenobiotic metabolism.

Estimation of genetic risk to humans based solely on a cell culture model system is impossible. A person is composed of hundreds of different types of cells contained within a complex matrix, and a cell culture, if it is to be homogeneous, it reflects only one cell. There is no guarantee this single cell will faithfully reflect the whole. For this reason and

for other reasons extrapolations between a cell culture approximation and a person are at best tenuous. Despite the hazards of extrapolation to human risk, cell culture systems for detecting genetic change are useful as experimental tools and useful for the screening potential mutagens.

This project studies some of human cell mediated xenobiotic metabolism involved in mutagen activation. Many potentially mutagenic xenobiotics require metabolism to electrophilic intermediates to be mutagenic. Many cell types in people contain the capacity to metabolize xenobiotics, but cells in culture rapidly lose this activity. Therefore mutation assays carried out using cultured cells require an extracellular metabolizing system, such as rat liver homogenates, to detect the mutagenic activity.

There are several advantages in using human cells which contain endogenous mutagen activating activity. Human cells containing human mixed function oxygenase activity represent an intact human cell drug metabolizing system. This system may respond differently than metabolizing systems from other species and cell types. Target cells with endogenous metabolizing capability avoid the necessity of an extracellular metabolizing systems, such as PMS or isolated primary cultures, and their inherent disadvantages.

A mutation assay utilizing human lymphoblasts competent for mixed function oxygenase activities has been developed. This assay was applied to the detection of mutagenic activity induced by a series of xenobiotics. And finally, some of the characteristics of cellular xenobiotic biotransformation were characterized. The cellular xenobiotic metabolism resembled in several aspects xenobiotic metabolism in cultured human peripheral lymphocytes although several aspects were novel.

II. Literature Survey

A. Mutation assays with metabolically competent target cells.

Many attempts have been made to couple drug metabolizing systems with mutation assays. The target cells commonly used in mutation assays do not express the cytochrome P-450 enzymes, therefore extracellular metabolizing systems have been incorporated into mutation assays. Four major types of metabolizing systems have been developed and coupled to mutation assays: 1) whole animal, 2) whole primary cells, 3) liver cell fractions, and 4) metabolically competent target cells.

There are several limitations with extracellular metabolizing systems. First, due competeing extracellular processes, an electrophilic intermediate may not be present at sufficient concentration to react with critical targets within the target cell. Second, most metabolizing systems are derived from rodent tissues, these systems may carry out qualitatively and quantitatively different reactions at different rates than the human enzymes. Three, the preparation of the metabolizing system is often not reproducible between independent preparations. And four, different metabolites are produced compared to intact cells (Ashurst and Cohen, 1980). Mutation assays in which the target cell contains endogenous metabolizing ability overcome these limitations. Inspite of these limitations, most mutation assays utilize target cells deficient in xenobiotic metabolism.

Several mutagenesis assay systems which utilize metabolism deficient target cells co-cultivated with primary tissue cells competent for xenobiotic metabolism have been developed. The development of these types

of systems is beyond the scope of this thesis, but these systems do deserve comment. Mutagenesis assay systems have been developed which use isolated primary tissue cells to provide metabolic capacity. Cells from several organs have been used, including rodent liver (Huberman and Sachs, 1974; Jones and Huberman, 1980; Jones et al., 1981; Langenbach et al., 1981b), rodent lung (Langenbach et al., 1981b), whole rodent embryos (Huberman and Sachs, 1974) and rodent bladder (Langenbach et al., 1981a). A variety of chemical mutagens have been tested in these systems and found to be active mutagens including polycyclic aromatic hydrocarbons (benzo(a)pyrene, 7,12-dimethylbenz(a)anthracene, 3-methylcholanthrene) (Huberman and Sachs, 1974; Langenbach et al., 1981b), a range of symetrical and asymetrical nitrosamines (including dimethyl nitrosamine, diethyl nitrosamine, diallyl nitrosamine, methyl-n-propyl nitrosamine, methyl-n-butyl nitrosamine, nitrosopiperazine, methylphenyl nitrosamine and others) (Jones and Huberman, 1980; Jones et al., 1981; Langenbach et al., 1981b), aflatoxin B_1 (Langenbach et al., 1981b) and aryl amines (Langenbach et al., 1981a). Whole cell mediated activation systems were found to be more sensitive for the activation of nitrosamines than tissue homogenate-mediated systems (Jones and Huberman, 1980). In addition, whole cell-mediated systems have shown a better correlation between potentcy of chemical mutagenicity and potentcy of chemical carcinogenicity (in the same species) than systems utilizing tissue homogenates to supply xenobiotic metabolism (Jones et al., 1981). This improved sensitivity correlation to in vivo end points implies that there is hope for the development of improved human cell mutagenesis systems with xenobiotic metabolism mediated by primary human cells. But the limited availability of human tissue specimens and the large interindividual variation of

metabolic capacity in the human population may complicate the general application of these types of human systems. Whole cell-mediated metabolic activation systems also suffer the inherent disadvantages of all extracellular metabolizing systems which were discussed earlier in this section.

One system has been developed which uses cells which contain oxidative xenobiotic metabolic capacity. Tong and Wiliams, (1978) have used an isolated rat liver epithelial cell line (ARL-6). These cells were treated with aflatoxin B_1 (1 uM x 24 hours). A five fold increase over the untreated control mutant fraction was observed. 2-Acetoaminofluorene, another chemical mutagen which requires activation, was also a mutagen in this system. The same investigators have extended this work (Tong and Williams, 1980) to further define the conditions for mutagen treatment and mutant selection. In this more recent report three other mutagens were tested: N-methyl- N'-nitro- N-nitrosoguanidine (MNNG) (which does not require metabolic activation), nitrosopyrrolidine (NP), and 3'-methyl-4'-dimethyl- aminoazobenzene (DAB) (the latter two chemicals require activation to be active mutagens). The authors used a three day exposure to the mutagen followed by 14 days of growth for phenotypic expression. Mutants were scored by colony formation in the presence of 10 ug/ml 6-thioguanine at 1×10^4 cells per cm^2. MNNG was found to be a potent mutagen - this chemical was used to define the length of phenotypic expresion. A DAB treatment of 5×10^{-5} M for 72 hours yielded a mutant fraction of 4.3×10^{-5} versus 7.5×10^{-6} for the untreated control culture. A NP treatment of 1×10^{-4} M for 72 hours yielded a mutant fraction of 8×10^{-5} versus 1.9×10^{-5} for the untreated control culture.

These reports provide an important demonstration - that target cells

with endogenous metabolic activity can be mutated with chemicals known to require metabolic activation. There are however several limitations to this system and the methodology used to measure mutation. The authors failed treat an adequate number of cells with mutagens, typically only 2 x 10^6 cells were treated per experimental point. The mean backgroud frequency was 11.8 x 10^{-6}, therefore only 23 ($\pm$ 10 for 95% confidence) mutants were present on the average. This low number of mutants resulted in large experimental fluctuations at low mutant fractions. The evaluation of relatively weak mutational responses was impossible using these protocols. The cell line used, ARL-6, is not of clonal origin and may not be homogeneous for metabolizing ability. ARL-6 cells are of rodent origin and may respond differently from human cells. Finally, the treatment period and phenotypic expression time are long and the cells require refeeding during mutant selection, this makes the assay more cumbersome to perform.

B. Benzo(a)pyrene metabolism and DNA binding.

Benzo(a)pyrene metabolism and DNA binding has been extensively studied. The production of specific metabolites and th foration of specific DNA adducts has been studied in a variety of different biological systems. This literature survey will be restricted to metabolism in rodent liver microsomes, metabolism in several human systems, and metabolism to DNA binding intermediates.

In rodent liver microsomes three major classes of metabolites are produced: trans-dihydrodiols, quinones, and phenols. In addition the arene oxide formation has been shown at the 7,8, the 9,10, or the 4,5 positions (Selkirk et al., 1974; Holder et al., 1974; Sims and Grover

,1974). These arene oxides may be converted to the corresponding phenols by non-enzymatic reactions, conjugated with glutathione or converted to the corresponding dihydrodiols through the action of epoxide hydrolases (Oesch, 1972). Three major trans-dihydrodiol benzo(a)pyrene metabolites are produced in rodent liver microsome, the 4,5, the 7,8 and the 9,10 trans-dihydrodiol BP. Several phenolic metabolites are produced in these incubations. Phenolic metabolites are poorly resolved under most chromatography conditions. Usually two phenol "peaks" are observed, these peaks co-migrate with 9-hydroxy BP and 3-hydroxy BP synthetic standards but may contain other phenolic isomers. Both phenolic and diolic compounds are conjugated to glucuronic acid, or sulfate before excretion (Booth et al., 1961; Nemoto and Gelboin, 1975; Autrup et al., 1978, 1982).

BP metabolism in human systems is different from BP metabolism in rodent liver. Several of the major BP metabolites in human systems have not been identified. Human liver microsomes produced trans 9,10-, 7,8-, and 4,5-dihydrodiols, quinones and phenols. In addition five unidentified metabolites were observed, two chromatographed in the region containing dihydrodiols, and three in the region containing quinones (Selkirk et al., 1975). Human lymphocytes produced different metabolite profiles depending on the incubation time with BP. During a 30 minute incubation no trans dihydrodiols were observed, the major metabolites were phenols and quinones. In addition, three unknown metabolites were observed, these metabolites co-chromatographed with the unknown metabolites observed in human liver microsomes (Selkirk et al., 1975). During a 24 hour incubation, human lymphocytes produce 4,5-, 7,8-, and 9,10- trans dihydrodiols, quinones and phenols and seven unidentified metabolites (Selkirk et al., 1975). These reports did not test for the presence of

cis-dihydrodiol BP metabolites, apparently the authentic standards were not available. Vaught et al, (1978) reported that the major BP metabolite produced by human peripheral blood lymphocytes were phenols. Quinones, trans-7,8-dihydrodiol BP, and one unidentified metabolite were also reported. The formation of the trans-7,8-dihydrodiol BP metabolites could be inhibited by the addition of 1 mM TCPO.

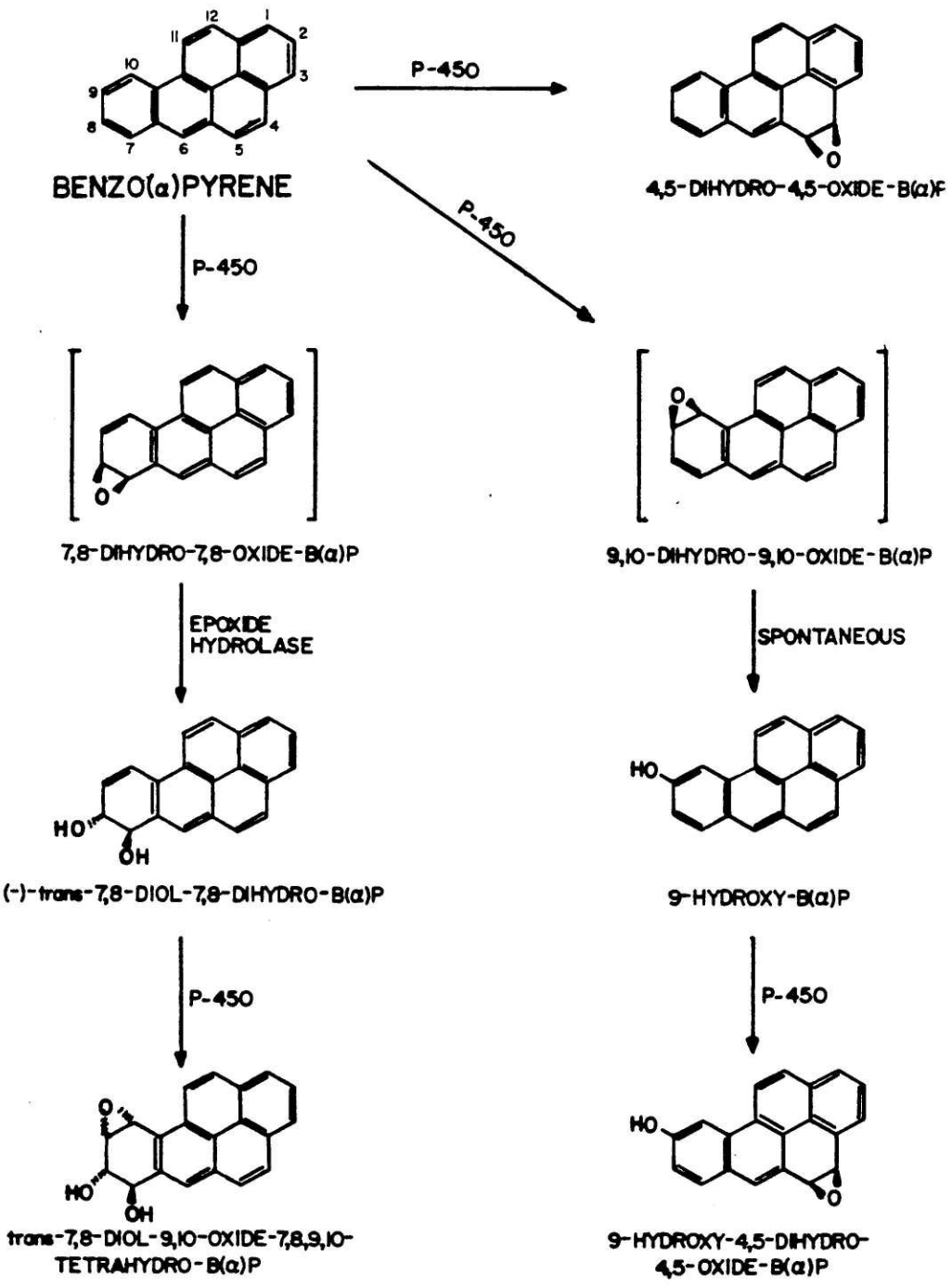
There are several reported pathways to BP binding to DNA. The major pathways of benzo(a)pyrene metabolism to DNA binding intermediates are outlined in figure 1. The three major pathways of benzo(a)pyrene metabolism to DNA binding intermediates are: oxidation at the K-region to form BP-4,5-oxide, oxidation of 9-hydroxy BP at the K-region to form 9-hydroxy BP-4,5-oxide, and oxidation at the "bay region". This "bay region" oxidation involves the production of BP-7,8-oxide, then hydrolysis of the arene oxide catalyzed by epoxide hydrolase to produce trans-7,8-dihydrodiol BP (the major isomer produced in rodent liver is drawn in figure 1). The trans-7,8-dihydrodiol BP is further oxidized in the 9,10 position to generate a vicinal diol epoxide. Two major isomers of the vicinal diol epoxide are produced, the syn isomer (epoxide and hydroxy group in the 7 position on the same side of the plane of the BP molecule) and the anti isomer (epoxide and hydroxyl group in the 7 position on opposite sides of the plane of the BP molecule). The syn diol epoxide is less stable than the anti diol epoxide. This decreased stability is due to a hydrogen bonding interaction between the epoxide and the hydroxyl group in the 7 position which labilizing the epoxide (Reference). Several other BP metabolites have been reported to bind to DNA, in particular BP-7,8-oxide, BP-9,10-oxide, and an unidentified quinone (Pelkonen et al., 1978). These pathways are quantitatively minor.

Figure 1.

Benzo(a)pyrene metabolic pathways to DNA binding intermediates

Three pathways of benzo(a)pyrene metabolism are outlined. The K-region oxidation of BP to produce BP-4,5-oxide. The K-region oxidation of 9-hydroxy BP to produce 9-hydroxy BP-4,5-oxide. And the bay region oxidation of trans-7,8-dihydrodiol BP to produce the vicinal diol epoxide BP.

METABOLIC PATHWAYS TO DNA BINDING



The specific metabolites bound to DNA vary with the experimental system. The "bay region" metabolism of BP to a vicinal diol epoxide has been reported to be the major pathway (greater than 90% of the radioactivity bound) to DNA binding in several biological systems including human bronchial explants (Jeffery et al., 1977), bovine bronchial explants (Jeffery et al., 1977), isolated viable rat hepatocytes (Ashurst and Cohen, 1980), cultured human colon (Autrup et al., 1978, 1982), cultured human esophagus, bronchus, and duodenum (Autrup et al., 1982), isolated perfused rat lung (Vahakangus et al., 1979), and rat lung and liver in vivo (Boroujerdi et al., 1981). In microsomal incubations with DNA the 9-hydroxy BP-4,5-oxide metabolite was the major DNA binding metabolite. This has been reported for rat liver microsomes (King et al., 1976; Lesca et al., 1981; Thompson et al., 1976), mouse liver microsomes (Pelkonen et al., 1978), and rat lung microsomes (Vahakangus et al., 1979). 9-Hydroxy BP-4,5-oxide bound to DNA has also been reported to be present in mouse and rat skin (Baer-Dubowska et al., 1981), isolated rat hepatocytes (Jernstrom et al., 1978), in rat lung in vivo (but not rat liver) (Boroujerdi et al., 1981). BP-4,5-oxide has been reported to be a relatively minor DNA binding metabolite in microsomal incubations (Pelkonen et al., 1978; Lesca et al., 1981) and has been reported to be present in mouse liver incuabations (Kahl et al., 1979) and in rat and mouse skin (Baer-Dubowska et al., 1981).

The trans-7,8-dihydrodiol,9,10-oxide BP intermediate reacts with the N-2 position of guanine (Jeffrey et al., 1977; Koreeda et al., 1978; Osborne et al., 1976), cytidine, adenosine (Meehan et al., 1977), phosphate groups (Koreeda et al., 1978; Osborne et al., 1976), and the N-7 position of guanine (Osborne et al., 1976) in DNA. The N-2 guanine adduct

of the vicinal diol epoxide was the major adduct formed in all reports. The N-7 guanine adduct was produced by the anti diol epoxide (but not the syn diol epoxide) and was present at approximately 1/3 the concentration of the N-2 guanine addduct. The N-7 guanine adduct of BP diol epoxide was extremely unstable (halflife of 3 hours) and has only been detected in in vitro incubations of synthetic diol epoxide with calf thymus DNA, the short halflife of this adduct means that the adduct would not survive the DNA isolation procedure required in biological incubations (King et al., 1979). The specific location of DNA binding of other BP metabolites has not been reported, but it is not unreasonable to expect the positions of binding to be qualitatively similar.

C. Human lymphoblast and lymphocyte mediated xenobitoic metabolism.

Many of the enzymes involved in oxidative xenobiotic metabolism are lost during growth in culture (Guzelian et al., 1977; Fahl et al., 1979). Most striking in this regard is the loss of the cytochrome P450 family of isozymes in hepatocytes when grown in primary culture. The loss of cytochrome P450 activity is about 80-90% after 24 hours (Guzelian et al., 1977; Fahl et al., 1979; Fry and Bridges, 1979. The total activity drops substantially, but some of the many oxidative reactions catalyzed by the P450 system remain reasonably close to in vivo levels (Guzelian et al., 1977; Fry and Bridges, 1979).

AHH activity is expressed in cultured peripheral human lymphocytes. This activity is extremely low in native lymphocytes, but can be stimulated by the addition of mitogens and PAH inducers (Whitlock et al., 1972). In the process of transformation from a mitogen-stimulated lymphocyte culture to an immortal human lymphoblastoid line the ability to

express cellular AHH activty is lost in a vast majority of cell culutres (H.L. Gurtoo, personal communication). The reason for this loss in unknown.

Benzo(a)pyrene hydroxylation by mitogen-activated human lymphocytes has been extensively studied (Busbee et al., 1972; Whitlock et al., 1972; Kellerman et al., 1973a,b; Rasco et al., 1978; Trell et al., 1976; Paigen et al., 1977; Vaught et al., 1978; Gurtoo et al., 1979). The bulk of these investigations examined a possible correlation between cancer incidence and AHH activity in mitogen-activated human lymphocytes. Several positive correlations have been reported (Kellerman et al., 1973b; Trell et al., 1976). Subsequent investigations have revealed that AHH activity was highly variable between different individuals and between independent samples from the same individual (Gurtoo et al., 1977). This inherent variability in lymphocyte cultures renders these correlations suspect.

Investigations into the xenobiotic biochemistry of human lymphocytes are limited. The oxidative metabolism of BP by mitogen-activated human lymphocytes has been discussed earlier in this section. Human lymphocytes produce predominantly phenolic metabolites and a limited array of dihydrodiolic metabolites (Selkirk et al., 1975; Vaught et al., 1978). Conjugation of these metabolites has not been examined.

Mitogen-activated human lymphocytes are capable of 7-ethoxyresorifin (ER) deethylation (Burke et al;., 1977b). This activity was detectable in 3-methylcholanthrene-induced lymphocytes but not redily detected in uninduced lymphocytes. No kinetic data were reported for ER deethylase activity in isolated lymphocytes, but the level of 3MC induced activity (using 10 uM ER as a substrate) was reported to range from 0.16 to 1.6

pmole resosufin per 10^6 cells per minute (range observed for 10 different individuals). The uninduced ER deethylase activity was only detectable in two individuals (activities of 0.04 and 0.08 pmole resorufin per 10^6 cells per minute) the other 8 individuals did not have detectable deethylase activity (less than 0.015 pmole resorufin per 10^6 cells per minute). Also, the authors report that ER deethylase activity increased with substrate concentration up to 2.5 uM. This implies that the apparent K_m in in the range of 1 uM.

Epoxide hydrolase activity in native and mitogen-stimulated human lymphocytes has been studied using benzo(a)pyrene-4,5-oxide as a substrate (Glatt et al., 1980). In native lymphocytes, the specific activities of epoxide hydrolase varied from 2.0 to 7.2 pmole per minute per mg protein (28 individuals). The specific activities in stimulated lymphocytes varied from 4.4 to 7.0 pmole per minute per mg protein (12 individuals). This specific activity was approximately 100 fold lower than human liver microsomes. Beta-naphthoflavone was capable of inducing this activity (range 1.8 fold to 2.6 fold among 13 individuals). Finally, the pH profile was reported to be broad with an optimum at pH 9.5.

III. Methodology

A. Materials

Cell culture supplies. Cell culture medium, RPMI medium 1640, and horse serum supplement were obtained from either Flow Laboratories, McLean, VA or GIBCO Inc., Grand Island, NY. Plastic tissue culture flasks were obtained from either Corning Glassworks, Corning, NY or Costar Plastics, Cambridge, MA.

Chemicals and enzymes. NADH (grade III), NADPH (type X), DB(a,c)A, DB(a,h)A, B(a)A, 2-AAF, EMS, gentamycin, kanamycin, deoxycytidine, hypoxanthine, aminopterin, thymidine, 6-thioguanine, DNase I (DN-CL), Alkaline phosphatase (Type III-S), Snake Venom Phosphodiesterase (type VII), sulfatase, and beta-glucuronidase were obtained from Sigma Chemical Co., St. Louis, MO. ANF and DMN were obtained from Eastman Chemical, Rochester, NY. BP, perylene and BNF were obtained from Aldrich Chemical, Co., Millwaukee, Wis. ICR-191 was obtained from Polysciences, Inc., Warrington, PA. 1-Methylphenanthrene was obtained from ICN / K and K, Plainview, NY. Arochlor 1254 was obtained from Analabs, North Haven, CT. Phenobarbitol was obtained from Mallinckrodt, St. Louis, MO. SKF-525 A was the generous gift from Dr. Kenneth Holden of Smith Kline and French Laboratories, Philadelphia, PA. Aflatoxin B_1 was the generous gift of Dr. Gerald Wogan of MIT. 7-Ethoxyresorufin and resorufin were the generous gift of Alan Klotz of MIT. Fluoranthene was the generous gift of Dr. John Babson of MIT. Lasiocarpine was the generous gift of Dr. Paul Newberne of MIT. Cyclopenteno(c,d)pyrene was the generous gift of Dr. Ercole Cavelieri of the Eppley Institute.

Radiochemicals. ^{3}H-BP (41.7 to 66 Ci per mmol), and ^{3}H-hypoxanthine (4.7 Ci per mmol) were obtained from New England Nuclear, Boston, MA. ^{3}H-BP-4,5-oxide (366 mCi per mmol), ^{14}C syn-(+)-trans-7,8-diol-9,10-epoxide-7,8,9,10-tetrahydro BP (^{14}C syn-diolepoxide BP) and ^{3}H anti-(+)-trans-7,8-diol,9,10-epoxide-7,8,9,10-tetrahydro BP (^{3}H anti-diolepoxide BP) (57 mCi per mmol and 389 mCi per mmol respectively) were obtained through the National Cancer Institute Chemical Carcinogen respository. ^{3}H-9-hydroxy BP was prepared from ^{3}H-BP (Sims, 1967). ^{3}H-BP was incubated with BNF induced rat liver microsomes, the metabolites extracted with ethyl acetate and separated on a silica gel G TLC plate (Analabs, Inc.) with a benzene:ethanol (9:1) mobile phase. The trans-9,10-dihydrodiol BP metabolite was located by fluorescence (and co-chromatography with the authentic standard). The plate was sprayed with concentrated HCl and incubated at 100 C for 10 minutes. Under the conditions the trans-9,10-dihydrodiol BP is converted to 9-hydroxy BP. The product was eluted with methanol.

Authentic benzo(a)pyrene metabolites. 3-hydroxy BP, 9-hydroxy BP, trans-4,5-dihydrodiol BP, trans-7,8-dihydrodiol BP, trans-9,10-dihydrodiol BP, BP-1,6-dione, BP-3,6-dione, and BP-6,12-dione were obtained from the National Cancer Institute Chemical Carcinogen Repository.

