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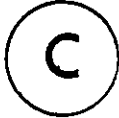
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ARYL HYDROCARBON HYDROXYLASE (AHH) ACTIVITY IN  
MOUSE, RAT, AND HUMAN MAMMARY TUMOURS

by



Michelle Elizabeth Mason

A Thesis  
Submitted to the Faculty of Graduate Studies  
through the Department of  
Biology in Partial Fulfillment  
of the requirements for the Degree  
of Master of Science at  
The University of Windsor

Windsor, Ontario, Canada

1980

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## ABSTRACT

### ARYL HYDROCARBON HYDROXYLASE (AHH) ACTIVITY IN MOUSE, RAT, AND HUMAN MAMMARY TUMOURS

by

Michelle Elizabeth Mason

AHH activity was measured in microsomes from diethylstilbestrol- and chemically-induced and spontaneously-arising mammary tumours from different strains of mice, dimethylbenz[a]anthracene-induced mammary tumours from Sprague-Dawley rats, and human breast tumours. Basal enzyme activities (pmoles 3-hydroxybenzo[a]pyrene formed/mg protein/min) ranged from 0.005-10.0 for mice, 0.005-0.5 for rat, and 0-40 for human tumours. Mean basal hepatic AHH activity in tumour-bearing mice was 200 and was 15 for tumour-bearing rats. Thus, some human tumours had higher activity than rat liver although most had very low or nondetectable AHH. AHH in mouse tumours increased 7 to 12-fold over basal levels when genetically "responsive" mice were injected with 80 mg/kg beta-naphthoflavone (BNF). No significant increase in AHH tumour activity was seen when genetically "non-responsive" mice were similarly treated. AHH activity in rat tumours was induced to levels 70-fold over basal activity after BNF-treatment. Addition of  $10^{-6}$ M alphanaphthoflavone (ANF) to the incubation medium partially inhibited AHH in most tumours from all 3 species, but occasionally caused apparent stimulation in human tumours. Basal AHH activity was consistently

enhanced in tumours from non-responsive mice in the presence of ANF. AHH in all tumours from BNF-treated animals was partially inhibited by ANF. There was no correlation between liver AHH and tumour AHH in the same animal nor any correlation between AHH and tumour weight or degree of necrosis. Human tumour AHH was not correlated with estrogen-receptor content. Mammary tumours of all species were very heterogenous in activity and multiple tumours in the same animal often differed widely in activity. Serial transplants significantly decreased variation in mouse tumour AHH activity indicating some selection by the host for stable sub-populations. Certain chemotherapeutic agents (cyclophosphamide) are substrates for mixed-function oxidases which may activate or inactivate drugs; thus, high AHH activity in certain human tumours may have important implications for the local metabolism of chemotherapeutic agents within the tumour itself. A study of a sample of human breast tumours assayed revealed a possible correlation between AHH activity and response to chemotherapy. In the future, a more definitive assessment of the possible prognostic value of monooxygenase assays on human tumours could be made when more time has elapsed to allow for evaluation of patients in relapse or remission.

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I am most grateful to Dr. A.B. Okey of the Department of Biology, University of Windsor, for the encouragement and guidance given to me throughout this study. His willingness to allow me to proceed with graduate studies during full-time employment with him is deeply appreciated.

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I would like to thank Dr. B. McDonald and staff at the Windsor Cancer Clinic whose willingness to help examine the clinical records of the breast tumour patients and welcomed advice aided in the final analysis of my results.

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# LIST OF ABBREVIATIONS

AHH	aryl hydrocarbon hydroxylase
ANF	alphanaphthoflavone (7,8-benzoflavone)
BA	benzanthracene
BNF	betanaphthoflavone (5,6-benzoflavone)
BP	benzo(a)pyrene
<u>BSA</u>	bovine serum albumin
CPA	cyclophosphamide
DES	diethylstilbestrol
DMBA	dimethybenzanthracene
DNA	deoxyribonucleic acid
DDT	dithiothreitol
EDTA	disodium ethylenediamine tetraacetate
GPO <sub>4</sub>	0.25M potassium phosphate/30% glycerol (pH 7.4)
KPO <sub>4</sub>	0.15M potassium chloride/0.25M potassium phosphate (pH 7.4)
3-OHBP	3-hydroxybenzo(a)pyrene
MC	3-methylcholanthracene
MFO	mixed function oxidase (oxygenase)
n	population size
NA	(data) not available
NADH	nicotinamide adenine-reduced
NADPH	nicotinamide adenine monophosphate-reduced
P	probability
PB	phenobarbitol

PAH	polycyclic aromatic hudrocarbons
pmole	picomole ( $10^{-12}$ mole)
r	correlation coefficient
mRNA	messenger ribonucleic acid
SD	standard deviation
SEM	standard error of the mean
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TDE-buffer	0.01M Tris/0.001M DTT/0.0015M EDTA (pH 7.4)
Tris	tris(hydroxymethyl)amino methane
UDP	uridine diphosphate
V	variance ( $S^2$ )
w/o	without
$\bar{x}$	mean of the population

## CHAPTER I

### INTRODUCTION

#### A. OVERVIEW OF THE ENZYME SYSTEM

Aryl hydrocarbon hydroxylase (AHH) is one of a group of membrane-bound microsomal monooxygenases that metabolize a wide variety of compounds including drugs, steroids, and polycyclic aromatic hydrocarbons (PAH). These microsomal mixed-function oxidases (MFO) require NADPH and oxygen, and contain different forms of cytochromes such as P-450 and P<sub>1</sub>-450. Because of the membrane-bound nature of these systems they have been difficult to purify and their reaction mechanism is not completely understood (Heidelberger, 1975). They are very non-specific and carry out aromatic ring hydroxylations, N-hydroxylations and oxidative demethylations of a great variety of substrates including endogenous substrates such as steroids and fatty acids, as well as xenobiotics such as polycyclic hydrocarbons and insecticides (Gillette et al, 1972).

The mixed-function oxidases are found in greatest quantity in the major organ of metabolism of xenobiotics, the liver, but are also detectable in other tissues especially those associated with portals of entry. Some examples include lung, kidney, intestine, adrenal, brain, uterus, lymph nodes, bone marrow, and mammary gland (Thorgeirsson and Nebert, 1977; Mattison and Thorgeirsson, 1978; Fysh and Okey, 1978).

The normal function of this system is to convert



lipid-soluble substances to more water-soluble substances which can be excreted as such or conjugated with more polar groups and then excreted, an overall process of "detoxification". Figure 1 demonstrates the complexity of the enzyme system. NADPH supplies reducing equivalents that ultimately reach a trimolecular complex composed of cytochrome P-450, a substrate such as benzo[a]pyrene (BP), and molecular oxygen. The incorporation of one atom of molecular oxygen into an aromatic substrate results in an arene oxide: with an alkyl substrate the result is an epoxide. These reactive intermediates can: 1) rearrange spontaneously to form a phenol, 2) be converted enzymatically to a trans-hydrodiol or glutathione conjugate, or 3) become covalently bound to cellular nucleic acids and proteins. The phenol can also be conjugated with UDP glucuronic acid. Conjugation of the products makes them more polar than the parent substrate, oxide, phenol, or dihydrodiol and, therefore, are more readily excreted (Thorgeirsson and Nebert, 1977).

The scheme outlined in Figure 1 illustrates the possible importance of steady state levels of reactive intermediates. Those not converted to inactive products by one of the above three pathways may interact with critical targets with toxicity, mutation, and cancer as possible end results. A delicate balance exists in each tissue, then, between enzymes that "activate" and those that "detoxify". This balance may be effectively altered by differences in genetics, age, hormonal status, nutritional balance, circadian rhythmicity, and enzyme stimulation or inhibition due to various drug-drug interactions.


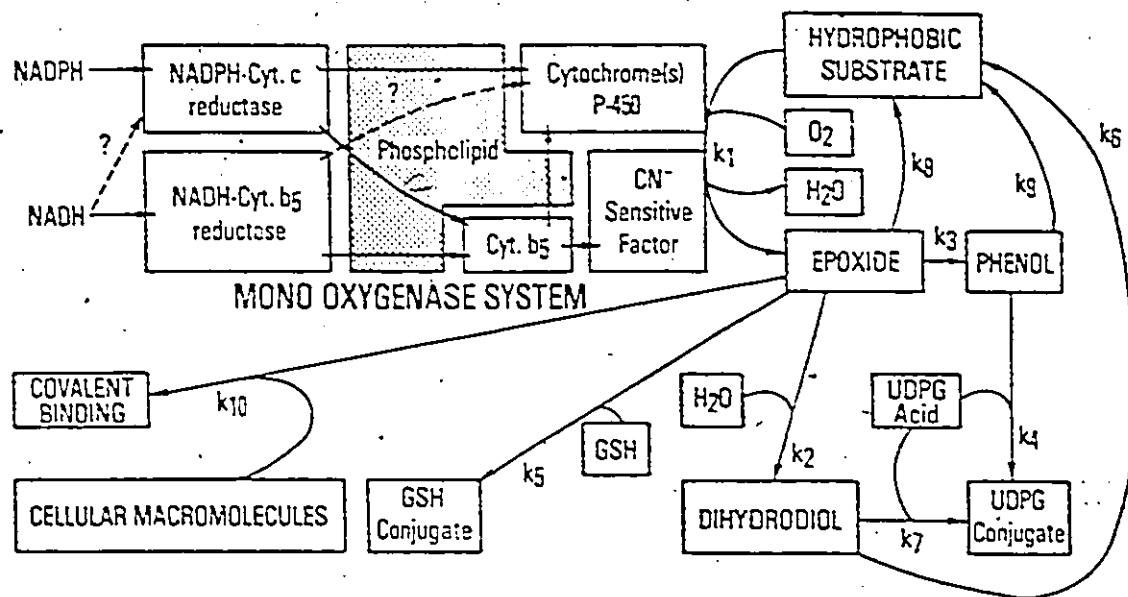


Figure 1. Scheme for the membrane-bound multi-component monooxygenase system(s) and the various possibly important pathways for metabolism of hydrophobic substrates. For any given substrate, the relative rates of  $k_1$  through  $k_{10}$  are currently not known and most likely differ among different tissues, strains, and species; age, nutritional balance, hormonal status, diurnal variations, and pH all possibly may be important factors in altering the various rates. Modified from Thorgeirsson and Nebert (1977).



Another property of this enzyme system is that it is highly "inducible". Many of the xenobiotics that act as substrates for the enzyme system are also good inducers of one or more forms of cytochrome P-450. Chemical structures of PAH commonly used for induction of MFO activity appear in Figure 1A. A decade ago, only two forms of cytochrome P-450 could be experimentally demonstrated (Conney, 1967); today, the number of forms of P-450 able to be experimentally characterized exceeds ten and as research techniques improve, many more may be found (Nebert and Jensen, 1979).

Generally, five basic criteria are used for characterization of the cytochrome P-450 form present: 1) functional characteristics based on differences in catalytic properties with specific reference to substrate specificity, 2) spectral characteristics, specifically the Soret peak position after preparations are reduced and combined with carbon monoxide, 3) the temporal expression of increased enzyme activities following treatment with inducing agents, 4) physical properties such as molecular weight obtained via electrophoresis, and, finally, 5) susceptibility to various enzyme inhibitors such as alphanaphthoflavone and metyrapone.

Generally, cytochrome P-450 denotes collectively all forms of membrane-bound hemoproteins associated with NADPH-dependent monooxygenases and capable of binding carbon monoxide when reduced. Cytochrome P<sub>1</sub>-450 is arbitrarily defined as the polycyclic aromatic-inducible form (subclass of P-450) most closely associated with PAH-induced AHH activity. The ontogenesis of inducible AHH corresponds well with increases in a


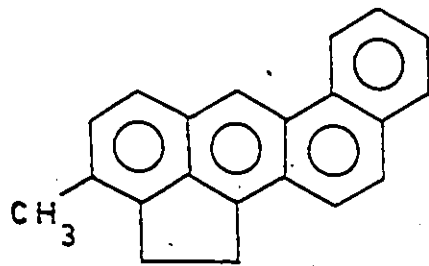
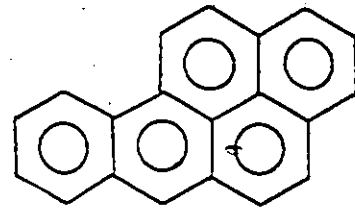


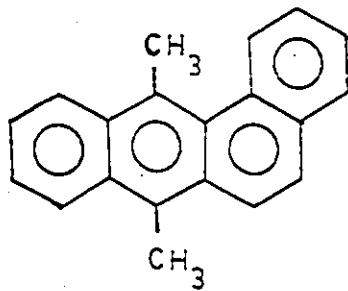
Figure 1A. Chemical structures of polycyclic hydrocarbons commonly used for induction of MFO activity.



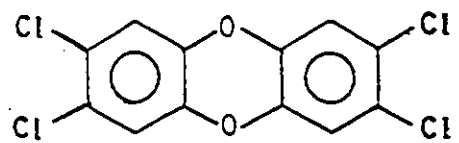
3-MC



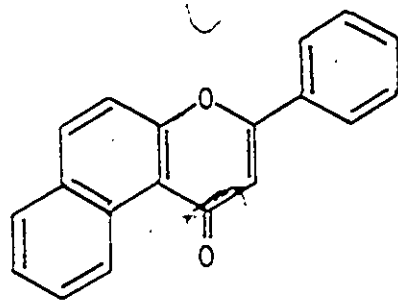
BP



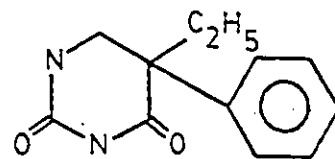
7,12-DMBA



2,3,7,8 TCDD



BNF



PHENOBARBITAL

56000-M<sub>r</sub> electrophoretic band. P<sub>1</sub>-450 is not considered the same as cytochrome P-448, arbitrarily defined as the polycyclic aromatic-inducible form most closely associated with the greatest hypsochromic shift in the Soret peak of the reduced hemoprotein-CO complex. Induction of the P-448 form of the cytochrome occurs later temporally and is characterized by increased synthesis of a 54000-M<sub>r</sub> electrophoretic band (Guenthner and Nebert, 1978).

In the following pages, references will be made basically to two different forms of the enzyme: that form of the enzyme found in 3-methylcholanthrene- (MC) or PAH-treated responsive animal strains designated cytochrome P<sub>1</sub>-450 and that form associated with cytochrome P-450 found in control or phenobarbital-treated animals. The two main criteria for characterization of the enzyme form present will be based on differences in catalytic properties with specific reference to benzo[a]pyrene and susceptibility to various classes of inhibitors.

Nebert and co-workers have done extensive work concerning the genetic regulation of these enzyme systems in inbred strains of mice (Nebert et al, 1978). One form of cytochrome P-450, cytochrome P<sub>1</sub>-450, is highly inducible by PAH such as benzo[a]pyrene and MC. This induction occurs only in genetically "responsive" inbred mouse strains, the prototype of which is the C57BL/6 (B6). Induction of this form of cytochrome (P<sub>1</sub>-450) by PAH is absent in liver and markedly decreased in other tissues of the "non-responsive" inbred mouse strains, such as DBA/2 (D2). This "responsiveness"

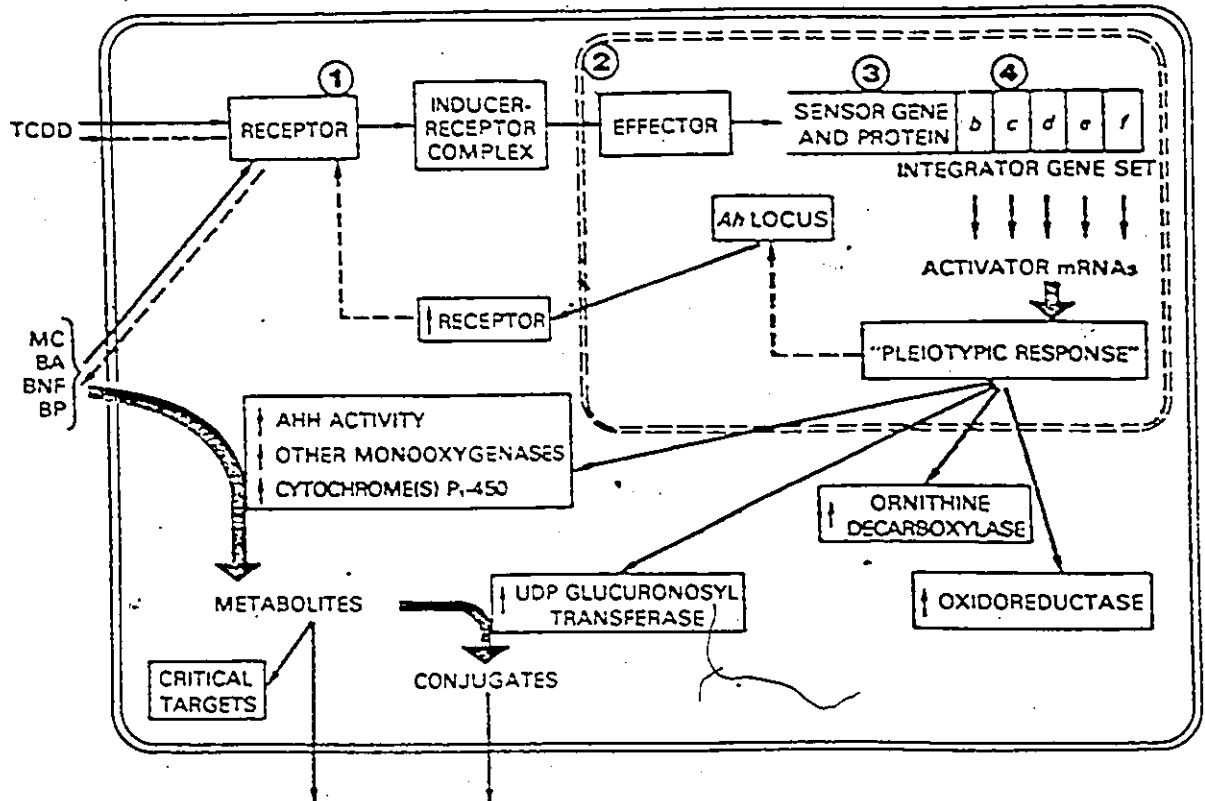
to aromatic hydrocarbons has been designated the Ah locus (Nebert et al, 1972).

An important product of the Ah (regulatory) locus in the mouse is a cytosolic receptor (Poland et al, 1976; Guenthner and Nebert, 1977; Carlstedt-Duke et al, 1979; Okey et al, 1979) capable of binding to certain PAH inducers. Such an inducer-receptor complex in some manner activates structural gene(s), thereby leading to an increase in synthesis of enzymes which metabolize the inducers and other related hydrophobic substrates as well. During this metabolic process, as previously described, reactive intermediates may be generated. The production of such intermediates is greater in "responsive" tissues than in "non-responsive" tissues resulting in genetic differences in cancer, mutagenesis, and toxicity. A schematic representation of these events is given in Figure 2.

A reliable, simple, and very sensitive assessment of aromatic hydrocarbon responsiveness following treatment of animals with PAH inducers is the aryl hydrocarbon hydroxylase enzyme assay. Using AHH induction as an indicator of phenotype at the Ah locus many inbred strains of mice have been studied; roughly half have been found to be "responsive". Extensive genetic crosses between the various mouse strains have shown the induction of AHH and cytochrome P<sub>1</sub>-450 to be expressed in different ways. Inheritance patterns of the Ah locus range from simple Mendelian inheritance where expression of AHH activity is inherited as an autosomal dominant trait, to additive or gene-dose inheritance, or to inheritance where



Figure 2. Hypothetical scheme depicting a cell in which polycyclic aromatic inducers evoke a pleiotypic response controlled by the Ah locus. TCDD, MC, BNF, and BP interact with the cytosolic receptors with differing affinities. The inducer-receptor complex, translocated into the nucleus, becomes the "effector", which interacts with DNA causing induction of the synthesis of a variety of enzymes. One protein which subsequently may be induced is the regulatory gene product of the Ah locus itself, the cytosolic receptor. Most inducers are in turn metabolized by the enzyme(s) induced and by other enzymes not shown. Reactive metabolites may bind covalently in the same cell or in other cells or other tissues to critical subcellular targets associated with tumours, mutation, and toxicity. Reactive and nonreactive metabolites and conjugated products as well, are excreted from the cell (Modified from Guenthner and Nebert, 1977).



lack of AHH induction is dominant. The simplest genetic model to explain the results from genetic crosses would require a minimum of six alleles and two loci (Thorgeirsson and Nebert, 1977). The important point to be made from this discussion is that the magnitude of AHH and cytochrome P<sub>1</sub>-450 induction by PAH appears to be genetically regulated in most tissues of the mouse (Nebert et al, 1975).

The liver is by far the most active site for the biotransformation of drugs and other xenobiotics to which the body is exposed. It was for this reason the tissue was of interest in the early stages of characterization of the enzyme system. It soon became apparent that the enzyme system was present at other sites in the body, especially those exposed to PAH, and was inducible not only in the liver but in the gastrointestinal tract, kidneys, skin, and lungs. The examination of AHH in extra-hepatic tissues may be of importance in local production of reactive metabolites in those tissues where tumours are frequently initiated.

#### B. ENZYME STUDIES IN TUMOURS

Examination of AHH activity in tumours has, so far, been concentrated on the "minimal deviation" hepatomas, Morris hepatomas (Watanabe et al, 1970), the ascites form of the Novikoff hepatoma (Saine et al, 1978), and transplantable solid liver tumours 5123 t.c., 5123 t.c. (H), and 7288 ct. c. (Strobel et al, 1978) have been examined for active MFO systems capable of metabolizing a variety of drugs and polycyclic hydrocarbon substrates. Generally, results indicated that the

enzyme systems present were, at best, 40 percent as active as those found in corresponding livers. The hepatomas did, however, possess an intact, inducible microsomal enzyme system capable of hydroxylating benzo[a]pyrene and other substrates. The aforementioned researchers felt that the presence and functionality of a drug-metabolizing enzyme system was an important finding and one that may have significance to successful chemotherapeutic management of metastatic disease. Many of the chemotherapeutic agents administered in treatment of cancer require metabolic activation via the mixed-function mono-oxygenase before their anti-tumour effects are elicited (Ohira et al, 1975 and Hilf et al, 1970). As the tumour system also functions in drug metabolism it may be a significant site of "second-pass" and local activation of some chemotherapeutic agents.

Because of the prevalency of human breast cancer, varied enzyme studies in human breast tumours have been undertaken (reviewed in Deshpande et al, 1977 and Coombes, 1978). The research in this area has been directed towards a search for biochemical parameters that will assist in predicting the clinical course of the disease. Much of this work has concentrated on serum enzyme levels in cancer patients as well as on the enzyme levels of the carcinoma itself. The most frequent alterations in plasma enzymes in cancer patients are due to secondary effects of the tumour on normal tissues, but occasionally they represent release of enzymes by the neoplastic tissue itself. Elevated plasma levels of enzymes such as sialyl transferase, galactosyltransferase, and alkaline phosphatase have been associated with breast cancer although not always

correlated with the course of the disease (Coombs, 1978). Human breast cancers are also associated with elevated glycolytic enzymes such as lactate dehydrogenase and glucose-6-phosphate dehydrogenase compared to normal breast tissue (Deshpande et al, 1977). Yet, again, the spread of the disease, as measured by clinical staging, does not influence the enzyme activities in the primary tumour. These problems are not surprising in view of the heterogeneity of cell type of breast tissue including stromal, ductal alveolar, and myoepithelial cells whose function and relationship to normal breast tissue is still undefined.

In the search for biochemical parameters that will aid in predicting the clinical course of breast cancer, one can conclude that there is no single tumour index that can accomplish this and further studies are required to evaluate the place of various biochemical markers in detecting metastases, predicting their response to chemotherapy and monitoring the course of the disease.

Because of its relevancy to the human breast cancer problem, the search for cytochrome P-450-mediated mono-oxygenase in mammary gland tumours is an important one. The mammary gland as a site of tumourigenesis in inbred laboratory strains of rodents, whether spontaneously-arising or chemically-induced, is a well established premise (Dao, 1964). The present study was undertaken to ascertain if a normally-functioning microsomal cytochrome P-450 mediated mono-oxygenase system was present in mammary gland tumours of mice and rats and in human breast biopsies sent to our laboratory by the Windsor Clinic of the

Ontario Cancer Foundation for estrogen-receptor analysis. Through the use of the AHH enzyme assay, it was hoped that the levels and ranges of the enzyme activity could be determined in these tumours. Through examination of the patterns of inducibility and inhibition of enzyme levels in the tumour microsomal suspensions, information regarding the genetic regulation of the enzyme system might be revealed. Finally, by means of relating human tumour AHH level to the patient's clinical response to treatment, it was hoped that an assessment could be made to determine if AHH activity in the primary tumour predicts the response to various chemotherapeutic regimes.

## CHAPTER II

### ARYL HYDROCARBON HYDROXYLASE ENZYME ASSAY

The aryl hydrocarbon hydroxylase enzyme assay represents a reliable, simple, and very sensitive means by which to determine the level of benzo[a]pyrene hydroxylation present in a sample. The fluorometric assay allows for an assessment of aromatic hydrocarbon responsiveness following treatment of animals with PAH inducers. Benzo[a]pyrene, the substrate for the assay, is metabolized by the mono-oxygenase system(s) to numerous oxygen-containing intermediates and products including covalently-bound metabolites (Nebert et al, 1975). It has been found empirically that the relative amount of 3-hydroxybenzo[a]pyrene formed is a reasonable estimate of benzo a pyrene metabolism in both liver and non-hepatic tissue. The fluorometric macroassay for AHH remains the best routine test for the Ah<sup>b</sup> allele. The procedure described below outlines the conditions that were derived for maximizing the enzyme activity detected in tumour microsomal suspensions.

#### A. GENERAL ASSAY PROCEDURES AND CONDITIONS

##### 1) Preparation of Tissues

Removal and storage of mouse, rat, and human mammary tumours will be discussed in subsequent chapters. Routinely, all tumour specimens were homogenized in 3 ml TDE buffer per g tissue using a Polytron PT-10 (Brinkmann Instruments, Westbury, N.Y.) at a setting of "5". Four pulses of fifteen

seconds duration with a forty-five second cooling period between pulses was the general tissue homogenization procedure used. The tumour homogenate was then centrifuged at  $10,000 \times g$  for 15 minutes and the supernatant from this was centrifuged at  $105,000 \times g$  for one hour to obtain the microsomal pellet. The microsomal pellet was then resuspended in  $GPO_4$  buffer using a short, gentle burst of the Polytron. Generally, for each g of tissue weight, 0.5 ml of  $GPO_4$  buffer was added to the microsomal pellet, with a minimum resuspension volume of 1.0 ml. All procedures unless otherwise indicated were carried out at  $0-4^\circ C$ . The protein concentration was routinely adjusted so that it was between 2.0-15.0 mg per ml as determined by the method of Lowry et al (1951) (see Appendix I).

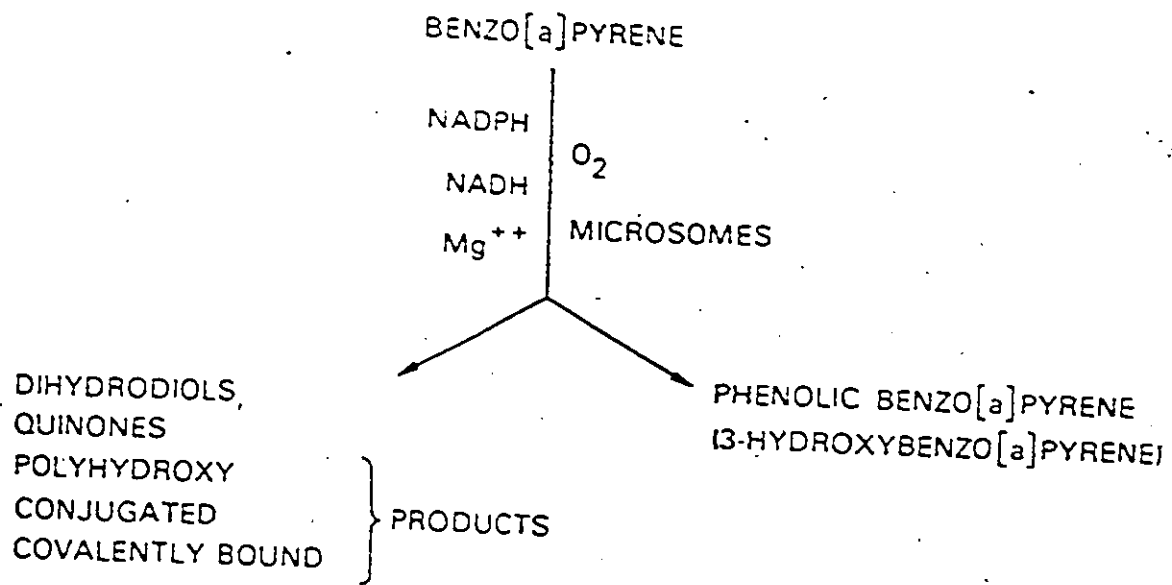
## 2) Enzyme Assay

AHH activity in tumour microsomes was measured essentially by the method of Nebert and Gelboin (1968). The reaction catalyzed by the aryl hydrocarbon hydroxylase system with BP as the substrate is illustrated in Figure 3. The reaction was carried out using 100  $\mu l$  of tumour microsomal suspension in a final incubation volume of 1.05 ml. The incubation mixture contained: 0.25 ml of 0.2M Tris (pH 7.5), 0.03 ml of 0.1M  $MgCl_2$ , 0.62 ml distilled water, 0.3 mg NADPH, 0.3 mg NADPH, and 0.7 mg bovine serum albumin (BSA).

Routinely, the in vitro inhibitor alphanaphthoflavone (ANF) was added in methanol to give a final concentration of  $10^{-6} M$  in the reaction vessel, at this stage. Where the effects of other inhibitors are being monitored, the final concentration will be indicated in the text.



Figure 3. Current concept of the aryl hydrocarbon hydroxylase assay. The substrate BP is oxygenated to arene oxides which rearrange nonenzymatically to phenols or are oxygenated by direct oxygen insertion to phenolic derivatives. The 3- and 9-phenols have the strongest fluorescence in alkali. Other oxygenated derivatives of BP, including dihydrodiols and quinones, are not measured by this assay. Adapted from Thorgeirsson and Nebert (1977).



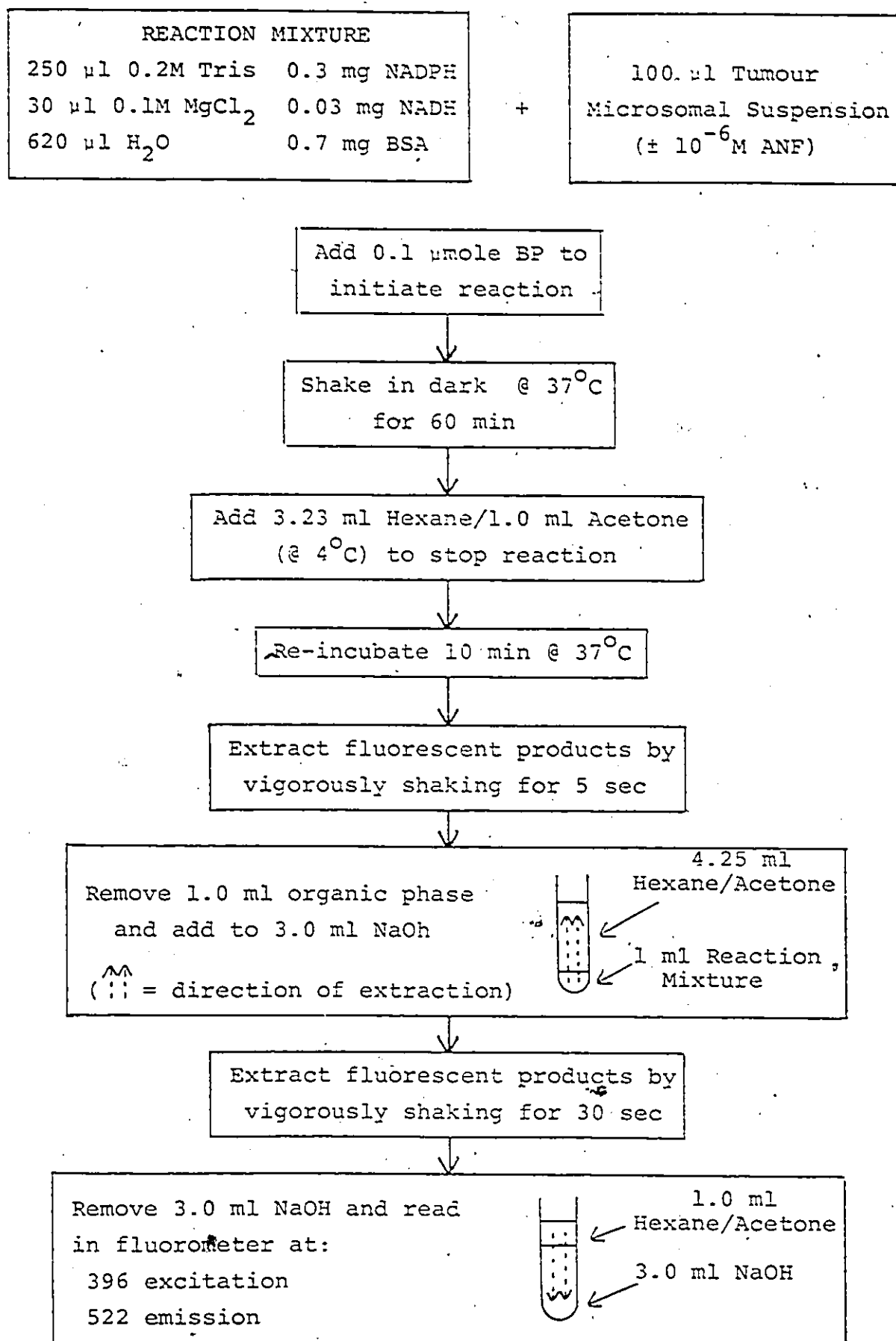
The reaction was initiated by adding 0.1  $\mu$ mole BP in 50  $\mu$ l of methanol to each reaction vessel. The mixture was then shaken in a warm water bath at 37°C for 60 minutes in air.

The reaction was stopped by adding 4.25 ml of cold hexane:acetone mixture (3.25:1.0) and the mixture was incubated for an additional 10 minutes at 37°C. A 1.0 ml aliquot of the 3.3 ml organic phase was extracted with 3.0 ml 1N NaOH. The concentration of the extracted, hydroxylated BP in the alkali phase was determined spectrophotofluorometrically with excitation at 396 nm and emission at 522 nm; 3-hydroxybenzo[a]pyrene, extracted under conditions identical to those of the samples, is used as a standard (see Appendix II). One unit of AHH activity is defined as that amount of enzyme catalyzing per minute at 37°C the formation of hydroxylated product causing fluorescence equivalent to 1 picomole of 3-hydroxybenzo[a]pyrene. Enzyme activities were determined in duplicate and generally were within 10 per cent of each other. The blank was prepared by adding microsomal suspension to the incubation vessel after the reaction was stopped with hexane:acetone. A schematic representation of the assay is given in Figure 4.

Microsomal suspensions prepared from mammary and liver tissues of tumour-bearing animals were prepared in the same manner. The enzyme assay of mammary microsomal suspensions was identical to that of tumour microsomal suspensions, whereas, only 50  $\mu$ l of liver microsomal suspension was added to the incubation mixture and samples were incubated for only

Figure 4. Schematic representation of AHH assay.

The sequence of events for performing the assay is outlined. Components of the incubation flask are indicated, as well as, the initiation of the incubation process by addition of the substrate, BP. After extraction of the phenolic metabolites, fluorescence can be read at excitation of 396 nm and emission at 522 nm.



10 minutes at 37°C.

## B. REQUIREMENTS AND KINETICS OF THE AHH REACTION

### 1) Incubation Mixture Requirements

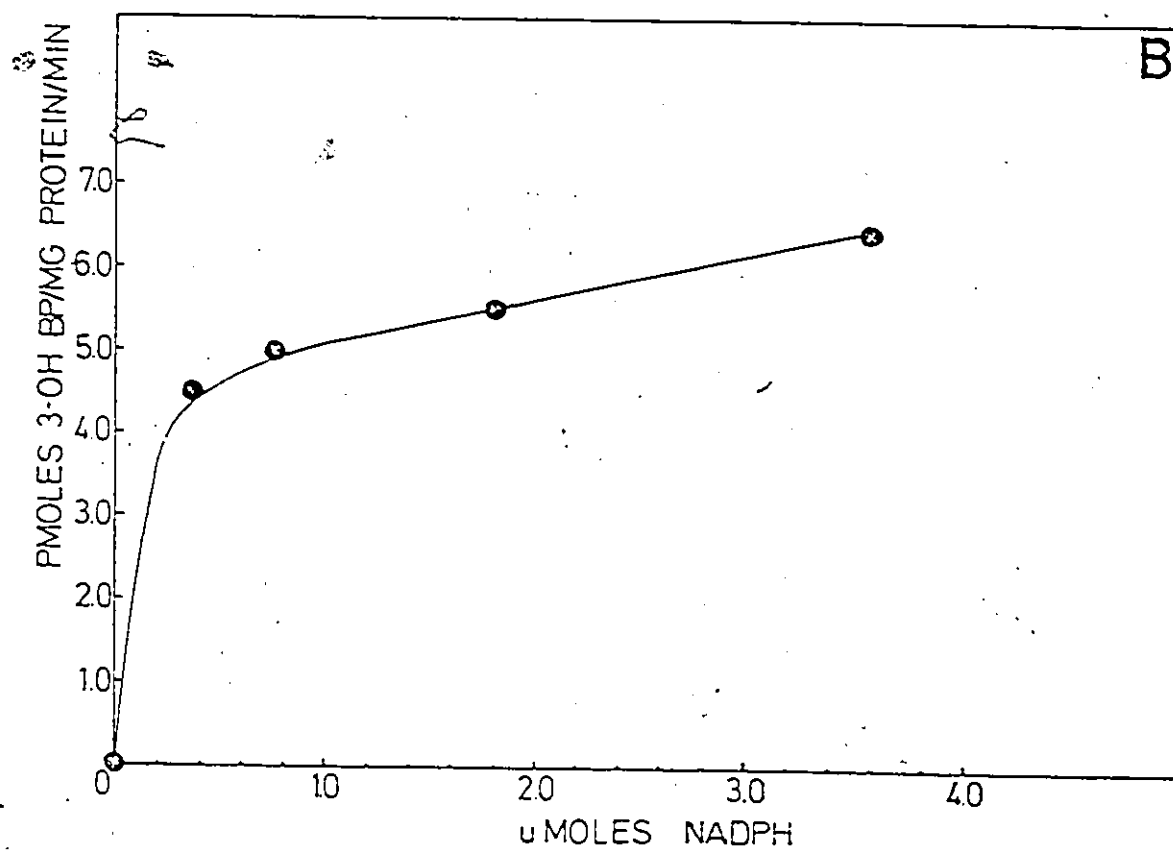
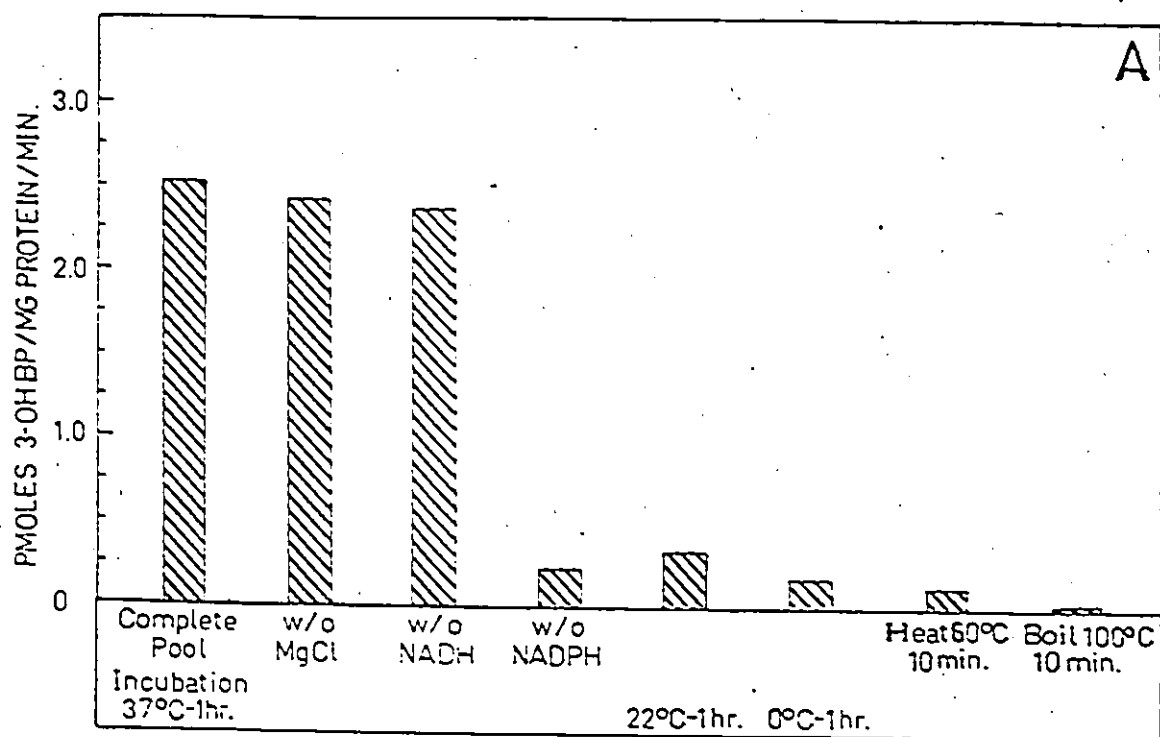
In Figure 5A, a summary of the conditions for the incubation procedure is given. The results in Figure 5A indicated the enzyme had an absolute requirement for NADPH whereas, lack of NADH in the incubation medium had no effect on AHH activity. This is in agreement with results from hepatic (Nebert and Gielen, 1972) and mammalian cell culture (Nebert and Gelboin, 1968) microsomal suspensions. Figure 5B shows the effect of increasing concentrations of NADPH on the reaction. It was thought that due to the long incubation period of one hour relative to that time used for liver microsomal suspensions, a depletion of co-factors might occur. As can be seen in Figure 5B, increasing the amount of NADPH available to the enzyme over the incubation period, did not dramatically increase AHH activity.

Lack of magnesium chloride in the incubation mixture had no effect on the enzyme activity as seen in Figure 5A. Nebert and Gelboin (1968) reported a divalent cation dependence in microsomal suspensions prepared from hamster fetal cells. The association of divalent cations is thought to be important for the integrity and functioning of biological membranes. Nebert and Gielen (1972) using mouse hepatic microsomal suspensions reported that non-dialyzed hepatic or non-hepatic tissues apparently have sufficient divalent cations nonspecifically associated with the microsomal membranes so that additional

Figure 5. Factors modifying tumour microsomal AHH activity. Microsomes were prepared from a DMBA-induced rat tumour; similar results were obtained using mouse and human tumour microsomes. Incubation time was 60 minutes at 37°C unless otherwise indicated. Each point represents the mean of duplicate determinations.

A. Heat inactivation was the result of pre-incubation of microsomal suspensions at 60°C and 100°C. The results of omitting co-factors from the reaction mixture are included. Incubation medium contained 0.72 mg microsomal protein.

B. Dependence of mouse tumour microsomal AHH activity on NADPH. Each incubation flask contained 0.86 mg protein and was incubated for 60 minutes at 37°C.





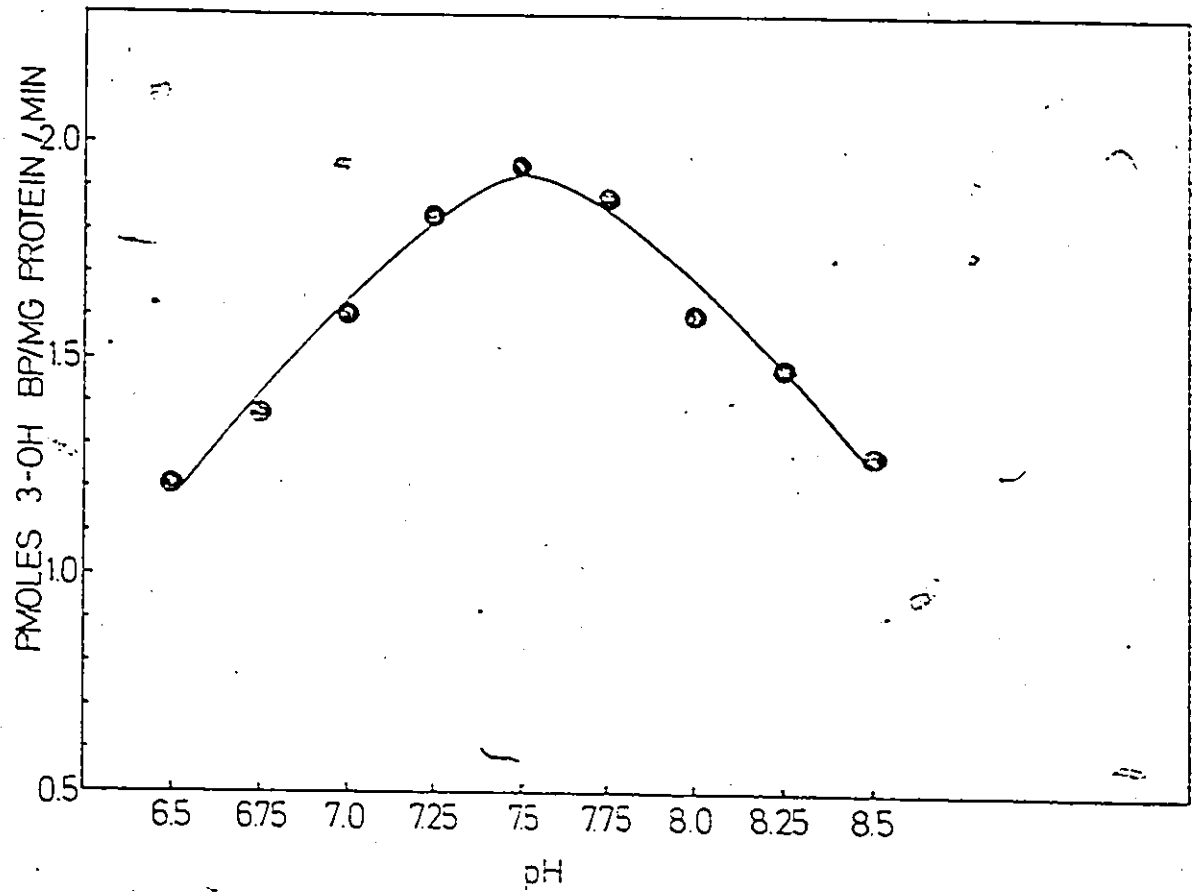
magnesium is not necessary. In additional experiments using microsomal suspensions from the livers of control and tumour-bearing mice, magnesium chloride independence was again demonstrated (data not shown). In light of these findings, lack of dependence of AHH activity on the presence of magnesium chloride is expected.

An additional prerequisite for maximal enzyme activity is determination of the most favourable pH for the reaction mixture. Adjusting the pH of the 0.2M Tris buffer used in the preparation of the reaction mixture yielded the distinct profile of the effect of pH on enzyme activity as seen in Figure 6. The range is fairly broad with AHH activity maximum at approximately pH 7.5. The enzyme activity is relatively consistently high between pH 7.0 and pH 8.0. This broad pH optimum for tumour microsomal preparations is similar to that found in liver and kidney (Gielen, Goujon, and Nebert, 1972) and in the mouse ovary (Mattison and Thorgeirsson, 1978). Mouse, rat, and human tumour microsomal preparations yielded similar patterns for pH dependency (rat and human data not shown).