B. Growth of cells

The isolation of AHH-1 cells is described in the results section. This cell line is a derivative of RPMI-1788 human lymphoblasts. AHH-1 cells are grown in RPMI medium 1640 supplemented with 5% horse serum. AHH-1 cells have stable, inducible aryl hydrocarbon hydroxylase activity. The serum dependence of AHH-1 cell growth, plating efficiency, and AHH

activity are summarized in table 1. All three parameters are stable between 5% and 20% horse serum. A horse serum concentration of 5% was chosen because cells form larger clones in this serum concentration compared to lower concentrations; this facilitates colony scoring.

AHH-1 cells are maintained in constant exponential growth by daily dilution to 2 - 4 x 10^5 cells per ml. The best cell growth was observed when cells were diluted routinely every 24 $\pm$1 hours. AHH-1 cells grow as single cells and loose multicell aggregates (2 to 100 cells) the aggregates were dissociated by agitation before cells were counted. AHH-1 cells grew best in plastic tissue culture flasks. Variable cell growth was observed when the cells were grown in either 500 ml bottles or spinner flasks. This variablity may be due to increased cell clumping under these conditions. AHH-1 cells doubling times ranged from 13.5 to 22 hours. Cell are counted on a model B Coulter counter.

C. Plating procedure

A microtiter plating technique has been recently developed in our laboratory (Furth et al., 1981). This technique involves seeding a known number of cells into 96 well microtiter plates. Cells grow in the wells and eventually form macroscopic colonies (typically after 10 to 14 days). The number of colonies is scored and Poisson statistics can be used to calculate the number of colony forming units per well (cfu/w). The probability of not observing a colony (the fraction of wells without colonies) can be expressed as $Pr(0)=e^{-x}$, where x is the number of cfu/w. The cloning efficiency can be calculated by dividing the cfu/w by the number of cells seeded into each well. Cloning efficiency is determined by seeding 1 to 5 cells per well and mutant frequency is determined by

Table 1. Serum dependence of AHH-1 cell parameters

% Serum	Relative Growth Rate	Relative AHH Activity*	Relative Plating Efficiency
2.5	0.88	0.76	0.59
5	0.93	0.91	0.78
7.5	0.93	1.0	0.98
10	0.95	0.94	0.80
12.5	0.92	0.97	1.0
15	1.0	0.98	0.98
20	1.0	0.92	0.89

* induced with 10 uM benzo(a)pyrene

seeding a known number of cells (up to 20,000) per well in the presence of selective agent. The plates are incubated for 14 days and colonies scored. The mutant fraction can be calculated according to the following equation:

mutant fraction = $AB(CD)^{-1}$

where A is the number of cfu/w in selective agent;

B is the number of cells per well in cloning efficiency plates;

C is the number of cfu/w in cloning efficiency plates; and

D is the number of cells per well in selective agent plates.

A detailed discussion of the statistical intrepretation of assay results can be found in Furth et al., 1981.

D. Gene-locus mutation assay

The methodology and basic format of human lymphoblast mutation assays has been described elsewhere (Thilly et al., 1980). This protocol was slightly modified for use with AHH-1 cells.

Stock cultures of AHH-1 cells were grown in plastic tissue culture flasks and diluted daily to 4×10^5 cells per ml. Prior to mutagen treatment pre-existing mutants in the population were eliminated by a 48 hour treatment with CHAT (deoxycytidine, 1×10^{-5} M; hypoxanthine, 2×10^{-4} M; aminopterin, 2×10^{-7} M; and thymidine, 1.75×10^{-5} M). Cells were then centrifuged (1000 x g for 5 minutes) and resuspended in medium plus THC (CHAT without the aminopterin). The THC treatment was necessary for the cells to recover from the toxic effects of the aminopterin. The cells were allowed to grow and the THC removed by normal dilutions due to cell growth. Culture volumes were scaled-up to prepare the desired number of cells for mutation measurement. Two days after the addition of THC the

cellular MFO activity was induced (if desired) by the addition of 1 x 10^{-5} M beta-naphthoflavone (BNF) in DMSO (final DMSO concentration 0.1%). Three days after the addition of THC the BNF (if present) was removed by cell centrifugation (twice at 1000 x g for 5 minutes). The cells were diluted to 1.5 x 10^5 cells per ml (48 hour mutagen treatment) or 4 x 10^5 cells per ml (24 hour mutagen treatment and aliquoted into replicate 150 cm^2 tissue culture flasks (100 ml per flask). Mutagen was then added in DMSO. The final DMSO concentration was kept constant within an experiment and never exceeded 0.3%. DMSO only was added to the negative control cultures and 10 uM BP was used as a positive control. All mutagen concentrations were tested in duplicate. Cultures were incubated with the mutagen for the desired length of time (24 or 48 hours), cell concentration determined, and 4 x 10^7 cells were centrifuged and resuspended in 100 ml of fresh medium in a new 150 cm^2 tissue culture flask. During the phenotypic expression period, 6 days, cell concentration was determined daily and the culture diluted to 4 x 10^5 cells per ml.

6-Thioguanine-resistant fraction was measured by a microwell plating technique which is described in Furth et al., 1981. AHH-1 cells were plated at 20,000 cells per well in the presence of 0.5 ug per ml 6TG and 2 cells per well in the absence of 6TG. Plates were incubated at 37 C in a humidified 5% CO_2 incubator for 14 days. The plates were the scored for the presence of absence of colonies in individual cells and the mutant fraction and confidence interval computed (Furth et al., 1981).

E. Benzo(a)pyrene Hydroxylase Assay

Benzo(a)pyrene hydroxylase (or AHH) activity was measured by the

production of fluorescent phenolic metabolites of benzo(a)pyrene (Nebert and Gelboin, 1968; Gurtoo et al., 1977).

Human lymphoblasts were centrifuged at 1000 x g for 5 minutes and resuspended in 10 ml of RPMI medium 1640 without serum. Cells were centrifuged again at 1000 x g for 5 minutes. Cells were resuspended in 1 ml of assay buffer (0.2 M sucrose, 0.05 M tris, 0.01 M $MgCl_2$, 1.5 mg/ml NADPH, 1.0 mg/ml NADH, pH 8.5) and benzo(a)pyrene was added in 50 ul acetone. The pH optimum of cellular AHH activity was broad with an optimum at pH 8.5 (figure 2). The cells were incubated at 37 C for 30 minutes. The incubation was terminated by the addition of 4 ml of hexane - acetone (3:1). The aqueous and organic phases were mixed throughly and allowed to separate. The organic layer was then removed and extracted with 2 ml of 0.5 N NaOH. Fluorescence in the basic extract was determined immediately on a Farrand model MK-1 fluorometer with excitation at 396 nm and emmision at 522 nm. The amount of phenolic benzo(a)pyrene metabolites was determined by comparison to a known amount of 3-hydroxybenzo(a)pyrene and expressed as 3-hydroxybenzo(a)pyrene equivalents. This assay is linear for up to 90 minutes and to cell concentrations up to 1 x 10^7 cell per ml. For routine measurements, a 30 minute incubation with 5 x 10^6 to 8 x 10^6 cells per ml and 100 uM benzo(a)pyrene was used.

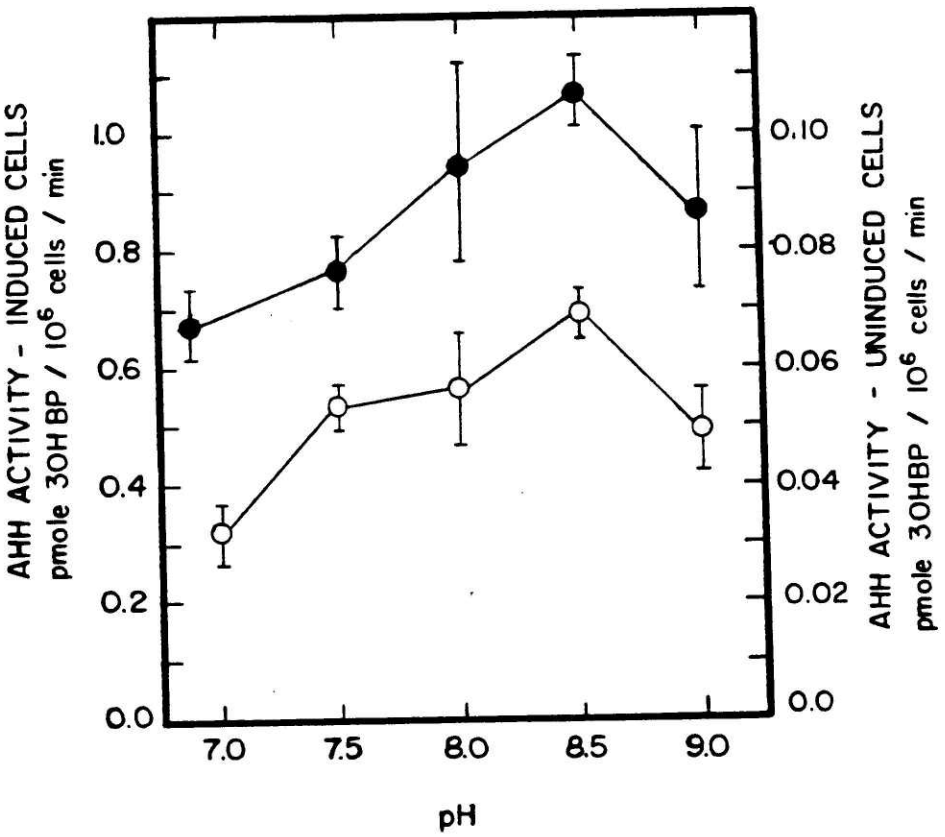
F. 7-Ethoxyresorufin deethylase assay.

7-Ethoxyresorufin deethylase activity was measured by the production of the fluorescent metabolite, resorufin (Burke and Mayer, 1974; Burke et al., 1977). Human lymphoblast cells were prepared as described for the benzo(a)pyrene hydroxylase assay. The substrate, 7-ethoxyresorufin, was added to a 2 ml cell suspension in 2 ul DMSO. Production of product,

Figure 2.

pH optimum for cellular AHH activity.

The optimum pH was determined for uninduced and DB(a,c)A induced AHH-1 cells. This optimum was pH 8.5 for both activities.


AHH ACTIVITY - INDUCED CELLS
pmole 3OH BP / 10^6 cells / min
1.0
0.8
0.6
0.4
0.2
0.0
0.10
0.08
0.06
0.04
0.02
0.0
AHH ACTIVITY - UNINDUCED CELLS
pmole 3OHBP / 10^6 cells / min
7.0
7.5
8.0
8.5
9.0
pH

resorufin, was monitored by fluorescence. The cell suspension (2 ml in buffered sucrose plus cofactors) was centrifuged (1500 x g for 30 sec), the supernatant fraction was decanted and fluorescence measured using a Farrand model MK-1 spectrofluorometer with excitation at 570 nm and emmision at 586 nm. The centrifugation step was necessary to eliminate cell interference due to light scatter at low substrate concentrations. Resorufin concentration was determined by comparison of fluorescence of cell-produced resorufin to a known concentration of resorufin standard.

G. Epoxide hydrolase assay.

Epoxide hydrolase activity was measured using BP-4,5-oxide as a substrate. The particular assay methodology was developed by Glatt et al., (1980) for the measurement of epoxide hydrolase activity in cultured human lymphocytes.

Human lymphoblast cells (8×10^6 cells per point) were centrifuged (1000 x g for 5 min) and resuspended in 0.4 ml of PBS. Cells were transfered to a tube containing 0.1 ml of 0.2 M glycine (pH 9.5) or 0.1 ml of 0.076 M sodium phosphate (pH 7.4). BNF-induced rat liver microsomes were used as a positive control and boiled cells were used as a negative control. ^{3}H-BP-4,5-oxide (366 mCi/mmole) was added to each tube (0.5 nmole in 25 ul acetonitrile) and the cell suspension incubated at 37 C for 15 minutes. The reaction was terminated by adding 3.5 ml of petroleum ether (Ligroine) and cooling the tubes on ice. If the incubation was carried out at pH 7.4, 0.2 ml of 0.2 M glycine (pH 9.5) were added to stablize the epoxide during extraction. DMSO was added to each tube to a final concentration of 50% (in the aqueous phase). The aqueous and organic phases were mixed by vortexing (3 times for 10 seconds each). The

aqueous and organic phases were separated by centrifugation (1000 x g for 5 minutes), the organic phase was removed and discarded. This extraction was procedure repeated twice more. The diol metabolites were extracted with 1 ml of ethyl acetate and the ethyl acetate phase extract was transfered to a scintillation vial. Aquasol (10 ml) was added to each vial and radioactivity measured using a Beckman model LS 3150 P liquid scintillation counter.

H. Analysis of Benzo(a)pyrene Metabolite Formation

Human lymphoblast cells (induced by treatment with 2 uM dibenz(a,c)anthracene for 24 hours) were prepared as described for the benzo(a)pyrene hydroxylase assay. Cells were incubated for 30 minutes under the conditions described for the benzo(a)pyrene hydroxylase assay. Radiolabeled benzo(a)pyrene (final concentration 25 uM) was used for metabolite determinations. Immediately before use ^{3}H-benzo(a)pyrene (specific activity 500 mCi per mmole) was purified in two steps. First the commercially available ^{3}H-benzo(a)pyrene (specific activity 41.7 to 66 Ci per mmole) was purified on a silica (Biosil, 200-325 mesh, Biorad Laboratories, Richmond, CA) column (in a 10 ml glass pipette) using a hexane mobile phase. After this step the radiometric purity of the ^{3}H-benzo(a)pyrene was approximately 99.5% as measured by HPLC. The silica column-purified ^{3}H-benzo(a)pyrene was added to unlabeled benzo(a)pyrene to a final specific activity of 500 mCi per mmole. The ^{3}H-benzo(a)pyrene was further purified by TLC. The compound was spotted on an Silica gel G glass TLC plate (Analabs, Inc., Newark, DE) and chromatographed in a benzene:ethanol (9:1) mobile phase. The benzo(a)pyrene was located by fluorescence and the spot scraped from the plate and eluted with methanol.

The silica gel was pelleted by centrifugation (1000 x g for 5 min) and the methanol removed. ^{3}H-benzo(a)pyrene concentration was determined by liquid scintillation counting. The radiometirc purity of the benzo(a)pyrene was 99.99+% after this step (determined by HPLC). The methanol was evaporated under a stream of nitrogen, the ^{3}H-benzo(a)pyrene dissolved in a small volume of acetone, and added to the cell suspension (final acetone concentration 2%). Cells were incubated for 45 minutes at 37 C. After incubation with benzo(a)pyrene, the cell suspension was then extracted three times with 2 ml of ethyl acetate. Under these conditions greater than 99% of the radioactivity was recovered in the organic phase. The combined ethyl acetate extracts were then dried with anhydrous magnesium sulfate, filtered and the ethyl acetate evaporated under a stream of nitrogen. The benzo(a)pyrene metabolites were then redissolved in a small volume of ethyl acetate and spotted on a Silica gel G TLC plate. The metabolites were separated from the parent compound using a benzene:ethanol (9:1) mobile phase. Authentic benzo(a)pyrene metabolite standards were also run to ensure proper separation. The area of the plate containing the metabolites (the origin to R_f 0.71) was scraped and eluted with methanol. The silica gel was pelleted by centrifugation and the methanol supernatant decanted. The methanol was evaporated under a stream of nitrogen and the metabolites were redissolved in 0.4 ml of methanol. The methanol concentration adjusted to 60% with water. Metabolites were separated using a Micromeritics model 7000 high performance liquid chromatograph equipped with an Ultrasphere ODS (4.6 mm x 250 mm) column (Altex, Inc., Berkeley, CA). Methanol concentration was initially 60% and was increased linearly to 100% methanol over 40 minutes. A constant flow rate of 1.00 ml per minute was maintained. Column

temperature was 25 C. Absorbance at 254 nm was monitored using a Micromeritics model 786 variable wavelength detector. Fractions were collected directly into 20 ml glass scintillation vials, 10 ml of aquasol (New England Nuclear, Boston, MA) was added to each vial and radioactivity measured using a Beckman model LS-3150 P liquid scintillation counter. This procedure is summarized in figure 3.

I. Analysis of benzo(a)pyrene binding to AHH-1 cell DNA.

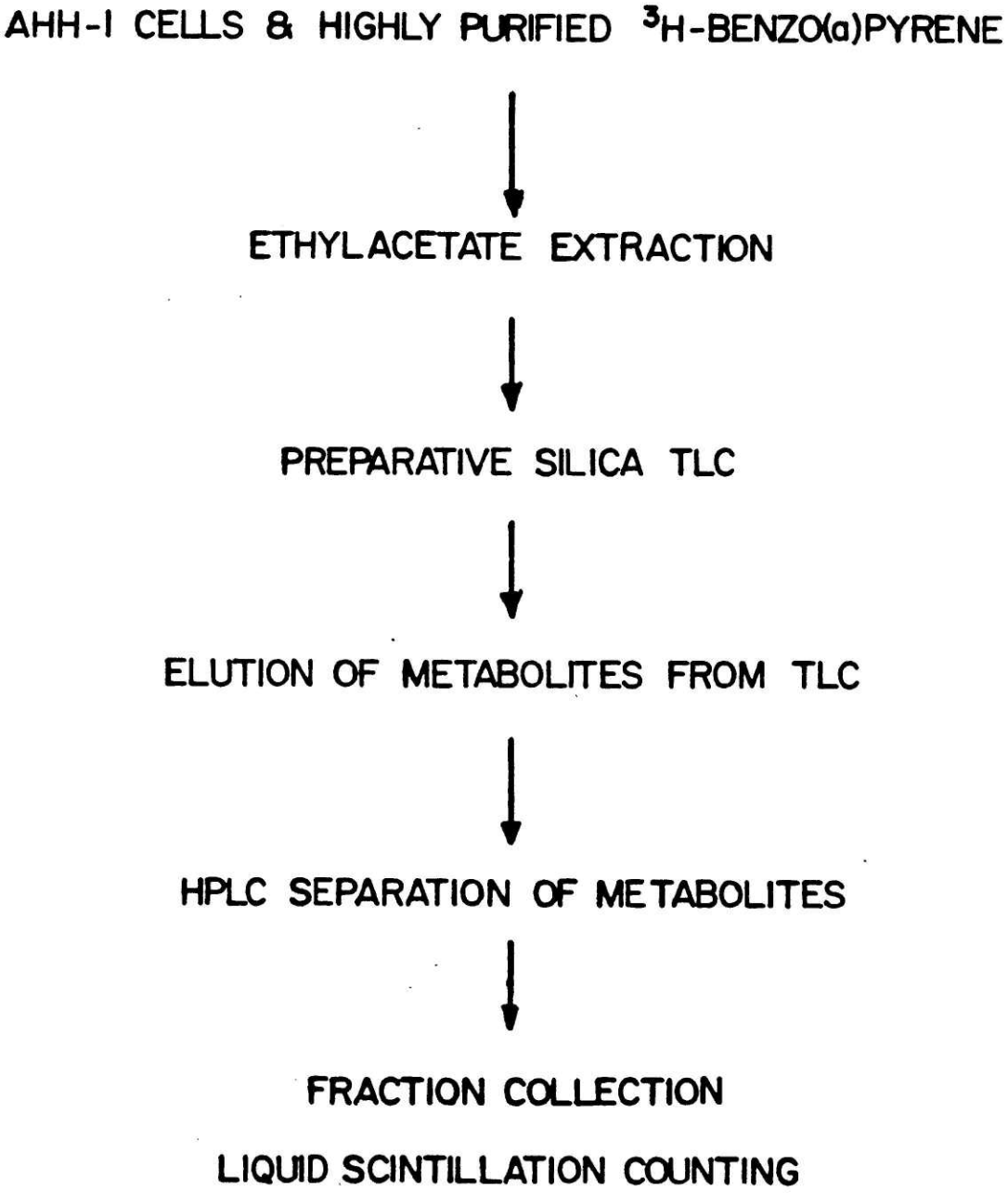
AHH-1 cells were induced by 2 uM dibenz(a,c)anthracene for 24 hours. Cells were centrifuged (1000 x g for 5 min) resuspended in RPMI medium 1640 and centrifuged again and resuspended at 1 x 10^7 cells per ml in RPMI medium 1640 containing 1.5 mg/ml of NADPH and 1 mg/ml NADH. ^{3}H-benzo(a)pyrene (41.7 to 66 Ci per mmole) was purified on a silica column as described for benzo(a)pyrene metabolite analysis. The ^{3}H-benzo(a)pyrene was adjusted to the desired specific activity (10 to 41.7 Ci per mmole) with unlabeled benzo(a)pyrene and added to the cell suspension in a small volume of acetone (final acetone concentration 2%). The final concentration of benzo(a)pyrene was 10-20 uM and was confirmed by liquid scintillation counting. Cells were incubated 3 hours at 37 C in the presence of labeled compound. Cells were then centrifuged (1000 x g for 5 min) and resuspended in 3.8 ml of 0.1 M NaCl, 0.05 M HEPES, 0.01 M EDTA (buffer A). The cells were lysed with 0.8 ml of 2% sodium lauryl sarcosine in buffer A. RNase A (1.2 mg in 0.4 ml of buffer A) was then added and then lysate incubated for 30 minutes at 37 C. Proteinase K (0.8 mg in 0.4 ml of buffer A) was then added and the lysate incubated for 120 minutes at 37 C. The cell lysate was extracted three times with 5 ml phenol:chloroform (1:1) equilibrated with buffer A and then extracted

Figure 3.

Procedure for benzo(a)pyrene metabolite analysis.

^{3}H-BP was purified in a two step procedure and incubated with AHH-1 cells. Metabolites were extracted and the bulk of the unmetabolized ^{3}H-BP removed in a preparative step. Metabolite composition was determined by HPLC separation and co-chromatography with authentic metabolite standards.

BENZO(a)PYRENE METABOLITE ISOLATION



three times with 5 ml of ethyl ether. DNA was precipitated with 10 ml cold ethanol. DNA was washed 5 times with ethanol and 15 times ethyl ether. Radioactivity was monitored in the ether washes and DNA was washed until no further radioactivity was washed from the DNA. Residual ether was removed in vacuo, and the DNA dissolved and enzymatically digested to deoxyribonucleosides according to the procedure of King et al., 1979. The DNA digest was loaded on a C_{18} Sep-pak (Waters Associates, Inc.) washed with 20 ml of 10% methanol and eluted with 10 ml of 90% methanol. When cells were treated with ^{3}H-BP, or calf thymus DNA was treated with ^{3}H-BP-4,5-oxide, approximately 25 to 35% for the radioactivity associated with the DNA hydrolysate eluted from the Sep-pak during the dilute methanol washes, the remainer of the radioactivity was removered in the 90% methanol wash. When cells or calf thymus DNA were treated with the radiolabeled BP diol epoxides, less than 10% of the radioactivity associated with the DNA hydrolysate eluted in the dilute methanol wash. The 90% methanol wash was prepared for HPLC analysis. The bulk of the methanol was removed in vacuo and the solvent composition adjusted to approximately 45% methanol.

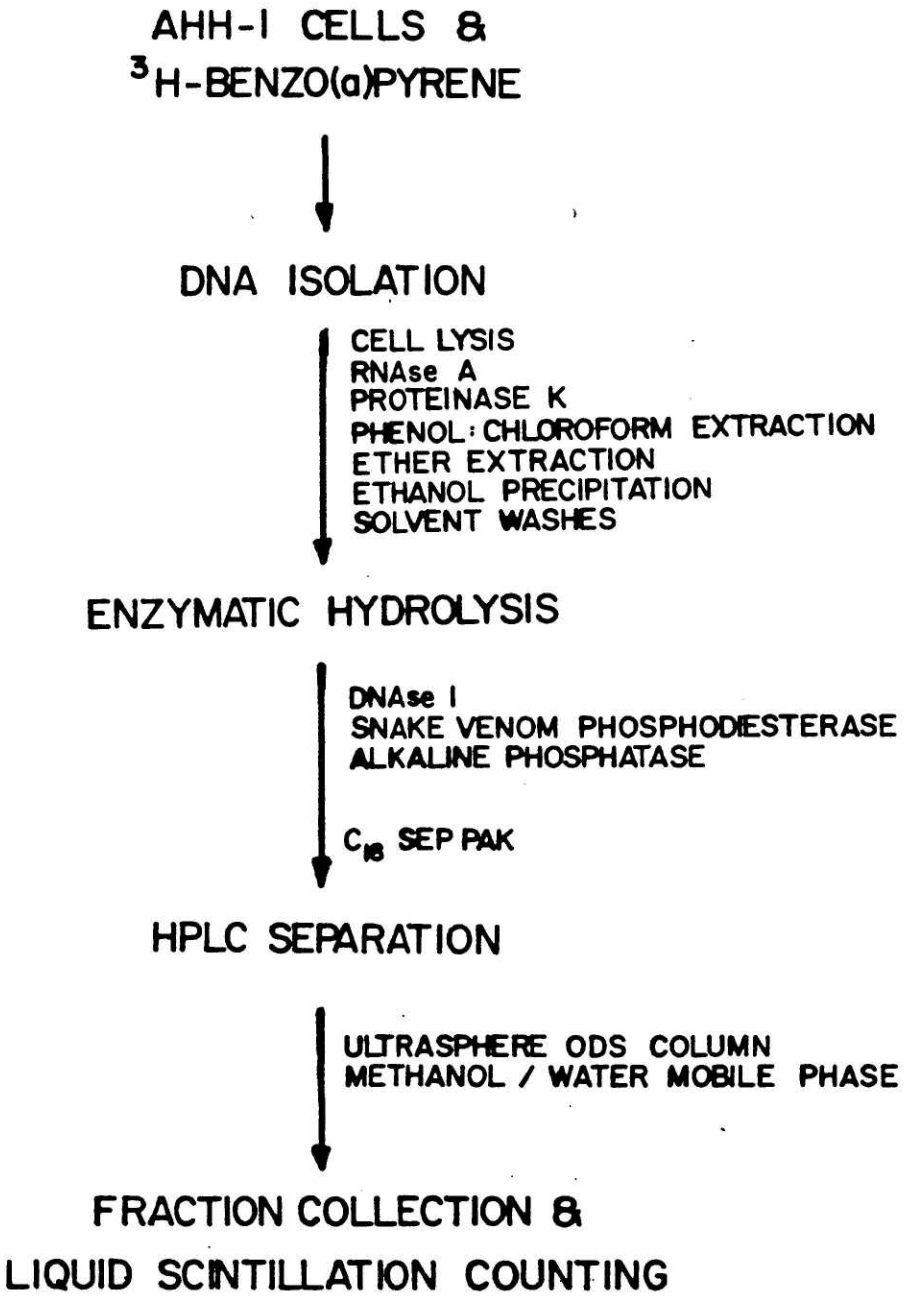
Deoxyribonucleoside-benzo(a)pyrene adducts were separated using an Ultrasphere ODS column (Panthananickal and Marnett, 1982; Eastman et al., 1981). All separations were performed at a column temperature of 24 to 27 C (except for the adduct analysis of anti- diol epoxide BP which was performed at 20 C) and a constant flow rate of 1.00 ml per minute. A methanol / water mobile phase was employed. Solvent conditions were adjusted depending on the polarity of the adduct species. The specific solvent conditions used are described in the appropriate figure ledgend. This procedure is summarized in figure 4.