## 2) Temperature Dependence

The thermolability of tumour microsomal AHH activity was studied by incubating the enzyme preparation at various temperatures. The enzyme activity showed a well-defined temperature dependence with respect to both pre-incubation and incubation periods. As indicated in Figure 5A, heating the microsomal suspension to 60°C or 100°C for 10 minutes prior to incubation for one hour almost completely inactivated

Figure 6. Dependence of mouse tumour AHH on pH of incubation medium. Each incubation flask contained 1.58 mg protein and was incubated for 60 minutes at 37° C. (Results were similar for rat and human tumour microsomal suspensions.)

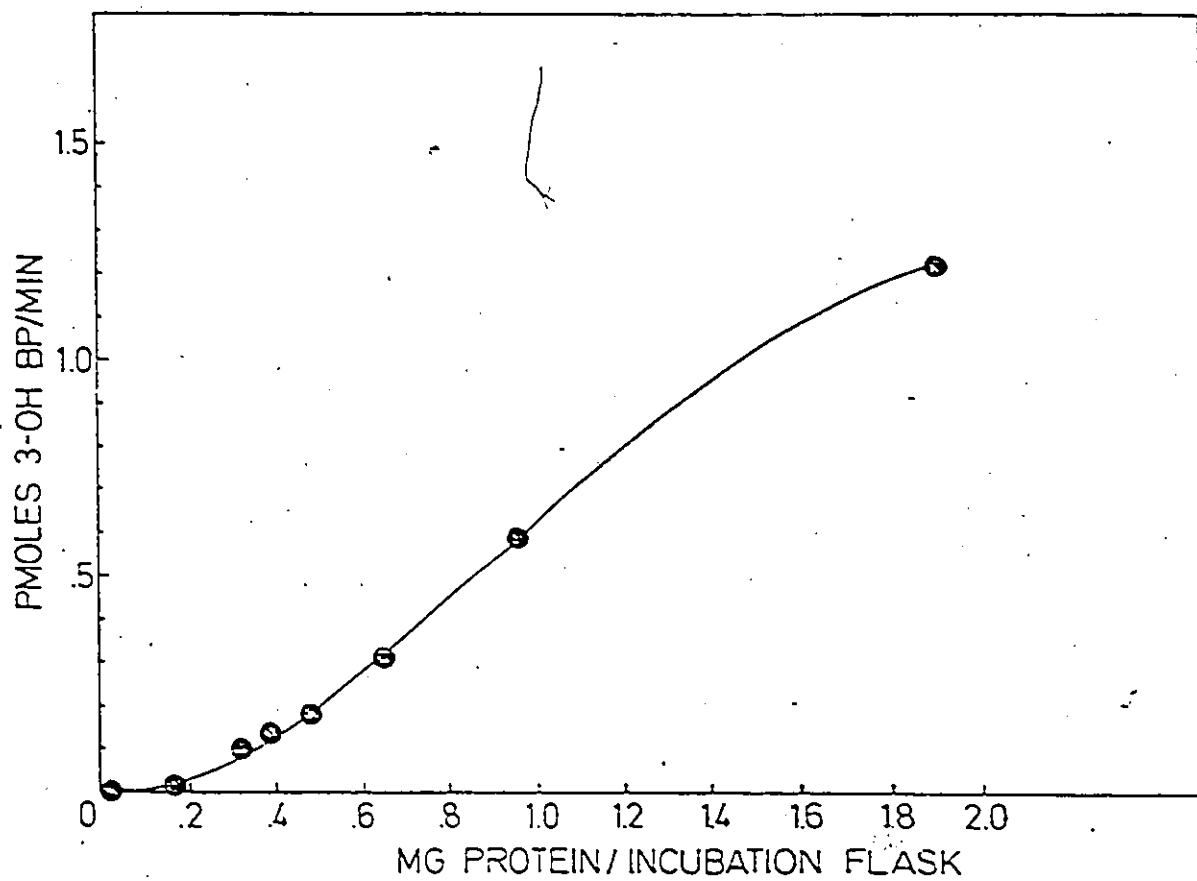


tumour AHH activity. Microsomal suspensions that were not pre-treated but were incubated at 0°C or at 22°C for one hour showed 4 per cent and 9 per cent, respectively, of control activity. This temperature dependence is similar to that reported by Nebert and Gelboin (1968) with hamster fetal cell microsomal suspensions and indicates the enzymic nature of the reaction.

### 3) Effect of Protein Concentration on Enzyme Activity

Figure 7 demonstrates the relationship between AHH activity and tumour microsomal protein. The relationship is a linear one, between 0.3-1.5 mg microsomal protein. The linear range for tumour microsomal preparations is somewhat broader than that given for mammary cell lines, hepatic or extrahepatic microsomes (Nebert and Gelboin, 1968; Gielen, Goujon, and Nebert, 1972; and Mattison and Thorgeirsson, 1978) but is quite likely attributable to the relatively low AHH activity found in tumour microsomal preparation as compared to other tissue types. The lack of linearity above 1.5 mg microsomal protein might be explained due to depletion of co-factors and lack of linearity below 300 µg, by approaching the limits of sensitivity of the assay with such low activity samples. Nebert and Gielen (1972) reported a lower hydroxylase activity in mouse hepatic microsomal suspensions if the reaction mixture contained less than 300 µg of total protein. The addition of bovine serum albumin improved the specific activity as it is known to improve the solubility of the substrate BP. This factor can be discounted in tumour

Figure 7. Dependence of mouse tumour AHH activity on microsomal protein concentration. The reaction was allowed to proceed for 60 minutes at 37°C. (Protein values do not include 0.7 mg BSA added to the incubation flask.)



microsomal incubations as all reaction vessels contained 0.7 mg BSA and absence or presence of BSA did not alter the AHH activity in those samples (data not shown).

#### 4) Effect of Incubation Time on AHH Activity

In order to insure that the reaction was performed under conditions that were linear with respect to time, enzyme activity was monitored at several time points during a two hour incubation period. Figure 8 shows representative graphs from tumour microsomal suspensions prepared from mouse, rat, and human mammary tumours. In spite of differences in absolute specific activity among the different tumours, profiles obtained were similar in appearance. As a result of the linearity of the reaction up to 60 minutes, a 60-minute incubation period was chosen to ensure that maximum AHH-produced metabolites were obtained from low activity samples such as tumours.

### C. DISCUSSION

After examination of various factors affecting the enzyme assay in tumour microsomal preparations, it can be concluded that the enzyme system resembles closely the system that has been extensively characterized in a variety of other tissues and strains (Nebert and Gelboin, 1968; Wiebel et al, 1971, 1973; Gielen et al, 1972; Burki et al, 1973; Mattison and Thorgeirsson, 1978; Fysh and Okey, 1978). Manipulations of assay variables elucidated the conditions under which to analyze the multiple samples of mouse, rat, and human mammary tumours. Having resolved those criteria, experiments were

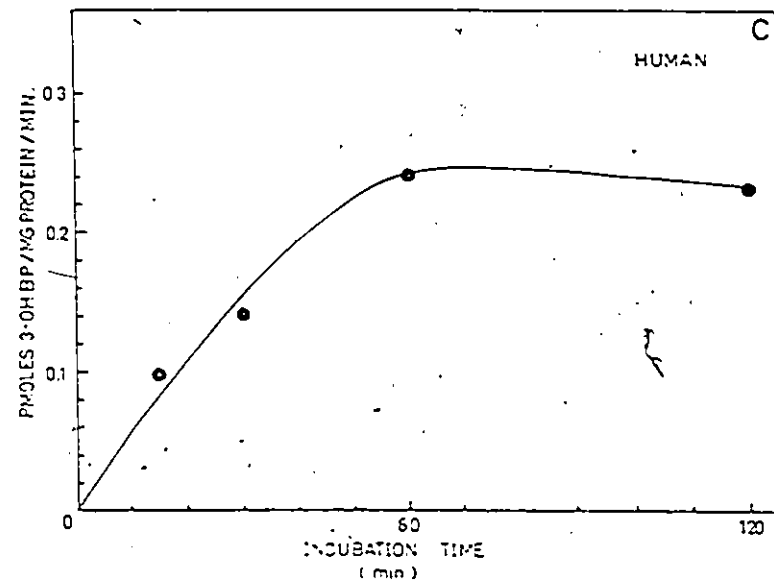
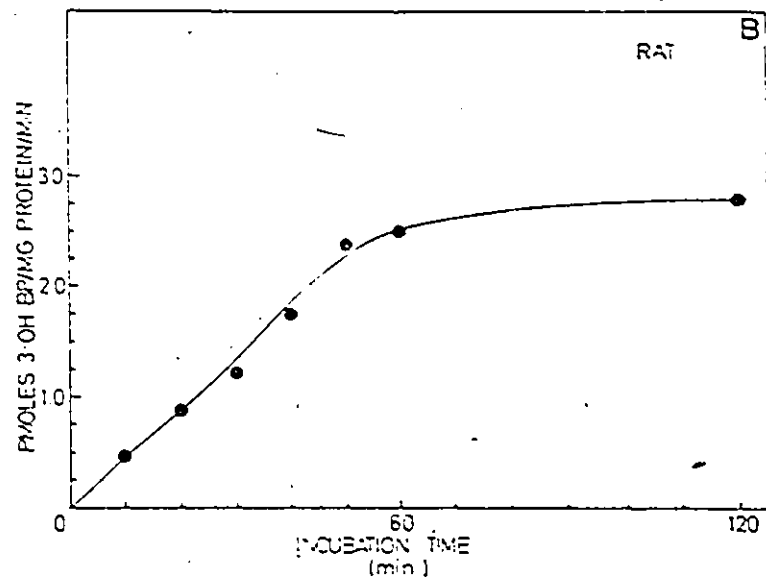
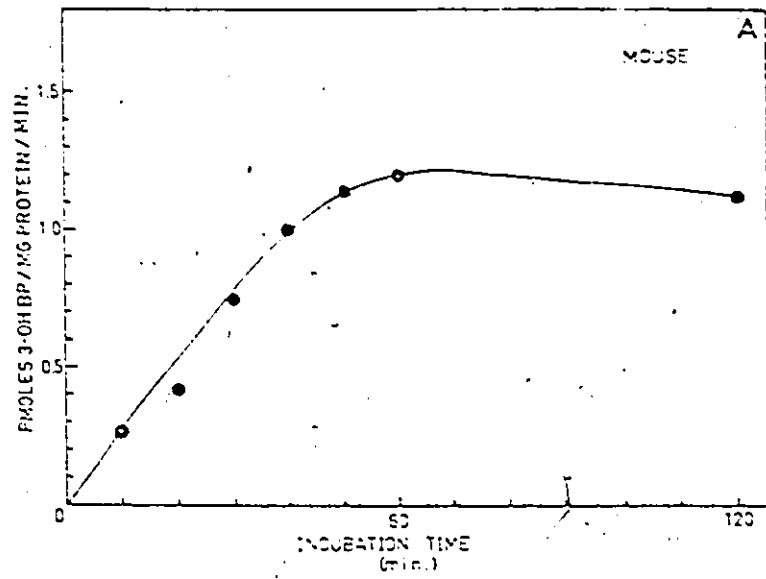
Figure 8. Effect of incubation time on tumour AHH activity.

A. Mouse Mammary Tumour. Each incubation flask contained 0.76 mg microsomal protein.

B. Rat Mammary Tumour. Each incubation flask contained 0.82 mg protein.

C. Human Mammary Tumour (specimen #1604). Each incubation flask contained 0.54 mg protein.





undertaken to further characterize the enzyme system present in mammary tumours with specific reference to levels and ranges of AHH activity, effects of inducing and inhibiting agents as well as any correlations between enzyme activity and host-associated parameters such as hormonal status and general health of the animal. The results are reported in the chapters following.

### CHAPTER III

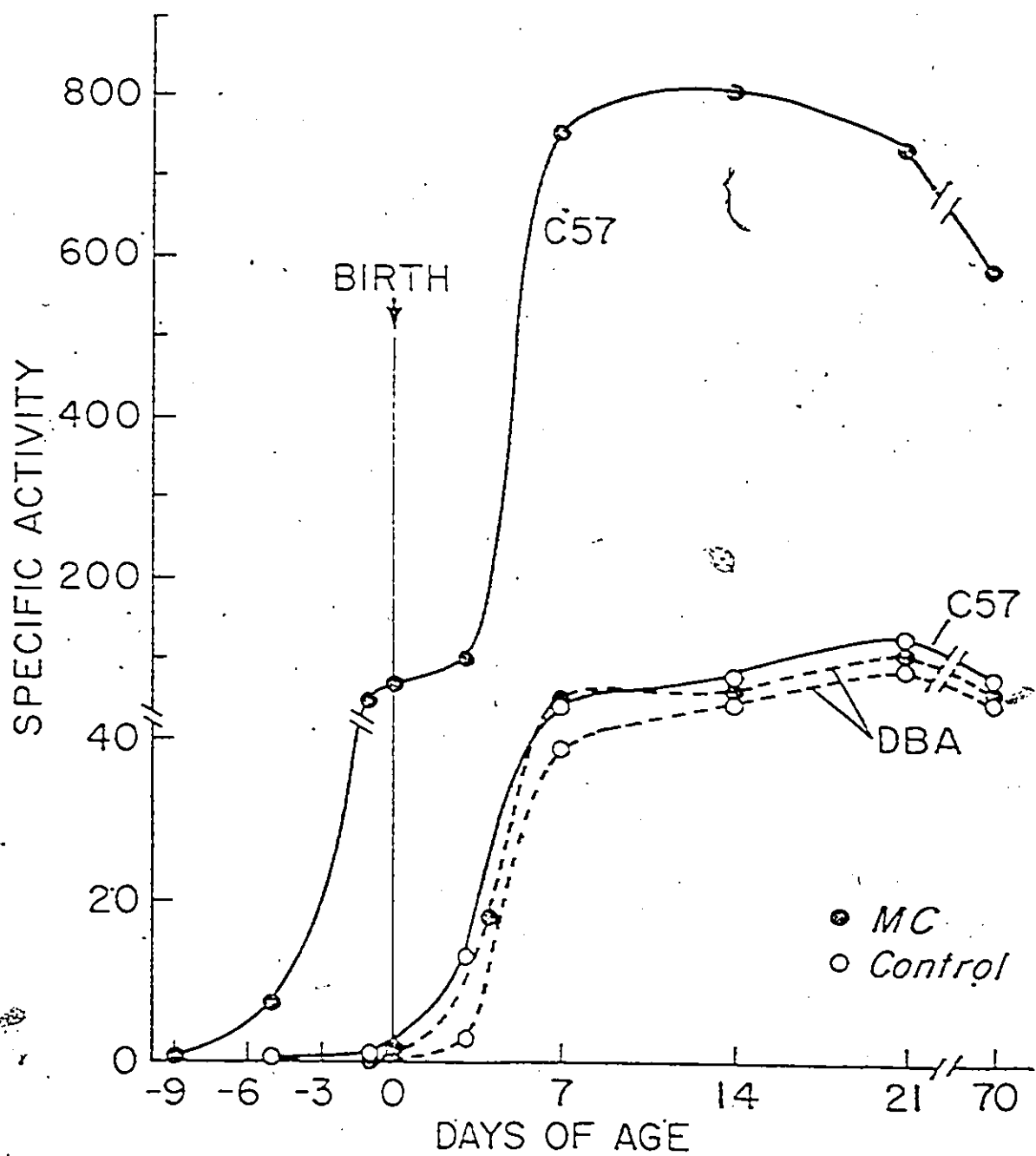
#### ARYL HYDROCARBON HYDROXYLASE IN MOUSE MAMMARY TUMOURS

The laboratory mouse has been the subject of extensive investigations regarding its drug-metabolizing enzyme systems (reviewed by Thorgeirsson and Nebert, 1977). Genetically homogeneous inbred strains of laboratory animals provide a model with which to examine the patterns of inheritance both of basal and induced AHH levels. The results of such studies using intercrosses and backcrosses between "responsive" (C57BL/6-type) and "non-responsive" (DBA/2-type) strains indicated that the genetic regulation of AHH induction in hepatic microsomes ("responsiveness") by polycyclic hydrocarbons is quite complex and controlled by at least three alleles at two nonlinked loci (Nebert et al, 1975).

Figure 9 demonstrates the extent of hepatic AHH induction by 3-methylcholanthrene in B6 and D2 mice as a function of age. As can be seen from the graph, the basal (or constitutive) form of the enzyme develops at the same rate in both mouse strains. The age-dependent response of AHH induction by MC never appeared in the "non-responsive" mouse strain as it does in the "responsive" mouse strain.

Although the predominant tissue of interest was liver, Nebert's group (Gielen et al, 1972) along with others (Wiebel et al, 1973; Burki et al, 1973; Abramson et al, 1977) became interested in extrahepatic tissues, particularly those associated with portals of entry such as lung, intestine, and

Figure 9. Hepatic levels of the basal aryl hydrocarbon hydroxylase (AHH) activity and of the enzyme activity in response to methylcholanthrene (MC) treatment of C57BL/6N (C57) and DBA/2N (DBA) mice, as a function of age. Each filled circle (●) represents the mean hydroxylase specific activity from individual livers of 6 to 15 mice 24 hours after the intraperitoneal administration of 80 mg/kg body weight MC. Each open circle (○) represents the mean activity from individual livers of 5 to 12 mice 24 hours after treatment with corn oil alone. The filled circles depicting AHH before birth represent the average specific activity found in 5 or more livers from a litter of fetuses whose mother had received MC 24 hours before. The filled circles on day zero indicate the mean activity from individual mice born within 24 hours after their mother received MC. Specific activity on the ordinate represents pmoles 3-OHBP formed per minute per mg protein of total liver homogenate. Taken from Nebert et al (1972).



skin. The enzyme levels in these tissues are of particular importance as they may activate (or inactivate) potentially harmful substances at their site of entry. Further, these tissues are targets for chemically-induced tumourigenesis (reviewed by Nebert and Jensen, 1979). Some values for AHH activity in mouse hepatic and nonhepatic tissues are summarized in Table 1.

It was shown that parental or dietary administration of known hepatic AHH inducers increases AHH in certain mouse tissues even in "hepatic-non-responsive" animals (Wiebel et al, 1973 and Burki et al, 1973). Genetic control of AHH induction may differ for hepatic and nonhepatic tissues. However, in inbred mouse strains there is a strong positive association between high hepatic AHH levels in induced, genetically "responsive" animals and high inducible levels in lung, skin, bowel, and kidney (Robinson et al, 1974).

Although it is well established that polycyclic hydrocarbons increase the incidence of mammary cancer in certain strains of mice (Russfield, 1966), mammary tissue was not examined for relative basal and induced levels of AHH activity until 1976 by Chuang and Bresnick. In an attempt to correlate the ease of MC-induced mammary tumourigenesis with the basal or induced specific activity of AHH, Chuang and Bresnick examined AHH levels in highly resistant strains such as the B6 mouse and a highly susceptible strain,  $A^+/K_i$ , as well as in intermediate types. Their results indicated little or no correlation between induced and basal levels (respectively) of AHH activity and the degree of susceptibility to MC-induced tumourigenesis.

Table 1. Inducibility of AHH in Hepatic and Extrahepatic Tissues in "Responsive" (C57BL/6N) and "Non-Responsive" (DBA/2N) Mouse Strains<sup>1</sup>

	C57BL/6N		DBA/2N	
	Basal	BA Induced <sup>2</sup>	Basal	BA Induced
Liver	51.67 $\pm$ 5.0 <sup>4</sup>	192 $\pm$ 16.67	30.66 $\pm$ 3.0	33.17 $\pm$ 5.17
Lung	6.93 $\pm$ 2.17	18 $\pm$ 3.3	1.87 $\pm$ 0.5	11.67 $\pm$ 1.0
Kidney	0.08 $\pm$ 0.01	4.7 $\pm$ 0.53	0.77 $\pm$ 0.01	0.27 $\pm$ 0.06
Skin <sup>3</sup>	2.1 $\pm$ 0.83	19 $\pm$ 6.33	1.2 $\pm$ 0.03	4.3 $\pm$ 0.43

1. From Wiebel et al, 1973.
2. Benzanthrane (100 mg/kg) injected i.p. in 0.2 ml corn oil 12 hours before sacrifice.
3. Induced by topical application of 300  $\mu$ g BA in 0.2 ml acetone to shaven backs of mice 16 hours before sacrifice.
4.  $\bar{x} \pm$  S.D. of specific activity (pmoles 3-OHBP/mg protein/minute).

They attribute the lack of correlation to the multiplicity and complexity of factors responsible for mammary tumourigenesis in the chemically-induced model. These factors include the host's hormone status, genetic constitution, and activation of latent mammary tumour viruses known to be involved in mouse mammary tumourigenesis.

Further work by this group (Chuang et al, 1977), using mammary cell lines, attempted to circumvent some of the above-mentioned factors. The cell lines arising from virgin or normal lactating tissue, malignant fibroblastic or epithelial tumours, and from hyperplastic alveolar nodules of mammary tissue were examined for their response to MC-type inducers in culture. The cells were examined for their response to an in vitro inhibitor, ANF, in addition to inducing agents. All lines were responsive to AHH induction by MC, and ANF produced 50 per cent inhibition in the specific activity of basal and induced AHH activity. Furthermore, one responsive tumour cell line, when innoculated into the non-responsive host strain, yielded mammary tumours which were not responsive to MC administered intraperitoneally.

The present study was undertaken to ascertain if AHH was present in a variety of mouse mammary tumours, arising from different methods of tumour induction. These included hormone-induced tumours, using DES, or chemically-induced tumours, using BP or DMBA, or tumours arising "spontaneously" in normal breeding inbred mice or found in captured wild Mus musculus. Tumour AHH activity was also examined in a



number of different strains of mice. In addition, serial transplantations of DES-induced tumours and spontaneously-arising tumours were performed so that the effect of such procedure on AHH activity could be evaluated. The effects of BNF and ANF were monitored in most tumours and AHH activity and responses to these agents compared to those seen in normal mammary tissue.

#### A. MATERIALS AND METHODS

##### 1) Animals and Tumour Induction

Mice used to provide a source of tumours for assay can be divided into three groups:

- a) C3H/HeJ mice - These mice were reared in our laboratory from stock originally purchased from The Jackson Laboratory, Bar Harbor, Maine. C3H/HeJ mice carry the mouse mammary tumour virus (MMTV<sup>+</sup>) which results in a very high incidence of mammary tumours (Gass et al, 1974): the so-called "spontaneous" tumours of a normal breeding population. Tumours in these mice routinely appeared between 9-12 months of age. Some of this stock, at four months of age, were isolated according to sex and begun on a diet containing 250 ppb of diethylstilbestrol (DES) into powdered mouse chow. Tumours in this group appeared at approximately 6 months of age and were removed up until one year of age. Previous histopathological investigators (Gass et al, 1974) have shown these tumours to be adenocarcinomas.

- b) Mus musculus - Female mice captured in the "wild" were maintained and bred under laboratory conditions. Upon tumour development the mice at approximately ten months of age were generously given to us by Dr. M. L. Petras.
- c) C3H/HeJ, RF/J, and DBA/2J - Inbred strains of female mice were obtained from The Jackson Laboratory for another study in our laboratory studying the susceptibility of different strains of mice to tumourigenesis by BP or DMBA. Starting at six weeks of age, mice were injected i.p. with 0.5 mg BP or DMBA over a 21 day period. Tumours first appeared at approximately six months of age and were removed up until one year of age.

Tumours generally were not allowed to exceed 10 per cent of the animal's body weight and normally weighed between 1-3 g. Removal at this stage generally ensured that the tumour was non-necrotic and firm and that the tumour had no apparent effect on the animal's general health (Kato et al, 1968). As the microsomal pellet obtained from DES-induced tumours was actually a "by-product" of cytosol preparation for use in estrogen-receptor analysis (Kuo, 1978 (thesis)), as part of this study, hormonal manipulations in the form of castration were sometimes performed on the animal. After castration the tumour was monitored for regression for up to two weeks before removal. DES, which has some inhibitive

properties on the AHH system, may or may not have been removed from the animal's diet. This will be indicated in figure legends.

## 2) AHH Induction in Mouse Mammary Tumours

In experiments testing the induction of AHH mice were injected i.p. with 80 mg BNF per kg body weight in corn oil for two consecutive days prior to sacrifice. In some mice, a section (0.5-1.0 g) of the tumour was removed under ether anesthesia and the incision closed with wound clips prior to injection of BNF. This served as the control enzyme value for this particular tumour specimen before induction by BNF.

## 3) Tissue Preparation

Tumour sections were removed, trimmed of necrotic portions, and rinsed in iced TDE buffer before freezing in a Revco ultracold freezer ( $-70^{\circ}\text{C}$ ) until day of assay. Animals were killed by cervical dislocation and tumours freed of any necrotic tissue and, as with livers removed from the same animal, minced and rinsed in iced TDE buffer. Frozen tumour sections were thawed on ice and processed as previously described in Chapter II to obtain microsomal suspensions. The samples were assayed as previously described in the same chapter.

## 4) Tumour Transplants

Tumour transplants<sup>2</sup> were done using DES-induced tumours and "spontaneously-arising" tumours of the C3H/HeJ strain. A portion of the tumour was assayed for AHH activity and the

remainder was cut into approximately 2 mm cubes weighing between 25-50 mg. These were rinsed in 0.9 per cent saline before transplantation. The mice used as transplant recipients were 6-8 week old female C3H/HeJ mice. Under ether anaesthesia small incisions (less than 1 cm) were made dorsally on each side of the vertebral column and a cube of mammary tumour placed subcutaneously and gently pushed forward to the axillary region. The incision was closed with wound clips. Virtually 100 per cent of the animals developed 2 mammary tumours 2-3 g in weight after an eight week period. These tumours were then assayed for AHH activity and, in some cases, used as a source for the next generation of transplants. AHH activity was then compared in "first" and "second" generation transplants to the original tumour.

## B. RESULTS

### 1) Mouse Mammary Tumour AHH Activity

The results obtained from the different strains of mice and sources of tumours are depicted in Figure 10. The results indicate that mouse mammary tumours have detectable MFO activity, as measured by the AHH assay (see Table 2). The basal level of tumour AHH activity in all tumour types appeared to exceed the basal AHH levels in normal mouse mammary tissue (Chuang and Bresnick, 1976). The degree of AHH induction by BNF was greater in all tumour types than in normal mammary tissue with the exception of tumours appearing

Table 2. Summary of AMH Activity in Mouse Mammary Tumours

	Basal AMH Activity <sup>1</sup>			Induced AMH Activity <sup>2</sup>		
	Control	$\pm 10^{-6}$ h AMF	% of Control Activity	Control	$\pm 10^{-6}$ h AMF	% of Control Activity
DES-Induced Tumours - C3H	0.601 $\pm$ 0.18 (34)	0.340 $\pm$ 0.107 (34)	45			
Spontaneous Tumours - C3H	1.718 $\pm$ 0.49 (12)	0.864 $\pm$ 0.25 (12)	50	21.18 $\pm$ 10.79 (4)	6.110 $\pm$ 2.60 (4)	29
Wild Chemically-Induced Tumours	1.726 $\pm$ 0.58 (18)	0.892 $\pm$ 0.26 (10)	50	11.72 $\pm$ 6.69	5.151 $\pm$ 2.77	46
Responsive - C3H	1.042 $\pm$ 0.24 (12)	0.634 $\pm$ 0.19 (12)	40			
Nonresponsive - B6A+BP	1.696 $\pm$ 0.35 (26)	1.552 $\pm$ 0.50 (26)	92	2.129 $\pm$ 0.69	1.189 $\pm$ 0.31	55

1. Specific activity - pmoles 3-OHMP/3 proteins/mililitre.

2. Induced - AMH activity after 80  $\mu$ g AMF/kg injected i.p., 48 hours prior to sacrifice.

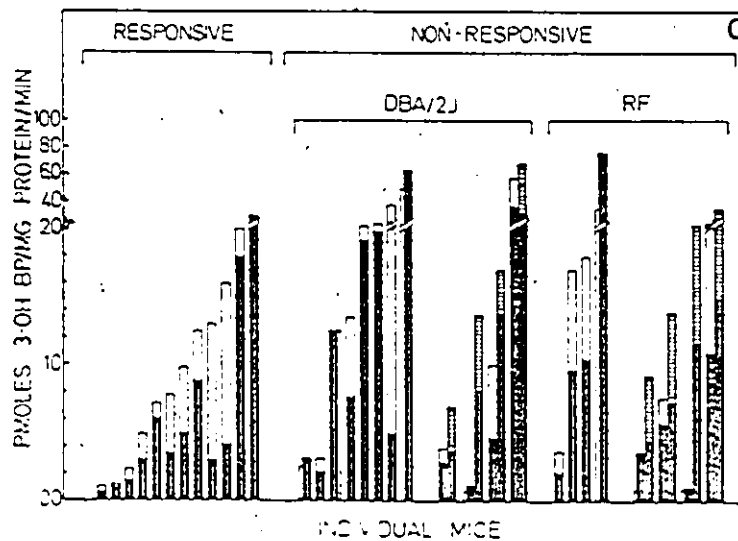
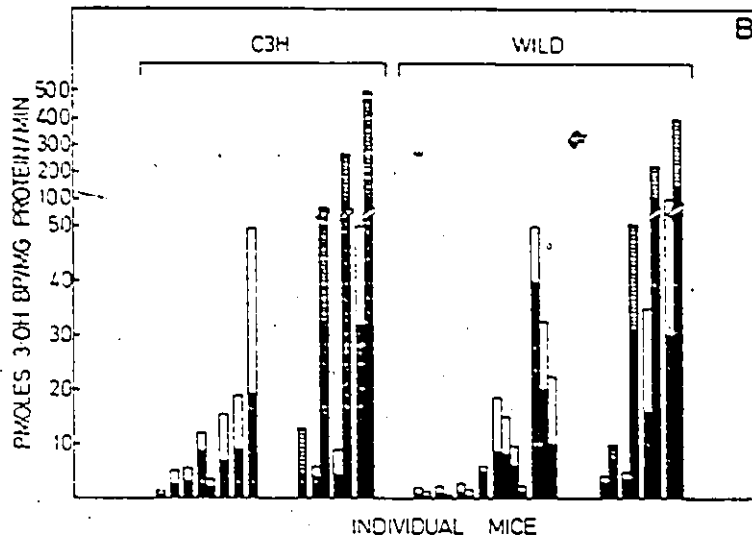
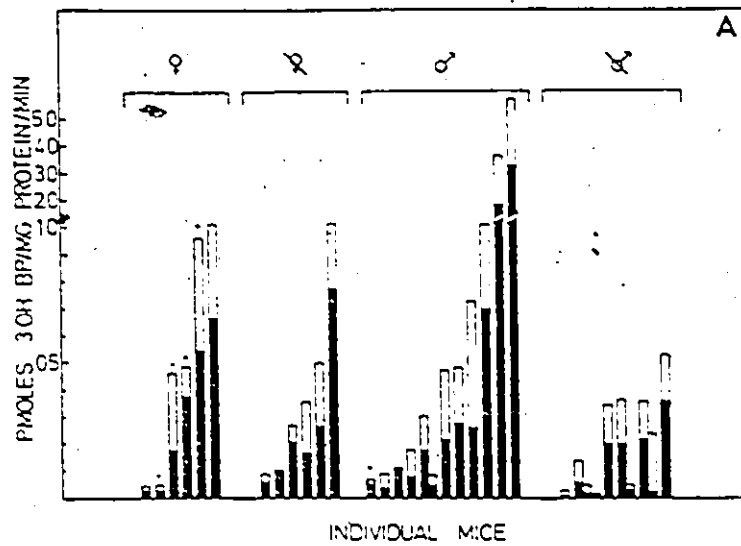
3. Mean value statistically greater than that in DES-induced tumours ( $p < 0.05$ ).

Figure 10. AHH activity in individual mouse tumours. Open bars ( □ ) represent AHH activity and lined bars ( ▨ ) represent BNF-induced activity. The solid portion of each bar ( ■ ) represents enzyme activity after addition of  $10^{-6}$  M ANF to the incubation medium. Contiguous bars represent multiple tumours from the same mouse. "Sectioned" tumours are represented by adjacent basal and induced values.

A. AHH activity in DES-induced mammary tumours of C3H/HeJ mice. The tumours are divided into 4 separate groups: those appearing on male or female mice and in castrated or intact mice. Mice still on the DES-diet at time of sacrifice are indicated by an asterisk.

B. AHH activity in "spontaneously-arising" mammary tumours. AHH activity was measured in spontaneously-occurring tumours of both an inbred laboratory strain, C3H/HeJ, and in wild mice. The BNF-induced tumours are set apart from the non-treated tumours.

C. AHH activity in BP- or DMBA-induced mammary tumours of responsive mice were all of the C3H/HeJ strain. None of this strain was treated with BNF. The non-responsive mice were either of the RF/J or DBA/2J strain. Mice that were "sectioned" then BNF-treated are separated from control mice.



in "non-responsive" strains of mice. Each tumour type will be discussed under separate headings:

- a) DES-Induced Mammary Tumours - The results of AHH activity in DES-induced mouse mammary tumours appears in Figure 10A. The results indicate that the degree of variation in range of activity in tumours was similar regardless of sex or hormonal manipulations. Whether the animal was still on DES-diet at time of sacrifice appeared to have no effect on AHH activity of the tumour. Statistical analysis of the data for each group (male or female, castrated or intact) indicated no significant differences in AHH activity (data not shown). Although no sex differences in drug metabolism are normally seen in the mouse (unlike the rat (Kato et al, 1974)), Nebert et al (1970) did report a lowering of basal AHH activity and magnitude of AHH induction by MC in hepatic tissues as a result of castration. Estradiol treatment was found to further lower AHH activity. The range of variability and small sample size preclude the detection of any such effects.

Since there were no significant differences between groups of mice and as all tumours were induced by DES diet, the data from both sexes and treatment groups were pooled and statistics summarized in Table 3 performed on the pooled data. Addition of ANF to the incubation mixture inhibited tumour



Table 3. Effect of DES Diet and Castration on AHH Activity  
in DES-Induced C3H/HeJ Mouse Mammary Tumours and  
Corresponding Livers

Treatment	Tumour	Liver
- off DES	$0.504 \pm 0.462$ (2) <sup>1</sup>	$128.72 \pm 78.24$ (2)
- on DES	$0.538 \pm 0.231$ (4)	$271.28 \pm 68.44$ (4)
- castrated <sup>2</sup>	$2.073 \pm 1.65$ (7)	$202.96 \pm 45.06$ (6)
- off DES	$1.235 \pm 0.576$ (10)	$107.03 \pm 20.78$ (10)
- on DES	$0.135 \pm 0.044$ (2)	(0)
- castrated	$0.208 \pm 0.057$ (10)	$151.68 \pm 17.65$ (6)

1.  $\bar{X} \pm \text{SEM}$  (n) specific activity (pmoles 3-OHBP/mg/minute)

2. - all castrated animals were not on DES-diet at time of assay.

AHH activity 55 per cent of control. This degree of inhibition is similar to that seen in nonhepatic tissues (Wiebel et al, 1973).

- b) "Spontaneously-Arising" Mammary Tumours - Data from two groups of spontaneous mouse tumours, from C3H and wild mice, were analyzed separately due to dissimilarities in enzyme patterns. As seen in Figure 10B, the range of activity and degree of inhibition was essentially the same for the two mouse types. The mean basal AHH activity given in Table 2 was the same for both mouse types. The two groups of mice differed in the degree to which AHH activity in the tumour could be induced by BNF; C3H mice induced-tumour AHH levels are 12-fold greater than their respective basal levels while tumours appearing in the wild mice were induced 7-fold over basal levels. The degree of inhibition by ANF, although greater in induced tumours from BNF-treated mice than in untreated tumours (30-35 per cent of control as compared to 50 per cent of control) was the same for both types of mice.

The enzyme levels of the "spontaneously-arising" tumours were higher than the basal levels seen in DES-induced tumours, but the degree of inhibition by ANF was roughly the same.

- c) Chemically-Induced Mammary Tumours - The two different

groups of chemically-induced tumours, those appearing in the "responsive" strain (C3H) and those in the "non-responsive" strains (RF and DBA), showed dissimilar patterns of enzyme activity. Although the non-responsive mouse tumours showed higher basal levels than did the responsive tumours, ANF had little effect (92 per cent of control) and in some samples, even enhanced AHH activity over control values (see Figure 10C) in the tumours from non-responsive mice. ANF appeared to inhibit AHH activity almost to the same degree (40 per cent of control) in chemically-induced tumours of the C3H strain as it did in DES-induced or spontaneous tumours of the same strain. No data were available for BNF-induction of AHH activity in chemically-induced tumours of the C3H strain as these mice were part of a concurrent laboratory study being conducted by K. C. Silinkas. Because of similarities in levels of AHH activity and response to ANF in tumours from DES-treated mice and in spontaneous tumours as compared to that seen in chemically-induced tumours, it is felt that an induction of AHH as a result of BNF treatment in chemically-induced tumours is a likely event. Assuming this to be true, the fold-inducibility ratio of 1.4 over basal levels as seen in chemically-induced tumours of the non-responsive strains appeared low.

Mice basically are classified into two categories in regard to genetic regulation of AHH: If AHH activity in liver is increased more than 2.5-fold over basal levels within 24 hours of treatment with an inducer, they are classified as aromatic hydrocarbon responsive. If AHH activity is increased less than 1.5-fold, mice are classified as AHH non-responsive. Based on this criterion, tumours appearing in non-responsive mice would be classified as non-responsive unlike other extrahepatic tissues such as kidney, lung, and skin (see Table 2) which have been reported to exhibit greater than 2.5-fold induction (Wiebel et al, 1973).

When the mean activity of the different mice tumour groups were statistically compared using the Student's "t" test, the AHH activity detected in "spontaneously-arising" tumours was significantly higher ( $p < .05$ ) than values detected in DES-induced tumours. No statistically significant differences were detected between other groups (from Table 2).

## 2) Tumour Production

Although most mice bore only one tumour, 20 per cent had two or more mammary tumours. A summary of activities in tumours of multiple tumour-bearing mice appears in Table 4. This provided a unique opportunity to study enzyme activity in separate distinct tumours on the same animal subject to the same host influences. In Figure 10, contiguous bars represent tumours from the same animal (where basal and

Table 4. Variation of AHH Activity in Multiple Mammary Tumours of the Mouse

Mouse	Individual Tumour AHH Activity		
	i	ii	iii
DES-Induced			
#26	0.054 <sup>1</sup>	0.353	
#27	0.353	0.240	
#28	0.301	0.086	
#30	0.008	0.039	0.134
Spontaneous			
#20	0.012	0.222	
#21	0.030	0.204	
#22	0.063	0.036	
#32	0.165	0.081	
#43	4.98	2.13	
#53	2.21	3.22	6.24
#55	0.177	0.948	1.49
#62	1.23	0.350	

1. Specific activity (pmoles 3-OHBP/mg protein/minute).

induced samples are adjacent, these represent "sectioned" tumours rather than multiple tumours). There was a large variation in activity within multiple tumours from the same animal. The tumours could be as close as 20 per cent of one another or as different as 20-fold greater in activity. Statistical analysis utilizing the ratio of variances seen in enzyme values from one mouse to that of the overall mouse tumour population, indicated no statistical differences in variation of multiple tumours from one mouse as compared to the variation seen in the overall tumour population.

### 3) AHH Activity in Livers of Tumour-Bearing Mice

Liver AHH data are available for DES-induced and spontaneously-occurring tumour-bearing mice and are summarized in Table 5. The basal levels of AHH in livers from C3H tumour-bearing mice, whether DES-induced or spontaneously-arising, were similar in AHH activity and ANF caused the same degree of enhancement of AHH activity in both groups of mice. This stimulation of basal enzyme activity in responsive strains of mice by ANF is a well-documented phenomenon (Goujon et al, 1972) and is attributable to a preponderance of cytochrome P-450 (the PB-inducible form) in untreated mice as compared to the presence of cytochrome P<sub>1</sub>-450 (the MC-inducible form) in BNF-treated mice. Contrasting with this was the degree of inhibition (40 per cent of control) seen in BNF-induced livers in the presence of ANF.

The basal levels of liver AHH activity in mice bear-

Table 5. Summary of AHH Activity in Livers of Tumour-bearing Mice

	Basal AHH Activity <sup>1</sup>			Induced AHH Activity <sup>2</sup>			Fold Induction
	Control	+ $10^{-6}$ M AHP	% of Control Activity	Control	+ $10^{-6}$ M AHP	% of Control Activity	
BES-Induced (C3H)	163,36119.73 <sup>3</sup> (26)	192,68121.98 (26)	120				
Spontaneous -C3H	102,70144.77	115,51153.50	115	1041.711188.9	671.461129.6	50%	10
-Wild	369,041138.7	528,681188.7	140	1111.451122.6	692.80118.64	62%	

1. Specific Activity - pmoles 3-OHP/ug protein/minute.

2. Induced - AHH activity after 80 ug AHP/kg injected i.p. 48 hours prior to sacrifice.

3.  $\bar{x} \pm$  SEM(n)

ing spontaneous tumours were different, with the "wild" mice demonstrating 3.5 times higher AHH activity than the C3H strain. The maximal activity was the same in both types of mice but because the basal enzyme level was higher in the "wild" mice, only a 3-fold induction over basal levels by BNF was seen as compared to a 10-fold induction over basal levels in livers of the C3H strain.

#### 4) Comparison of Tumour Microsomal Protein Yields and DNA Content

Total microsomal protein yield was determined as a product of protein concentration of the microsomal suspension times the volume of the suspension divided by the tumour weight. This calculation generated approximately the same value for all tumour groups, whether DES- or chemically-induced or spontaneous (10 mg microsomal protein per g tumour). This value was somewhat lower than that derived for mouse livers (14 mg microsomal protein per g liver).

Some of the mouse tumours were assayed for DNA concentration using the ethidium-bromide method of Beers and Wittliff (1975). AHH activity was expressed per  $\mu\text{g}$  DNA as well as per mg microsomal protein. Although this generated a slightly different specific activity for each tumour, the fold-inducibility ratio of induced AHH levels over basal levels was 11-fold when expressed per mg protein and 10.5-fold for  $\mu\text{g}$  DNA. The enzyme values expressed per  $\mu\text{g}$  DNA were highly correlated ( $r = 0.93$ ,  $p < 0.05$ ) with values expressed per mg protein. The levels and ranges of tumour



AHH activity were the same when expressed per  $\mu$ g DNA as with mg protein when compared between groups of tumours.

#### 5) AHH Activity in Serially-Transplanted Tumours

Tumour transplants were done on four groups of mice using tumours from different sources. The conditions and results are summarized in Table 6. By inspection it appeared that the variation in the transplanted tumour AHH activity was as great and the range of levels as wide as in the original tumour population. Statistical analysis by comparison of ratios of variances from the original "parent" population to that of the transplant samples yielded an F value significant at the 0.05 level. This indicated that the enzyme values from transplanted tumour samples had significantly less variation than that of the "parent" tumour population. However, the "second generation" transplants had no less variation in activity than did the "first generation" transplants.

Other trends in the transplanted tumour samples included elevated AHH activity in the "second generation" transplants as compared to the original tumour population from both DES-induced and spontaneously-arising tumours after a drop in activity at the "first generation" transplant step. Repeated transplants using spontaneously-arising tumour as a source seemed to select for a tumour population showing stimulation of AHH activity in the presence of ANF whereas all other tumour transplants reflect the original tumour's susceptibility to inhibition by ANF.

Table 6. AMP Activity in Serially-Transplanted Mouse Tumours

Tumour Source	Source AMP		1st Passage AMP		Tumour Source		2nd Passage AMP	
	Control	$10^{-6}$ M AMP	Control	$10^{-6}$ M AMP	Control	$10^{-6}$ M AMP	Control	$10^{-6}$ M AMP
DES-Induced (♀) #31	0.068	0.051	0.031±0.11 (7)	0.167±0.04 (7)				
DES-Induced (♀) #54	0.546	0.327	2.42±1.41 (2)	1.43±0.31 (2)				
Spontaneous (♀) #60	0.549	0.458	0.413±0.09 (12)	0.752±0.17 (12)	1.131	2.06	0.772±0.31 (6)	1.80±0.27 (6)
DES-Induced (♀) #61	1.070	0.086	0.590±0.11 (9)	0.552±0.11 (9)	1.498	1.27	1.714±0.40 (11)	1.26±0.22 (11)

1. - AMP activity (picoles 3-OMP/mg protein/minute) in tumour used for transplantation.

2. - AMP activity in tissue tumours from first transplantation step.

3. - AMP activity in tissue tumours from first transplantation step.

4. - AMP activity in tumour used as source (from first transplantation) for second transplantation step.

5. - AMP activity in tumours of second transplantation step.

6) Lack of Correlations Between Tumour AHH Activity and Tumour Weight, Degree of Necrosis, or Corresponding Liver AHH Activity

Mouse tumours were examined for any parameters that might explain some of the variation in AHH activity observed.

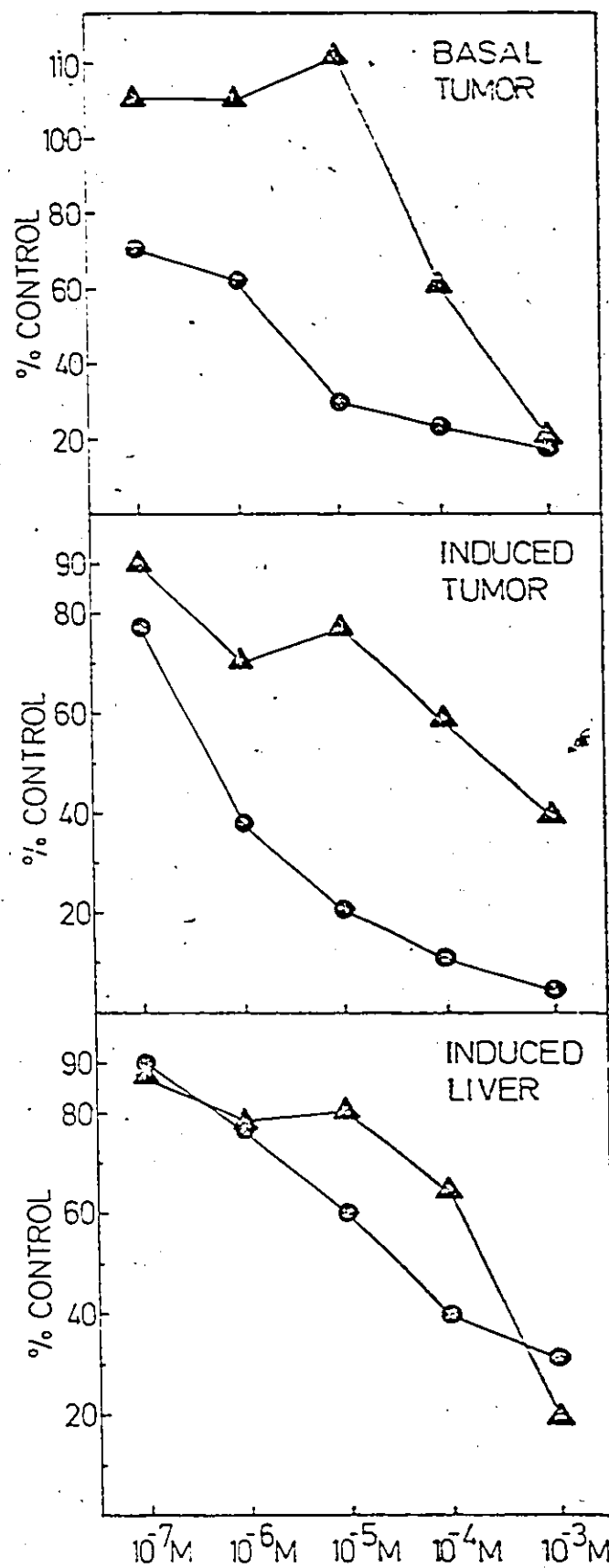
As tumours progressed in size they became more necrotic in the mouse. Tumours were graded for degree of necrosis based on a subjective scale where "0" denoted a healthy, firm, pink tumour and "3", a very necrotic, bloody, soft tumour and "1" and "2" intermediate states. No correlation existed between tumour AHH activity and tumour weight ( $r = -0.08$ ) nor was any detected between tumour AHH activity and degree of necrosis ( $r = 0.05$ ). Similarly, no significant correlation was detected between tumour AHH activity and AHH activity in corresponding livers of tumour-bearing mice.