Figure 4.

Procedure for benzo(a)pyrene-DNA adduct analysis.

AHH-1 cells were incubated with purified ^{3}H-BP. Cells were lysed, RNA and protein digested, and remaining protein removed by organic solvent extractions. The DNA was precipitated with cold ethanol and washed extensively with organic solvents. Finally, the DNA was redissolved, hydrolysed, and analyzed by HPLC.

CELLULAR DNA ADDUCT ANALYSIS



J. Production of standard benzo(a)pyrene-DNA adducts.

^{3}H Benzo(a)pyrene-4,5-oxide, ^{3}H anti-diolepoxide-BP and ^{14}C syn-diolepoxide-BP were reacted with calf thymus DNA in either water or acetone - water (1:1 or 1:2) pH 5 according to published methods (Blobstein et al., 1975; Weinstein et al., 1976; Jennette et al., 1977). The unbound hydrocarbon was removed by ethyl ether extractions (4 to 10 times). Nucleic acids were precipitated with ethanol. Precipitated DNA was washed as described for the isolation of the cellular DNA adducts. Modified DNA was enzymatically hydrolysed, purified using a C_{18} Sep-pak and analyzed by HPLC as described for the cellular BP-DNA adducts.

K. Inhibition of AHH activity by monoclonal antibody.

Specific monoclonal antibody against rat liver 3-methylchloanthrene-induced cytochrome P450 and non-specific antibody were obtained in collaboration with Dr. H. Gelboin (National Institutes of Health, Bethesda, MD). AHH-1 cells were induced with 2 uM DB(a,c)A for 24 hours. Cells were washed as described for the AHH assay. AHH-1 MFO activity was an intracellular activity, therefore the cells were lysed to allow the interaction with the antibody. AHH-1 cells (1×10^{8} cells per ml) were lysed by mild vortexing (5 seconds) in 50 mM tris pH 8.5. The 0.1 ml of the cell lysate was added to 0.85 ml of AHH assay buffer (including cofactors). Antibody in 0.025 ml PBS was added to the lysate, the cells were incubated for 15 minutes at room temperature. The substrate, BP (50 uM final concentration) was then added in 0.025 ml acetone, the mixture incubated at 37 C for 30 minutes and BP-phenol fluorescence determined by organic extraction followed by alkaline

extraction as described earlier. Activity was compared to controls without antibody (PBS only) and controls with non-specific antibody.

V. RESULTS AND DISCUSSION

A. Isolation of the AHH-1 cell line

The most important step in developing a mutation assay in human lymphoblasts containing endogenous MFO activity is the isolation of a cell line with desirable physical and biochemical properties. The first, and most obvious property is that the cell line contain adequate levels of endogenous MFO activity. It is also advantageous that the activity be inducible by the "classical" inducers of MFO activity such as polycyclic aromatic hydrocarbons. Second, the cell line should be homogeneous for MFO activity. This ensures that there is not a resistant subpopulation which may have a selective advantage during highly toxic treatments. Such selection may be observed as a decrease in mutation induction as the relative survival decreases. Third, the cell line should have a high cloning efficiency. This allows the measurement of mutant fraction using the existing methods of drug-resistant colony formation in 96 well microtiter plates. And fourth, gene-locus mutation should be measured at a locus which is sensitive to all types of mutations (base-pair, frameshift, additions, and deletions) and has a short phenotypic expression. At the beginning of this project their was no reported human lymphoblast cell line which fulfilled all of these requirements. Therefore the RPMI-1788 cell line was chosen because it had been reported to contain a reasonably high basal and induced levels of AHH activity (Freedman et al., 1979a,b).

A sample of RPMI-1788 cells was obtained from Dr. H.L. Gurtoo of Roswell Park Memorial Institute. This cell line was characterized and

found to have the following properties:

Cellular AHH activity was inducible. This activity could be induced about 3.5 fold by pretreatment with 10 uM benzo(a)pyrene for 48 hours. The RPMI-1788 cell line cloned with a 20% efficiency in microtiter plates when grown with a 10% horse serum supplement. Higher cloning efficiencies were observed when the cell were grown with a 10% fetal calf serum supplement (30%). The sample of RPMI-1788 cells obtained from Roswell Park was found to be contaminated with mycoplasma (species unknown). The laboratory which performed the mycoplasma tests classified the level of contamination as moderate (100 to 200 organisms per cell). And finally, RPMI-1788 cells were non-homogeneous for AHH activity. Single cell subclones were isolated and characterized for BP-induced inhibition of growth (a parameter which has been shown to be proportional to AHH activity (Hankinson, 1979)) and were found to have a hypersensitive subpopulation.

Because of the importance of the RPMI-1788 cell line and the unavailability of samples of RPMI-1788 cells free of mycoplasma contamination, a cure for the mycoplasma contamination was attempted. The basic strategy for this cure was selection of a single cell clone which contained no mycoplasma. RPMI-1788 cells were grown in growth medium containing 200 ug/ml gentamycin and 100 ug/ml kanamycin. Both of these antibiotics have been shown to have anti-mycoplasmal activity and are stable in cell culture medium. The antibiotic concentrations chosen were the highest which still permitted good cell growth (80 to 90% of growth rate in antibiotic-free medium). A spinner culture was grown for 10 generations (a 1000-fold increase in cell number) in gentamycin-kanamycin medium. This amount of proliferation was chosen because it would be

expected to decrease the average number of mycoplasma per cell to less than one and was brief enough (7 days) that selection for antibiotic-resistant mycoplasma was unlikely. After growth of the spinner culture in gentamycin-kanamycin, the cells were plated in microtiter plates at low density (0.2 cells per well). Colonies were isolated and characterized for BP-induced inhibition of cell growth. A total of 47 colonies were isolated and characterized. Of the 47 colonies, one was found to be hypersensitive to BP-induced growth inhibition (see figure 5). This clone was designated AHH-1. AHH-1 cells were recloned at low cell density twice more in gentamycin-kanamycin medium to eliminate the mycoplasma contamination. At each step of this cloning procedure BP-induced growth inhibition was measured and was found to be stable trait. The final subclone was chosen because it formed large, homogeneous colonies in microtiter wells with high efficiency (40 to 60%). This cell line was used in the development of a mutation assay at the HGPRT locus.

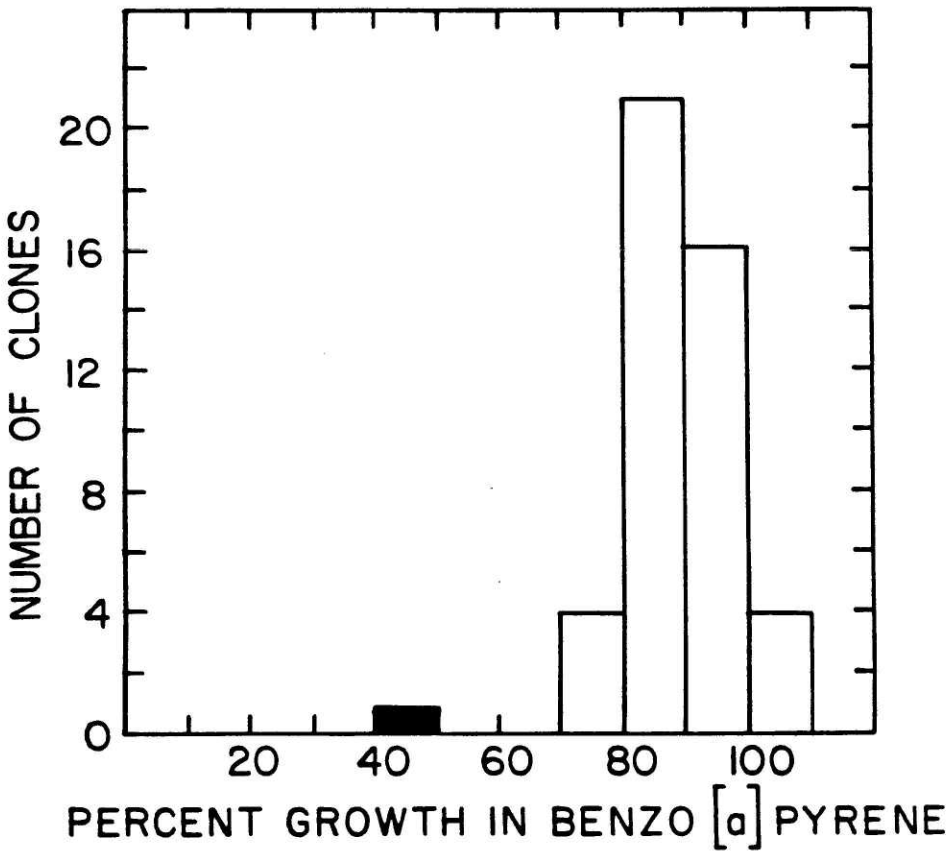
The AHH-1 cell line has been routinely tested for mycoplasma contamination (greater than 40 tests performed over 18 months) by two independent laboratories, Flow Laboratories, McLean, VA and Bioassays, Inc., Woburn MA. All tests have been negative.

Figure 5.

Distribution of benzo(a)pyrene induced

toxicity with RPMI-1788 subclones

One subclone of the RPMI-1788 human lymphoblast cell line was found to be hypersensitive to benzo(a)pyrene induced inhibition of cell growth. This subclone was designated AHH-1.


NUMBER OF CLONES
0
4
8
12
16
20
20
40
60
80
100
PERCENT GROWTH IN BENZO [a] PYRENE

B. Development and application of mutation assay at the HGPRT locus.

1. Development of mutation assay.

The development of a gene-locus mutation assay involved several steps. The experimental parameters, selective agent concentration, cell plating density, and phenotypic expression time, were optimized. The putative mutant colonies were isolated and characterized to verify that they are truely stable mutants. Systemic bias for or against the mutant phenotype was tested.

Experiments of this type measuring mutation at the HGPRT and TK loci in other human lymphoblast cell lines had previously been carried out in our laboratory. But initial efforts to isolate 6-thioguanine-resistant colonies from AHH-1 cells failed. This initial work, which was performed immediately after the isolation of the cell line and while the achievement of a long-term cure of the mycoplasma contamination was still under investigation, was ambiguous. It was conceivable that the cells contained a very low level contamination (below detection by mycoplasma assay detection) which was inhibiting 6TG-resistant colony formation. There was, of course, no proven mycoplasma free AHH-1 culture for controls. The discovery which made mutation assay development possible was that the AHH-1 derivative of RPMI-1788 cells clump in the presence of high concentrations of 6-thioguanine (5 ug/ml) which have been routinely used in our laboratory for mutant selection in other human lymphoblast cell lines. This phenomena inhibited colony formation at the cell density (20,000 cells per well) used to measure mutant colony formation with human lymphoblasts. Further investigations revealed that 6TG resistant AHH-1

colony formation was strongly inhibited at cell densities above 2000 cells per well. Subsequent work optimizing selective conditions overcame this limitation by decreasing the 6-thioguanine concentration to 0.5 ug/ml. Utilizing this lower selective agent concentration cell densities up to 20,000 cells per well could be used. The dependence of mutant recovery on 6-thioguanine concentration was examined in detail. Cells were plated at two densities, 2,000 and 20,000 cells per well (Table 2). At 2,000 cells per well, the observed mutant fraction was constant in the range of 0.1 to 5 ug/ml although some growth of wild type cells was observed at 0.1 ug/ml. Above 5 ug/ml mutant colony formation was inhibited. At 20,000 cells per well mutant recovery was inhibited at 6TG concentrations above 1 ug/ml. In this same experiment, a 6-thioguanine resistant - mixed mutant population was plated at 2 cells per well. This population was not sensitive to the toxic effects of 6-thioguanine up to 5 ug/ml.

The final assay parameter determined was the time required for phenotypic expression of 6-thioguanine resistance. AHH-1 cell cultures were treated with ICR-191 for 24 hours. The cultures were resuspended in growth medium and allowed to grow. At different times after treatment the 6-thioguanine-resistant fraction was measured. ICR-191 induced mutation was fully expressed after 6 days of growth and the mutant fraction remained stable for the length of this experiment. These results are presented in figure 6.

Seven BP-induced 6TG-resistant colonies were isolated, purified and grown for 20 generations without selective conditions. After 20 generations the mutant phenotype was examined. All 6-thioguanine resistant clones remained 6-thioguanine resistant, i.e. grew with comparable growth rates with and without 6-thioguanine present in the

Table 2.

Dependence of mutant recovery on 6-thioguanine concentration.

AHH-1 cells were plated under three conditions: 1) ICR-191-treated culture (0.5 ug/ml for 24 hours) plated at 2,000 cells per well, 2) ICR-191-treated culture plated at 20,000 cells per well, and 3) 6-thioguanine resistant cells (BP-induced mixed mutant population) plated at 2 cells per well.

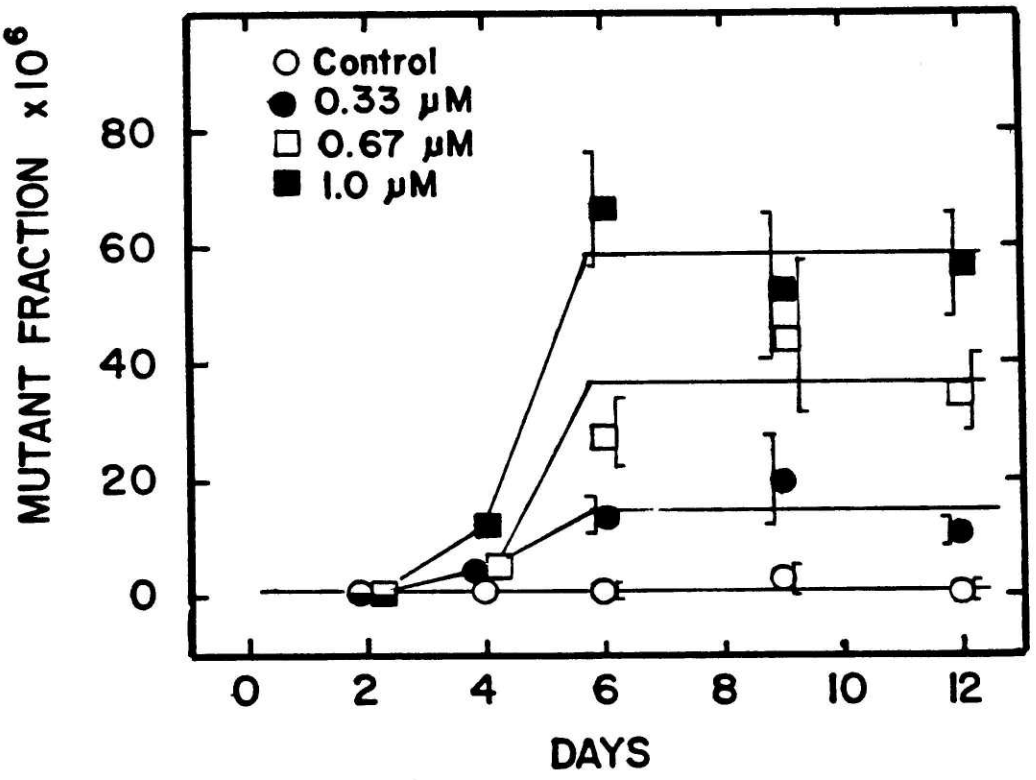
	RELATIVE MUTANT RECOVERY		
6TG Concentration	2,000 c/w	20,000 c/w	2 c/w
0.0 ug.ml			1.00
0.1 ug/ml	1.21		
0.3 ug/ml	0.81	0.91	
0.5 ug/ml		1.00	
1 ug/ml	1.14	0.85	1.17
2 ug/ml		0.14	
3 ug/ml	0.81	¼0.04	
5 ug/ml	1.01		0.78
10 ug/ml	0.48		0.96
20 ug/ml	0.03		0.91

c/w - cells per well in 96 well microtiter plates.

Figure 6.

Phenotypic lag for 6-thioguanine resistance.

ICR-191 treated cultures were plated at different days after mutagen treatment. 6-Thioguanine resistant was fully expressed by 6 days after treatment and remained stable up to 12 days after treatment.


MUTANT FRACTION x10⁶
Control
0.33 μM
0.67 μM
1.0 μM
80
60
40
20
0
0
2
4
6
8
10
12
DAYS

medium, but did not grow in the presence of CHAT (table 3). Therefore the mutant phenotype was a stable characteristic for at least 20 generations of growth in the absence of selective pressure. In addition, the mutant phenotype was confirmed by the measurement of cellular HGPRT activity. HGPRT activity was measured using a DEAE filter binding technique (Thilly et al., 1976). None of the 6TG resistant colonies had detectable HGPRT activity (table 3).

There are four possible processes which may bias the results of the quantitative mutation assay. The apparent mutant fraction may be altered by differential survival during toxic treatment, differential growth rates between mutant and wild type cells under non-selective conditions, differential plating efficiencies between mutant and wild type cells, or selective mutation of subpopulations with increased MFO activity.

Mutant selection by preferential survival during mutagen treatment would be most important for highly toxic treatments. Benzo(a)pyrene treatment is not highly toxic to AHH-1 cells, the relative surviving fraction is above 70%. Therefore differences in relative survival would little influence the apparent mutant fraction. A more important influence for BP-induced mutation may be the level of cellular MFO activity. This will be addressed later in this section. ICR-191 was moderately toxic to AHH-1 cells. The relative survival of both wild-type and a mixed ICR-191 - induced mutant (8 independent colonies) populations was tested. No significant differential survival was observed (figure 7).

Seven benzo(a)pyrene induced 6-thioguanine resistant mutants were isolated and characterized. All seven clones had similar growth rates under non-selective conditions compared to wild type cells. This indicates that there is no selective pressure for or against the mutant

Table 3.

6-Thioguanine resistant clones - doubling times under selective conditions and HGPRT activity.

Clone	T_2 Control	T_2 6TG	T_2 CHAT	HGPRT Activity*
BP-1	19.1 hr	20.8 hr	ng	<0.5%
BP-2	17.3 hr	18.2 hr	ng	<0.5%
BP-3	20.0 hr	21.7 hr	ng	<0.5%
BP-4	16.0 hr	18.4 hr	ng	<0.5%
BP-5	19.1 hr	20.4 hr	ng	<0.5%
BP-6	18.9 hr	17.9 hr	ng	<0.5%
BP-7	24.3 hr	26.4 hr	ng	<0.5%
Wild type	16.4 hr	ng	20.5 hr	100%

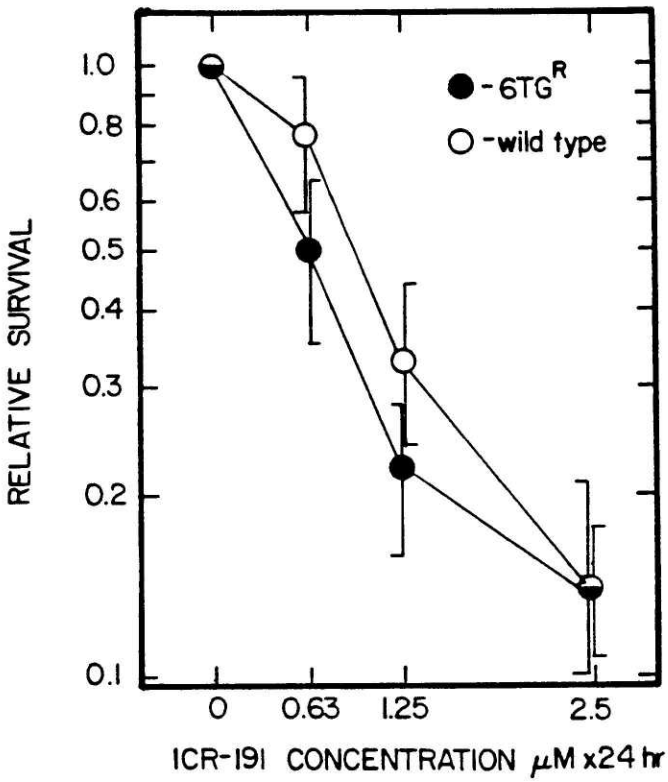
ng - no growth observed for three days of culture.

* The lowest detectable HGPRT activity using this assay system was 0.5% of the wild type activity.

Figure 7.

Relative survival of wild-type and 6-thioguanine resistant cells after ICR-191 treatment.

No differential cell survival was observed between wild-type AHH-1 and 6-thioguanine resistant cells.


RELATIVE SURVIVAL
1.0
0.8
0.6
0.5
0.4
0.3
0.2
0.1
● - 6TG^R
○ - wild type
0
0.63
1.25
2.5
ICR-191 CONCENTRATION μM x 24 hr

cells due to differential growth rates. One clone, BP-7, grew with a slower doubling time than the rest; this clone also had a low plating efficiency. These results are summarized in table 4.

The 6-thioguanine resistant clones which were tested and wild type cells had comparable plating efficiencies. This indicates that there is no selective pressure for or against the mutant cells due to differential plating efficiencies. These results are summarized in table 4.

Finally, the BP-induced 6-thioguanine resistant colonies were characterized for AHH activity. The level of AHH activity in cultured cells has been correlated to their relative sensitivity to BP toxicity (Hankin, 1979). Specifically, the higher the cellular AHH activity the more sensitive the cell to BP toxicity. It may be reasonable to extend this observation to the induction of gene-locus mutation. In particular, cells with more AHH activity may be more mutable. Benzo(a)pyrene may preferentially induce mutants from a "super-metabolic" subpopulation. The mutant clones contain comparable AHH activity compared to wild type cells. The variability was within the normal range observed for measurements made on independent cultures. A possible cause for this variability will be discussed later. The mean basal and induced AHH activity of the 6-thioguanine resistant colonies was similar to that of wild type cells (table 5). Therefore BP-induced 6TG resistant cells were not from a subpopulation with higher capacity to metabolize benzo(a)pyrene.

Table 4.

6-Thioguanine resistant clones -

plating efficiency

Clone	Plating Efficieny	Plating Efficiency With 6TG	Plating Efficiency With 6TG & 2000 c/w
BP-3	0.22	0.24	0.23
BP-4	0.33	0.34	0.34
BP-5	0.15	0.21	0.20
BP-6	0.33	0.26	0.34
BP-7	0.06	0.06	0.07
Wild type	0.25	$<10^{-3}$	$<10^{-5}$

Table 5.

6-Thioguanine resistant clones-

AHH activity

Clone	AHH activity Basal*	AHH activity Induced*
BP-1	0.20	1.64
BP-2	0.28	2.36
BP-3	0.28	1.88
BP-4	0.16	2.76
BP-5	0.12	0.72
BP-6	0.16	1.88
BP-7	0.28	1.84
Wild type	0.28	2.04
Mean $6TG^R$	0.21	1.87

* in pmole 3OHBP per 10^6 cells per minute

2. Application of mutation assay

When developing a new model system it is important to determine the limits of the system. The versatility of the mutation assay utilizing AHH-1 cells was demonstrated by testing a series of xenobiotics for their ability to induce gene-locus mutations without the addition of an extracellular metabolizing system. With the exception of BP, the cellular xenobiotic metabolites were not studied. It is reasonable to assume in these mutation induction measurements the xenobiotic was in fact mutagenic via cellular metabolism. Since the xenobiotic metabolism is an intrinsic property of AHH-1 cells experiments separating and selectively eliminating the xenobiotic metabolism were impossible. Metabolism-requiring mutagens were tested under two conditions, uninduced and beta-naphthoflavone preinduced cellular MFO activity.