7) Effect of Cytochrome P-450-Mediated Mono-Oxygenase Inhibitors on Tumour AHH Activity

The effects of two different inhibitors of AHH activity were examined, over a range of concentrations, on basal and induced tumour microsomal suspensions. A microsomal preparation from a BNF-induced liver of a tumour-bearing mouse was examined for comparison. The results are given in Figure 11.

Several laboratories have been involved with elucidation of the mechanism of action of ANF inhibition of AHH activity. Zampaglione and Mannering (1973) reported that ANF inhibition of AHH activity in extrahepatic tissues (intestinal mucosa and adrenal) was associated with a preponderance of microsomal cytochrome "P-448" ( $P_1$ -450). Furthermore, experiments with partially

Figure 11. Effect of ANF or metyrapone on AHH in microsomes from mammary tumour or liver from a C3H/HeJ mouse. AHH activity is expressed as per cent of the control flask to which 50  $\mu$ l methanol, the solvent for both inhibitors, was added. The level of AHH activity after addition of ANF to the incubation medium is represented by the closed circles (●) and metyrapone, by closed triangles (▲). The basal tumour incubations contained 140  $\mu$ g microsomal protein, the induced tumour, 430  $\mu$ g protein, and induced liver, 560  $\mu$ g protein per incubation flask.



purified and reconstituted microsomal preparations indicated that the inhibitory and stimulatory effect of the flavone resides in the cytochrome component of the enzyme system.

There is some evidence (Goujon et al, 1972) that benzoflavones are substrates for the microsomal mixed-function oxygenase system. Spectral analysis of the reaction by Goujon indicated that inhibition of AHH activity by ANF might be attributed to ANF competing with the polycyclic hydrocarbon (BP) substrate for the enzyme active site. Kinetic analysis of the reaction over a wide concentration range of ANF revealed anomalies suggesting that the inhibitor may interact with the enzyme system in more than one way.

Where ANF represents a class of inhibitors affecting primarily cytochrome  $P_1$ -450, metyrapone represents a class of inhibitors affecting primarily cytochrome P-450. These two inhibitors were chosen in the hopes of distinguishing between the two forms of AHH thought to be present in mouse tumours. Results from inbred mouse strains could then serve as a reference when inhibitor effects were examined in human mammary tumours. As expected from results of the routine addition of ANF to the incubation medium, the  $P_1$ -450 form of the enzyme predominated in both basal and induced mouse mammary tumours as well as in induced mouse liver. Both basal and induced tumour microsomes compared closely with the pattern observed in induced mouse liver.

The induced form of the enzyme seems to be more susceptible to inhibition by ANF than does the basal form, with 50 per cent of control inhibition occurring at  $2 \times 10^{-6}M$  for

the basal form. This argument is strengthened by the stimulation of basal enzyme activity at low concentrations of metyrapone and incomplete inhibition of induced forms at the lower concentrations in the presence of metyrapone. Apparently, instead of competing with the  $P_1$ -450 form of the enzyme, it is interfering with the substrate interaction with the enzyme. Comparing the effect of metyrapone on the basal form of the enzyme and on the induced form, it is apparent that BNF causes induction of cytochrome P-450 in addition to the predominantly-formed cytochrome  $P_1$ -450, if one assumes that metyrapone and ANF are capable of distinguishing the two forms of the enzyme.

#### D. DISCUSSION

The microsomal suspensions from mouse tumours whether DES- or chemically-induced or spontaneously-arising, possess low levels of AHH activity, typically 2-6-fold greater than levels reported in normal mouse mammary tissue but approximately 1.0 per cent of the activity reported for the mouse liver.

The capacity of mouse tumour AHH activity to be enhanced by BNF treatment is equal to normal mouse mammary tissue, ranging from 3-12-fold. This upper limit compares to the fold-inducibility ratios seen in mouse liver although absolute AHH activity in mouse mammary tumours is much lower. The exception to this is the tumours from the non-responsive strain, which based on their response to BNF, would have to be classified as non-inducible.

The degree of inhibition by ANF observed in the constitutive or basal form of the enzyme (between 40-50 per cent of control) was very consistent for all mouse tumour types, with the exception of tumours from the non-responsive mouse strains. In tumours from "non-inducible" mice ANF had little or no effect, except after the animal had been treated with BNF. AHH activity then was inhibited 50 per cent of control values as compared to 30-35 per cent of control observed in tumours from BNF-treated responsive mice.

Although consistency of response to both inducing and inhibiting agents existed within groups of mice tumours, with the exception of tumours from the non-responsive strains, different levels of basal and induced AHH activity existed when groups of tumours were compared. The basal AHH activity in the DES-induced tumours was approximately one-half of that observed in the spontaneously-occurring tumours of the C3H strain and wild mouse. The AHH activity in chemically-induced tumours were intermediate in value.

Tumours in a single strain of mice, C3H, therefore, had different enzyme activities depending on the tumourigenic agent. Although DES itself is a strong inhibitor of hepatic mono-oxygenase activity (Goujon et al, 1972), only 6 out of 34 animals were still on a DES diet at the time of assay. Although Allaben and Gass (1978) reported a significant increase in hepatic benzo[a]pyrene hydroxylase in rats fed 250 ppb DES in their diet no such increase was seen in the mice in this study. A search of the literature revealed no information on the long-term effects of prolonged feeding of



DES on MFO activity in extrahepatic tissues of the mouse. The fact that DES can have an effect on hepatic AHH activity suggests that the stilbene nucleus interacts with hepatic cytochrome P-450. In view of the findings by Herbst et al (1975) associating DES therapy of mothers during pregnancy and vaginal adenocarcinoma in the offspring at puberty, a study of the metabolic activation of DES and interaction with cytochrome P-450, especially that present in mammary tissue, could reveal some information about the mechanism of mammary tumourigenesis in the C3H mouse.

It was observed that BNF-induced enzyme levels in spontaneously-occurring tumours were greater in the C3H strain than levels in wild mice. As no data were available regarding the degree of BNF induction in DES-induced tumours or chemically-induced tumours of the C3H strain, it is not known whether the above difference is a result of genetic differences between two mouse types or due to activation of a latent mammary tumour virus in the C3H mouse.

Regardless of differences seen in tumour types, the form of enzyme found in the tumours of responsive mouse strains resembled the form characterized in other extrahepatic tissues from both control and BNF-treated animals (Gieleń et al, 1972 and Wiebel et al, 1973). This conclusion is based on response to BNF treatment in the mouse and the presence of ANF in the incubation medium. It is concluded then that the mechanism of induction by BNF (see Chapter I) and the earlier described competitive process by which ANF inhibits AHH activity was the same for neoplastic tissue as it was for normal tissue.

Whereas tumour enzyme activity patterns from responsive strains of mice conformed to those reported in the literature for extrahepatic tissues, the tumour enzyme activity observed in non-responsive tumours did not. Although basal enzyme levels in tumours of non-responsive mice compared closely to levels seen in other tumour types, the enzyme in this strain responds differently to both BNF and ANF. As indicated in Table 1 extrahepatic tissues such as skin, lung, and kidney of non-responsive mice are inducible by MC-type inducers, but the liver is not. Tumours from these mice cannot collectively be classified as AHH-inducible tissues as they demonstrated a fold-inducibility ratio of only 1.4, although individual tumours sometimes demonstrated up to 7-fold induction. This is in agreement by results obtained by Chuang and Bresnick (1976) using normal mammary tissue from non-responsive strains of mice (AKR/J and A<sup>+</sup>/Ki). Normal mammary tissue from AKR/J mice showed 1.4-fold induction after MC treatment and the A<sup>+</sup>/Ki strain, 1.9-fold induction. Assuming the same genetic regulation of AHH activity in non-responsive strains examined in this study (DBA/2J and RF/J), neoplastic transformation has not caused this altered response, the same pattern presenting itself in normal mammary tissue. This suggests similarities in the basal form of the enzyme found in liver and in normal and neoplastic mammary tissues of non-responsive strains of mice.

This similarity is strengthened by the fact that the basal form of the enzyme found in tumour tissue showed the same response to ANF as does the liver (Goujon et al, 1972), where little or no inhibition is seen. A discrepancy exists,

however, when the effect of ANF on MC-treated liver and BNF-treated tumour is compared. Goujon et al (1972) reported that ANF had no effect on AHH activity in MC-treated livers of DBA/2N mice, whereas this study revealed (see Table 2) that the AHH activity in tumour microsomes from BNF-treated non-responsive mice was inhibited to almost 50 per cent of control activity after BNF treatment.

Another study by Chuang and Bresnick (1977) using mammary cell lines, found that a cell line derived from a spontaneous adenocarcinoma in A<sup>+</sup>/Ki mice (a non-responsive strain) showed AHH induction when exposed to BA in cultures. However, when the same tumour cells were innoculated into A<sup>+</sup>/Ki mice, the AHH activity of the resulting mammary tumour was unaltered by MC treatment. Some aspect of the host environment must be acting to regulate the form of AHH enzyme expressed in non-responsive mice and the mode of regulation differs in extrahepatic tissues such as skin, bowel, and lung as compared to liver, normal mammary tissue, and tumour. Although data concerning the effect of ANF on the BNF- or MC-treated forms of non-responsive normal mammary tissue are lacking, perhaps in the process of tumorigenesis regulation has been altered so that ANF can inhibit AHH present in transformed mammary tissue.

Analysis of liver AHH activity in tumour-bearing mice revealed no differences from AHH activity in livers of mice reported in the literature. The basal and induced levels as well as the patterns of induction and inhibition are as reported by Goujon et al (1972). One anomaly of interest was

the slightly elevated basal AHH levels observed in wild mice which had been kept under laboratory conditions for some time. This may reflect differences in genetic constitution. Little is known presently about genetic regulation of cytochrome P-450-mediated mono-oxygenases in wild populations of mice. In this study, induction by BNF yielded comparable maximal values in both C3H and wild mice so that the fold-inducibility ratio for wild mice was only 3-fold whereas C3H mice showed 10-fold induction.

In an attempt to explain variation seen in AHH activity in mouse mammary tumours, correlations between tumour weight, degree of necrosis, and liver AHH activity were examined. The lack of correlation to tumour weight or degree of necrosis indicates that some other variable not examined may better explain variation observed.

Studies indicate that a range of enzyme activities exist for any tissue from an inbred animal (Thorgeirsson and Nebert, 1977); mammary tumour tissue was no exception. Nebert and Gielen (1972) reported for mice that there was a high correlation in the magnitude of enzyme induction in the liver and the kidney. This, however, was not the case for tumour and liver AHH activity measured in this study. It is not known if this might be attributed to the tumourigenic process.

Analysis of AHH activity in multiple tumours from the same mouse revealed that there was no significantly less variation in these tumours when compared to the overall mouse tumour population. Although differences in enzyme activity were observed in tumours produced by different induction

methods such as DES- or chemically-induced or spontaneously-arising, one might expect that tumours developed as a result of one process and moderated by the same host influences might have demonstrated less variability than that of the overall tumour population. A consideration of the theory of the monoclonal origin of tumours may aid in the understanding of results.

The concept that neoplasms frequently develop as a clone from a single cell of origin is one with widespread acceptance (reviewed by P.C. Nowell, 1976). A model for tumour evolution would include tumour initiation in a normal cell by an induced change making it neoplastic thus imparting to it a selective growth advantage over normal lines. This step would be followed by neoplastic proliferation. As a result of genetic instability in the expanding tumour population additional mutant cells may arise. Most of these variants will be eliminated due to metabolic disadvantages or immunologic destruction but one subline with selective advantage will become the precursor to a new predominant sub-population. Over time there is sequential selection of sublines which are increasingly abnormal. As this sequence is not completely random, different tumours will exhibit some similarities as they grow; but divergence also occurs as local conditions in each neoplasm differently affect the emergence of variant sublines. Thus, although the site of initiation (mouse mammary gland) and the initiating agent (DES or BP) are identical in each group, conditions within each neoplasm will dictate the characteristics of the fully developed tumour due to selective pressures. Yet,

because of the similarity of initial circumstances surrounding tumourigenesis, tumours produced do display some common qualitative characteristics such as response to inducing and inhibiting agents.

In the tumour transplant studies the variance observed in AHH activity of transplanted tumours was less than that of the overall or "parent" tumour population. This could be interpreted as evidence of selection of a stable sub-line of the original tumour leading to less variability in AHH activity in the transplanted tumours. Although no definite conclusions can be made concerning factors affecting AHH activity in mouse mammary tumours, it appears that, regardless of the characteristics of the original transformed cell, the host can exert some moderating influences on the tumour cell population with the outcome of stabilization of tumour properties detected.

One must consider the multitude of factors governing tumourigenesis in the mouse mammary gland to accept the variation observed in AHH activity of mouse tumours. Conditions within each neoplasm and host, regardless of similarity of strain or tumourigenic process will dictate the characteristics of the tumour. It is perhaps more surprising, considering the variety of strains and tumourigenic procedures, that enzyme levels and patterns of response to inducing and inhibiting should compare so closely. The lack of similar patterns in the non-responsive strains only follows from what is known about differences in the genetic regulation of the Ah locus in these strains.

Of importance, then, is that the mouse mammary gland has MFO activity and it is inducible in responsive strains. Although results by Chuang and Bresnick (1976) indicate that it is unlikely that the presence of an active MFO system affects the tumourigenic process in the mouse mammary gland, its presence might have some relevancy and experimental value for a study of the mammary gland as a site of potential activation of anti-neoplastic agents used in the management of human breast cancer.

## CHAPTER IV

### ARYL HYDROCARBON HYDROXYLASE

#### IN RAT MAMMARY TUMOURS

Aryl hydrocarbon hydroxylase has been extensively characterized in several rat tissues. Research has included studying the effects of classes of inducers and various inhibiting agents (Wiebel et al, 1971; Ciaccio and DeVera, 1975; Dent et al, 1977; Kitchin and Woods, 1978; Fysh and Okey, 1978). These investigators found the enzyme system present in hepatic tissue as well as lung, kidney, ovary, and mammary tissues. It was demonstrated that the enzyme was inducible following MC treatment of the animal. ANF was the most effective flavone derivative in distinguishing between two forms of AHH present in these tissues; hepatic AHH activity in MC-treated rats (associated with cytochrome P<sub>1</sub>-450) is strongly inhibited by ANF whereas the form of enzyme found in control or PB-treated rats (cytochrome P-450) is not inhibited by ANF and in some cases may exhibit "stimulation" of AHH activity. Results are summarized in Table 7.

The type of enzyme stimulated by ANF predominates in the livers of immature rats (data not shown) and of normal male rats. This type is inducible by PB. A second form which is inhibited by ANF comprises a large fraction of AHH activity detectable in normal adult females and is inducible by PAH. This form also predominates in extrahepatic tissues (Wiebel et al, 1971).



Table 7. Summary of AHH Activity in Different Rat Tissues

Rat Tissue	Basal AHH		MC-Induced AHH <sup>1</sup>		PB-Induced AHH	
	Control	+ANF <sup>2</sup>	Control	+ANF	Control	+ANF
Liver (♀)	18.6 <sup>3</sup>	4.7	306	42.0	not determined	
(♂)	100	142	20.77	10.39	same as control <sup>4</sup>	
Kidney <sup>5</sup>	0.24	0.132	25.63	9.74	same as control <sup>6</sup>	
Lung <sup>5</sup>	0.84	0.42	20.77	10.39	same as control <sup>4</sup>	
Ovary	1.10		3.87		not determined <sup>7</sup>	
Mammary - normal	0.04	0.025	0.81	0.37	same as control <sup>8</sup>	
- lactating	0.40		1.2		not determined <sup>9</sup>	

1. Induced AHH as a result of 80 mg MC (or BNF)/kg i.p.
2. ANF added in vitro to give final concentration of  $10^{-6}$  M.
3. Specific activity (pmoles 3-OHBP/mg protein/minute).
4. From Wiebel and Gelboin (1975).
5. No sex differences detected.
6. From Wiebel et al (1971).
7. From Mattison and Thorgeirsson (1978).
8. From Fysh and Okey (1979).
9. From Dent et al (1978).

Mammary tissue in rodents is highly susceptible to carcinogenesis by PAH, but has not undergone extensive investigation concerning its capacity to metabolize xenobiotic compounds. Huggins et al (1959) reported on the rapid induction of mammary tumours by 7,12-dimethylbenz-[a]anthracene (DMBA) and functional characteristics of the tumours which were evoked. More research has followed, especially concerning the hormonal regulation of initiation of mammary tumourigenesis in the rat (Dao, 1962). Ciaccio and DeVera (1975) reported that BP induced rat mammary gland AHH and Dent et al (1977) described the induction of mammary AHH in lactating rats fed polybrominated biphenyls. Fysh and Okey in two separate studies characterized the ontogeny of rat mammary AHH (1978) and examined AHH in mammary tissues throughout the course of pregnancy and lactation (1979) in an attempt to associate the age at which rats are most susceptible to PAH carcinogenesis with the mammary gland's ability to metabolize PAH's via AHH.

It was the purpose of this study to examine the AHH enzyme in rat mammary tumours induced by a single 15 mg dose of DMBA given orally. Initially, the enzyme activity was compared in two different buffer systems, TDE and  $KPO_4$ . The levels and range of AHH activity was then studied in tumour microsomal suspensions as well as in microsomes from normal mammary tissue and liver of tumour-bearing rats. The effects of an MC-type inducer, BNF, were determined, using a surgical procedure whereby both control and induced AHH levels were obtained from the same tumour. The form of enzyme present

was determined by the addition of ANF to the incubation medium. Finally, comparisons were made between AHH of normal mammary tissue to that of neoplastic tumours.

#### A. MATERIALS AND METHODS

##### 1) Animals and Induction of Mammary Tumours

Rats used were 50-day-old Sprague-Dawley female purchases from Holtzman Company, Madison, Wisconsin. After a single oral dose of 15 mg DMBA dissolved in corn oil, 100 per cent of rats had developed mammary tumours within 2-4 months, many at multiple sites. Animals were examined weekly for tumour formation. Upon tumour detection, the tumour was never allowed to exceed 10 per cent of the animal's body weight before removal and most often weighed between 4-8 g at removal. Removal at this stage generally ensured that the tumour was non-necrotic and firm and that the tumour had no apparent effect on the animal's general health (Kato et al., 1968).

##### 2) Induction of AHH Activity

In experiments testing the induction of AHH, rats were injected intraperitoneally with BNF (80 mg/kg body weight in corn oil) for two consecutive days prior to sacrifice. In later experiments, as earlier described for the mouse (Chapter III), under ether anaesthesia, a section of the tumour was removed (1-2 g in weight) and the incision closed with wound clips prior to induction. Two rats underwent a similar surgical procedure but without BNF induction, to ascertain the effect of "tumour-sectioning" on the basal AHH levels.

### 3) Tissue Preparation

Initial studies with the DMBA-induced tumours included a comparison of AHH activity in tumours homogenized in two different buffer systems, TDE and  $\text{KPO}_4$ . As microsomal suspensions for both mouse and human mammary tumours would be obtained as a by-product of estrogen-receptor analysis performed on these specimens (McGuire et al, 1975) and the fact that the requirements for estrogen-receptor analysis precludes the use of any buffer but TDE, it was necessary to ascertain that the type of buffer had little or no effect on tumour AHH activity.

Animals were killed by cervical dislocation and the tumours, mammary tissue (plus regional subcutaneous fat), and livers were removed. The tumour tissue was freed of any necrotic zones and, as with mammary and liver tissue, was minced, weighed, and divided into equal portions and rinsed in TDE or  $\text{KPO}_4$  buffer. The "tumour-sections" removed two days previously (if the animals were BNF-treated) were trimmed of any necrotic zones prior to freezing in a Revco ultra-cold freezer ( $-70^\circ\text{C}$ ) until the day of assay of the remaining tissues. Frozen tumour samples were thawed on ice and processed, along with freshly-excised tumour, mammary, and liver tissues, as previously outlined in Chapter II.

## B. RESULTS

### 1) Rat Mammary Tumour AHH Activity

The results (see Figure 12) indicated that DMBA-

induced rat mammary tumours have MFO activity as measured by the AHH assay. The basal levels were low but reproducible. Average induction by BNF was inducible approximately 70-fold above basal activity. A mean value for enzyme activity in control and BNF-treated tumours is given in Table 8. Addition of  $10^{-6}$  M ANF to the microsomal incubation mixture partially inhibited (20-70 per cent of control activity) or had no effect on basal AHH activity. The BNF-induced AHH activity was consistently inhibited to approximately 30 per cent of control activity by the addition of ANF.

Table 8 also gives a comparison of enzyme activity in the two different buffer systems. The activity was generally higher in TDE buffer with induced AHH activity 25 per cent higher and control values 40 per cent higher in TDE than in  $KPO_4$  buffer. Statistical analysis of the data using Student's t test showed no significant differences in buffers at a significance level of 0.05 in all tissues except induced mammary and induced liver where significantly higher values were obtained in TDE buffer. As a result of this, all incubation manipulations and enzyme studies were performed using tumour microsomal suspensions prepared in TDE buffer. All values quoted refer to AHH activity using TDE buffer.

## 2) Tumour Production

Tumour induction with DMBA often produces multiple tumours in the same rat (Huggins et al, 1959). Although 20 out of 35 rats examined for AHH activity bore only one tumour, the remainder had 2 or 3 tumours with the mean tumour number

Figure 12. AHH activity in individual rat mammary tumours.

A. Open bars ( $\square$ ) represent basal AHH activity and those that are lined ( $\equiv$ ) represent BNF-induced AHH activity. The solid portion ( $\blacksquare$ ) of each bar represents enzyme activity after addition of  $10^{-6}$  M ANF to the incubation medium. Contiguous bars represent multiple tumours from the same rat.

B. Tumours in this section differ from 'A' as they are "sectioned" tumours. A portion of the tumour was removed (depicted as basal levels) and the rat was BNF-treated and the induced tumour removed 24 hours later. Multiple tumours from the same rat are BNF-induced and are contiguous with the "sectioned" tumour.