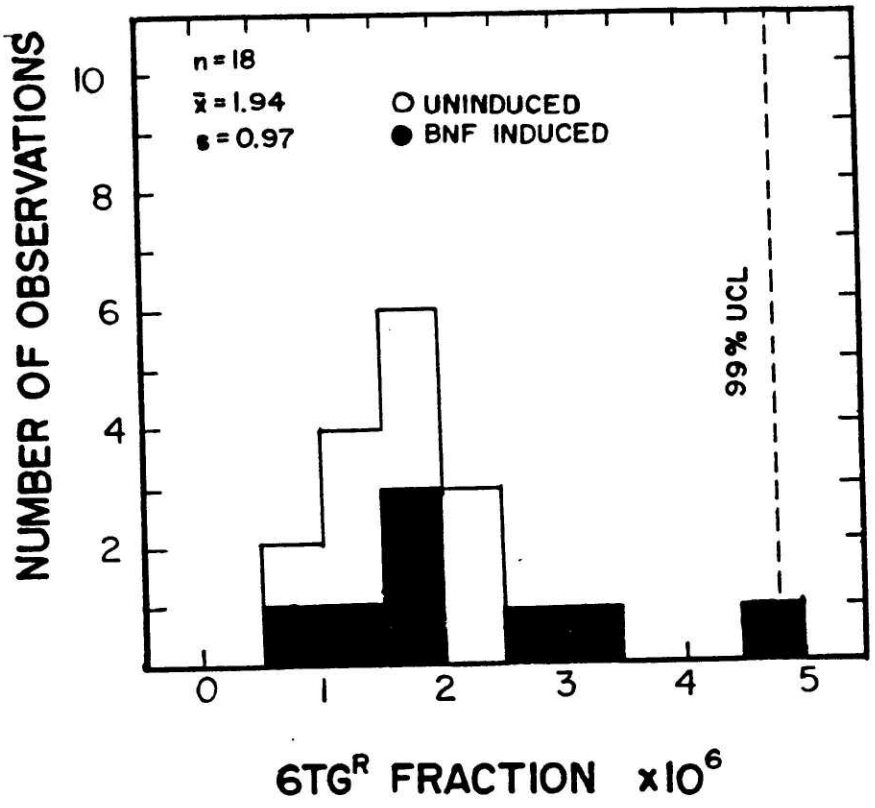
In this series of experiments a positive response was defined as a observed mutant fraction which was greater than the 99% upper confidence limit of the historical negative control observations (figure 8). This upper confidence limit was 4.8×10^{-6}. Negative control values for both uninduced and beta-naphthoflavone induced cultures were used for this calculation.

These investigations were designed to be a screening process. The desired endpoint was a yes or no result regarding mutagenicity. Therefore a few (two to five) relatively high mutagen concentrations were used. The results of this screening process are presented in table 6.

The direct acting mutagens, EMS and ICR-191, were found to be potent mutagens. This was expected, the metabolic competance of AHH-1 cells should influence very little the mutagenicity of these compounds. AHH-1

Figure 8.

Distribution of negative control values. Independent negative control values (mean of duplicate determinations) were complied and mean and standard deviation calculated. A positive response was defined as greater than the mean plus three standard deviations or 4.8×10^{-6}.


n = 18
x̄ = 1.94
s = 0.97
O UNINDUCED
● BNF INDUCED
99% UCL
NUMBER OF OBSERVATIONS
10
8
6
4
2
0
1
2
3
4
5
6TG^R FRACTION x10^6

cells were found to be about equally sensitive to the mutagenic effects of these chemicals compared to TK6 human lymphoblasts (EMS, Penman et al., 1980; ICR-191, Skopek et al., 1978).

Nine compounds which require cellular oxidative metabolism were tested; seven, (BP, cyclopenteno(c,d)pyrene, 1-methylphenanthrene, lasiocarpine, dimethylnitrosamine, 2-acetoaminofluorene, and aflatoxin-B_1) were active; two, (perylene and fluoranthene) were not active.

The overall magnitude of the induction of gene-locus mutation by metabolism requiring mutagens in AHH-1 (induced mutant fraction compared to the background) was, with the exception of CEPE and aflatoxin B_1, relatively weak. The induced mutant fractions were 3 to 6 fold above the negative control values. One interpretation of these weak responses is that the cellular MFO activity is the rate determining step for the induction of gene-locus mutation. Investigations into the relationship between BP mutagenesis and metabolism have revealed a correlation between the amount of BP metabolism and the amount of induced mutation (figure 21). This interpretation can be tested through the selection of AHH-1 variants with higher MFO activity and comparison between mutagen sensitivities.

BP induced statistically significant mutation at 0.3 uM x 24 hours (compared to experimental negative control) or 1 uM x 24 hours (compared to historical distribution of negative controls).

DMN was not mutagenic to uninduced AHH-1 cells but was mutagenic to BNF-induced AHH-1 cells. A small increase in mutant fraction was observed with DMN-treated uninduced AHH-1 cells, this response was not significant when compared to the historical negative controls.

CEPE was an extremely potent mutagen for both uninduced (CEPE is a

weak inducer of AHH activity) and BNF-induced AHH-1 cells. The apparent shape of the CEPE dose-response curve deserves comment. The apparent decrease in mutant fraction when the mutagen concentration was increased from 3 uM to 10 uM was unexpected. This decrease may be attributed to the apparent destruction of cellular metabolic capacity by the mutagen exposure. In particular, at the end of the treatment cells exposed to 10 uM CEPE for 48 hours did not contain detectable ER deethylase activity while cells exposed to 1 uM or 3 uM CEPE for 48 hours had slightly induced levels of ER deethylase activity (2.1 fold and 2.7 fold respectively). CEPE mutagenesis will be studied further.

Lasiocarpine, a pyrrolizidine alkaloid, aflatoxin B_1, a mycotoxin, and 1-methylphenanthrene, a major soot component were tested and found to be mutagenic. The sensitivity AHH-1 cells to chemical mutagenesis varied between mutagens, AHH-1 cells were most sensitive to aflatoxin B_1 and least sensitive to lasiocarpine. Each individual mutagen induced approximately the same mutagenic response in cells which had their MFO activity either uninduced or induced by pretreatment with BNF. The concentrations of mutagen necessary to induce a positive response were different for each mutagen, lasiocarpine, 87 and 118 uM; Aflatoxin B_1, 0.7 and 1.1 uM; and 1-methylphenanthrene, 60 and 31 uM (values for uninduced and induced cells respectively (table 7)).

2-Acetoaminofluorene was tested only in uninduced cells and was found to be an active mutagen.

The two compounds which did not induce gene-locus mutation in AHH-1 cell deserve comment. Perylene, a potent mutagen for bacteria (with rat liver mediated metabolism) is not mutagenic to TK6 human lymphoblasts (with rat liver mediated metabolism) (Penman et al., 1980). The negative

result for perylene may represent the refractory nature of human cells to perylene mutagenesis. Higher perylene concentrations could not be tested with AHH-1 cells because of solubility limitations.

The negative result for fluoranthene was unexpected and may be due to the low levels of cellular epoxide hydrolase activity. This activity is necessary for the generation of fluoranthene metabolites capable of inducing mutation and binding to DNA (J. Babson and G.N. Wogan personal communication).

The relative sensitivity of AHH-1 cells and TK6 cells to the mutagenic effects of the tested compounds has been calculated (TK6 cells were treated in the presence of 5% vol/vol rat liver PMS.). The criteria for comparison is the mutagen concentration where the dose-response curve or the extrapolation between the lowest tested dose and the negative control crosses the 99% upper confidence limit of the historical negative control values. This comparison between cell lines requires qualification, the region of the AHH-1 cell dose response curve near the 99% upper confidence limit has not been characterized for CEPE and AFB_1. In addition, the lowest mutagen concentration tested may be in an apparent plateau in the dose-response curve. Therefore a rather large extrapolation was necessary. This may result in an erroneously high estimate of the significant mutagen concentration. These results are summarized in table 7. The purpose of table 7 is to provide the reader with a comparison between the mutational response observed with AHH-1 cells and the mutational response observed in a metabolism deficient cell type treated in the presence of PMS. Comparisons between the relative sensitivity of the two cell types should not be made because there are several important differences between the two assay systems. The PMS

preparation (5% vol/vol) contains much higher levels of xenobiotic metabolizing capacity compared to AHH-1 cells, therefore more of the particular xenobiotic may have been metabolized. The extent of xenobiotic metabolism and DNA adduct formation was studied for only BP. In addition, there were large differences in exposure times, the PMS preparation allows only short exposures, while with AHH-1 several day long exposures were possible.

TK6 cells (+ PMS) and AHH-1 cells were approximately equisensitive to the mutagenic effects of 2AAF and BP. AHH-1 cells were less sensitive to AFB_1, fluoranthene, and 1-methylphenanthrene; and more sensitive to CEPE. DMN and lasiocarpine have not been tested in TK6 cells.

Table 6.

Xenobiotics tested for mutagenicity in AHH-1 cells.

MUTAGEN	CONCENTRATION X TIME	METABOLIC CAPACITY*	RELATIVE SURVIVAL	MUTANT FRACTION x 10^6 MEAN ± STD. DEV.
CONTROL	DMSO solvent only	uninduced	1.00	1.9 ±0.4
EMS	50 uM x 24 hours	uninduced	0.48	6.3 ±1.3
EMS	100 uM x 24 hours	uninduced	0.36	20 ±5
control	DMSO solvent only	uninduced	1.00	1.6 ±0.5
ICR-191	0.63 uM x 24 hours	uninduced	0.65	62 ±11
ICR-191	1.25 uM x 24 hours	uninduced	0.48	116 ±17
ICR-191	2.5 uM x 24 hours	uninduced	0.13	147 ±28
control	DMSO solvent only	BP is an	1.00	1.6 ±0.5
BP	0.3 uM x 24 hours	inducer	0.87	3.4 ±0.5
BP	1 uM x 24 hours		1.02	4.9 ±0.9
BP	3 uM x 24 hours		0.74	6.1 ±1
BP	10 uM x 24 hours		0.82	12.7 ±1.5
BP	30 uM x 24 hours		0.75	18.2 ±2.1

* induced - cells were pretreated with 10 uM beta-naphthoflavone for 24 hours prior to mutagen treatment. Cells were centrifuged twice to remove the inducer before the addition of the mutagen.

Table 6 continued.

MUTAGEN	CONCENTRATION X TIME	METABOLIC CAPACITY	RELATIVE SURVIVAL	MUTANT FRACTION x 10^6 MEAN ± STD. DEV.
CONTROL	DMSO solvent only	uninduced	1.00	2.1 ±0.5
DMN	5 mM x 48 hours	uninduced	0.98	3.5 ±0.7
DMN	14 mM x 48 hours	uninduced	0.92	3.5 ±0.7
DMN	28 mM x 48 hours	uninduced	0.81	4.1 ±1.3
control	DMSO solvent only	induced	1.00	3.2 ±0.6
DMN	5 mM x 48 hours	induced	1.12	4.0 ±0.7
DMN	14 mM x 48 hours	induced	1.00	8.0 ±2.0
DMN	28 mM x 48 hours	induced	0.87	8.6 ±2.1
control	DMSO solvent only	CEPE is a	1.00	2.4 ±0.6
CEPE	1 uM x 48 hours	weak inducer	0.61	71 ±6
CEPE	3 uM x 48 hours	(2 to 3	0.63	78 ±9
CEPE	10 uM x 48 hours	fold)	0.59	22 ±2
CEPE	25 uM x 48 hours		0.54	34 ±4
control	DMSO solvent only	induced	1.00	0.8 ±0.3
CEPE	25 uM x 48 hours	induced	0.34	74 ±7
CEPE	50 uM x 48 hours	induced	0.27	64 ±7

Table 6 continued.

MUTAGEN	CONCENTRATION X TIME	METABOLIC CAPACITY	RELATIVE SURVIVAL	MUTANT FRACTION x 10^6 MEAN ± STD. DEV.
CONTROL	DMSO solvent only	uninduced	1.00	0.7 ±0.3
LSCP	100 uM x 48 hours	uninduced	1.30	5.4 ±0.6
LSCP	300 uM x 48 hours	uninduced	0.89	6.2 ±1.3
LSCP	600 uM x 48 hours	uninduced	0.82	6.0 ±1.4
control	DMSO solvent only	induced	1.00	2.8 ±0.7
LSCP	10 uM x 48 hours	induced	1.03	3.9 ±1
LSCP	100 uM x 48 hours	induced	0.91	4.3 ±1
LSCP	300 uM x 48 hours	induced	0.81	12.7 ±2.1
control	DMSO solvent only	uninduced	1.00	1.1 ±0.4
1MP	25 uM x 48 hours	uninduced	0.54	4.4 ±1.2
1MP	50 uM x 48 hours	uninduced	0.38	4.1 ±0.8
1MP	100 uM x 48 hours	uninduced	0.31	7.8 ±1.1
control	DMSO solvent only	induced	1.00	2.0 ±0.5
1MP	25 uM x 48 hours	induced	0.47	3.6 ±1.2
1MP	50 uM x 48 hours	induced	0.35	8.9 ±1.5
1MP	100 uM x 48 hours	induced	0.29	7.8 ±1.4

Table 6 continued.

MUTAGEN	CONCENTRATION X TIME	METABOLIC CAPACITY	RELATIVE SURVIVAL	MUTANT FRACTION x 10^6 MEAN ± STD. DEV.
CONTROL	DMSO solvent only	uninduced	1.00	2.4 ±0.6
AFB_1	6.8 uM x 48 hours	uninduced	0.56	27 ±3
AFB_1	13.6 uM x 48 hours	uninduced	0.45	20 ±2.5
AFB_1	20.4 uM x 48 hours	uninduced	0.36	35 ±4
control	DMSO solvent only	induced	1.00	4.8 ±0.9
AFB_1	6.8 uM x 48 hours	induced	0.52	17 ±2
AFB_1	13.6 uM x 48 hours	induced	0.43	20 ±2.5
AFB_1	20.4 uM x 48 hours	induced	0.35	25 ±2.7
control	DMSO solvent only	uninduced	1.00	2.0 ±0.9
Fla	10 uM x 48 hours	uninduced	1.05	1.3 ±0.7
Fla	20 uM x 48 hours	uninduced	0.93	1.0 ±0.6
Fla	40 uM x 48 hours	uninduced	0.75	2.8 ±1
Fla	50 uM x 48 hours	uninduced	0.67	3.2 ±0.9
Fla	100 uM x 48 hours	uninduced	0.45	3.2 ±1.3
conrol	DMSO solvent only	induced	1.00	1.8 ±0.7
Fla	40 uM x 48 hours	induced	0.70	3.7 ±1.3

Table 6 continued.

MUTAGEN	CONCENTRATION X TIME	METABOLIC CAPACITY	RELATIVE SURVIVAL	MUTANT FRACTION x 10^6 MEAN ± STD. DEV.
CONTROL	DMSO solvent only	uninduced	1.00	1.8 ±0.3
2AAF	25 uM x 48 hours	uninduced	0.73	4.8 ±1.2
2AAF	50 uM x 48 hours	uninduced	0.50	3.8 ±1.1
2AAF	75 uM x 48 hours	uninduced	0.37	6.9 ±1.7
2AAF	100 uM x 48 hours	uninduced	0.33	7.7 ±0.7
control	DMSO solvent only	uninduced	1.00	0.7 ±0.3
Per	10 uM x 48 hours	uninduced	0.93	3.2 ±0.75
Per	20 uM x 48 hours	uninduced	0.95	1.9 ±0.5
control	DMSO solvent only	induced	1.00	2.8 ±0.65
Per	10 uM x 48 hours	induced	0.89	2.7 ±0.6
Per	20 uM x 48 hours	induced	0.89	2.7 ±0.6

Abbreviations: 2AAF, 2-acetoaminofluorene, AFB_1, aflatoxin-B_1; BP, benzo(a)pyrene; CEPE, cyclopenteno(c,d)pyrene; DMN, dimethylnitrosamine; EMS, ethyl methanesulfonate; Fla, fluoranthene; LSCP, lasiocarpine; 1MP, 1-methylphenanthrene; Per, perylene.

Table 7.

Minimum mutagen concentrations necessary for a postive mutagenic response - AHH-1 cells and TK6 cells

Mutagen concentrations are the concentration where the dose-response curve crosses the 99% upper confidence limit of historical negative control values. For compounds which were not found to be mutagenic the highest concentration tested (in parentheses).

	AHH-1 CELLS		TK6 CELLS	
Mutagen	Uninduced	Induced	+ 5% vol/vol PMS	Reference
BP	1×10^{-6} M		1×10^{-6} M	(a)
CEPE	3×10^{-8} M		5×10^{-8} M	(b)
DMN	(2.8×10^{-2} M)	7×10^{-3} M		
LSCP	8.7×10^{-5} M	1.2×10^{-4} M		
1MP	6×10^{-5} M	3.1×10^{-5} M	6×10^{-6} M	(a)
AFB_1	7×10^{-7} M	1.1×10^{-6} M*	5×10^{-9} M	(c)
Fla	(1×10^{-4} M)	(4×10^{-5} M)	2×10^{-6} M	(a)
2AAF	2.5×10^{-5} M		1×10^{-4} M	(c)
Per	(2×10^{-5} M)	(2×10^{-5} M)	(2.2×10^{-5} M)	(d)

* observed negative control was abnormally high (4.8×10^{-6}). Therefore the minimum effective concentration was calculated on the basis of a background of 2.4×10^{-6}, the negative control value observed for uninduced cells.

(a) Barfnecht et al., 1982a
(b) Barfnecht et al., 1982b
(c) D.A. Kaden personal communication
(d) Penman et al., 1980

C. Characterization of xenobiotic biotransformation

The characterization of AHH-1 cell mediated xenobiotic biotransformation has been divided into two parts. Part one, analysis of cellular xenobiotic capacities using the model substrates benzo(a)pyrene and 7-ethoxyresorufin (ER) (sections 1 through 6). And two, characterization of the biotransformation of the polycyclic aromatic hydrocarbon, benzo(a)pyrene (section 7).

1. Induction of AHH-1 cellular mixed function oxidase activity.

The range of inducers of cellular MFO activity was determined. In the liver *in vivo*, MFO activity can be induced by a variety of xenobiotics. Phenobarbitol is a classic inducer of cytochrome P450 and induces primarily a demethylase activity although it does have some effect on AHH activity (Conney *et al.*, 1973). Arochlor 1254 induces a wide range of P450 associated activities (Alvares and Kappas, 1977) which tends to resemble the pooled microsomes of phenobarbitol and 3-methylcholanthrene treated animals (Ryan *et al.*, 1977). In fact, the different halogenated biphenyl cogeners present in the arochlor 1254 mixture seem to function as inducers of either phenobarbitol or 3-methylcholanthrene P450 forms (Poland and Glover, 1977; Goldstein *et al.*, 1977) although controversy concerning the purity of the polychlorinated biphenyls still remains (Goldstein *et al.*, 1978). Recent evidence indicates that in the case of TCDD and other polyhalogenated substances, a cytosolic receptor exists for translocation to the nucleus (Greenlee and Poland, 1979; Okey *et al.*, 1979). Neither the induction capabilities of TCDD nor the presence of a

TCDD receptor have been determined. However, arochlor 1254 does not induce cellular AHH activity as will be shown below.

The compounds in table 8 have been tested for their ability to induce AHH activity in AHH-1 cells. The fluorescent AHH assay outlined earlier was used to determine activity levels. Two indicies of inducing ability have been calculated: fold induction over uninduced control levels and ability to induce relative to a benzo(a)pyrene induced control which was induced and assayed under identical conditions. The fold induction (or induction ratio) tends to vary depending on parameters such as cell doubling time during induction (this will be discussed later in this section). The relative inducing efficiency of a xenobiotic compared to a simultaneous benzo(a)pyrene control yields more consistent results on independent days since the both the experimental and control cultures are subject to the same influences.

The AHH activity of AHH-1 cells could be induced 10 to 20 fold by pretreatment with polycyclic aromatic hybrocarbon inducers of cytochrome P450 activity. Benzo(a)pyrene, dibenz(a,c)anthracene, dibenz(a,h)anthracene, benz(a)anthracene and beta-naphthoflavone were found to be potent inducers of AHH activity after a 24 hour pre-incubation with the inducing agent. Several other PAH's were found to be weak inducers, chrysene, DMBA, and perylene induced activities 2 to 4 fold over basal levels. Arochlor 1254 and phenobarbitol, two inducers of cytochrome P450 activity <u>in vivo</u>, did not induce AHH activity in AHH-1 cells. The negative result encountered for arochlor 1254 must be qualified; arochlor 1254 bound to cells may have inhibited the AHH activity. The commonly used inducer of AHH activity <u>in vivo</u>, 3-methylcholanthrene (3MC), has not yet been tested for induction of AHH activity. This compound will be

TABLE 8.

Inducers of AHH activity. Cells were incubated in the presence of the inducer for 24 hours prior to the measurement of AHH activity.

Compound	Range Concentration	Fold induction	Induction Relative to benzopyrene
arochlor 1254	3-30 uM	1.3	0.07
B(a)A	3-30 uM	14.2	1.03
BP	10 uM	13.8	1.00
BNF	3-30 uM	11.4	0.82
chrysene	1-10 uM	3.7	0.37
DB(a,c)A	3-30 uM	20.1	1.45
DB(a,h)A	1-10 uM	13.6	1.5
DMBA	1-10 uM	2.1	0.2
fluoranthene	3-30 uM	1.0	n.a.
perylene	1-10 uM	2.7	0.27
phenanthrene	0.1-10 uM	0.8	n.a.
phenobarbitol	1 mM	1.2	n.a.
pyrene	0.1-10 uM	1.0	n.a.
triphenylene	0.1-10 uM	0.7	n.a.

n.a. Not applicable. The AHH activity was at or below the level observed in untreated, control cultures.

tested in the future. Two inducers were chosen for routine induction of cellular MFO activity. Beta-naphthoflavone was used because it was non-mutagenic and therefore useful as an inducer of MFO activity in mutation assays. Dibenz(a,c)anthracene was used because it was the most potent inducer and preferred over DB(a,h)A because the latter was difficult to dissolve in DMSO.

The concentration response for benzo(a)pyrene induction of AHH activity was examined in detail. Benzo(a)pyrene was found to be a potent inducer of AHH activity. Increases in AHH activity were observed over a wide concentration range. A significant induction of AHH activity was observed after a 24 hour treatment with 1×10^{-8} M benzo(a)pyrene and an apparent plateau in induction was observed at concentrations greater than 1×10^{-5} M. These results are presented in figure 9.

The time course of AHH activity was determined. This parameter was determined for two reasons: 1) to ensure that during mutation assays the mutagen treatment was long enough to induce AHH activity (if relevant); 2) to determine the appropriate time for making biochemical measurements on the induced AHH activity. AHH-1 cells were diluted to a low density and benzo(a)pyrene (10 uM) was added at different times. After the addition of the inducer the cultures were left unperturbed until AHH activity was measured. AHH activity was measured on all cultures simultaneously. AHH activity was induced quickly. Significant induction was observed after a one hour delay. AHH activity increased linearly with time and reached at 24 hours and remained stable (in the presence of the inducing agent) for up to 72 hours. On the basis of this data, a 24 hour treatment was chosen for routine work. These results are summarized in figure 10.

As mentioned earlier, the fold induction of cellular MFO activity

Figure 9.

Concentration dependence for benzo(a)pyrene mediated induction of AHH activity.

Cells were treated with different concentrations of benzo(a)pyrene for 24 hours. Cells were washed and AHH acvtivity measured. Each point represents the mean of four independent determinations (two independent experiments) confidence limits represent one standard deviation.

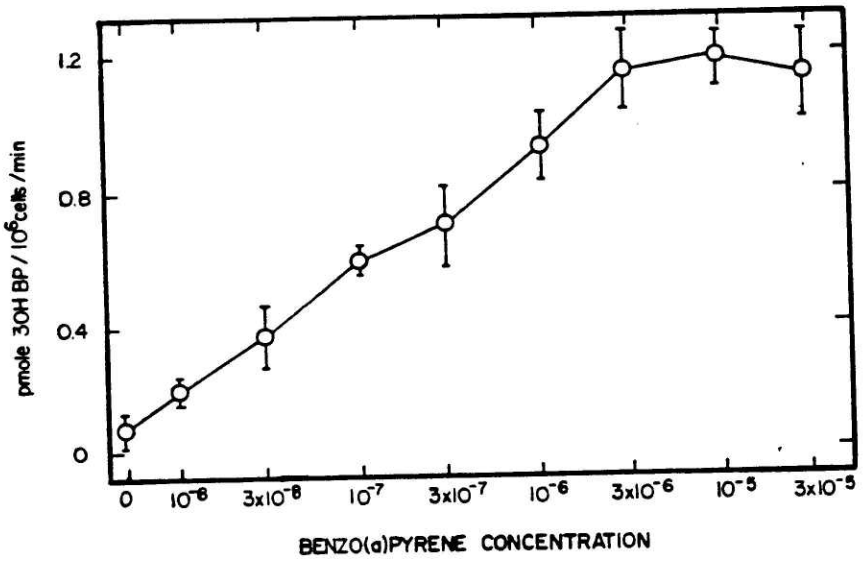
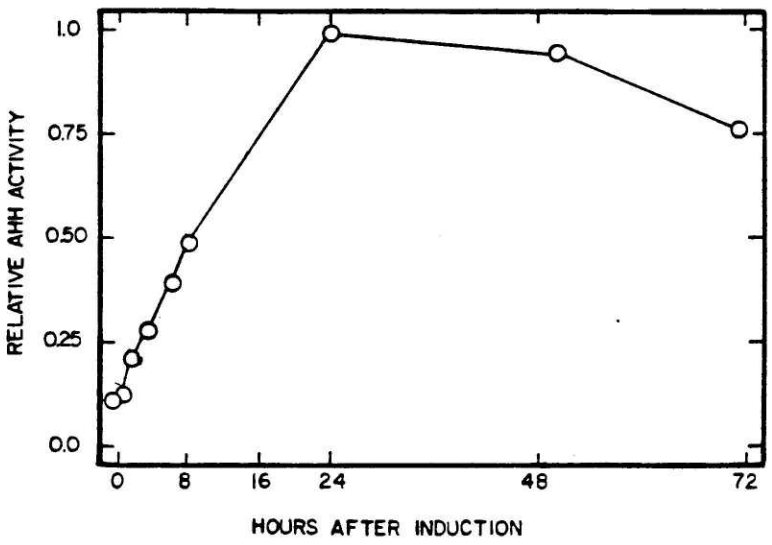

1.2
0.8
0.4
0
pmole 3OH BP / 10^6 cells / min
0
10^-8
3x10^-8
10^-7
3x10^-7
10^-6
3x10^-6
10^-5
3x10^-5
BENZO(a)PYRENE CONCENTRATION

Figure 10.