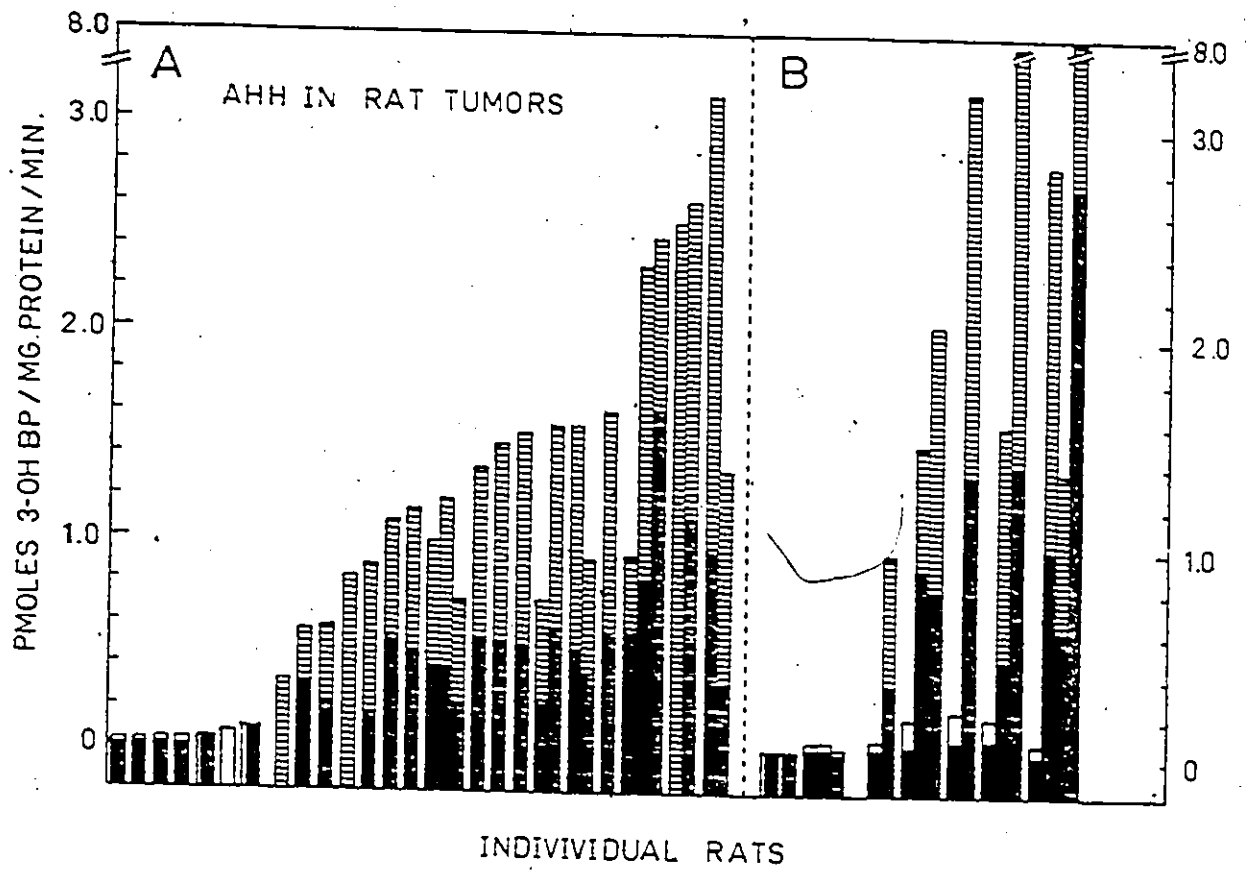


Table 8. Summary of AMH Activity in Rat Mammary Tumours and Corresponding Mammary and Liver Tissues

Rat Tissue	AMH Activity <sup>1</sup> in TDE Buffer				AMH Activity in KPO <sub>4</sub> Buffer			
	Control	+ 10 <sup>-6</sup> M AMF	% of Control Activity	Fold-Inducibility	Control	+ 10 <sup>-6</sup> M AMF	% of Control Activity	Fold-Inducibility
Tumour								
- Basal	0.049±0.01 (16)	0.026±0.01 (16)	54	67X	0.030±0.01 (10)	0.017±0.01 (10)	58	81X
- Induced <sup>2</sup>	3.351±1.50 (36)	0.995±0.33 (36)	30		2.452±0.52 (12)	0.870±0.16 (12)	36	
Mammary								
- Basal	0.024±0.01 (8)	0.126±0.01 (8)	52	104X	0.015±0.01 (4)	0.015±0.01 (4)	105	114X
- Induced <sup>3</sup>	2.50±0.32 (24)	0.481±0.05 (24)	19		1.727±0.18 (24)	0.497±0.06 (24)	29	
Liver								
- Basal	13.06±3.80 (8)	6.46±1.15 (8)	50	19X	11.74±3.44 (8)	6.07±1.10 (8)	52	
- Induced <sup>3</sup>	510.58±22.17 (24)	291.80±18.87 (24)	50		467.51±28.06	250.88±17.76	40	

1. Specific activity X 1 SEM (n) - protein 3-OHMP/mg protein/minute.

2. Induced AMH activity after 80 mg DMF/kg injected i.p. 40 hours prior to sacrifice.

3. Mean activity in TDE buffer significantly higher (p < 0.05) than same tissue in KPO<sub>4</sub> buffer.



per rat equalling 1.57. This provided, as with the mouse, an opportunity to study enzyme activity in separate, distinct tumours subject to the same host influences, both endogenous and exogenous.

In Figure 12, contiguous bars represent tumours from the same animal (where basal and induced determinations are adjacent, these represent "sectioned" tumours rather than multiple tumours). AHH activity in multiple tumours from the same rat, as summarized in Table 9, varied, ranging from as close as 4 per cent of one another to as far apart as one tumour having 6-fold greater activity than another tumour from the same rat. However, the ratio of variances from tumour activity in the overall tumour population to the variance seen in activity in one animal indicated no significant differences in variation within a rat versus between rats.

### 3) AHH Activity in Normal Mammary Tissue

The AHH activity detected in normal rat mammary tissue was approximately half that level observed in tumour tissues. These values are included in Table 8. As with tumour AHH activity, the basal levels detected in mammary levels are low but reproducible and the activity was inducible by BNF-treatment as reported by Fysh and Okey (1978). AHH activity was inducible approximately 100-fold by BNF treatment. Addition of ANF to the incubation mixture inhibited control microsomal activity to approximately 50 per cent of control and inhibited activity in microsomes from BNF-treated rats to approximately 20 per cent.

A comparison of mammary AHH activity in the dif-

Table 9. Variation of AHH Activity in Multiple Mammary Tumours of the Rat<sup>1</sup>

Rat	Individual Tumour AHH Activity		
	i	ii	iii
#9	3.113 <sup>2</sup>	2.601	
#11	0.704	1.502	
#12	2.504	2.601	
#17	1.003	2.311	2.441
#18	1.517	0.886	
#19	2.809	1.333	8.575
#22	0.984	1.158	0.708 <sup>c</sup>
#29	1.549	7.025	
#33	1.462	2.114	

1. All rats were BNF-treated (80 mg/kg)
2. Specific Activity (pmoles 3-OHBP/mg protein/minute)

7

ferent buffers gave the same results as with tumours. Activity was higher in TDE buffer, control activity 40 per cent higher ( $p > 0.05$ ) and induced activity 30 per cent higher ( $p < 0.05$ ).

#### 4) AHH Activity in Rat Liver Tissue

The enzyme levels in livers of tumour-bearing rats given in Table 8 do not differ significantly from those reported in the literature for non-tumourous animals (Wiebel and Gelboin, 1975). As with tumour and mammary tissue, liver AHH activity is higher in TDE buffer than in  $KPO_4$  buffer. Only induced AHH activity is significantly higher (0.05 level of significance) in TDE buffer.

#### 5) Correlations Between AHH Activity in Tumours and Tumour Weight, Mammary, and Liver AHH Activity

As not all tumours were of the same size at removal, a possible correlation between tumour size and AHH activity might be expected. It has been noted in DMBA-induced tumours that as they progress in size they become less hormone dependent than they were originally (Dao, 1964), so it was possible that some change in AHH activity might occur over time as well. Table 10 indicates that no correlation exists between tumour size and AHH activity.

Earlier studies with extrahepatic tissues indicated that AHH activity in hepatic tissue correlated with activity seen in extrahepatic tissues (reviewed in Thorgeirsson and Nebert, 1977). Values in Table 10 indicate that no such correlation existed for tumour AHH activity and both mammary and

Table 10. Correlation Coefficients of Basal Rat Mammary  
Tumour AHH Versus:

	r	n
Tumour Weight	-0.165	16
Mammary AHH Activity - Basal	0.053	8
- Induced	-0.096	24
Liver AHH Activity - Basal	-0.301	8
- Normal	0.105	24

liver activity. Further, no significant correlation existed between mammary and liver AHH activity ( $r = -0.14$ ) so lack of correlation is not a result of the tumourigenic process as normal mammary gland demonstrated the same relationship.

### C. DISCUSSION

The microsomal suspensions from DMBA-induced Sprague-Dawley rat mammary tumours possess low but significant and reproducible levels of AHH; basal AHH activity was approximately 250 times lower than that of the corresponding liver (see Table 8), whereas induced enzyme levels approached that of uninduced liver.

The capacity of the enzyme activity to be enhanced by MC-type inducers such as BNF and the susceptibility to inhibition by ANF resemble those activity patterns as described for the adult female rat liver (Wiebel and Gelboin, 1975).

The increased susceptibility of the induced enzyme form found in both tumour and mammary microsomes to inhibition by ANF is worthy of note. Nebert and Gielen (1972) reported a similar phenomenon in MC-induced kidney and hepatic microsomes from responsive mice. They postulated that the greater sensitivity of the inducible hydroxylase to ANF indicates that the heme portion of cytochrome P-450 active sites found in control microsomes is either not as accessible to the flavone inhibitor or it differs in its spin-state, compared with that form (cytochrome P<sub>1</sub>-450) found in the MC-responsive animal (Nebert et al, 1972).

Analysis of all three tissue types (tumour, mammary, and liver) demonstrated TDE to be a suitable buffer for homogenizing tissues for the analysis of AHH activity. Activity generally was higher in TDE buffer than in  $\text{KPO}_4$  buffer. While the constituents of TDE buffer were not studied separately, dithiothreitol (a sulfhydryl compound that acts to stabilize disulfide bridges in hormone-receptor complexes) may act in an analogous manner in microsomal suspensions to stabilize the AHH complex during microsomal preparation and assay. Although EDTA is a chelating agent which reversibly binds  $\text{Mg}^{2+}$  and other divalent cations, thus inhibiting enzymes requiring such ions for activity (Lehninger, 1970), experimental manipulations summarized in Chapter II indicated that there were adequate quantities of divalent cations associated with microsomal membranes. Therefore, inclusion or omission of  $\text{Mg}^{2+}$  in the incubation mixture had no pronounced effect on enzyme activity (see Figure 5A). A search of the literature revealed no extensive comparison of buffer effects on AHH activity and widespread use of the  $\text{KPO}_4$  buffer system probably arose from the fact that it constitutes the major intracellular buffer of living organisms and it is a commonly used laboratory buffer.

Analysis of the data given in Table 8, performed by taking a ratio of variances of different tissues and comparing this number to an F value at a significance level of 0.05, revealed tumours to have no greater degree of

variability in AHH activity than was observed in liver or mammary tissue of tumour-bearing rats. The wide range of enzyme values detected in rat mammary tumours can be compared to AHH activity observed in rat mammary tissue during developmental stages (Fysh and Okey, 1978). Watanabe et al, (1970) reported a similar distribution of AHH enzyme levels in Morris hepatomas and postulated that the variation might be covered within the ranges occupied by fetal and neonatal rat liver as well as adult liver.

Similarities between malignant and embryonic cells have served as the basis for the theory that one probable cause of cancer is the expression of fetal genes in a mature cell (Maugh and Marx, 1975). Two of the three principal characteristics of malignancy - sustained cell division and cell migration - are also characteristic of embryonic cells. The third characteristic is a reversion of structure and metabolic activity to a more primitive state. This theory is the outcome of the research surrounding the study of fetal antigens or markers present in cancer. Hence, the detection of AHH levels in mammary tumours equal to levels observed at early stages of rat mammary development could be interpreted as possibly supporting the hypothesis as outlined above that oncogeny may represent blocked or reverted ontogeny.

The gross effects of tumourigenesis on the host is well documented (Bertino, 1977). These include weight loss and cachexia quite likely as a result of the tumour's ability

to compete for key nutrients within the host with the resulting metabolic abnormalities. Certain malignancies, in addition, can cause deficiencies in the immune system resulting in enhanced susceptibility to various infections. As well, although not the case for DMBA tumourigenesis in the rat due to the noninvasive properties of the tumours (Dao, 1964), organ function in other models can be compromised due to invasion by cancer cells. All these factors taken together with the fact that tumours do not appear in the rat after an identical latency period and were not all removed at the same point in their development, could serve as explanation of some of the variation seen in tumour AHH activity. These factors would be difficult to separate and assess. Quite likely, it is a combination of many of the above factors which contribute to the range of enzyme activity observed in tumours.

When the AHH activity of the control mammary tissue in Table 8 is compared to the literature values reported in Table 7, the values compare closely. However, when induced levels are compared, the mammary tissue of tumour-bearing rats appears to be more inducible (100-fold as compared to 60-fold) in control mammary tissue from rats of approximately the same age. The reason for this increased susceptibility to BNF-induction in tumour-bearing animals is not clear. Watanabe et al (1970) reported a similar phenomenon in Morris hepatoma-bearing rats. They found the host liver in a hepatoma-bearing rat to be more sensitive to environmental controls than a normal rat liver. These



controls included day-night regimes, feeding schedules, and per cent protein in the diet. The enhanced susceptibility seen in mammary tumour-bearing rats to BNF-treatment may reflect some alteration in the organizational properties of the enzyme system as a result of the original tumour-causing administration of DMBA.

The livers of the tumour-bearing rats showed the same enhanced susceptibility to BNF-treatment as did the mammary gland. Inspection of basal enzyme levels and comparison to values reported in the literature (see Table 7) indicated that the basal activity is not depressed in tumour-bearing rats, but the greater fold-induction seen in these animals is a result of higher induced AHH activity. Literature values for fold-inducibility in control rat liver range from 17-fold in 30 day-old female rats to 30-fold in 70-100 day-old female rats. The 40-fold inducibility ratio observed in tumour-bearing rats is greater than these ratios. An alteration at some level of the organization of the enzyme system as a result of the single oral dose of DMBA might, again, explain this finding.

Strittmatter (1979), in a study examining the levels of enzyme activities in the livers of hepatoma-bearing rats, reported that the microsomal drug-metabolizing activities and associated cytochrome P-450 and NADPH-cytochrome C reductase activity showed significant decreases in hepatoma-bearing rat livers as compared to normal livers. His work characterizing the Reuber H-35 hepatoma generally

showed the same pattern of moderate decreases of specialized functions, compared to normal liver, that is commonly seen in hepatomas. He conjectured that a wide diversity in the direction and magnitude of changes that have been reported previously for host tissues appears to reflect the varying effects of different tumour types and periods of tumour growth. Although mammary tissue and liver of mammary tumour-bearing rats show no such decrease in AHH activity, it may simply reflect the noninvasive nature of the tumour and the effect of earlier removal before the tumour could adversely affect the host.

In an attempt to explain the range of activities detected in mammary tumours, correlations between tumour weight and both mammary and liver AHH activity were determined. The lack of correlation is reminiscent of that seen in mouse mammary tumours. Considering the incomplete understanding of the functioning of the microsomal P-450 system and factors affecting it, the lack of explanations is not surprising. A comparison of variances and lack of significant differences within multiple tumours in the same rat as compared to between rats accentuated this lack of complete understanding. Initially it might have been expected that the enzyme activity arising in tumours induced by one chemical in an inbred strain of laboratory rats might show less variability than discovered. If one considers the monoclonal origin of tumours as discussed in Chapter III, the results are not surprising. Thus, although the site of initiation

(rat mammary gland), and the initiating agent (DMBA) for tumourigenesis are identical in all rats studied, conditions within each neoplasm will dictate the characteristics of the fully developed tumour due to selective pressures. Yet, because of the similarity of initial circumstances surrounding tumourigenesis, tumours produced do display some common qualitative characteristics such as response to the inducing agent, BNF, and in vitro inhibitor, ANF.

The relevance of diversity of response to these two agents will become more apparent when response is discussed in the inherently diverse human tumour population.

## CHAPTER V

### AHH IN HUMAN BREAST TUMOURS

The differential susceptibility of the human population to chemical carcinogens might be partially attributed to individual differences in carcinogen metabolism. Examination of the levels and regulation of carcinogen-metabolizing enzymes, such as the microsomal mixed-function oxidases, in humans may aid in the understanding of the genetic and environmental factors which effect an individual's susceptibility to cancer.

Various levels of mixed-function oxidases have been found in humans by in vivo assays of drug metabolism including measurements of cytochrome P-450 and AHH in adult and fetal liver (Schoene et al, 1972 and Pelkonen, 1977), placenta (Welch et al, 1968), lung (McLemore et al, 1978), blood components such as lymphocytes (Kellerman et al, 1973 and Paigen et al, 1979), monocytes (Bast et al, 1974), and macrophages (McLemore et al, 1977) as well as a variety of fetal tissues including adrenal glands, testes, kidney, and intestine (Rifkind et al, 1978). In Table II, the tissue distribution of AHH in man and in the rat is compared.

Interest in this area was sparked by Kellerman's (1973) original report claiming genetic polymorphism for AHH inducibility. By examining a population of healthy subjects for AHH induction by MC in cultured (mitogen-activated) lymphocytes, Kellerman et al (1973) reported a trimodal distribution of AHH inducibility, using a "low",

Table 11. A Comparison of Tissue Distribution of AHH in Man and Rat

Tissue	Presence in rat <sup>a</sup>	man	Inducibility in man by PAH	Activity in man <i>in vitro</i>
<b>Adult</b>				
liver	+++	+++	+	Comparable to exp. animals < 1% of liver
lungs	+	+	+	
kidney	+	?	?	
adrenal	+	?	?	
intestine	+	?	?	Measured in newborn foreskin tissue culture
skin	+	+	+	
spleen	+	?	?	
blood (cells)	+	+	+	
muscle	-	?	?	Mitogen-treated lymphocytes; monocytes
brain	-	?	?	
Placenta	+	+	+	Present in smokers; strongly dependent on stage of pregnancy and individual
<b>Fetus</b>				
liver	-	+	-	Two-4% of adult liver
lungs	-	(+)	-	
kidney	-	(+)	-	Extremely low or absent
adrenal	(+)	++	(+)	
intestine	-	-	-	Higher than in liver
skin	-	-	-	
spleen	-	-	-	
blood (cells)	?	-	?	
muscle	?	?	?	
brain	-	-	?	

1. Taken from Pelkonen (1976).

"intermediate", and "high" designation for the degree of inducibility. The data gave an excellent fit to the Hardy-Weinberg equilibrium and were consistent with a hypothesis of two alleles at a single locus or monogenic control. They also found that lung cancer patients had a different distribution of AHH inducibility with a preponderance of intermediate and high AHH inducibility phenotypes, and concluded that the allele for high AHH inducibility conferred an increased risk to lung cancer.

A flurry of papers followed the original report by Kellerman, many criticizing the results as they were unable to reproduce them (Kouri et al, 1974; Paigen et al, 1977; Fletcher et al, 1978) and some concurring with the results (Emery et al, 1978; Brandenberg et al, 1978; Gahmberg et al, 1979). Much of this problem in reproducing the results resides in the effect various culture and assay conditions have on lymphocyte AHH activity and inducibility.

As mitogen activation of the lymphocytes is required before appreciable amounts of AHH induction can occur, some felt (McLemore et al, 1978) that the results could be an artifact of mitogen stimulation and a consequence of inconsistent responsiveness of lung cancer patients' lymphocytes to mitogens. This group and others tried to circumvent this problem by using human lung tissue and pulmonary macrophages as well as blood monocytes (Bast et al, 1974). Unfortunately, the comparative degree of difficulty in obtaining these alternative tissues has prevented their widespread use.

Paigen et al (1977, 1979) criticized the results of Kellerman stating that the higher AHH inducibility of patients with lung cancer is a consequence of the disease itself and not representative of AHH inducibility that existed before cancer. Being generally accepted that AHH levels and inducibility ratios are genetically determined, Paigen and co-workers used the progeny of cancer patients and compared AHH inducibility ratios to matched controls. They also selected patients with respiratory cancer whose tumours were completely resected and who had been disease-free for a period of time. In this way, inducibility ratios should not be a product of the disease state, and their lymphocytes should respond to mitogens and produce enzyme activity similar to that shown by controls. Although they found variation in AHH inducibility which they attributed to genetic differences, they reported a unimodal distribution of AHH inducibility in humans, not trimodal, as reported by Kellerman et al (1973).

In a recent paper, Gurtoo et al (1979), in an attempt to avoid the uncertainties of factors surrounding various culture and assay conditions, tried to examine physical properties of the basal and induced enzyme forms of human AHH by studying the specificity of induction and inhibition and other enzymological properties in human lymphocytes. Inducible phenotypes could be more readily identified from the noninducible phenotypes if some qualitative difference in the intrinsic physical properties of

the basal and induced enzyme could be detected. Although they found that basal and induced forms are qualitatively similar and differ only quantitatively in comparable un-induced and PAH-induced lymphocytes, similar studies with human liver indicated a marked interindividuality in different samples (Buening et al, 1977).

All of the above research only served to strengthen the theory that AHH inducibility was indeed an inheritable trait. No one could confirm the trimodal distribution of AHH inducibility reported by Kellerman; in fact, most found a unimodal distribution indicating a polygenic control of AHH inducibility. As Paigen et al (1977) discussed, this is not surprising as it is difficult to observe trimodal distribution unless the genetic differences between homozygous parents are great and the coefficient of variation of the assay small; neither condition applies to the determination of AHH inducibility. Some researchers still claim to have observed a preponderance of high inducibility phenotypes in cancer patients (Emery et al, 1978; Brandenburg et al, 1978 and Gahmberg et al, 1979) whereas Paigen's group along with others consistently found no such relationship. Obviously, more studies with different experimental designs are needed to confirm the relationship between AHH inducibility and susceptibility to cancer in man.

These conflicting reports do not diminish the critical importance of further testing the metabolism of polycyclic aromatic hydrocarbons for genetically determined



individual variation in AHH activity and determining the relationship of such polymorphisms to human cancer risk. The present study was undertaken to determine individual variation in AHH levels of human mammary tumours. Some characteristics of the predominant form of the enzyme present ascertained by the use of in vitro AHH inhibitors will be reported. As specimens were obtained as biopsies for estrogen-receptor analysis, it was impossible to study the experimental induction of enzyme levels as in animal models. Although the relationship of any polymorphisms to cancer risk could not be ascertained due to the nature of the study, it was hoped that some useful relationship between AHH levels and data contained therein could be detected upon examination of clinical data and response to chemotherapy.

#### A. MATERIALS AND METHOD

##### 1) Source of Human Tumours

The human tumours were breast tumour specimens received from local hospitals for estrogen-receptor analysis. The specimens were transported to our laboratory on ice within 2 hours of removal. The tumours were trimmed of any adhering fat, minced and weighed before storage (less than 2 months) in liquid nitrogen ( $-226^{\circ}\text{C}$ ), until time of assay. The tumours routinely weighed approximately 1 g though specimens as small as 50 mg and as large as 10 g were received.

##### 2) Tissue Preparation

On day of estrogen-receptor analysis the frozen

tumour was pulverized with a Thermo-Vac tissue pulverizer (Thermo Vac Industries, Copiague, New York) and the resulting powder placed in iced TDE buffer. The tumour was then processed as described in Chapter II. After centrifugation at  $105,000 \times g$  for one hour, the resuspended microsomal pellet was frozen in a Revco ultra-cold freezer ( $-70^{\circ}\text{C}$ ) until day of assay. This period never exceeded two months in duration. Microsomes from mouse tumours handled in this manner showed no apparent loss of AHH activity. On the day of assay, the frozen microsomal suspension was thawed on ice and assayed as previously described in Chapter II.

### 3) Testing of Inhibitor Effects on AHH Activity

Human tumour microsomes were routinely incubated with  $10^{-6}\text{M}$  ANF in vitro to determine the pattern of inhibition as with animal tumours. In addition, in order to better elucidate the form of AHH present, in some human tumours with adequate microsomal suspension volume and detectable AHH activity, other in vitro inhibitors were tested. A range of concentrations from  $10^{-3}$  -  $10^{-7}\text{M}$  of compounds such as ANF and metyrapone, was the usual range employed.

## B. RESULTS

### 1) Human Tumour AHH Activity

The 216 human tumours assayed displayed a wide range of enzyme activities with 25 per cent of the samples assayed yielding non-detectable or near-zero values (less than  $0.1 \text{ pmoles 3-hydroxybenzo[a]pyrene/mg protein/minute}$ ).

If one were to separate the range of values into arbitrary groups, 15 per cent of the tumours assayed had no activity, 25 per cent had levels comparable with the range observed in uninduced rat mammary tumours (0-0.1 pmoles 3-hydroxybenzo[a]pyrene/mg protein/minute), and 60 per cent of human tumours and AHH activity within the range of activities detected in uninduced mouse mammary tumours (0-2.0 pmoles 3-hydroxybenzo[a]pyrene/mg protein/minute). The 40 per cent of human tumours which have activity greater than 2 pmoles 3-hydroxybenzo[a]pyrene would compare to induced rat and mouse mammary tumour AHH activity, although there is certainly some overlap in these ranges. Owing to the heterogeneity of the group studied whether due to different pathologies of tumours or patients' histories, it is impossible by simple inspection to determine which of the tumour enzyme activities, if any, represent "induced" values versus basal values. Although the validity of such a number is questionable, the mean AHH activity in human tumours is  $0.85 \pm 0.263$  (SEM) pmoles 3-hydroxybenzo[a]pyrene/mg protein/minute. This mean value, keeping in mind the lack of distinction between basal and induced forms, brings the activities of human tumours roughly in line with basal mouse mammary levels. This mean value represents approximately 10 per cent of that AHH activity found in human adult liver (Pelkonen, 1976), the activity of adult human livers representing 30-60 per cent of rat liver activity. Individual AHH activity in human breast

tumours is shown in Figure 13A.

Included in Figure 13A are the results of the addition of  $10^{-6}$  M ANF to the incubation medium. As indicated, there was marked interindividuality for the stimulating and inhibiting effects of ANF in different tumour samples. In 8 per cent of the tumour samples with detectable AHH activity, ANF had no effect on enzyme activity. In 22 per cent of the samples, ANF had an enhancing effect on AHH activity and in 47 per cent of the specimens, an inhibiting effect was detected. Of particular interest is the mean value of AHH activity ( $0.50 \pm 0.180$  (SEM)) in tumours where enhancement in the presence of ANF occurred as compared to that of  $1.94 \pm 0.848$  (SEM) in those samples where inhibition occurred. Hence, the levels of enzyme activity in the group where ANF inhibited AHH activity might be induced from patients who might have been induced by drug treatment or smoking etc.

By using inhibitors showing preference to one form of AHH (Goujon et al, 1972), an attempt was made to differentiate between the basal AHH activity associated with cytochrome P-450 and that associated with cytochrome P<sub>1</sub>-450, the induced form. Figure 14 shows the results of a concentration range of inhibitors, including ANF and metyrapone, in several tumour specimens. As can be seen from the three samples chosen, there is marked individuality in response to inhibiting agents, suggesting variation in the form of cytochrome P-450 found in the human population.

Figure 13. AHH activity in individual human breast tumours.

A. Each bar represents AHH activity in a single breast tumour specimen expressed per mg protein. Where values have been grouped for "low" activity samples, the range of AHH activity detected and the number of breast tumours is indicated. The effect of  $10^{-6}$  M ANF on each specimen is represented by the solid portion of each bar (■).

(Note: Where ANF stimulates AHH, the basal level will be less than the solid portion of each bar.)

B. Each bar represents AHH activity in a single breast tumour specimen expressed per  $\mu$ g DNA. "Low" activity values have been grouped as above. The corresponding AHH activity expressed per mg protein is directly above (in 'A') that same specimen expressed per  $\mu$ g DNA. In some cases, DNA determinations were not available for each human breast tumour specimen.

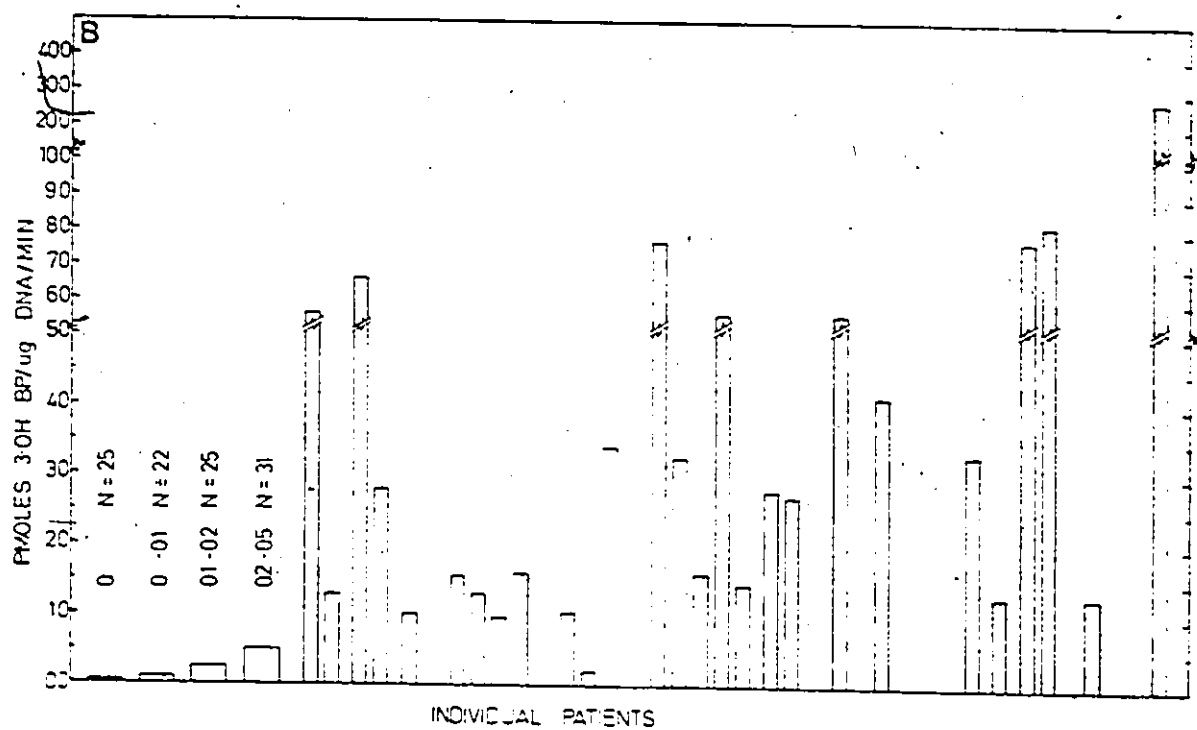
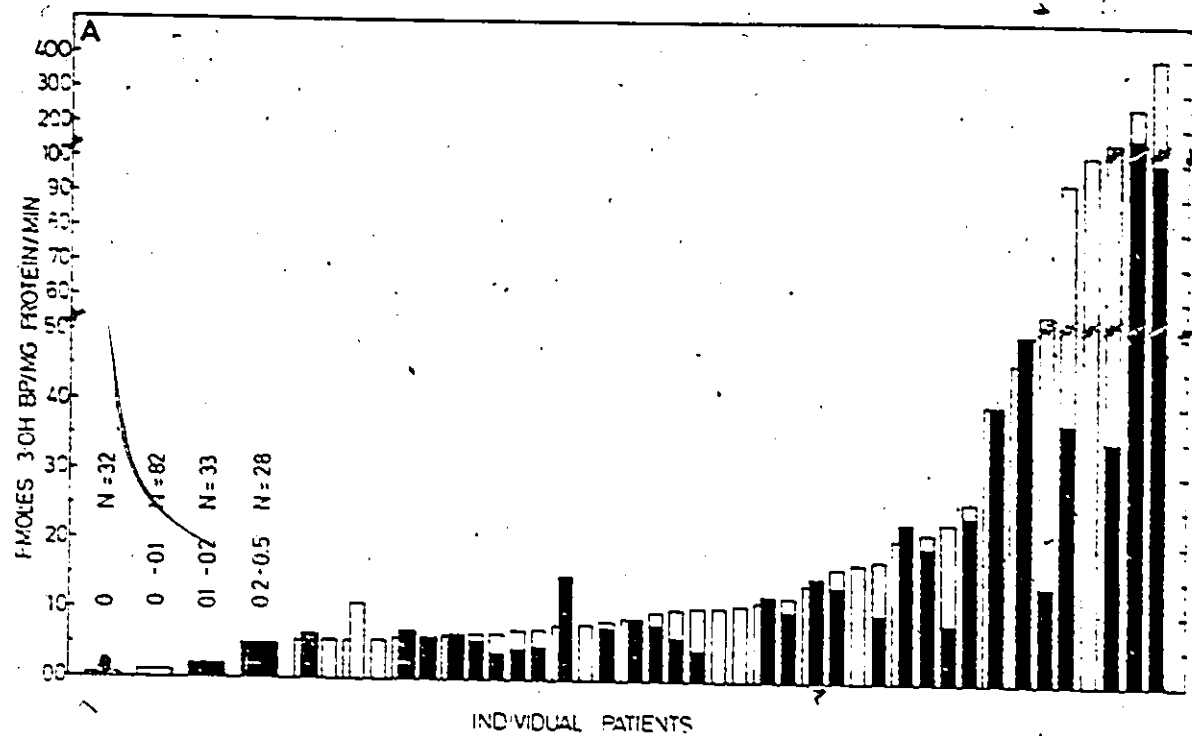
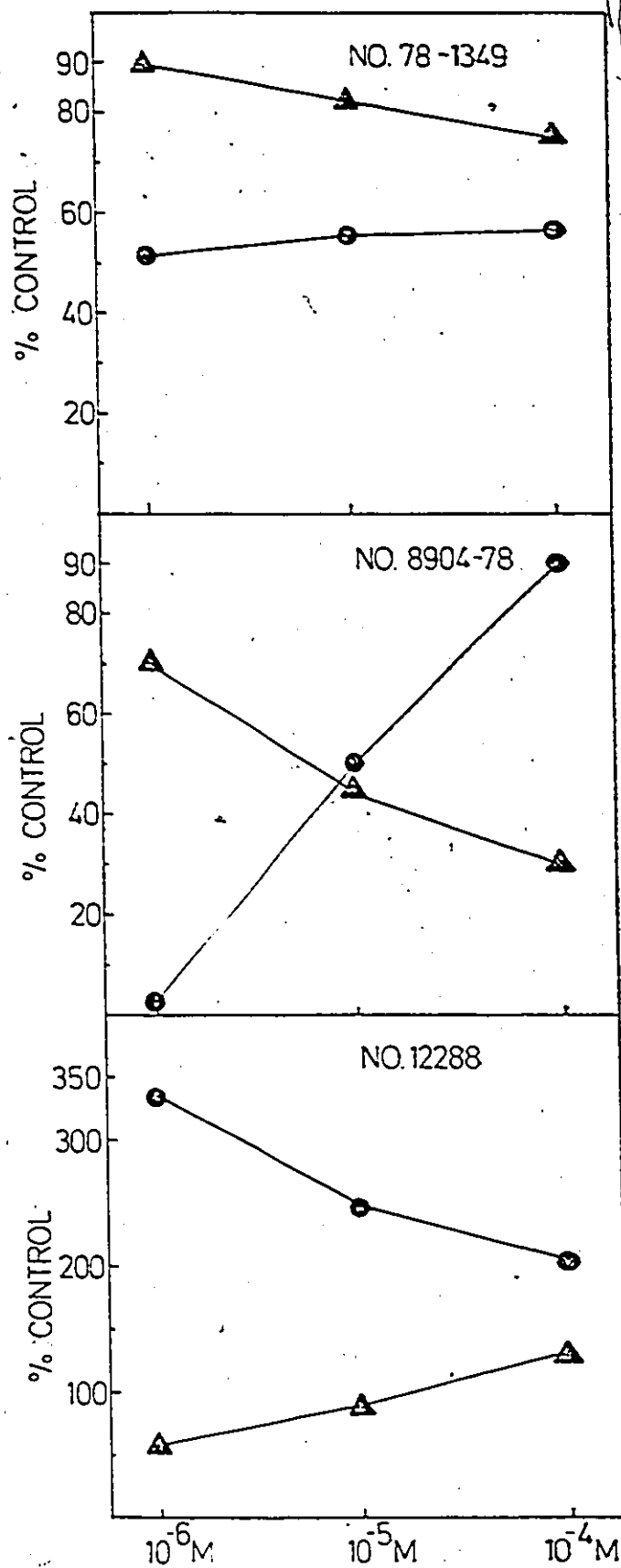


Figure 14. Effect of ANF and metyrapone on individual human breast tumours. The AHH activity is expressed as per cent of the control flask, to which 50  $\mu$ l methanol, the solvent for both inhibitors, was added. The level of AHH activity after addition of ANF to the incubation medium is represented by the closed circles (●) and metyrapone, by closed triangles (▲). The incubation flask for specimen #78-1349 contained 0.430  $\mu$ g microsomal protein, #8904-78 contained 0.672  $\mu$ g microsomal protein, and #12288, 760  $\mu$ g microsomal protein.





## 2) Specific Activity Expressed per Mg Protein of $\mu$ g DNA

DNA determinations were done on most human tumour specimens by the method of Beers and Wittliff (1975) (see Appendix III). Due to the polyploidy of human tumour specimens, it was thought that the range of enzyme activity expressed in terms of  $\mu$ g DNA instead of mg protein might yield a different distribution pattern. When enzyme activity expressed in terms of mg protein were arranged in ascending order (Figure 13A), the corresponding activity per unit DNA do not increase similarly (Figure 13B). The highest enzyme values whether expressed per mg protein or per  $\mu$ g DNA generally remain high.

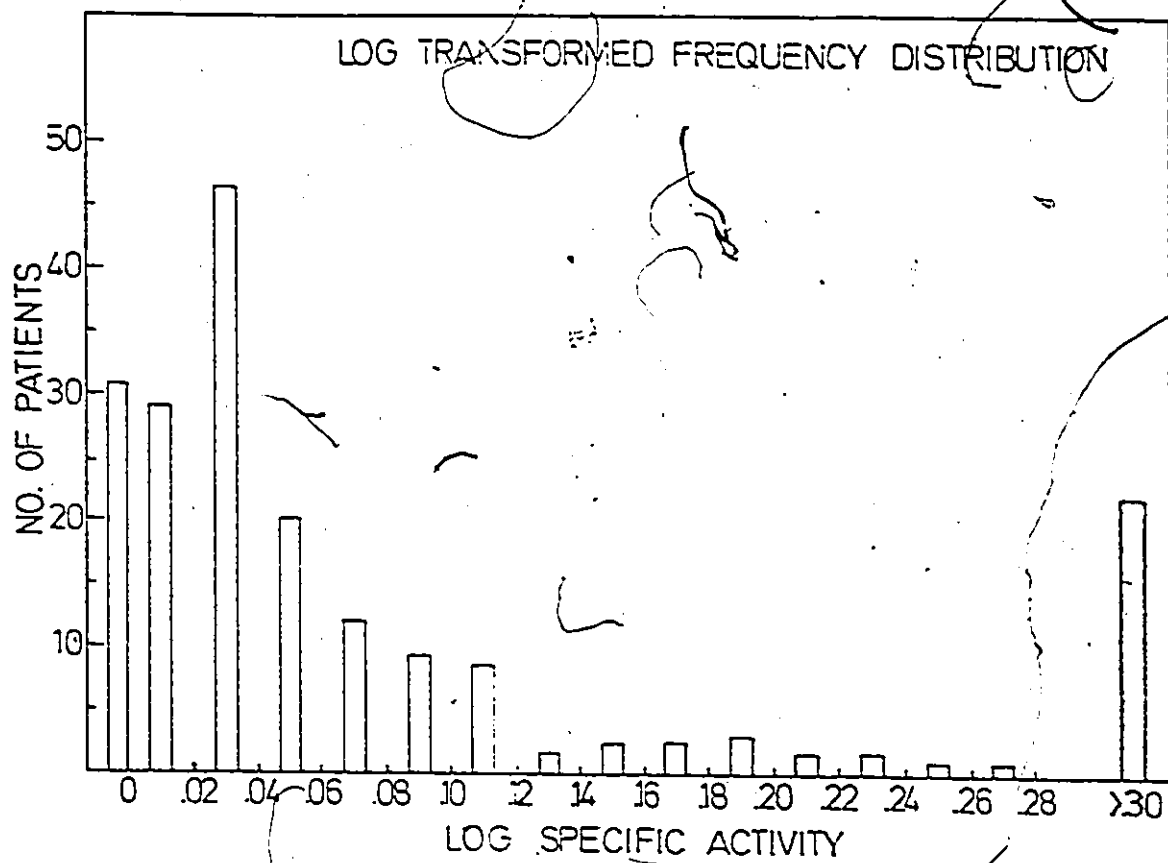
## 3) Frequency Distribution of AHH Activity in Human Tumours

A frequency distribution of the  $\log_{10}$  of the specific activity per mg protein is given in Figure 15. Attempts to normalize the distribution of AHH activity was unsuccessful in human tumours, in contrast to rat and mouse tumours. The relative abundance of nondetectable and near-zero values had the effect of causing the distribution curve to be highly skewed to the left.

## 4) Microsomal Protein Yield

The microsomal protein yield routinely was approximately 3.3 mg microsomal protein per g tumour, one-third of that measured in mouse tumours. This could be a result of marked fibrosis, the so-called scirrhus-type carcinoma

Figure 15. Frequency distribution of AHH activity in human breast tumours. The  $\log_{10}$  of the AHH activity for each human breast tumour was determined and frequency distribution plotted.



prevalent in human tumours, seen only rarely in rat tumours and not evident in mouse tumours. As this tumour type is very dense, a lower protein yield results.

#### 5) Correlation of AHH Activity with Estrogen-Receptor Levels

As indicated earlier, the primary reason breast tumours were sent to our laboratory was for estrogen receptor analysis. Currently, a controversy exists in the literature with some researchers claiming that absence of estrogen receptor correlates with a positive response to chemotherapy (Lippman et al, 1978) whereas, other groups claim that tumours that are estrogen receptor-positive respond more favourably to chemotherapy (Kiang et al, 1978). Yet another group claims that favourable results with chemotherapy occurred equally among patients with hormonal response and those with no response (Samal et al, 1978). Because of this controversy and due to the readily available data concerning levels of estrogen receptor, a correlation coefficient was determined for AHH activity and estrogen receptor levels in human tumours. With a correlation coefficient of  $r = -0.03$ , it appears that there is no relationship between AHH activity and estrogen receptor levels in the human breast tumours studied. Li et al, (1976) reported a similar lack of correlation between estrogen receptor levels in human mammary tumours and steroid metabolism (testosterone).

#### 6) Relationship Between AHH Activity and Pathology of Human Tumours

In an attempt to explain the range of enzyme levels detected in human tumours, an attempt was made to relate enzyme levels to the pathological classification of the tumour. Upon examination of the clinical records it became apparent that a broad range of descriptive terms, depending upon the pathologist in attendance, were applied to the breast tumours ranging from a "poorly differentiated carcinoma" to a "benign cystosarcoma phylloides" (a rare form of breast cancer). Although other researchers (Kern, 1979) reported a correlation of estrogen receptor levels with morphologic and cytologic features and preliminary survival data, because of ambiguity in the clinical records, it remains, at present, difficult to determine the relationship, if any, between pathology and enzyme levels.

#### 7) Relationship Between AHH Activity and Response to Chemotherapy

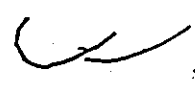
As many chemotherapeutic agents require metabolic activation for expression of their anti-neoplastic activity, one of the more promising aspects of the research was to determine if MFO activity in the primary tumour had any predictive abilities in terms of response to various chemotherapeutic agents.

Although the understanding of metabolic activation and the mechanism of action of many chemotherapeutic agents remain unknown, the metabolism (alkylation) of cyclophosphamide

(CPA), an alkylating agent used in many anti-cancer drug regimes, is known to be catalyzed by hepatic microsomal cytochrome P-450 system (Ohira et al, 1975). Procarbazine and nitrosoureas, though not so commonly used as CPA, are also substrates for the cytochrome P-450 dependent enzymes (Reed and May, 1978). As other chemotherapeutic agents are investigated, more may be found to require activation by the cytochrome P-450 system.

It is known that CPA requires enzymatic oxidation in vivo to generate alkylating moities. The drug is activated by hepatic microsomal enzymes and the active metabolite reaches target sites through systematic circulation (Alberts et al, 1978). If AHH is present in sufficient quantities in the target sites, human breast tissue, for example, it may be important in the local activation of CPA. Active intermediates formed in situ might more quickly and efficiently exert their cytotoxic effects upon rapidly dividing cancer cells.

It was with this concept in mind that two separate sub-populations of human breast tumours were examined for possible correlation of AHH activity and response to chemotherapy. A random sample of patients whose biopsies had been performed 14-54 months before AHH assay analysis was chosen, 20 of whom had nondetectable levels of AHH activity and 20 others who had "high" AHH activity (greater than 0.5 pmoles/mg protein/minute).



Some of the characteristics of the two populations appear in Appendix VI. Examination of the sub-populations, with respect to treatment given and objective and subjective response revealed only 1 out of 20 patients in the "low" AHH activity to be "evaluable" and 3 out of 20 in the "high" AHH activity group. Evaluable patients were those who were put onto a chemotherapy regime (usually cyclophosphamide-methotrexate-5-fluorouracil (CMF)) after remission so that a response could be assigned. Generally, all patients received some form of radiation treatment immediately after surgery; this may constitute the only treatment the patient would receive. Others were placed immediately onto the Ontario Cancer Foundation Adjuvant Therapy program consisting of irradiation plus CMF treatment. Other patients who were not evaluable were those on some form of hormone treatment. Of the 4 patients out of 40 who were "evaluable", 3 patients with "high" AHH activity did appear to respond to chemotherapy after remissions following surgery on the primary tumour. The one patient in the "low" AHH activity group did not respond to chemotherapy and died with autopsy revealing extensive bone metastases.

### C. DISCUSSION

The presence of PAH-metabolizing enzymes in different human tissues and their ability to be induced is a discovery of great impact (Nebert and Gelboin, 1968) as it may represent an important protective mechanism by which

Carcinogenic or noxious compounds are inactivated. Animal studies have demonstrated a resistance to hydrocarbon carcinogenesis after the induction of hydrocarbon-metabolizing enzymes (Silinskas and Okey, 1975). Although enzyme activity examined in this report was in neoplastic mammary tissues, some important comparisons can be made to normal human tissues and the relevancy to chemotherapeutic response discussed.

It is difficult to assess the factors contributing variability in human tumours as the patients from whom they were obtained represent a diverse group. At least two factors may contribute to the variability as they are known to affect drug metabolism in man: cigarette smoking and disease of the patient (Conney and Burns, 1972). Other investigators have reported on this diversity of enzyme levels so prevalent in man (Pelkonen, 1976) regardless of tissue type. Although a survey of enzyme levels alone is of little value in assessing the nature of AHH in man, the use of inhibitors has proved useful.

Work by Pelkonen (1976) and others (Rifkind et al, 1978) with fetal and adult human livers, showed that hepatic AHH was not inhibited by ANF, thus, exhibiting properties typical of cytochrome P-450-linked AHH or the "basal" form. The form of enzyme present in the placenta, which has been reported to be induced in smoking mothers and strongly inhibitable by ANF, exhibits characteristics of P<sub>1</sub>-450-linked AHH. The enzyme systems studied also differed with



respect to response in other inhibitors. Adult and fetal hepatic AHH were inhibited by SKF-525 and metyrapone, whereas, the placental enzyme was not. Concurrent studies with fetal adrenal gland AHH showed it to behave more like the hepatic system. Additional studies on substrate specificity, drug-induced spectral interactions, and other properties of adult hepatic and fetal hepatic, and adrenal and placental mono-oxygenase systems point to a difference between hepatic enzyme systems and that in the placenta of humans (Pelkonen et al, 1977).