Time course of AHH activity induction.

AHH activity was induced linearly with time after a one hour delay. AHH activity was maximal at 24 hours after induction and remained stable until 72 hours.


RELATIVE AHH ACTIVITY
1.0
0.75
0.50
0.25
0.0
0
8
16
24
48
72
HOURS AFTER INDUCTION

varied (observed inducibilities from 10 to 30 fold) between independent experiments. This variability can be partially accounted for by fluctuations in cell growth during induction. The level of benzo(a)pyrene induced AHH activity was found to depend on cell doubling time. The shorter the doubling time the higher the induced AHH activity. These results are presented in figure 11.

2. Inhibitors of AHH-1 cellular mixed funciton oxidase activity.

The cellular AHH activity and ER deethylase activity was inhibited by known inhibitors of cytochrome P-450 MFO activity (Wiebel et al., 1972). DB(a,c)A-induced AHH activity was inhibited by alpha-naphthoflavone (7,8-benzoflavone), carbon monoxide, and SKF-525 A and destroyed by boiling (table 9). Basal and DB(a,c)A-induced ER deethylase activity is inhibited by ANF, and SKF-525 A (table 10 and figures 12 and 13). The kinetics of the inhibiton of ER deethylase activity by ANF was characterized.

3. Kinetic analysis of AHH-1 cellular mixed function oxidase activity.

The kinetic parameters for two mixed function oxygenase associated activities, BP hydroxylation and 7-ethoxyresorufin (ER) deethylation have been examined. ER deethylation is associated primarily with the PAH inducible cytochrome P-450 activities (Burke and Mayer, 1975; Burke et al., 1977a). BP hydroxylase activity is associated with basal, phenobarbitol induced, and most active in PAH induced rat liver microsomes.

The apparent K_m and V_{max} for basal (figure 14) and induced (figure 15) BP hydroxylase activities were 2.1 uM, 0.07 pmole/10^6 cells/min, and

Figure 11.

Dependence of AHH activity on cell doubling time

BP induced AHH activty was compared on independent days in independent cultures and cell doubling time calculated during induction of AHH activity. The range of doubling times represents culture variability. Cell culture conditions were not manipulated to alter cell doubling times.

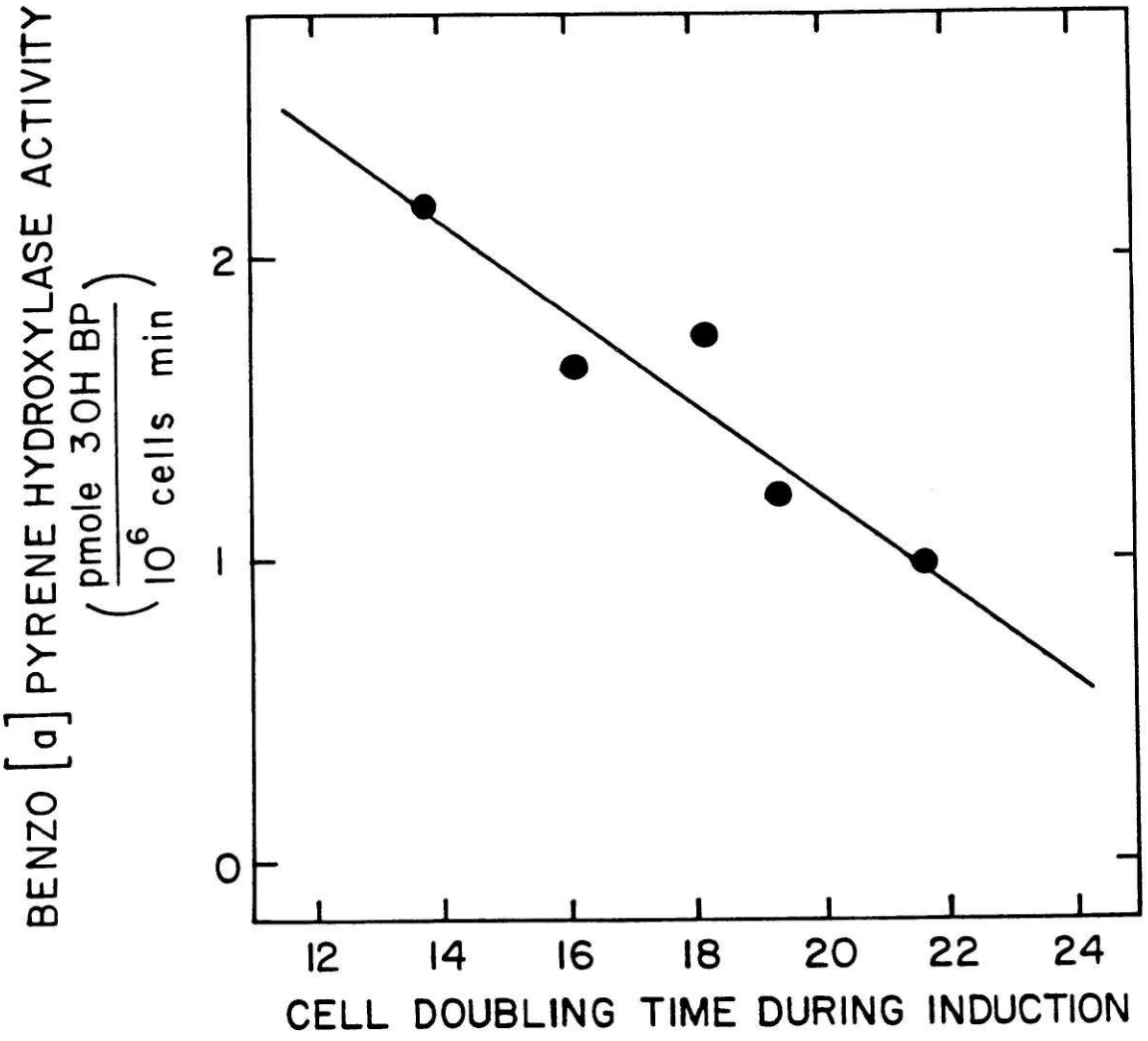

BENZO [a] PYRENE HYDROXYLASE ACTIVITY
(pmole 3OH BP / 10^6 cells min)
0
1
2
12
14
16
18
20
22
24
CELL DOUBLING TIME DURING INDUCTION

TABLE 9.

Inhibitors of dibenz(a,c)anthracene-induced AHH activity.

Conditions	Percent AHH activity*
no inhibitor	100 ±4.6
boiled cells	not detectable
carbon monoxide	1 ±3.8
alpha-naphthoflavone (10^{-7} M)	90 ±10
alpha-naphthoflavone (10^{-6} M)	46 ±1.8
SKF-525 A (10^{-6} M)	92 ±8
SKF 525 A (10^{-5} M)	79 ±2.8

* expressed as mean ± standard deviation.

TABLE 10.

Inhibitors of 7-ethoxyresorufin deethylase activity.

ER deethylase activity was measured as described in methodology section. Inhibitor was added to cell suspension in DMSO (0.1% final concentration). ER concentrations, induced cells 2 uM, uninduced cells 1 uM.

Inhibitor	DB(a,c)A-induced % Activity*	Uninduced % Activity
none	100 ±14	100 ±0
ANF (10^{-9} M)	97 ±9	98 ±5
ANF (10^{-8} M)	93 ±13	80 ±2
ANF (10^{-7} M)	68 ±14	30 ±2
ANF (10^{-6} M)	19 ±4	2.3 ±6
ANF (10^{-5} M)	2.4 ±0.6	1.1 ±5
ANF (10^{-4} M)	not detectable	not detectable
none	100 ±2.4	100 ±6
SKF-525 A (10^{-7} M)	102 ±2	97 ±1.4
SKF-525 A (10^{-6} M)	104 ±1.4	96 ±3
SKF-525 A (10^{-5} M)	87 ±2	84 ±3
SKF-525 A (10^{-4} M)	64 ±2.4	40 ±1.4
Boiled cells (5 min)	not detectable	

* % Activity expressed as mean ± standard deviation.

8.6 uM, 2.2 pmole/10^6 cells/min respectively. For comparison proposes, the reported apparent K_m for the basal and DB(a,h)A induced RPMI-1788 cell BP hydroxylase activity were 0.8 uM and 1.8 uM respectively (Freedman et al., 1979b). The apparent V_{max} for BP hydroxylase activity for basal and DB(a,h)A induced RPMI-1788 cells were 0.01 and 0.08 pmole per 10^6 cells per minute respectively (Freedman et al., 1979b). These apparent V_{max} values from the Lineweaver-Burke analysis conflict with the RPMI-1788 cell activity levels for basal and induced BP hydroxylase activty of 0.11 $\pm$0.02 and 0.33 $\pm$0.11 pmole per 10^6 cells per minute respectively which were reported in a companion paper (Freedman et al., 1979a). This apparent conflict was not addressed by the authors, the simplest explanation was that the authors errored in labeling the Lineweaver-Burke plot legends. The apparent K_m of mitogen-activated human lymphocyte basal and 3MC induced AHH activity were 3 uM to 2 uM and 4 uM to 8 uM respectively (range of three individuals), the basal and 3MC induced apparent V_{max} were 0.02 to 0.03 pmole per 10^6 cells per minute and 0.05 to 0.22 pmole per 10^6 cells per minute respectively (Gurtoo et al., 1979). The AHH-1 cell apparent K_m's resemble those reported for human lymphocytes and the AHH-1 cell V_{max}'s were somewhat higher.

The apparent K_m and V_{max} for basal (figure 12) and induced (figure 13) ER deethylase activities were 40 nM, 0.07 pmole/10^6 cells/min, and 1 uM, 1 pmole/10^6 cells/min respectively. No detailed kinetic data are available for human lymphocytes with this substrate, but the levels of ER deethylase activity observed in AHH-1 cells was comparable to that observed in 3MC-induced isolated lymphocytes (Burke et al., 1977b).

The kinetics of alpha-naphthoflavone (ANF) inhibition of ER deethylase activity were examined in detail. ANF was a competitive

inhibitor of the basal activity (K_i 3 nM) (figure 12) and a linear-mixed type of inhibitor (predominantly non-competitive) of the induced activity (K_i 0.3 uM) (figure 13).

The observed differences in the kinetic parameters indicates that the basal and induced activities are probably different enzymes.

4. Inhibition of AHH activity by a monoclonal antibody against cytochrome P450.

Implicit in these studies was that the cellular metabolic capacity was a cytochrome P-450 enzyme. Spectral evidence for the presence of cytochrome P450 has been elusive. It has been reported that non-cytochrome P-450 enzymes, in particular prostaglandin synthetase can metabolize BP when it acts as a hydroperoxidase during its catalytic cycle. These reactive oxygen intermediates (such as hydrogen peroxide and superoxide) would not be capable of ethoxyresorufin deethylation, which is a known P-450 process. Hydroxyl radical would be capable of deethylating ER by direct insertion into the carbon hydrogen bond. But due to the high reactivity of hydroxyl radical and the wealth of possible targets available, it is unlikely that this specific insertion into ER would be an efficient process. It is probably sufficient to measure known cytochrome P-450 processes as indirect proof for the presence of the enzyme, but happily, more direct evidence has become available. The cellular BP metabolizing activity is inhibited not only by classical inhibitors of cytochrome P-450 (carbon monoxide, ANF, SKF-525 A), but also by monoclonal antibody against rat liver 3-methylcholanthrene-induced cytochrome P-450 (gift for Dr. H. Gelboin) (figure 16).

Figure 12.

Linweaver-Burke plot of uninduced
7-ethoxyresorufin deethylase activity

Uninduced AHH-1 cells were incubated with different concentrations of ER and the production of resorufin was monitored by fluorescence (open symbols). The addition of 1×10^{-8} M ANF to the incubation mixture resulted in inhibition of the deethylase activity (closed symbols). This inhibition was compitetive.

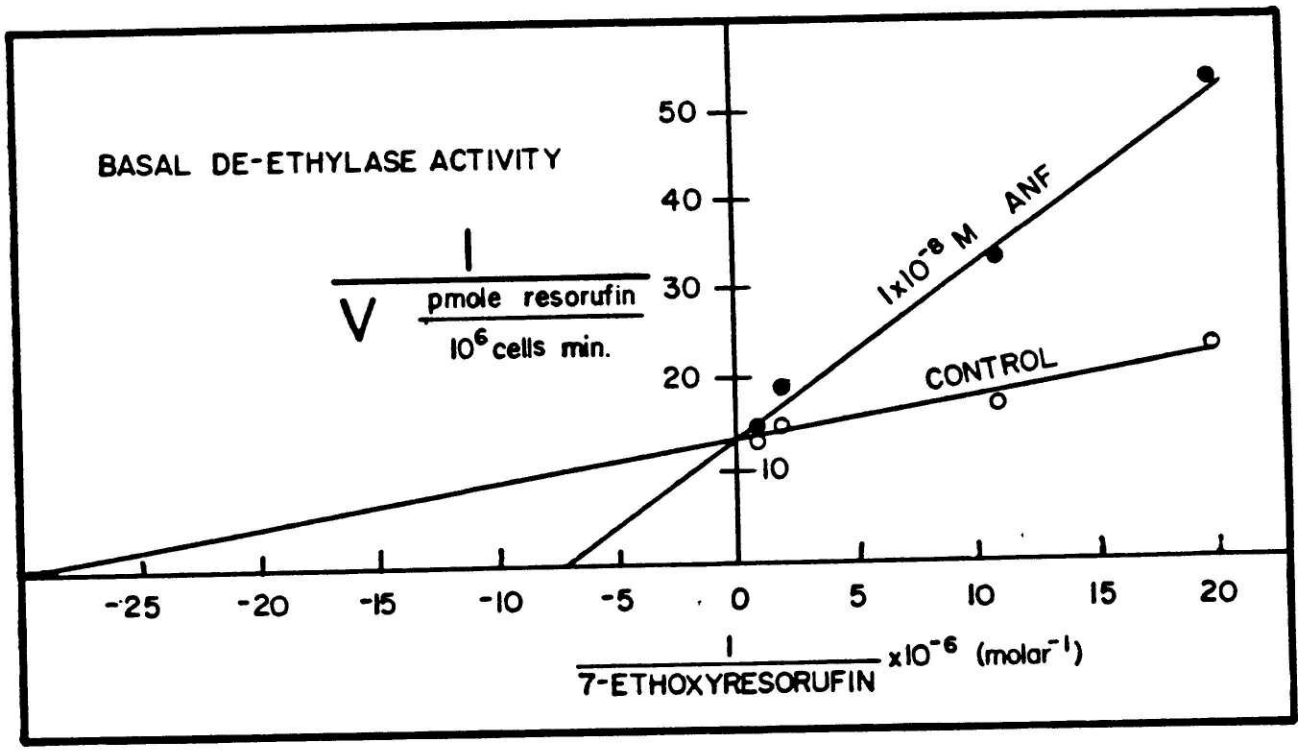

BASAL DE-ETHYLASE ACTIVITY
1/V pmole resorufin / 10^6 cells min.
50
40
30
20
10
1x10^-8 M ANF
CONTROL
-25
-20
-15
-10
-5
0
5
10
15
20
1/7-ETHOXYRESORUFIN x10^-6 (molar^-1)

Figure 13.

Linweaver-Burke plot of induced
7-ethoxyresorufin deethylase activity

DB(a,c)A-induced AHH-1 cells were incubated with different concentrations of ER and the production of resorufin was monitored by fluorescence (open squares). The addition of 1×10^{-7} M (open circles) and 1×10^{-6} M (closed circles) ANF to the incubation mixture resulted in inhibition of the deethylase activity. ANF was a linear-mixed type of inhibitor (predominantly non-competitive).

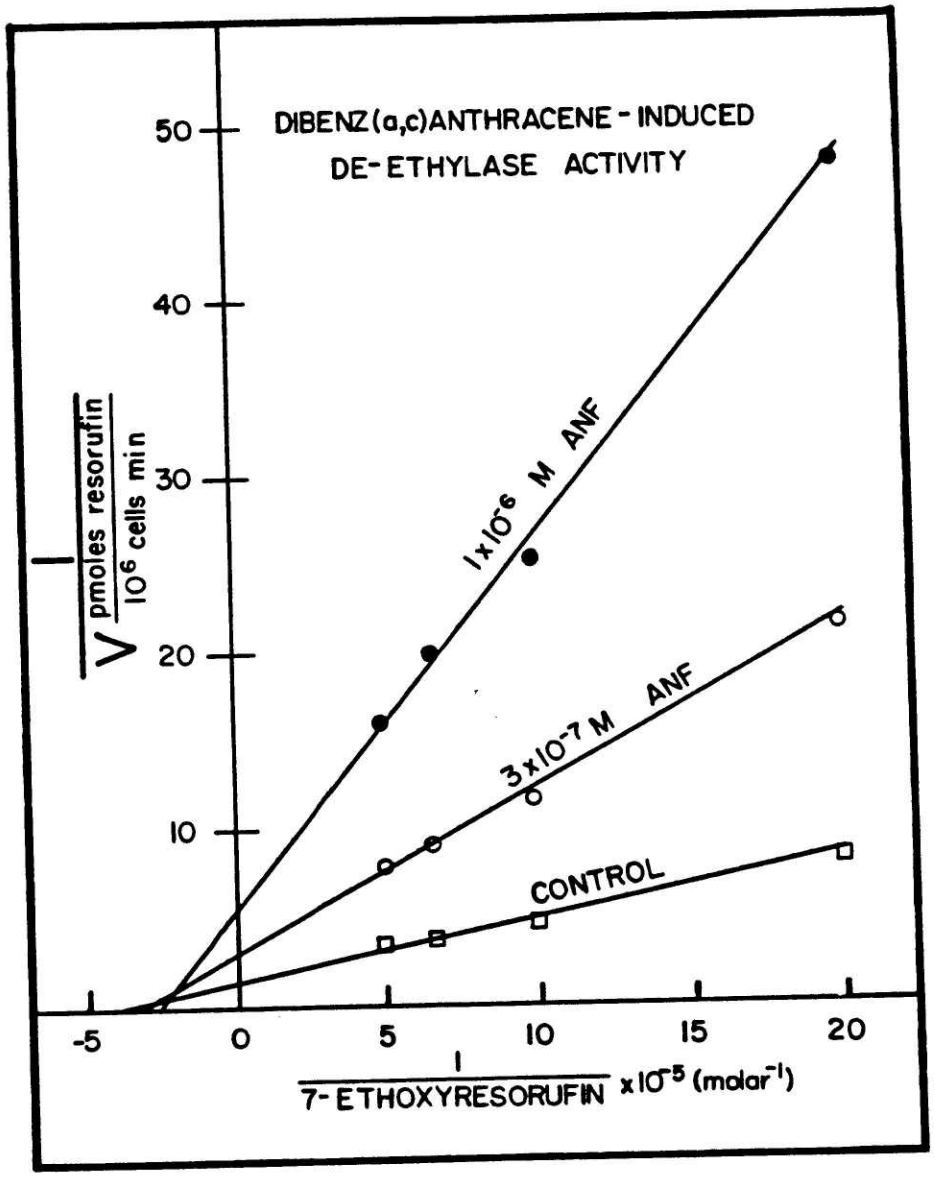

DIBENZ(a,c)ANTHRACENE - INDUCED
DE-ETHYLASE ACTIVITY
1/V pmoles resorufin / 10^6 cells min
50
40
30
20
10
1x10^-6 M ANF
3x10^-7 M ANF
CONTROL
-5
0
5
10
15
20
1/7-ETHOXYRESORUFIN x10^-5 (molar^-1)

Figure 14.

Lineweaver-Burke plot of uninduced

benzo(a)pyrene hydroxylase activity

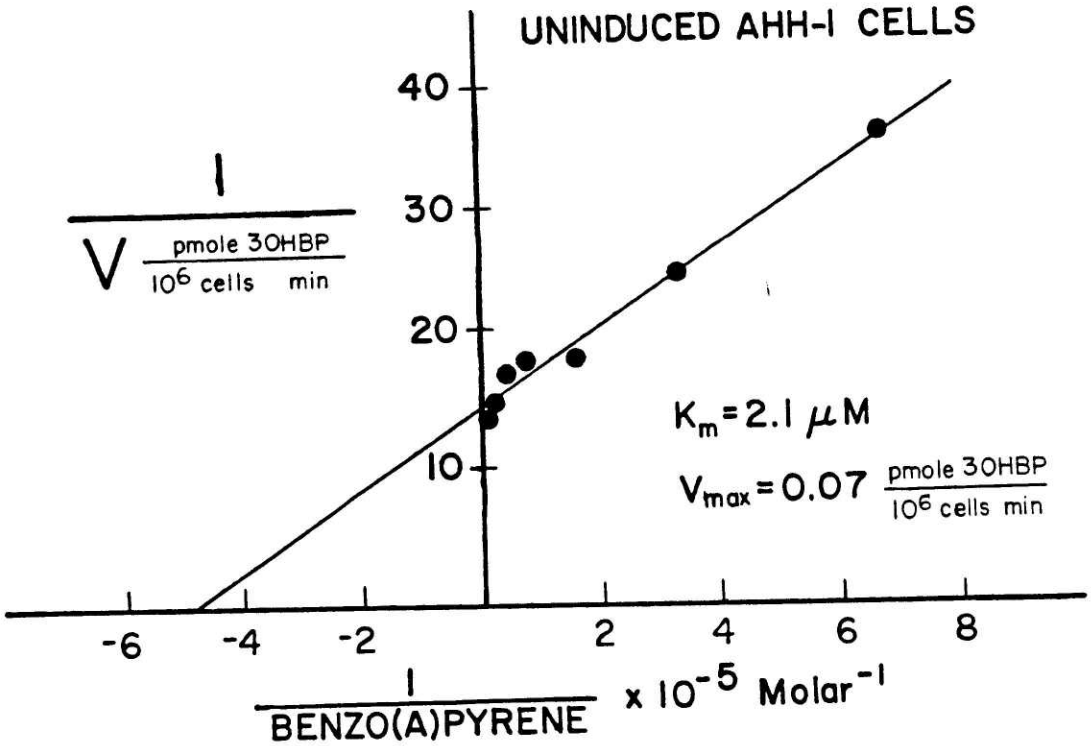

UNINDUCED AHH-1 CELLS
40
30
20
10
$\frac{1}{V \frac{\text{pmole 3OHBP}}{10^6 \text{ cells min}}}$
$K_m = 2.1\ \mu M$
$V_{max} = 0.07 \frac{\text{pmole 3OHBP}}{10^6 \text{ cells min}}$
-6
-4
-2
2
4
6
8
$\frac{1}{\text{BENZO(A)PYRENE}}$ x 10^{-5} Molar^{-1}

Figure 15.