Pelkonen and co-workers (1975) have done additional studies in human fetal cell culture which has the advantage of being able to study both basal and induced levels. The enzyme in fetal culture was inducible by exposure to PAH in vitro. The basal enzyme in control cultures was not inhibited by ANF, whereas, the induced enzyme was. The hepatic fetal culture system exhibited the same characteristics as the liver microsomal system. In their study of fetal cell cultures and fibroblast cultures, they have found a unimodal type of distribution of induced AHH activity suggesting polygenic control of AHH induction in these systems.

Other investigators (Buening et al, 1977) reported marked individuality in the activating and inhibiting effects of ANF in different human liver samples obtained from surgical biopsy or surgery. The authors felt that this individuality may result both from the presence of multiple forms of mono-oxygenases in varying amounts and proportions in the

different liver samples and from a selective effect of ANF on certain mono-oxygenases. The discrepancy in responses reported by this group and those reported by Pelkonen et al, (1977) may simply reflect a bias in subjects sampled as Pelkonen reported his group contained no heavy smokers.

Gurtoo et al (1977), in a recent study examining the induction and inhibition of AHH in fresh mitogen-stimulated human lymphocytes, reported that AHH was not inducible by PB and was consistently inhibited by ANF even at higher concentrations ( $10^{-3}$  M). In their study over a range of concentrations SKF-525 and metyrapone produced moderate inhibition to moderate stimulation of AHH activity though not to the same degree as ANF. The inhibitor patterns reported suggest that lymphocyte AHH, both basal and induced, is essentially cytochrome P<sub>1</sub>-450 linked. The differences in response to inhibitors between adult livers and lymphocyte could reflect inherent differences in tissue types or artifacts as a result of mitogen stimulation of human lymphocytes.

Based on the above discussion, the pattern of AHH response to ANF in human tissues has been reported in other tissues but, interestingly enough, never have all patterns of stimulation and inhibition been reported in the same tissue as has been observed in human breast tumours. This could be a result of two factors: heterogeneity of human breast tumours or lack of distinction between basal and induced forms of the enzyme in human tumours.

In earlier discussions on mouse and rat mammary

tumours, the same general pattern of response to ANF was reported. The human tumour population tested may well represent a much more heterogenous group of samples than either mice or rats. The tumours are arising in a more genetically diverse host than are inbred strains of laboratory animals. Further, we can only guess as to the original tumour-initiating cause whether viral, chemical, or hormonal or any combination of these factors in the human patients.

If more details of the patients' histories were known, the varied response to ANF could be attributed to differences in the form of enzyme present, basal or induced. As reported, the mean enzyme activity in the group where ANF caused inhibition was 4-fold higher than that seen in the group where ANF stimulated AHH activity. A 4-fold induction is reminiscent of the fold-induction seen in mouse tumours. It would be difficult to classify the human tumours based only on this factor.

Attempts to understand the mechanism(s) producing the variability in human mammary tumours, as with animal tumours, proved not very successful. Estrogen receptor levels in the overall population did not correlate well with AHH activity. In the sub-populations of 20 "low" and 20 "high" AHH activity samples, there appeared to be no significant correlation between AHH activity and degree of histopathological differentiation of the tumours ( $r = 0.045$ ) or staging of the disease ( $r = 0.002$ ), which reflects degree of axillary metastases.

When two sub-populations of human breast tumours were examined for possible correlation between AHH activity,

analysis revealed that no significant difference in response to chemotherapy could be attributed to "high" or "low" AHH activity in the tumours. The few patients in each sub-population that were "evaluable" and demonstrated the proposed response based on AHH activity (patients with "high" AHH activity tumours responding more favourably to chemotherapy than those with "low" AHH activity) indicated that a more comprehensive study may have some potential in evaluation of predictive abilities of AHH in patient response to chemotherapy.

As in any clinical test, the elapsed time for follow-up period must be great enough so that any remissions and response to chemotherapy can be determined. In this study, over time, more patients may become "evaluable" under criteria established for this study. In the future, patients may undergo a relapse, with assignment to chemotherapy as a possible outcome or those presently on chemotherapy will, in time, be able to be assessed as to their response to treatment. As in all retrospective studies, the researcher has had little control over the experimental design. It would be easier to judge any possible correlation between AHH activity and response to chemotherapy if the study had been designed to test simple treatment effects of various chemotherapy regimes in "high" and "low" AHH activity patients. The significance of the presence of high levels of AHH activity in human breast tumours will probably require the passage of several years before an accurate assessment can be made. The eventual computerization of records will make

such a study easier and allow more patients to be examined.

In summary, the results demonstrate that some human breast tumours possess detectable levels of AHH activity that vary in range from non-detectable to levels equal to those of uninduced rat liver. The higher levels of activity resemble those seen in "induced" mouse and rat mammary tumours. Competitor studies with ANF revealed a heterogeneous response to the inhibitor indicating the presence of multiple forms of cytochrome P-450. A survey study of samples of "low" and "high" activity tumours indicated that examination of levels of AHH in human breast tumours might be a promising method by which to predict response to chemotherapy. A more detailed analysis of the clinical records at a later date might shed more light on this interesting possibility.

## CHAPTER VI

### CONCLUSIONS

1) Mouse, rat, and human mammary tumours have MFO activity as measured by the AHH assay.

2) Mouse mammary tumours possess higher levels of AHH activity varied depending on the mode of tumour-induction: "spontaneously-arising" tumours have significantly higher levels than DES tumours or chemically-induced tumours. Treatment of genetically "responsive" mice with BNF resulted in a 10-fold increase of AHH activity. In "responsive" mice, ANF consistently inhibited AHH activity both in untreated and BNF-induced tumours.

3) Tumours in "non-responsive" mice did not demonstrate "induced" AHH activity after BNF treatment. Basal tumour AHH was not inhibited by ANF whereas BNF-induced tumour AHH was inhibited to 50 per cent of control in "non-responsive" mice.

4) Serial transplantations of mouse tumours indicated that variation in AHH activity decreased in successive transplants as compared to the original tumour population.

5) Basal AHH levels in rat tumours were comparable to levels reported for normal rat mammary tissue. Treatment of rats with BNF caused a 70-fold increase in mean tumour AHH activity. ANF consistently inhibited tumour AHH in BNF-treated rats.

6) AHH activity in human breast tumours was variable ranging from non-detectable levels found in most tumours

to values as high as non-induced rat liver. ANF in the incubation flask inhibited AHH activity in some human tumours and caused apparent stimulation in others. This may represent evidence for multiple forms of cytochrome P-450 in the human population.

7) Examination of clinical records revealed no correlation between AHH activity in human breast tumours and estrogen receptor concentration. A preliminary study of a sample of the human tumour population revealed some evidence for a possible correlation between tumour AHH activity and patient response to chemotherapy.

## APPENDICES

### I. Statistics

All statistical tests and repetitive calculations were performed on a Texas Instruments model SR-52 programmable calculator.

A. Standard deviation of X by the  $N^{-1}$  method was computed using the following formulae:

$$SD_X = \sqrt{\frac{\sum_{i=1}^n x_i^2 - n\bar{X}^2}{n-1}} \quad \text{where} \quad \bar{X} = \frac{\sum_{i=1}^n x_i}{n}$$

Standard error of the mean of X by the N method was computed as follows:

$$SEM_X = \frac{SD_X}{\sqrt{n}}$$

Variance of the data was calculated by:

$$V = (SD_X)^2$$

The coefficient of variation, representing the relative spread of distribution of data points, was calculated as follows:

$$C.V. = \frac{100\% SD_X}{\bar{X}}$$



### B. Least Squares Regression Line

The linear least-squares fit of input data points (x,y) were calculated using the following formulae:

$$\text{slope} = m = \frac{n \sum xy - (\sum x) (\sum y)}{n \sum x^2 - (\sum x)^2}$$

$$\text{intercept} = b = \frac{\sum y - m \sum x}{n}$$

The least square line of y on x has the equation:

$$y = mx + b$$

### C. Correlation Coefficient

The correlation coefficient of input data points (x,y) was calculated as follows:

$$r = \frac{\sum xy - \frac{\sum x \sum y}{n}}{\sqrt{\left( \sum x^2 - \frac{(\sum x)^2}{n} \right) \left( \sum y^2 - \frac{(\sum y)^2}{n} \right)}}$$

### D. Student's "t" Test

The value of t was determined from the equation:

$$t = \frac{\bar{X} - \bar{Y}}{\sqrt{\left( \frac{(n_x - 1) SD_x^2 + (n_y - 1) SD_y^2}{n_x + n_y - 2} \right) \left( \frac{1}{n_x} + \frac{1}{n_y} \right)}}$$

$$\text{where } \bar{X} = \frac{\sum x}{n_x} \text{ and } \bar{Y} = \frac{\sum y}{n_y}$$

$\overline{SD}_x$  = Standard deviation of x and  $\overline{SD}_y$  = Standard deviation of y. Degrees of freedom =  $N_x - N_y - 2$ .

#### E. F-Test for Comparison of Variances

Variation from different populations were compared by means of the following ratio:

$$F = \frac{V_1}{V_2} \quad \text{where } V_1 > V_2$$

where  $DF_1$  = degrees of freedom for upper variance

$$V_1 = N_1 - 1$$

where  $DF_2$  = degrees of freedom for lower variance

$$V_2 = N_2 - 1$$

#### F. One-Way Analysis of Variance (ANOVA)

The following formulae were used in testing the differences between population means of k treatment groups, where each group i ( $i = 1, 2, \dots, k$ ) consists of  $n_i$  observations  $x_{ij}$  ( $j = 1, 2, \dots, n_i$ ):

$$\text{Sum of observations in group } i = \sum_{j=1}^{n_i} x_{ij}$$

$$\text{Total Sum of Squares} = SS = \sum_{i=1}^k \sum_{j=1}^{n_i} x_{ij}^2 - \frac{\left( \sum_{i=1}^k \sum_{j=1}^{n_i} x_{ij} \right)^2}{\sum_{i=1}^k n_i}$$

$$\text{Treatment Sum of Squares} = TSS = \sum_{i=1}^k \frac{\left( \sum_{j=1}^{n_i} x_{ij} \right)^2}{n_i} - \frac{\left( \sum_{i=1}^k \sum_{j=1}^{n_i} x_{ij} \right)^2}{\sum_{i=1}^k n_i}$$

Error Sum of Squares = ESS = SS - TSS

The value of the F statistic is:  $F = \frac{DF_2 \times TSS}{DF_1 \times ESS}$

where  $DF_1$  = treatment of degrees of freedom =  $k - 1$

$DF_2$  = error degrees of freedom =  $\sum_{i=1}^k - k$

## II. DNA Measurement by Ethidium-Bromide Technique

Beers and Wittliff (1975) have outlined a rapid method of determining DNA in mammary gland homogenates using the ethidium-bromide technique. This method was applied to determination of DNA in mammary tumour tissues.

### A. Reagents

- 1) SDS Homogenizing Buffer: This buffer contains 0.05M Tris-HCl, 1.95M NaCl, 0.025M EDTA, and 0.1% lauryl sodium sulfate (SDS) at pH 8.0. To prepare 6.05 g Tris (THAM), 113.1 g NaCl, and 7.30 g EDTA were combined and made up to 1L. The buffer was brought to pH 8.0 with concentrated NaOH. SDS was added and mixed; it remains as a flocculent precipitate.
- 2) Washing Buffer: This buffer is identical to the homogenization buffer except that it contains no SDS.
- 3) Dilution Buffer: This buffer contains 0.05M Tris-HCl, 0.2M NaCl, and 0.025M EDTA at pH 8.0. To prepare, combine 6.05 g Tris, 11.60 g NaCl, and 7.30 g EDTA and make up to 1 L. Bring to pH 8.0 with concentrated NaOH.

- 4) Ethidium-Bromide Solution: Ethidium-bromide is added at a concentration of 40 mg per litre of dilution buffer.
- 5) Ribonuclease: 20 mg ribonuclease per ml dilution buffer is prepared so when 20  $\mu$ l is added to the extracted sample, 400  $\mu$ g ribonuclease is present.
- 6) DNA Standards: DNA standards are made up in the washing buffer using calf thymus DNA. The samples are made up 10-fold more concentrated so that upon dilution with the dilution buffer, the final concentration series ranges from 10  $\mu$ g/ml to 100  $\mu$ g/ml. A stock concentration of 1 mg/ml is made and dilutions performed so that initial concentrations before dilution range from 0.1 - 1.0 mg/ml.

B. Procedure

- 1) To homogenate or nuclear pellet from 0.5 - 1.0 g tumour, add 3.0 ml homogenizing buffer.
- 2) Using the Polytron PT-10 at a setting of "6", the homogenate, plus buffer is resuspended for 10 seconds.
- 3) Heat suspension at 60°C for 1 hour.
- 4) Cool for 10 minutes at 0°C to precipitate the SDS; centrifuge for 10 minutes at 10,000 x g and collect the supernatant.
- 5) Wash pellet with 2.0 ml washing buffer; centrifuge for 10 minutes at 10,000 x g and pool with previous

supernatant.

- 6) Add 2.0 ml homogenizing buffer to the pellet and extract again for 1 hour at 60°C. Chill as before, centrifuge, and combine this supernatant with previous one.
- 7) Wash the pellet with 2.0 ml of washing buffer. Centrifuge and combine supernatant with above.
- 8) Remove two 0.1 ml aliquots and dilute each to 1.0 ml with dilution buffer.
- 9) Add 1 ml ethidium-bromide solution to each aliquot and read the fluorescence against standards.
- 10) Add 20  $\mu$ l ribonuclease (20 mg/ml) to hydrolyze RNA.
- 11) Incubate samples at 37°C for 1 hour along with appropriate standards.
- 12) DNA content is determined from the difference in the fluorescence of the ribonuclease-treated sample and that read before the addition of ribonuclease.

#### C. Technical Notes

The fluorescence of each sample was determined by exciting at 365 nm and reading the emission at 590 nm. Ethidium-bromide binds quantitatively to DNA and RNA by intercalating with the bases of these polymers. After this occurs, the fluorescence of the dye is enhanced 20-25-fold and this increase, when assayed at concentrations of salt greater than 0.1M, was specific for nucleic acid polymer.

### III. Lowry Technique for Protein Measurement

#### A. Reagents

- 1) Reagent A: Dissolve 20 g  $\text{Na}_2\text{CO}_3$  (sodium carbonate) in 900 ml distilled water before adding 0.2 g sodium potassium tartrate. Make up to 1 litre.
- 2) Reagent B: Dissolve 0.5 g  $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$  in 100 ml distilled water to make up a 0.5% solution.
- 3) Reagent C: Alkaline copper sulfate solution. Mix 50 ml reagent A with 1 ml reagent B just prior to use. Discard after 1 day.
- 4) Folin Reagent: Dilute 50 ml Folin-Ciocalteu reagent (nN) with 70 ml distilled water.
- 5) BSA (5 mg/ml): Make up a stock solution of BSA at 5 mg/ml by adding 5 g BSA and bringing up to 1 L with water.

#### B. Procedure

- 1) Pipet 100  $\mu\text{l}$  of microsomal suspension into test tube after appropriate dilution.
- 2) Add 900  $\mu\text{l}$  distilled water and mix.
- 3) Prepare blanks in the same manner by adding 100  $\mu\text{l}$  distilled water in one and 100  $\mu\text{l}$   $\text{GPO}_4$  buffer in another.
- 4) Standards are prepared in the same manner; 100  $\mu\text{l}$  of each BSA standard ranging from 50 - 500  $\mu\text{g}$  BSA is added.

- 5) Pipet 5 ml of reagent C to each tube, mix and let stand for 10 minutes.
- 6) After 10 minutes, add 0.5 ml Folin reagent, mix immediately and allow colour development at room temperature for 30 minutes.
- 7) After 30 minutes, measure absorbance at 660 nm against the distilled water blank. Subtract the  $\text{GPO}_4$  buffer blank from the unknown and determine the concentration from a standard BSA protein curve, established using BSA solutions over a range of concentrations. Correct value for dilution if required.



#### IV. 3-Hydroxybenzo[a]pyrene Standardization Curve

In order that the relative fluorescence from microsomal suspensions can be quantified, a standard curve using purified 3-hydroxybenzo[a]pyrene (a generous gift from Dr. E. W. Nebert) was constructed (Nebert, 1978).

##### A. Reagents

1) Stock solution of 3-hydroxybenzo[a]pyrene:

A stock solution of  $2 \times 10^{-4} \text{ M}$  was made up by adding 0.536 mg / 10 ml methanol.

Dilutions were done such that a concentration range of  $2 \times 10^{-4} \text{ M}$  to  $2 \times 10^{-8} \text{ M}$  yielded a range of 1-10,000 pmoles of 3-hydroxybenzo[a]pyrene per incubation flask.

2) Reaction Mixture: The 3-hydroxybenzo[a]pyrene was added to the reaction mixture containing co-factors and microsomal protein as described in Chapter II.

##### B. Procedure

1) The microsomal suspension was heated to  $60^{\circ}\text{C}$  for 10 minutes to destroy any enzyme activity. This ensures that the 3-OHBP is not converted enzymatically to nonfluores-

cence and concentration of 3-OHBP is not underestimated.

- 2) The reaction mixture is prepared in the normal manner as outlined in Chapter II using heat-inactivated microsomal suspensions.
- 3) Upon addition of 3-OHBP to the reaction mixture, the normal incubation period of 60 minutes at 37°C is followed after which the usual incubation and extraction procedures are performed.

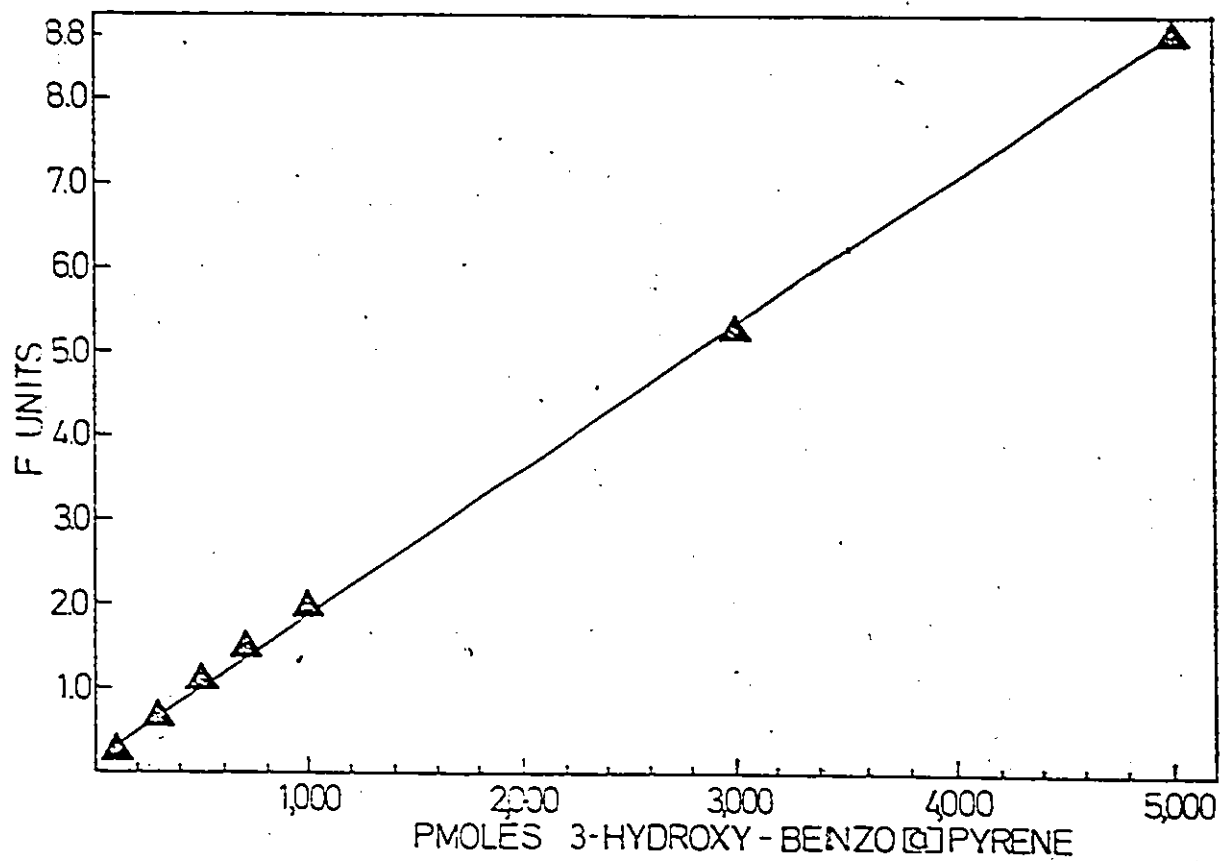
### C. Results

The resultant standard curve indicates a lack of linearity at low 3-OHBP concentrations (see Figure 16). This lack of linearity is not inherent in the fluorometer itself as direct addition of 3-OHBP alone to 1.0N NaOH results in a linear calibration curve (Nebert, 1978). Similar standard curves are obtained when no microsomes are present in the reaction mixture as when the samples are not incubated. The nonlinearity is attributed to insufficient extraction at low 3-OHBP concentrations. This lack of linearity becomes important in tissues with low AHH activity and sets the limits of the assay at 0.1 pmole 3-OHBP/mg/minute.

Note: Because of the difficulties in obtaining, using, and storing authentic 3-OHBP as a standard,

Figure 16. 3-Hydroxybenzo[a]pyrene Standard Curve.

The curve depicts the relative fluorescence as a function of 3-OHBP concentrations added to the reaction mixture and incubated for the 60 minute time period. Heat-inactivated mouse mammary tumour microsomal suspension was used with 560  $\mu$ g microsomal protein added to each incubation flask.



it is desirable to be able to use a readily available standard to calibrate the machine each day.

Quinine sulfate, a stable compound, fluoresces strongly in 0.1N  $H_2SO_4$ , at the peak excitation and emission wave-lengths of 3-OHBP. A standard curve using a concentration range of quinine sulfate from 0.001-20  $\mu g/ml$  can be constructed for this purpose.

V. Characteristics of "High" and "Low" AHH Activity Tumour  
Sub-Populations

Characteristics	High AHH	Low AHH
Median Age (years)	54	70
Per Cent ER Positive	10	25
Disease-Free Interval (months)	28.8	28.0
Disease Staging - Stage I	35 %	21 %
Stage II	40 %	57 %
Stage III	5 %	11 %
Stage IV	20 %	11 %
Degree of Tumour Differentiation		
- poorly differentiated	57 %	46 %
- moderately differentiated	7 %	0 %
- Well differentiated	36 %	54 %

# VI. Chemicals Used and Suppliers

Acetone	- Fisher <sup>a</sup>
Albumin, bovine serum (BSA)	- Sigma <sup>b</sup>
Alphanaphthoflavone (ANF)	- Sigma
Beta naphthoflavone (BNF)	- Sigma
Cupric Sulfate	- Fisher
Deoxyribonucleic acid (DNA)	- Sigma
Diethylstilbestrol (DES)	- Sigma
Dimethylbenzanthracene (DMBA)	- Sigma
Dithiothreitol (DTT)	- Sigma
Ether (anaesthesia grade)	- Fisher
Ethidium-bromide	- Sigma
Ethylene diamine tetra-acetate	- Sigma
Folin-Ciocalteu Reagent - 2N	- Sigma
Glycerol	- Sigma
Hexane	- Fisher
Lauryl sodium sulfate (SDS)	- Sigma
Magnesium chloride	- Fisher
Nicotinamide adenine monophosphate, reduced (NADPH)	- Sigma
Nicotinamide adenine, reduced (NADH)	- Sigma
Potassium phosphate	- Fisher
Ribonuclease I	- Sigma
Sodium carbonate	- Fisher
Sodium chloride	- Fisher
Sodium hydroxide	- Fisher

Sodium tartrate

-- Fisher

Tris (tris(hydroxymethyl)aminomethane)

-- Fisher

a = Sigma Chemicals, St. Louis, Missouri, U.S.A.

b = Fisher Scientific Company, Toronto, Ontario, Canada..



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