Lineweaver-Burke plot of induced

benzo(a)pyrene hydroxylase activity

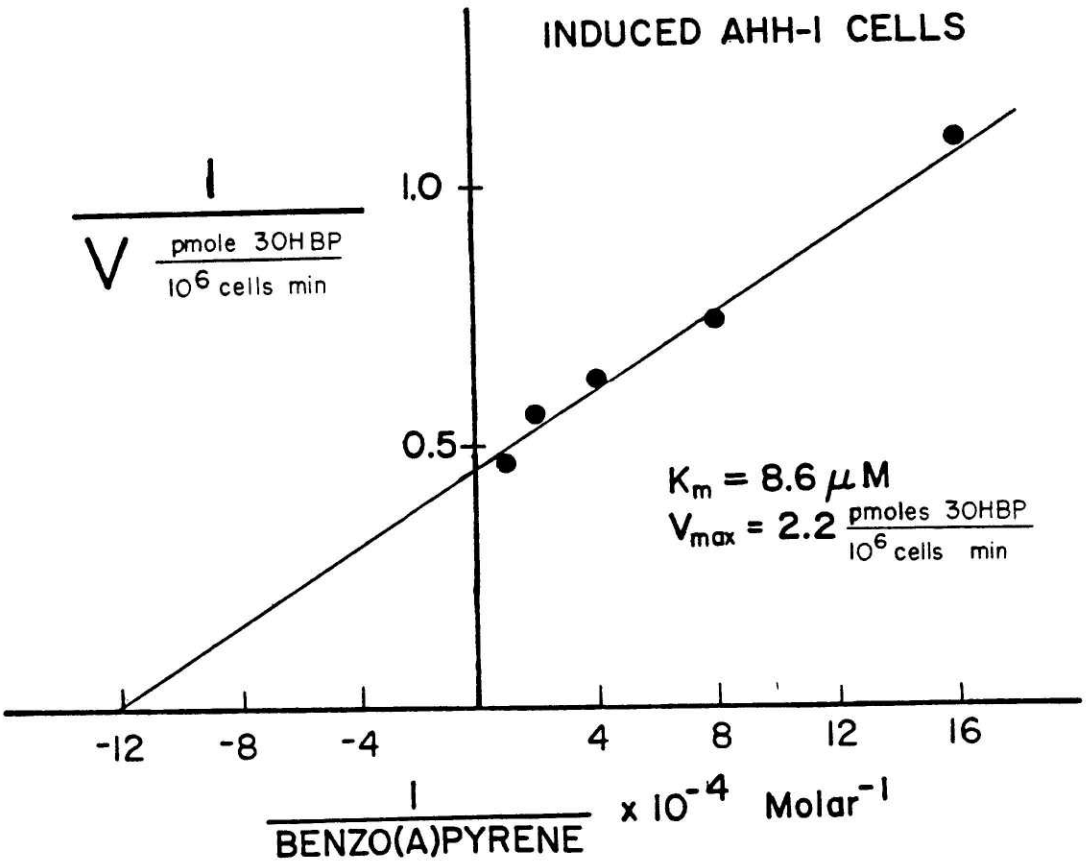
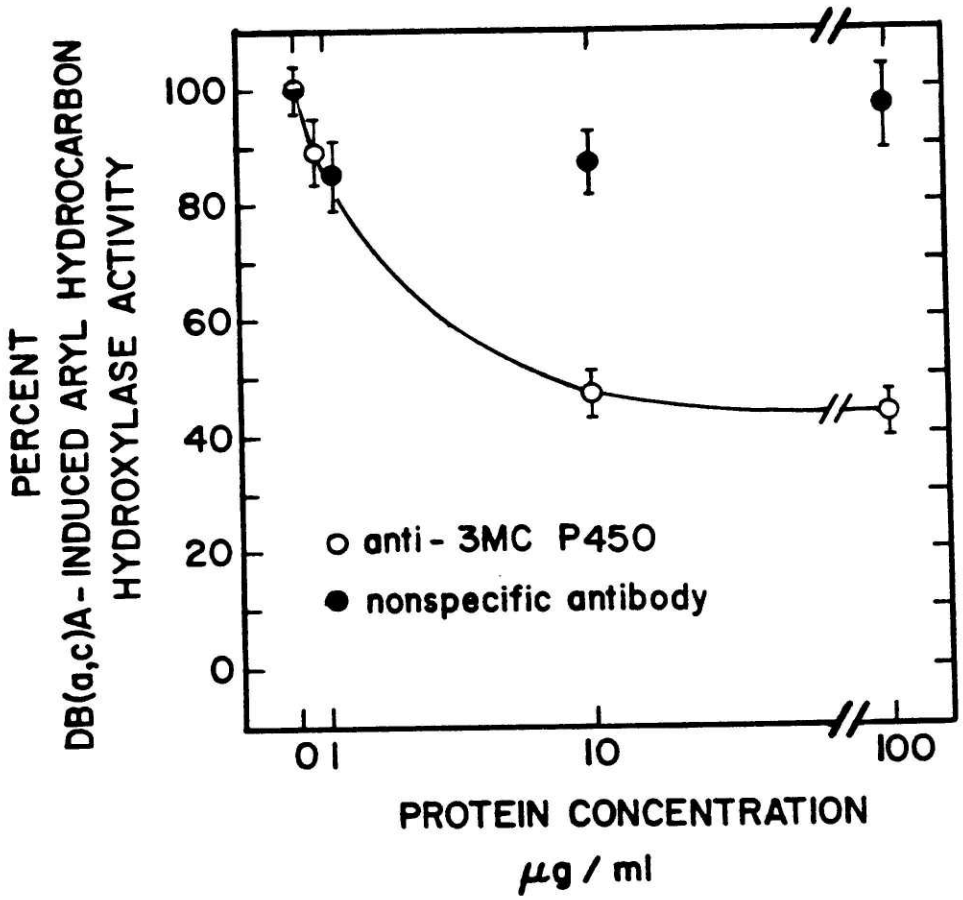

INDUCED AHH-1 CELLS
1
V pmole 3OHBP / 10^6 cells min
1.0
0.5
$K_m = 8.6\ \mu M$
$V_{max} = 2.2$ pmoles 3OHBP / 10^6 cells min
-12
-8
-4
4
8
12
16
1 / BENZO(A)PYRENE x 10^{-4} Molar^{-1}

Figure 16.

Inhibition of induced benzo(a)pyrene hydroxylase activity by monoclonal antibody against 3MC induced rat liver cytochrome P450

DB(a,c)A-induced benzo(a)pyrene hydroxylase activity was inhibited by anti-3MC induced rat liver cytochrome P450 monoclonal antibody (open symbols). The maximum inhibiton was 55%. The hydroxylase activity was not inhibited by a nonspecific monoclonal antibody (closed symbols).


PERCENT
DB(a,c)A-INDUCED ARYL HYDROCARBON
HYDROXYLASE ACTIVITY
100
80
60
40
20
0
0 1
10
100
o anti-3MC P450
● nonspecific antibody
PROTEIN CONCENTRATION
μg / ml

5. Characterization of AHH-1 cellular epoxide hydrolase activity.

The cellular epoxide hydrolase activity was also examined. In these studies BP-4,5-oxide was used as a substrate. BP-4,5-oxide is an excellent substrate for the microsomal epoxide hydrolase in rodent liver (Leutz and Gelboin, 1975; Schassmann et al., 1976; Oesch and Golan, 1980) and human lymphocytes (Glatt et al., 1980). BP-4,5-oxide is an extremely poor substrate for the cytosolic epoxide hydrolase in rodent liver (Oesch and Golan, 1980; Ota and Hammock, 1980; Gill and Hammock, 1980). The cytosolic epoxide hydrolase activity has not been studied in human tissues (Kenji Ota, personal communication).

Benzo(a)pyrene metabolite studies have demonstrated that no detectable trans-dihydrodiol-BP metabolites were produced by AHH-1 cells. One interpretation was that epoxide hydrolase activity was not a important metabolic pathway for BP. The level of cellular epoxide hydrolase activity was measured using BP-4,5-oxide as a substrate.

AHH-1 cells did not have detectable epoxide hydrolase activity with BP-4,5-oxide as a substrate (table 11). The level of cellular activity was measured at pH 9.2 (the optimum for the microsomal epoxide hydrolase (Glatt et al., 1980)) and measured at pH 7.4 (the optimum for the cytosolic epoxide hydrolase (Gill and Hammock, 1980)). The AHH-1 cell epoxide hydrolase activity was less than 0.006 pmole per 10^6 cells per minute at pH 9.2 and less than 0.02 pmole per 10^6 cells per minute at pH 7.4. The presence of a cytosolic epoxide hydrolase activity in AHH-1 cells has not been determined.

AHH-1 cell epoxide hydrolase activity was much lower than that observed in isolated human lymphocytes (a reported range of 2.0 to 7.2

TABLE 11.

Epoxide hydrolase measurements.

Epoxide hydrolase activity was measured using BP-4,5-oxide as a substrate.

Conditions	Observed Counts Per Minute[a]	Activity[b]
Boiled cells pH 9.2	2530 ± 140 (n=5)	
Cells pH 9.2	2250 ± 180 (n=5)	(0.006)
Microsomes pH 9.2 (0.27 mg protein)	79700 ±5400 (n=4)	52 ± 4
Boiled cells pH 7.4	4200 ± 440 (n=5)	
Cells pH 7.4	3600 ± 500 (n=5)	(0.02)
Microsomes pH 7.4 (0.27 mg protein)	79500 ±11000 (n=3)	51 ± 7

[a] - expressed as mean ± standard error of the mean.

[b] - expressed as the observed activity in pmole per mg protein per minute (microsome positive control incubations) or the lowest activity in pmole per 10^6 cells per minute which could have been detected with 95% confidence (cellular incubations).

pmole per minute per mg protein (2 x 10^6 cells per mg protein) using the same protocol) (Glatt et al., 1980).

6. Characterization of AHH-1 cellular conjugating ability.

Two other xenobiotic metabolizing activities were examined by "indirect measurement". In particular, the extent of beta-glucuronidation and sulfation of BP phenolic metabolites produced by DB (a,c)A- induced AHH-1 cells was measured by de-conjugation and fluorescence measurement. Beta-glucuronide conjugates were de-conjugated by treatment with 10,000 Fishman units of beta-glucuronidase and sulfate conjugates were de-conjugated by treatment with 10 units sulfatase. Subsequent extraction and measurement of fluorescence revealed that a 12% increase in fluorescence was observed after beta-glucuronidase treatment. This increase was statistically significant ($p < 0.05$). No change in fluorescence was observed after sulfatase treatment (table 12).

These measures indicate that benzo(a)pyrene phenols are not extensively conjugated (sulfate or beta-glucuronide) by AHH-1 cells. The extent of conjugation of other BP metabolites was not measured.

TABLE 12.

Conjugation of benzo(a)pyrene phenols.

BP phenols produced by dibenz(a,c)anthracene-induced AHH-1 cells. Cells were incubated with BP for 30 minutes. The pH was then adjusted to 4.5 and enzyme added (experimental points) or buffer only (control points). Samples were incubated at 37 C for 10 additional minutes, extracted and fluorescence measured.

Conditions	Percent Fluorescence*
control	100 ±4.5
10,000 fishman units beta-glucuronidase	112 ±7.1
10 units sulfatase and 10 mM d-saccharic acid 1,4-lactone	98 ±15

* expressed as mean ± standard deviation.

7. Mutation and metabolism studies with benzo(a)pyrene as a model compound.

The metabolism, DNA binding, and mutagenicity of the model compound BP were studied in detail. For these experiments a novel set of mutagen treatment conditions were used. The constraints on these conditions were, one, the cellular MFO activity must be stable (to decrease variability); two, the cells must be treated with BP at high cell density (to conserve expensive radiolabeled compounds and to facilitate biochemical measurements); three, physiological cell culture conditions must be maintained (to ensure cell viability); and four, a statistically significant induction of gene-locus mutation must be observed (the treatment has some toxicological significance). All four criteria were satisfied using the following conditions. Beta-naphthoflavone or DB(a,c)A induced AHH-1 cells were given a brief treatment (1.5 to 3 hr) with BP at high cell density (7 to 9 x 10^6 cells per ml) in cell culture medium (pH 7.5) with cofactors.

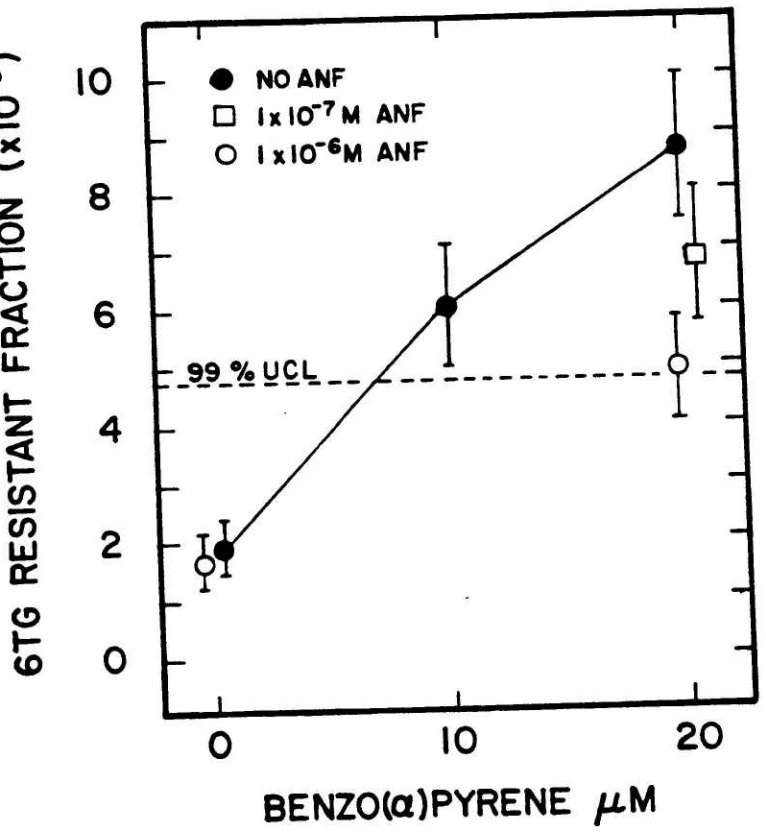
a. Mutagenicity of benzo(a)pyrene.

BP is mutagenic under these conditions (figure 17). Both BP concentrations tested, 10 uM and 20 uM, induced statistically significant (compared to the internal control and historical control distribution) increases in 6-thioguanine-resistant fraction. The induction of 6-thioguanine resistance by 20 uM BP could be inhibited by the addition of 1 x 10^{-7} M and 1 x 10^{-6} M alpha-naphthoflavone. This inhibiton was expected given the effects of ANF on AHH activity and ER deethylase activity. This mutation inhibition observation was not characterized

Figure 17.

Mutagenicity of benzo(a)pyrene to AHH-1 cells.

AHH-1 cells were induced by BNF pretreatment (10 uM for 24 hours). The cells washed and exposed to BP for 1.5 hours. ANF, and inhibitor of mixed function oxigenase activity, was added to the culture in DMSO. Points are the mean of two independent experiments performed in duplicate. Confidence limits are one standard deviation.


6TG RESISTANT FRACTION (x10⁻⁶)
10
8
6
4
2
0
NO ANF
1x10⁻⁷M ANF
1x10⁻⁶M ANF
99 % UCL
0
10
20
BENZO(α)PYRENE μM

further because the nature of the BP-DNA adducts were not known. Upon a more complete characterization of the BP-DNA adduct species, it may be worthwhile to characterize the effect of ANF on not only mutagenicity, but also the relative amount of individual DNA adducts. Is the formation of all adducts inhibited equally? From these studies an increased understanding of which adducts may be the most efficient in inducing gene-locus mutations may be gleaned. A more detailed discussion of these proposed experiments is presented in the future work section of this thesis.

b. Benzo(a)pyrene metabolism.

The production of fluorescent phenolic metabolites was monitored concurrently with the measurement of gene-locus mutation. The phenol production was linearly related to the induced mutation (figure 18). It is surprising that this crude approximation of cellular metabolism showed such a strong correlation to a complex endpoint such as gene-locus mutation, especially since the BP-DNA profile separated by HPLC was highly complex (figure 19). The cellular metabolites were examined in more detail by HPLC separation. Phenolic metabolites were the major products of oxidation (figure 20). When these phenols were further resolved (figure 21), three components were observed. The major component co-chromatographed with the 9-hydroxy-BP, the second component is as yet unidentified but may be the 7-hydroxy-BP, and the final component (the small hump in the trailing edge of the second peak) co-chromatographs with the 3-hydroxy-BP. The second most abundant BP metabolite co-chromatographs with the cis-4,5-dihydrodiol-BP. This metabolite may have resulted from the spontaneous hydrolysis of BP-4,5-oxide. This

Figure 18.

Benzo(a)pyrene phenol production during mutagenicity assay.

Benzo(a)pyrene phenol production during BP treatment was measured as described in methodology section. Points are the mean of two independent experiments performed in duplicate. Confidence limits one standard deviation.

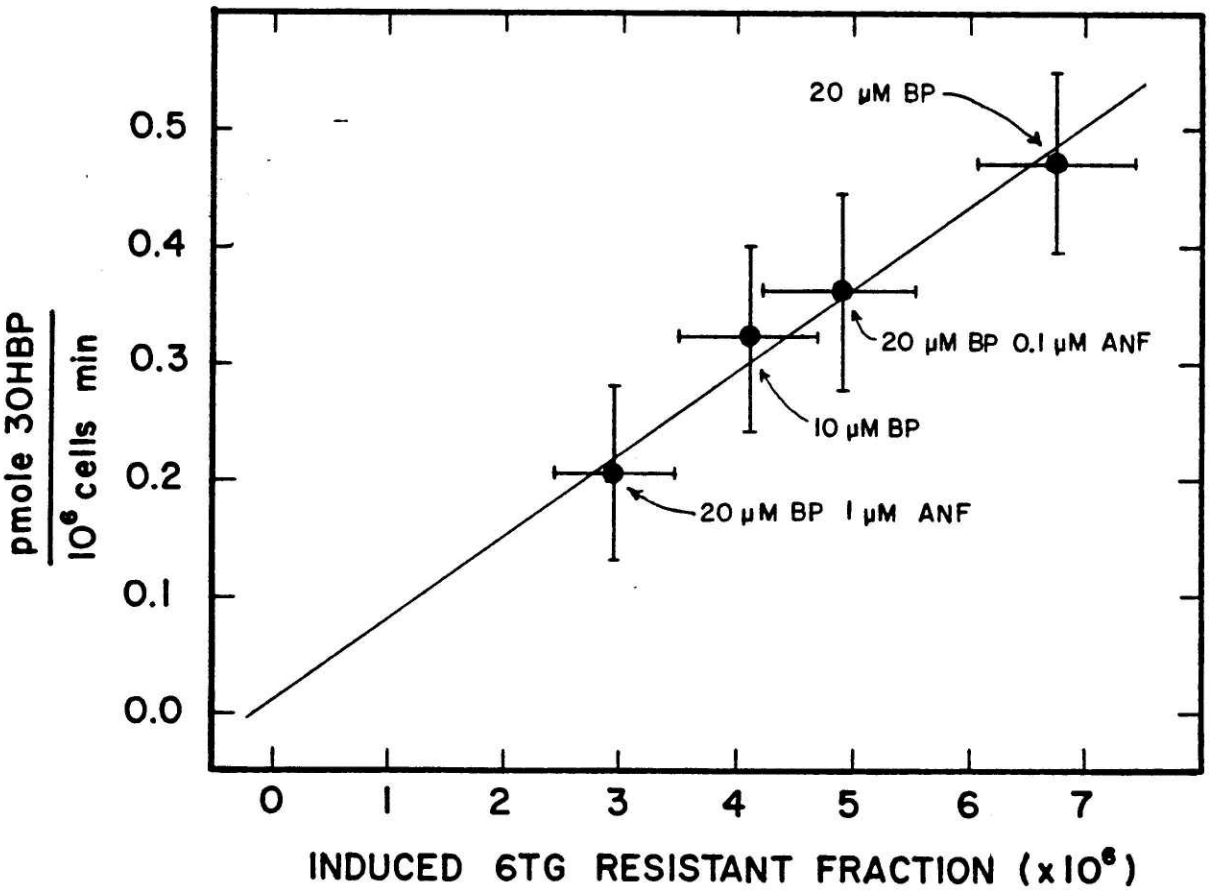

20 μM BP
20 μM BP 0.1 μM ANF
10 μM BP
20 μM BP 1 μM ANF
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
pmole 30HBP / 10⁶ cells min
INDUCED 6TG RESISTANT FRACTION (x10⁶)

Figure 19.

Benzo(a)pyrene-DNA adduct profile.

AHH-1 cells were treated with ^{3}H-BP, cellular DNA isolated, hydrolysed, and the deoxyribonucleoside-BP adducts separated by HPLC. Chromatography solvent conditions, 45% methanol for 45 minutes, followed by a linear gradient for 45 to 60% methanol over 60 minutes, then held at 60% methanol. Fractions collected every 0.5 minutes. Note: flag marker at 80 minutes has been added for comparison purposes. The chromatography conditions for analysis of BP-4,5-oxide adducts were different, higher initial methanol concentrations were used. Therefore the bulk of the adducts eluted rapidly (figure 23). A flag has also been added to this figure at the same relative place in the chromatogram.

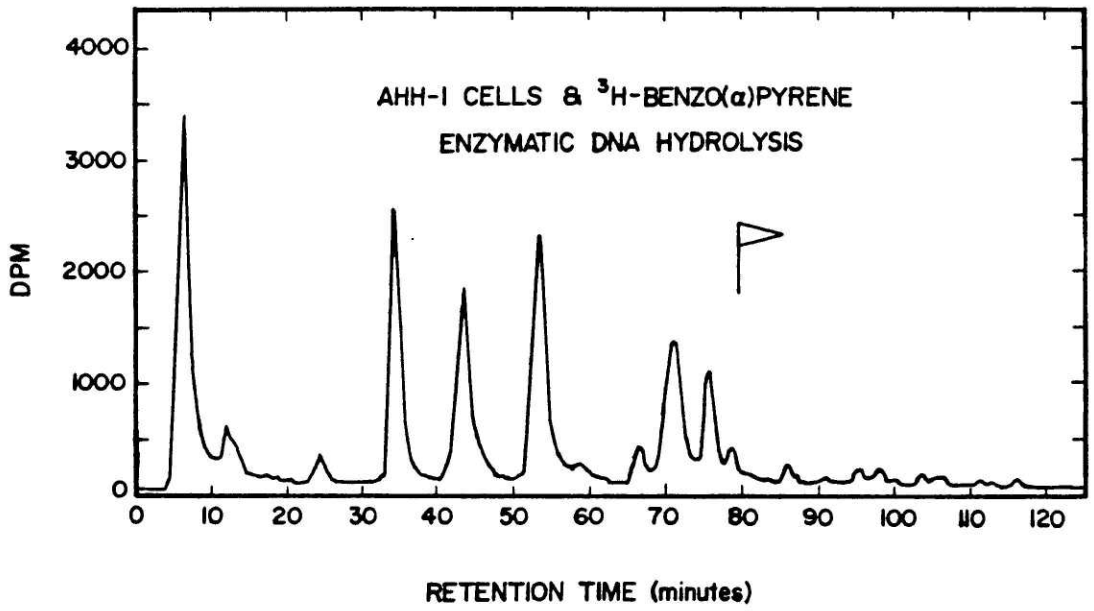

AHH-1 CELLS & ^{3}H-BENZO(α)PYRENE
ENZYMATIC DNA HYDROLYSIS
DPM
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
90
100
110
120
RETENTION TIME (minutes)

Figure 20.

Benzo(a)pyrene metabolites produced by induced AHH-1 cells.

AHH-1 cell benzo(a)pyrene metabolites were produced and analyzed as described in methodology section. The predominant metabolites produced were benzo(a)pyrene phenols. (Chromatography solvent conditions, linear methanol gradient, 1% per minute initially 60% methanol. Fractions collected every minute.

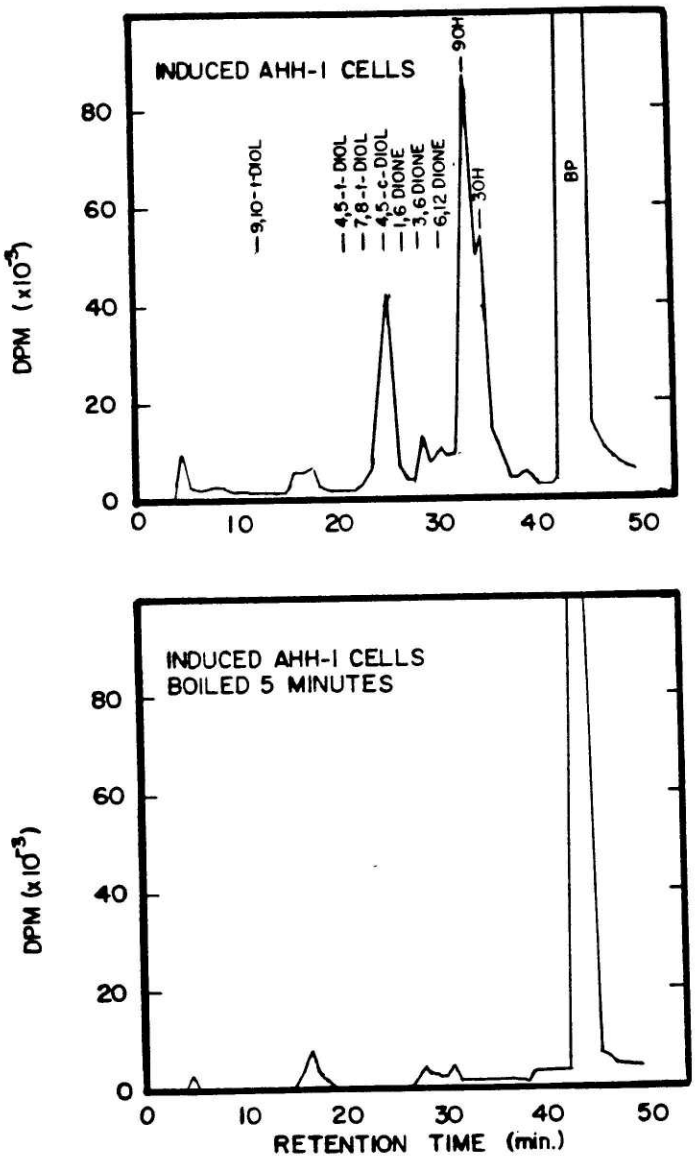
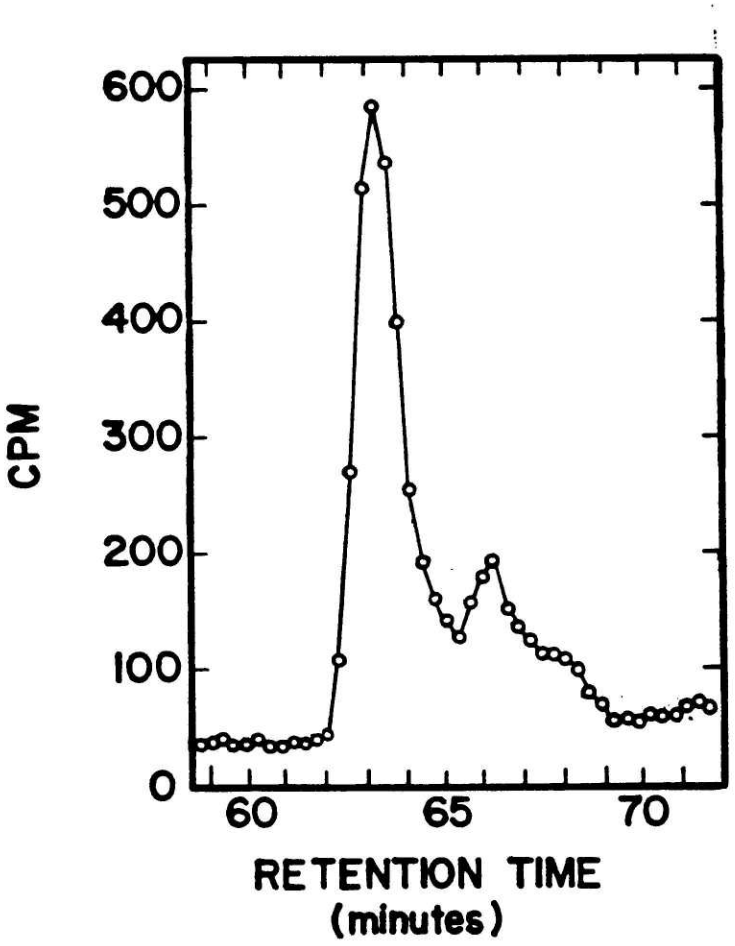

INDUCED AHH-1 CELLS
9,10-1-DIOL
4,5-t-DIOL
7,8-t-DIOL
4,5-c-DIOL
1,6 DIONE
3,6 DIONE
6,12 DIONE
9OH
3OH
BP
DPM (x10⁻³)
0
20
40
60
80
0
10
20
30
40
50
INDUCED AHH-1 CELLS
BOILED 5 MINUTES
DPM (x10⁻³)
RETENTION TIME (min.)

Figure 21.

Separation of benzo(a)pyrene phenols.

Benzo(a)pyrene phenols were produced as in figure 20. Chromatography solvent conditions, linear methanol gradient, 0.25% per minute, initially 60% methanol. Fractions collected every 0.3 minutes starting 59.6 minutes into the run.


600
500
400
300
200
100
0
CPM
60
65
70
RETENTION TIME
(minutes)

hydrolysis should yield both the cis and trans dihydrodiol isomers, therefore it is unclear why the cis isomer was observed and not the trans. The cis-4,5-dihydrodiol BP metabolite observed in cellular incubations chromatographed 2 minutes before the earliest quinone standard. It is possible that an unknown quinoe metabolite could have the came retention characteristics as the cis-4,5-dihydrodiol BP. Spectra data for this metabolite has been elusive. A small amount of quinones were produced, these were not characterized further. Two minor identified metabolites were observed, an early eluting peak (6 minute retention time) and a peak which chromatographed between the trans-9,10-dihydrodiol-BP and trans-4,5-dihydrodiol-BP standards (18 minute retention time). No trans-dihydrodiol metabolites were observed. This BP metabolite profile, phenols and quinones produced but no trans-dihydrodiols, resembles that observed in isolated human lymphocytes (Selkirk et al., 1975). (Selkirk also reports several metabolites in the region of the chromatogram contaning dihydrodiols. These metabolites did not co-chromatograph with the trans- 9,10-, 7,8-, or 4,5-dihydrodiol-BP standards. One could speculate that these metabolites were cis-dihydrodiols.)

BP metabolism in AHH-1 cells resembles that reported for both RPMI-1788 cells and human lymphocytes. RPMI-1788 cells produced predominantly phenols and quinones, no trans-dihydrodiol BP metabolites were observed (Freedman et al., 1979a). In addition, an unidentified peak was observed eluting between the quinones and trans-dihydrodiol BP standards. This peak could be cis-4,5-dihydrodiol BP. Human lymphocytes produce BP phenols, quinones, and a limited array of trans-dihydrodiols (Selkirk et al., 1975; Vaught et al., 1978).

Based on the observed AHH-1 cell BP metabolite profile several

hypotheses as to the BP-DNA adducts were put forward. The trans-7,8-dihydrodiol-BP was not observed, therefore the trans-7,8-diol-9,10-epoxide-BP metabolites or bay region metabolites which have been reported to be the major pathways to DNA binding in vivo were unlikely. Although a cis-7,8-diol-9,10-epoxide BP metabolite can not be ruled out with the metabolite information (in particular the unknown peak in the dihydrodiol region). The cis-7,8-dihydrodiol-9,10-epoxide BP has been reported to bind to DNA in microsomal incubations (Pelkonen et al., 1978), but has not been reported in biological incubations with BP. The fact that they have not been identified does not mean they were not present because under some chromatography conditons, such as LH20 chromatography, cis-diolepoxide BP-DNA adducts and trans-diolepoxide BP-DNA adducts co-migrate (Pelkonen et al., 1978). The cellular metabolite profile does implicate K-region metabolism. The cis-4,5-dihydrodiol BP was a metabolite implicating the presence of BP-4,5-oxide. BP-4,5-oxide can bind to DNA and adducts have been reported in incubations with liver microsomes and in mouse and rat skin in vivo (Baer-Dubowska, et al., 1981). The 9-hydroxy-BP metabolite was a major metabolite, 9-hydroxy-BP can be further metabolized in the K-region forming 9-hydroxy-4,5-oxide-BP, this metabolite binds to DNA and is the major metabolite in incubations of liver microsomes (King et al., 1976) and also has been reported to be present in vivo (Baer-Dubowska et al., 1981). The presence or absense of individual DNA binding metabolites was investigated by examining the BP-DNA adduct profile in AHH-1 cells and comparing that addduct profile to the adduct profile generated by the reaction of synthetic BP metabolite standards with DNA.

c. Benzo(a)pyrene-DNA adduct analysis.

The hypothesized pathways of BP metabolism to DNA binding intermediates in AHH-1 cells have been tested. The K-region pathways to DNA binding were observed, the bay region pathways to trans-7,8-diol-9,10-epoxide-BP were not observed. The criteria for declaring whether a specific metabolite was bound to cellular DNA was co-chromatography of the cellular adduct(s) with the adduct(s) formed when the metabolite standard was reacted with calf thymus DNA. Chromatography was carried out using an Ultrasphere ODS column which has been reported to be superior for the separation of BP-DNA adducts (Panthananickal and Marnett, 1981).

The cellular BP-DNA adduct profile was complex, 10 major and 10 minor peaks were resolved by HPLC. The overall binding level after treatment with 20 uM BP for 1.5 hours (or 10uM BP for 3 hours) was 2 adducts per 10^7 basepairs. This treatment induced a mutant fraction of 7×10^{-6}. If 100% adduct recovery and a target size for 6-thioguanine resistance of 1000 basepairs are assumed, approximately 28 adducts were present on the DNA per mutation. The overall recovery of DNA adducts was not determined (the recovery during the HPLC analysis was 80+%). In addition, BP metabolites can produce labile N^7-guanine adducts which would not be recovered using this DNA isolation procedure (Osborne et al., 1978). The contribution of these labile adducts to the overall BP-DNA binding in AHH-1 cells or any cellular system is unknown.

The cellular DNA adduct peak at a 25 minute retention time co-chromatographed with the DNA adduct produced from the incubation of ^{3}H-9-hydroxy-BP with microsomes and DNA (figure 22). This peak accounted for 2% of the total DNA adducts (table 13).

The minor adduct peaks present at retention times greater than 80 minutes appear to be produced by BP-4,5-oxide. The cellular BP-DNA adducts and the highly complex adduct profile produced from the reaction of BP-4,5-oxide with calf thymus DNA co-chromatograph (figure 23). This series of peaks accounted for 7% of the total DNA binding (table 13).

The early eluting (6 minute retention time) tritium peak (18.5% of the total counts bound to DNA) elutes with the unmodified deoxyribonucleosides. The identity of this peak was not investigated. This peak may represent tritium exchange from the BP into DNA and/or BP adducts attached to incompletely hydrolyzed portions of DNA.

Finally, the two major metabolites which have been reported to bind to rodent liver DNA in vivo, the syn- and anti-(+)trans-7,8-diol-9,10-epoxide- 7,8,9,10-tetrahydro BP do not appear to be binding to DNA in AHH-1 cells (figures 24 and 25 respectively). As a control, BP tetrols were produced by incubation of the anti- diol epoxide BP in water at pH 5. These tetrols did not cochromatograph with any cellular or standard metabolite DNA adduct peaks (a retention time of 38 minutes under the conditions described in figure 25).

d. Summary of benzo(a)pyrene biotransformation studies.

These investigations of AHH-1 cell mediated benzo(a)pyrene biotransformation are summarized in two flow diagrams. Under the incubation conditions described earlier, 0.4 to 0.9% of the initial BP was metabolized, 0.001 to 0.003% of the initial BP bound to DNA (figure 26). This low total BP metabolism (less than 1%) is a strong argument for isolating variants with higher xenobiotic metabolizing capacity - biochemical measurements would be much easier. Approaches to the

Figure 22.

Chromatography of AHH-1 cell DNA-^{3}H-BP adducts and 9-hydroxy-BP adducts.

DNA adducts were produced as described in methodology section. Chromatography solvent conditions, isocratic 45% methanol. Fractions collected every 0.5 minutes (cellular adduct) and every 1 minute (9-hydroxy-BP adduct).

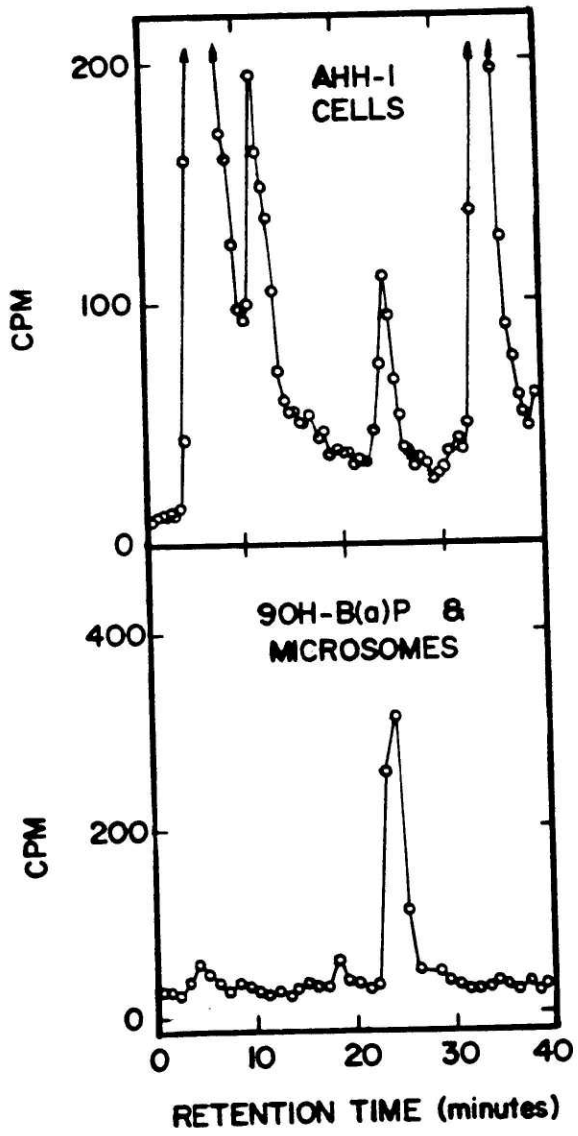

AHH-1
CELLS
200
100
0
CPM
9OH-B(a)P &
MICROSOMES
400
200
0
CPM
0
10
20
30
40
RETENTION TIME (minutes)

Figure 23.

Chromatography of AHH-1 cell DNA-^{3}H-BP adducts and DNA BP-4,5-oxide adducts.

DNA adducts were produced as described in methodology section. Chromatography solvent conditions, 60% methanol isocratic for 20 minutes, followed by a linear methanol gradient 0.2% per minute. Fractions collected every minute. Under these conditions the major cellular adduct peaks eluted rapidly and were resolved into only two peaks. Note: marker flag has been added for comparison purposes with the cellular adduct profile in figure 19. In this run higher initial methanol concentrations were used. The flag is located in the same relative place in the adduct profile in both figures.

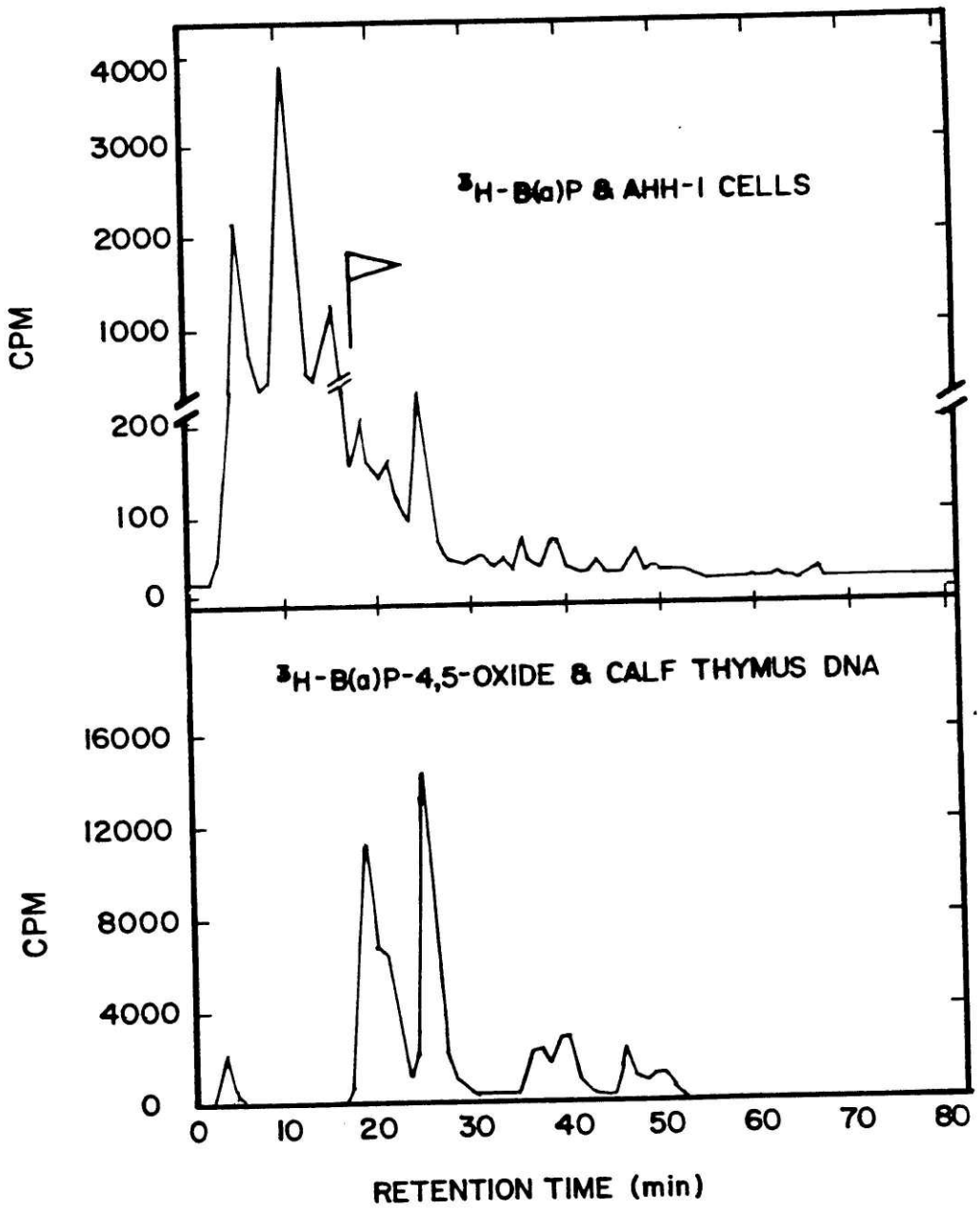

4000
3000
2000
1000
200
100
0
CPM
^{3}H-B(a)P & AHH-1 CELLS
^{3}H-B(a)P-4,5-OXIDE & CALF THYMUS DNA
16000
12000
8000
4000
0
CPM
0
10
20
30
40
50
60
70
80
RETENTION TIME (min)

Figure 24.

Chromatography of AHH-1 cell DNA-^{3}H-BP adducts and ^{14}C *syn*-(+)-trans-7,8-diol-9,10-epoxide-7,8,9,10-tetrahydro-BP DNA adducts.

DNA addducts were produced as described in methodology section. ^{3}H-BP cellular adducts and ^{14}C *syn*-diolepoxide-BP adducts were co-injected. Chromatography solvent conditions, 45% methanol for 45 minutes, followed by a linear gradient to 60% methanol over 60 minutes. Fractions collected every 0.5 minutes.

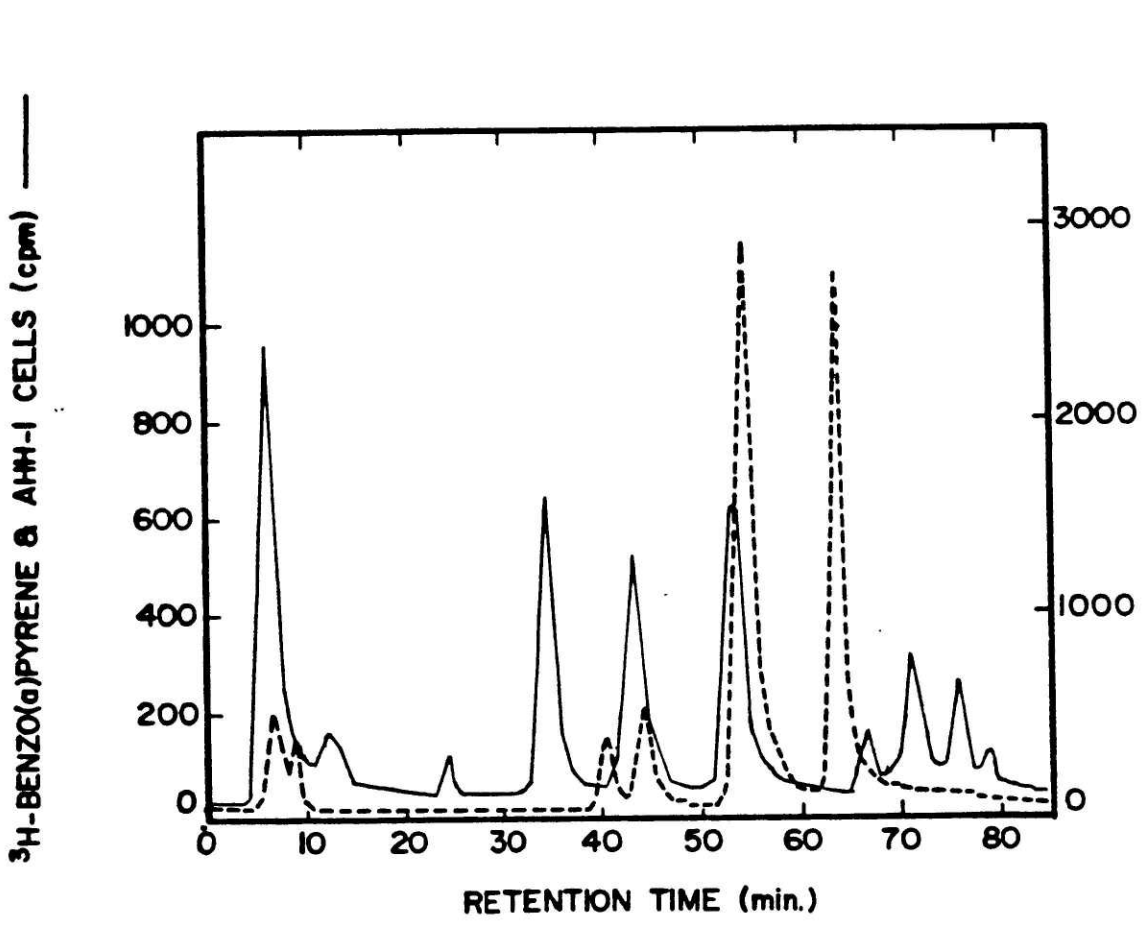

^{3}H-BENZO(a)PYRENE & AHH-1 CELLS (cpm) ——
^{14}C-sym-DIOL EPOXIDE-BENZO(a)PYRENE & DNA (cpm) - - - -
0
200
400
600
800
1000
0
1000
2000
3000
0
10
20
30
40
50
60
70
80
RETENTION TIME (min.)

Figure 25.

Chromatography of AHH-1 cell DNA-^{3}H-BP adducts and ^{3}H anti-(+)-trans-7,8-diol-9,10-epoxide-7,8,9,10-tetrahydro-BP DNA adducts

DNA addducts were produced as described in methodology section. Chromatography solvent conditions, 45% methanol for 45 minutes, followed by a linear gradient to 60% methanol over 60 minutes. Fractions collected every minute. Note: chromatography was performed at a column temperature of 20 C, therefore slightly longer retention times were observed when compared to all other DNA adduct chromatography (which was performed at 24 to 27 C).

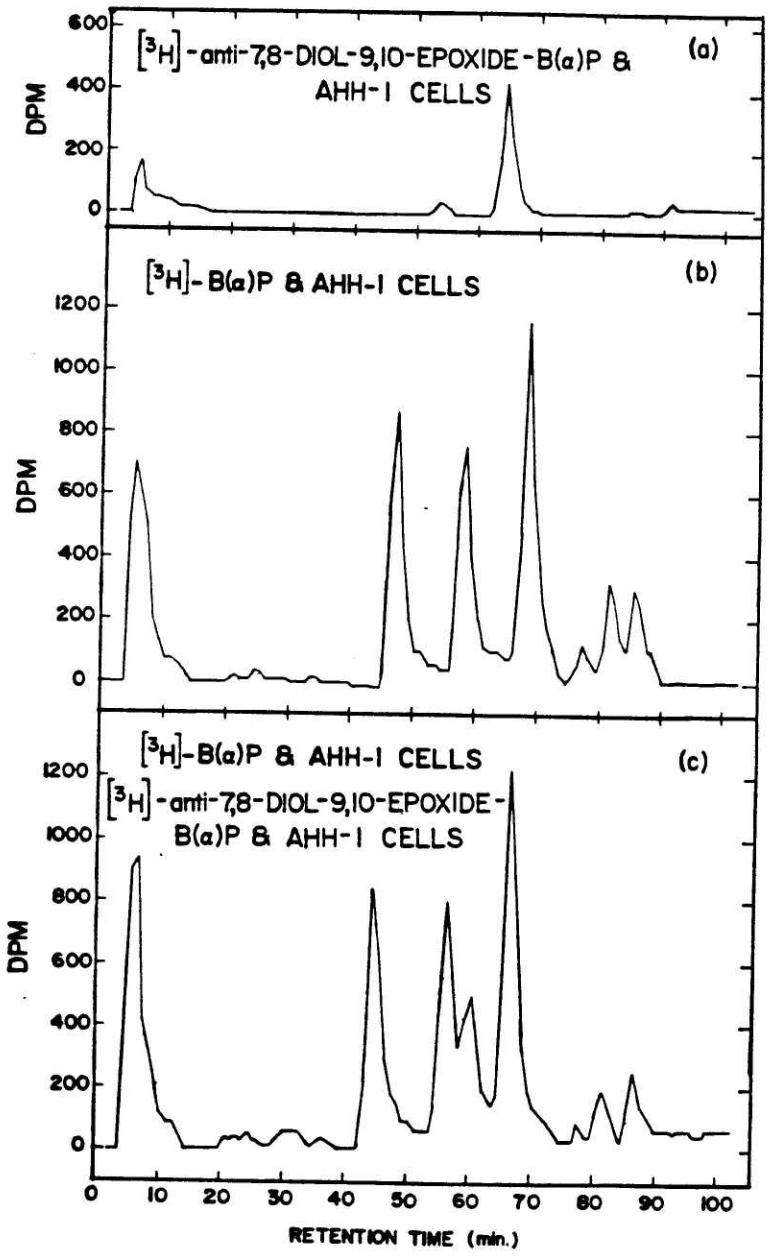

[3H]-anti-7,8-DIOL-9,10-EPOXIDE-B(α)P & AHH-1 CELLS
(a)
[3H]-B(α)P & AHH-1 CELLS
(b)
[3H]-B(α)P & AHH-1 CELLS
[3H]-anti-7,8-DIOL-9,10-EPOXIDE-B(α)P & AHH-1 CELLS
(c)
DPM
RETENTION TIME (min.)

Table 13.

Relative amounts of individual ^{3}H-benzo(a)pyrene

AHH-1 cell DNA adduct species.

Retention Time	Relative Amount (%)	BP Metabolite Bound
6 minutes	18.5	retention time of unmodified deoxyribonucleosides
12 minutes	4.6	
24.5 minutes	2.1	9-hydroxyBP + metabolism
34 minutes	12.9	
43.5 minutes	12.9	
53 minutes	17	
67 minutes	2.6	
71 minutes	10.8	
75.5 minutes	5.5	
78.5 minutes	2.1	
80 + minutes	7.1	BP-4,5-oxide
background*	3.9	
Total	100	

* background - cpm which were above background (liquid scintillation counter) but did not chromatograph as peaks.

isolation of variants with higher xenobiotic metabolizing capacity are outlined in the suggestions for future research section. The after treatment wth 20 uM BP for 1.5 hours the specific BP-DNA binding level was 2 adducts per 10^7 base pairs, or approximately 28 adducts per mutation (assuming 100% recovery of DNA adducts and a target of 1000 base pairs for 6-thioguanine resistance).

The production of specific cellular metabolites and the contribution of the two identified DNA binding intermediates to the total DNA binding is presented in figure 27. (Values reported in figures 26 and 27 are the observed range.) The majority of the BP metabolites (84 to 94%) have been identified. These metabolites result from oxidation of the parent compound. Cellular epoxide hydrolase activity does not appear to be an important pathway for benzo(a)pyrene metabolism, the spontaneous hydrolysis appears to be a faster process. The apparent low epoxide hydrolase activity present in AHH-1 cells may actually be a blessing. It may be possible to select variants of AHH-1 cells which are epoxide hydrolase proficient and therefore resistant to epoxide toxicity. These variants allow the study of the differences in metabolism, DNA binding, and mutagenicity between epoxide hydrolase deficient and proficient isogenic cell types. These experiments are outlined in the suggestions for future research section.

The majority of the BP-DNA adduct profile was uncharacterized. The K-region metabolites, BP-4,5-oxide and 9-hydroxy BP-4,5-oxide, were found bound to DNA. These two metabolites accounted for 9% of the total adducts. The syn- and anti- trans-7,8-diol-9,10-epoxide BP metabolites were not bound to cellular DNA. The BP-DNA adduct profile in cultured human lymphocytes has not been reported, therefore comparisons to cultured

human lymphocytes were not possible.

Figure 26.

Flow diagram of benzo(a)pyrene metabolism and DNA binding.

Flow diagram of the percentage of the total ^{3}H-BP which is metabolized and the percentage which binds to cellular DNA. Values represent the observed range (metabolism - 4 independent determinations, DNA binding - 6 independent determinations).

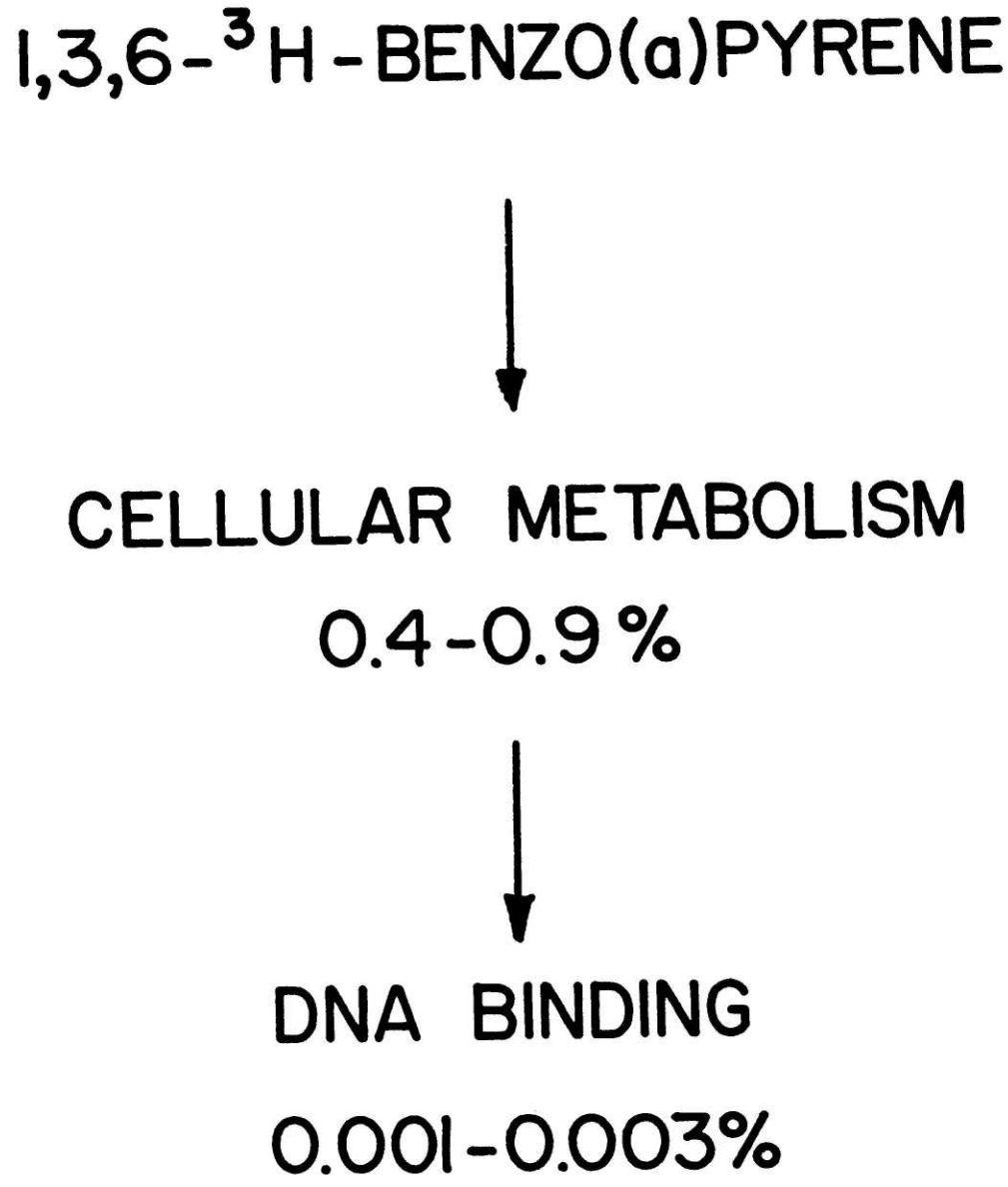
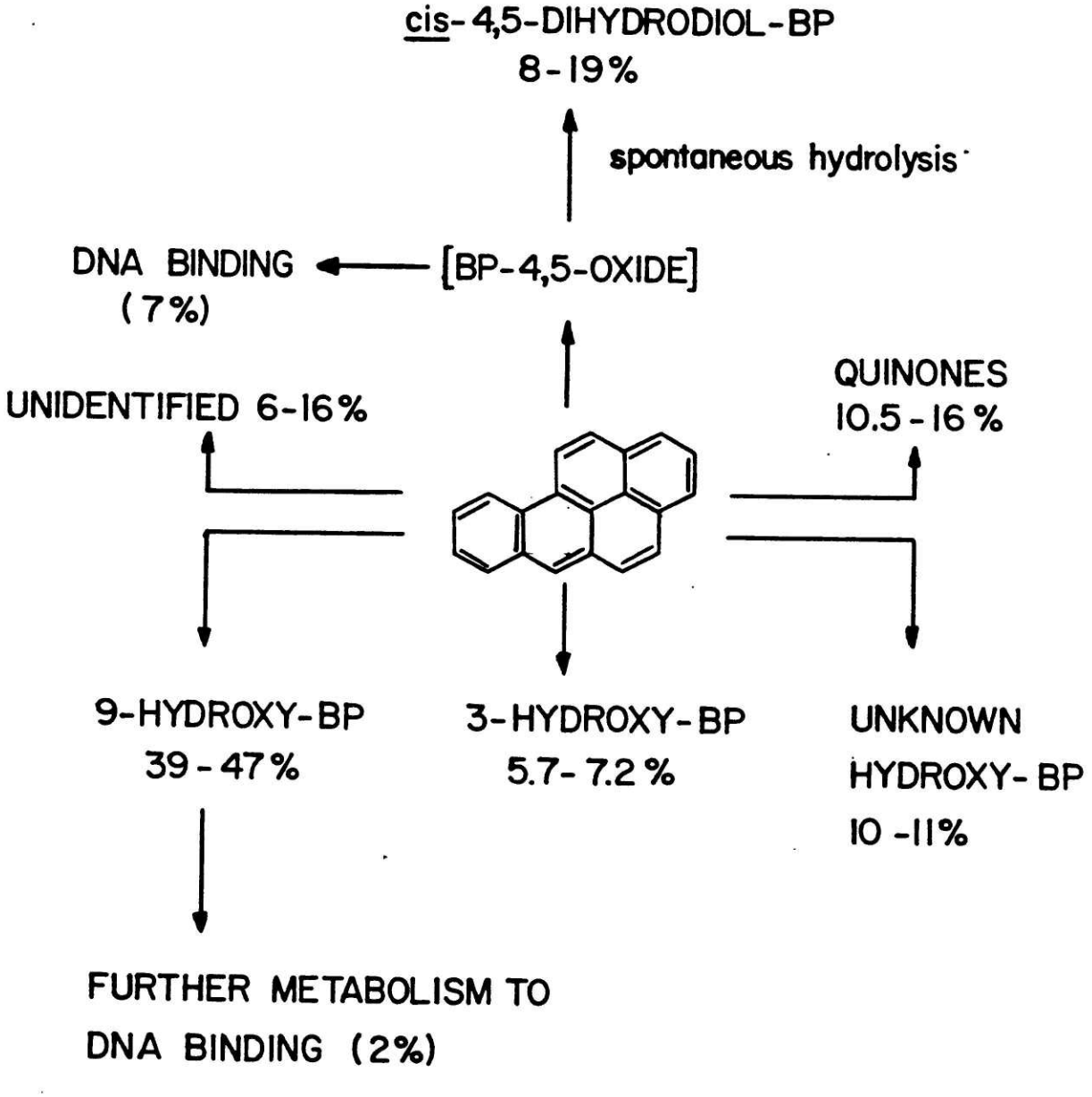

1,3,6-^{3}H-BENZO(a)PYRENE
CELLULAR METABOLISM
0.4-0.9%
DNA BINDING
0.001-0.003%

Figure 27.

Flow diagram of specific ^{3}H-benzo(a)pyrene metabolites.

The relative amount of specific ^{3}H-BP metabolites is diagrammed. Values represent the observed range (three independent determinations).


cis-4,5-DIHYDRODIOL-BP
8-19%
spontaneous hydrolysis
DNA BINDING
(7%)
[BP-4,5-OXIDE]
UNIDENTIFIED 6-16%
QUINONES
10.5-16%
9-HYDROXY-BP
39-47%
3-HYDROXY-BP
5.7-7.2%
UNKNOWN
HYDROXY-BP
10-11%
FURTHER METABOLISM TO
DNA BINDING (2%)

IV. Summary and conclusions

A gene-locus mutation assay utilizing human lymphoblasts competent for xenobiotic metabolism has been successfully developed. These human lymphoblasts were sensitive to induced mutagenesis by several structurally diverse xenobiotics. The feasibility of using this assay system as a general screen for chemical mutagenicity remains to be investigated. The inability to activate fluoranthene to a mutagen is disquieting and may signal a deficiency in the cellular metabolic capacity.

This assay system represents a useful model system for the study of human cell mediated xenobiotic metabolism and and the influence of these processes on gene-locus mutation. Greater understanding of these processes will be obtained through the future selection of variants of the parent cell line which contain altered xenobiotic metabolic capacity.

AHH-1 cells were found to be a relatively epoxide hydrolase deficient and mixed function oxygenase proficient cell. These characteristics were reflected in the benzo(a)pyrene metabolite profile and consistent with the partial characterization of the BP-DNA adduct profile.

Many aspects AHH-1 cell xenobiotic metabolism resembled human periphal lymphocyte xenobiotic metabolism. The observed kinetic parameters for AHH activity and ER deethylase activity, and the predominant metabolism of BP to phenols. Several aspects of benzo(a)pyrene biotransformation by this cell line were unique. In particular, trans-dihydrodiol BP metabolites were not observed while cis-dihydrodiol BP metabolites were observed. The uniqueness of the metabolite profile extends into the BP-DNA adduct profile. Two previously

reported BP-DNA adduct species were observed (9-hydroxy-4,5-oxide BP mediated and BP-4,5-oxide mediated DNA binding), but the trans-diolepoxide-BP mediated DNA adducts were not observed. Characterized adducts represent only 9% of the total bound material. This is the first reported observation of major BP-DNA adducts isolated from biological incubations which were not BP-4,5-oxide, 9-hydroxy-4,5-oxide BP, or syn- or anti- trans-diolepoxide BP mediated. The future characterization of the novel BP-DNA adducts will prove very interesting.

V. Suggestions for future work

AHH-1 cells represent a novel and useful system for the study of chemical-biological interactions. Because this system is novel, a host of future research possibilities are open. Three projects are outlined below. These projects have developed directly out of this research.

Probably the most important aspect of AHH-1 cells is that configuration of cellular xenobiotic metabolism and other cellular processes, critical targets, etc. is not disrupted. Therefore studies with this intact cell system may be more relevant to the analogous processes which occur *in vivo*. Because AHH-1 cells are of human origin, the relevant *in vivo* processes occur in people. AHH-1 cells do not represent all cell types present *in vivo*, but they may reflect some cells. This is, of course, preferred over systems which reflect *no* cells present *in vivo*. An example of the type of investigations which are possible is outlined below.

AHH-1 cells, in addition to being competent for xenobiotic metabolism, are phagocytic. These two characteristics can be used for studying the effects of xenobiotics present in particulates. AHH-1 cells can be exposed to soot particulates, a relevant route of soot exposure. The cells will phagocytize a portion of the particulates, metabolize xenobiotics which are eluted from the particulates, and thus be exposed to possible genetic hazard in this way. This type of investigation is impossible in cells which are not metabolically competent.

Outlined below are three projects which have been spawned directly from the results obtained in the course of this project. These projects

are; one, the isolation of variants in xenobiotic pathways; two, further characterization of BP-DNA adducts; and three, further characterization of ANF inhibition of BP mutagenicity.

A. Isolation of variants with altered xenobiotic metabolism.

The isolation of isogenic variants with altered xenobiotic metabolism will be a useful tool for estimating the influence of different xenobiotic metabolism pathways on mutagenicity. Several techniques will be suggested for the isolation of variants with either 1) greater MFO activity, 2) less MFO activity (with the possibility of eliminating specific isozymes), or 3) greater epoxide hydrolase activity.

All selection of variants should be from a AHH-1 cell population which has been enriched for mutants. ICR-191 is an useful mutagen for this purpose because ICR-191 induces a high mutant fraction with very little toxicity. Also, ICR-191 is not clastogenic, so variants are more likely to have a normal chromosome complement.

The basis scheme for variant selection will be either increased survival after a toxic treatment or treatment with a specific agent which causes the variants to be either more or less fluorescent. Variants can be selected with the former technique by enrichment with sequential toxic treatments, and selected with the later technique using a fluorescence activated cell sorter.

Variants with higher epoxide hydrolase activity can be selected via resistance to the toxic effects of epoxide. In particular, if a variant with higher microsomal epoxide hydrolase activity is desired, treatment with either styrene oxide or BP-4,5-oxide (two substrates for microsomal epoxide hydrolase) can be performed. Cells with more epoxide hydrolase

activity will have increased survival. This selective scheme may also select variants which are resistant to epoxides for other reasons, such as higher glutathione transferase activity, or perhaps inability to transport the epoxide across the cell membrane. These would be detected in the characterization and would also be useful and interesting variants.

Variants with higher MFO activity can be isolated by fluorescence activated cell sorting. A technique for the isolation of variants with greater BP metabolizing ability from a mouse hepatoma population has been reported (Miller and Whitlock, 1981). In this technique, the rapid loss of benzo(a)pyrene fluorescence was the basis for variant selection. Some work measuring 7-ethoxyresorufin (ER) deethylation via cytofluorometry has been performed. 7-Ethoxyresorufin is a weakly fluorescent neutral molecule and not retained by cells, it is deethylated by mixed function oxygenases to resorufin. At physiological pH, resorufin is highly fluorescent, has a net negative charge and is retained by the cell. Cells with higher deethylase activity will contain higher amounts of resorufin and therefore be more fluorescent. Preliminary studies comparing the fluorescence of uninduced and DB(a,c)A-induced AHH-1 cells were encouraging, DB(a,c)A induction caused a 10 fold increase in intracellular resorufin fluorescence (figure 28). By sorting populations which are either induced or uninduced a whole series of variants can possibly be isolated, constitutive induced, super-inducible (induced isozyme(s) have an apparent K_m of 1 uM), super-uninduced (uninduced isozyme(s) have an apparent K_m of 40 nM), etc.

Finally, variants with lower amounts of total MFO activity, or variants lacking the specific isozyme(s) necessary to activate specific compounds to toxic forms can be selected by resistance to the appropriate

xenobiotic. The basic technique would be the same as the isolation of variants with higher epoxide hydrolase activity. Cross resistance between independent isolates with the same xenobiotic and different xenobiotics can be examined.

B. Further characterization of benzo(a)pyrene-DNA adduct profile.

Approximately 91% of the benzo(a)pyrene-DNA adducts are at this time uncharacterized (or 72% if the early eluting counts are neglected). Characterization of this material should be performed. In particular, the specific base adducted and the position of adduction can be identified by methylation with ^{3}H-dimethyl sulfate, perchloric acid hydrolysis, and ion-exchange chromatography. The speculated presence of cis-7,8-dihydrodiol-BP and 7-hydroxy-BP in the cellular BP metabolite profile should be tested when the standards become available. If the presence of these metabolites is confirmed, the possibility of cis-7,8-dihydrdiol-BP mediated DNA adduct(s) should be tested (if the radiolabeled standards become available). And the possibility of a 7-hydroxy-BP mediated DNA adduct(s) should be tested (the radiolabeled standard is available through the NCI Chemical Carcinogen repository). These novel pathways are outlined in figure 29.

C. Further characterization of ANF inhibition of benzo(a)pyrene mutagenicity.

The further analysis of ANF inhibiton of benzo(a)pyrene mutagenicity may be useful. In particular, the characterization of the relative mutagenicity observed under specific conditions and the relative amounts of different BP-DNA adduct species (i.e. peaks separated by HPLC).

Preliminary experiments have demonstrated that the addition of ANF resulted in an inhibition of DNA binding, and that all adduct species were not inhibited equally. If a series of different BP and ANF concentration were examined for induced mutation and inhibition of specific adduct species, a series of $\underline{n}$ equations in $\underline{n}$ unknowns can be generated. These equations would have the form:

$$\text{Relative mutation} = \sum_{i=1}^{n} (x_i)\ (\text{relative amount of adduct}_i)$$

Solving these equations the coefficients (x_i) for each adduct can be obtained. This investigation is one way to determine if all the benzo(a)pyrene adducts equally mutagenic or are some adducts more mutagenic than others.

Figure 28.

Cellular fluorescence during 7-ethoxyresorufin treatment.

Uninduced or DB(a,c)A-induced AHH-1 cells were treated with 2 uM 7-ethoxyresorufin. After 15 minutes cells were analyzed using a flow cytofluorograph (Ortho Instruments, Westwood, MA). Incubation conditions, 2 x 10^6 cells per ml in RPMI medium 1640 treated with 2 uM ER for 15 minutes at 37 C. Analysis conditions, Argon laser, excitation wavelength 514 nm, emmision wavelength 570 nm to 590 nm.

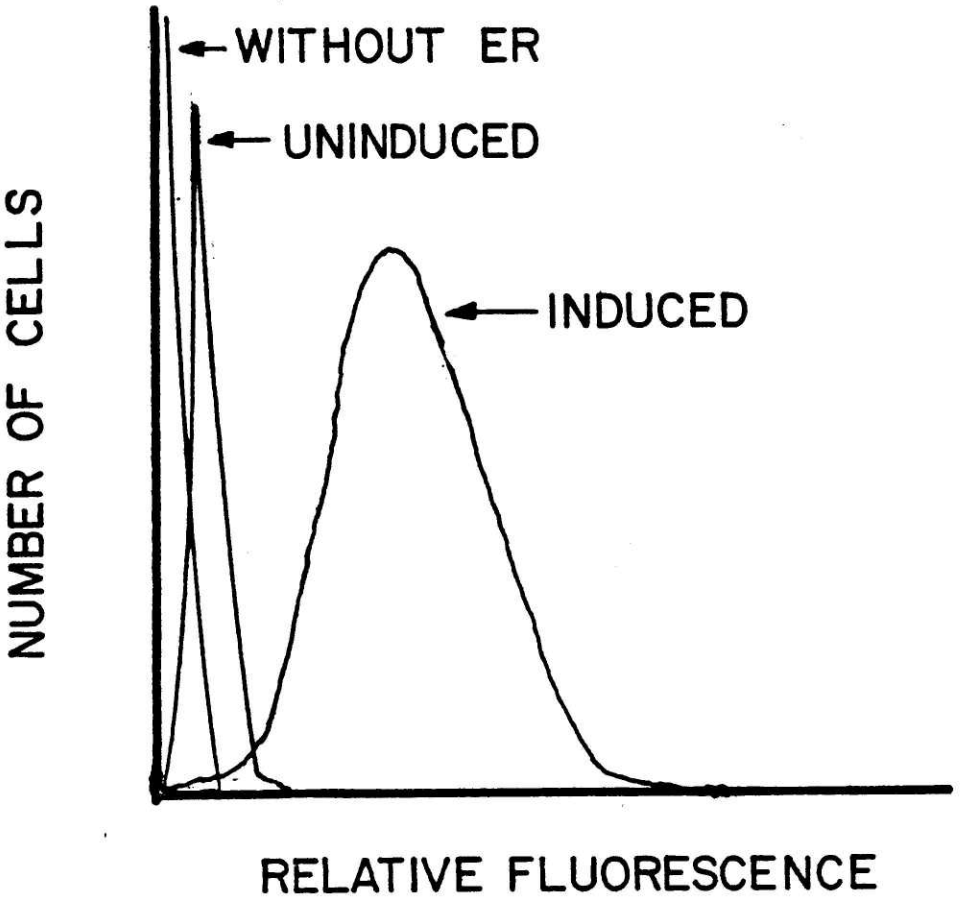
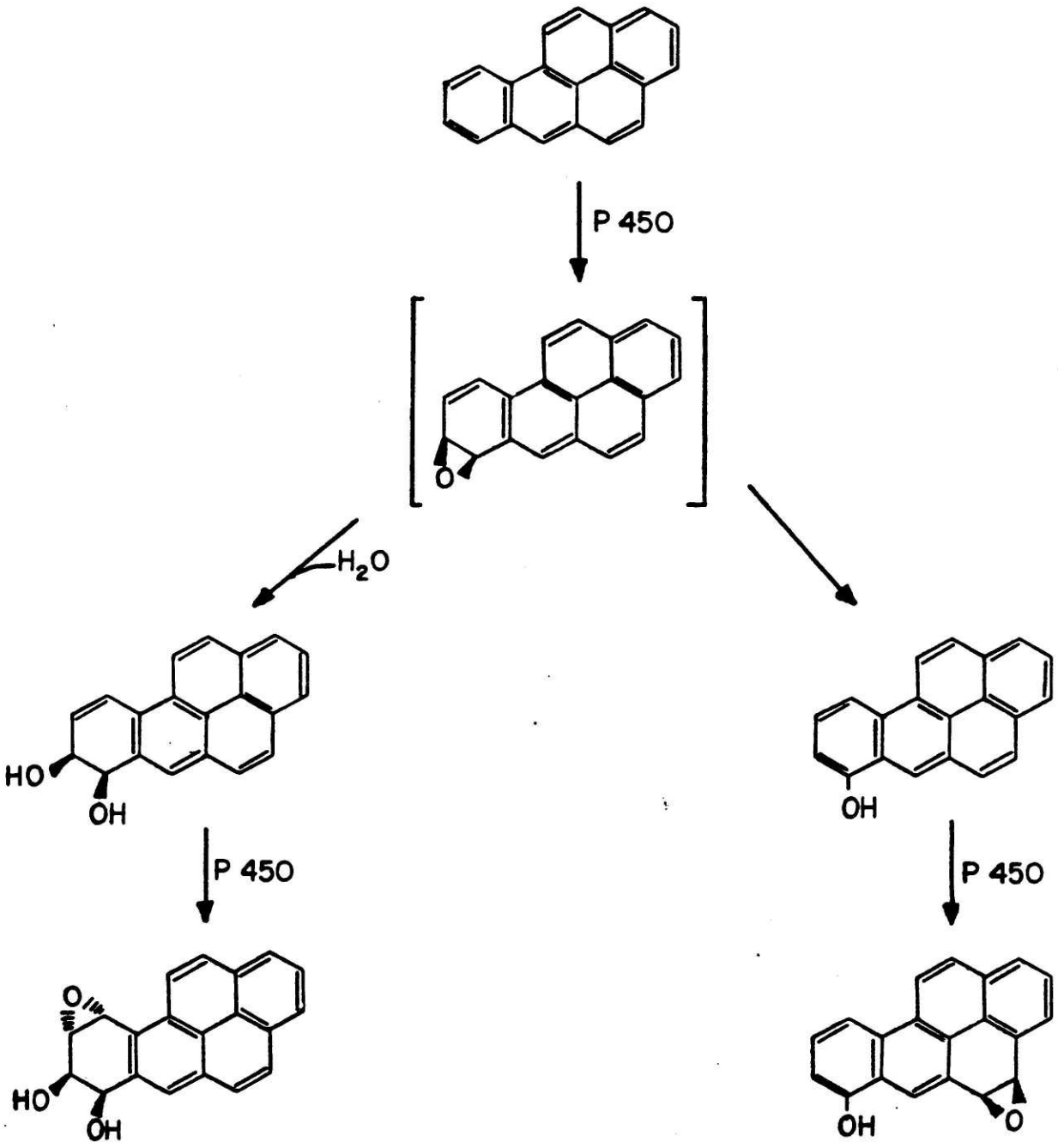

WITHOUT ER
UNINDUCED
INDUCED
NUMBER OF CELLS
RELATIVE FLUORESCENCE

Figure 29.

Proposed novel metabolic pathways for AHH-1 cell mediated

Binding of benzo(a)pyrene to cellular DNA

SPECULATED PATHWAYS TO DNA BINDING



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BIOGRAPHICAL NOTE

Charles Louis Crespi was born on a cold Chicago January evening in 1957. Eight months after the blessed event, the Crespi family moved to the sleepy bedroom community of Downers Grove, IL.

Charles first attended school in the United Kingdom. Upon returning to the United States, Charles attended St. Mary's school for grades one through three, the Avery Coonley School for grades three through eight and then a local "prep school", Benet Academy. After four years of preparation, Charles, was accepted to MIT. Charles eventually settled in MacGregor House, an all male undergraduate dormitory, and became a chemistry major.

Prof. William G. Thilly was a faculty resident in MacGregor. In the summer of 1977, Charles started working for Prof. Thilly. Charles received a S.B. in chemistry in June, 1979 and a S.M. in toxicology under Prof. Thilly also in June, 1979.