INDUCTION OF ARYL HYDROCARBON HYDROXYLASE IN *AMBYSTOMA TIGRINUM*

THESIS

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Fulfillment of the Requirements

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By

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Aryl hydrocarbon hydroxylase (AHH) was induced 15-fold in Ambystoma tigrinum by intraperitoneal injection of 3-methylcholanthrene in corn oil, or 10-fold by addition of aromatic polycyclic hydrocarbons to the aqueous environment of the neotene animal. The cytochrome P-450-associated microsomal enzyme is similar to the inducible, one-gene, autosomal-dominant system typical in the laboratory mouse and man. Differences in optimal temperature for enzyme induction and activity were noted in organ culture of human and Ambystoma tissues, and ratios of benzpyrene metabolites differed between Ambystoma and Mus. The half life of enzyme activity induced in vivo was related to the excretion of hydrocarbon metabolites.

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CHAPTER I

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH), one of major groups of chemical carcinogens (63), induce the synthesis of aryl hydrocarbon hydroxylase (AHH) in the tissues of a host of animals, including man (32, 33, 65, 66, 68, 70, 71). Aryl hydrocarbon hydroxylase, also known as benzo(α)pyrene-3-hydroxylase (E.C.1.14.1.1.), is a mocrosomal mixed function oxygenase (61) which is capable of metabolizing a number of exogenous contaminants of food, air, and water such as carcinogenic polycyclic aromatic hydrocarbons (PAH), ploychlorinated biphenyls and pesticides, and endogenous compounds such as steroid hormones (26, 28, 34). Metabolism of PAH may either decrease or potentiate the biochemical activity of those compounds known to be carcinogenic (36, 37, 38, 49).

Because of its role in the biotransformation of PAH carcinogens, much study has been centered on developing an understanding of the AHH system in an attempt to delineate the interaction between living systems and such chemical carcinogens.

To understand the relationship between AHH induction and tumorigenesis, several questions must be answered. Initially, how are PAH related to cancer? Secondly, what is the relationship between PAH metabolism by AHH and tumorigenesis, and thirdly, what are the genetic properties of AHH in animal systems?

The Relationship of PAH to Tumorigenesis

The relationship between PAH and tumorigenesis can be explained in terms of three broad theories: viral oncogenesis, the immunological theory, and the somatic mutation theory.

Although viral oncogenesis was demonstrated as early as 1908 by Ellerman and Bang (9) who showed that cell-free filtrates of a chicken leukemia virus would transmit the disease, this field of research received little attention until 1932 when viral oncogenesis was demonstrated in rabbits. This was the year that the Shope papilloma virus was discovered and that the filtrates of this virus caused new papillomas to appear on previously wart-free rabbits (86). It is now known that of the 5 groups of DNA viruses at least 3 are oncogenic (50). In 1944, Rous reported that application of chemical carcinogen to rabbits along with the Shope Papilloma virus resulted in rapid production of skin papillomas (82, 83).

Duran-Reynals, working with fowl pox virus, found that skin tumors resulted when chickens containing the virus were treated with methylcholanthrene (20). Tanaka (88), working with several human viruses of the herpes group, found that they induced tumors in mice which had been painted with solutions of chemical carcinogens in quantities deemed too small to be tumorigenic. From these and other findings, the viral oncogene concept of neoplasia was developed and reported in revised form by Todaro and Huebner in 1972 (90, 98). The current viral oncogene theory hinges on the presence of latent viruses within the host genome that are activated by carcinogens, including the PAH.

In 1964, Green postulated a change in the pattern of antigenic lipoproteins in membranes by carcinogens resulting in a loss of immunological identity of the tissue (35). However, it is the opinion of Boyland and others that carcinogenesis is difficult to fit into the general pattern of immune response (6). It now appears more probable that an absence or aberrance of immune response is necessary for developing neoplastic cells not to be immunologically attacked and removed from the host animal.

Boveri (8) reported the association of cancer with chromosome aneuploidy in somatic cells; however, Little and Tyzzer (57) first recognized the similarity between

the causes of mutation and malignant change and demonstrated the role of genes in the initiation, perpetuation and transplantation of tumors. This led to the formulation of the somatic mutation theory of tumorigenesis which has, and still does, occupy a central, though controversial, position in basic cancer research. That there seems to be some correlation between mutagenicity and carcinogenicity of chemical compounds gives much credence to this theory (21, 63). Some investigators initially believed otherwise. Burdette (11) in his review concluded that:

> "those investigators who have tested a wide range of different compounds have found no parallelism between mutagenicity and carcinogenicity . . ."

As a consequence of recent advances in molecular biology it has become possible to describe the mutation process in terms of chemical reactions and to assess whether these are likely to occur with carcinogens or their metabolites (22, 23, 24, 25, 27). Fahmy (21) classifies chemical carcinogens into two well defined groups on the basis of their mutagenicity in cellular genetic systems: (a) gene mutators and (b) gene inactivators. The second group is typified by the more potent carcinogens, the PAH and aromatic amines. These agents were found to be inactive as far as initiating point

mutations and large chromosome breaks but do induce small chromosome deletions in D. melanogastor.

Brookes and Lawley (10) reported covalent bonding of genic DNA to various hydrocarbons as early as 1964. Fahmy proposed two molecular mechanisms for the interaction of PAH with living systems: (a) the covalent bonding of the carcinogen to a chromosomal locus in a manner that physically interferes with its replication, or (b) the physical absorption of the carcinogen within the chromosomal site and subsequent reaction with or inactivation of enzymes involved in macromolecular synthesis (21). The first mechanism, covalent bonding, could occur with PAH since these compounds or their metabolies do react with nucleic acids and proteins (62). The second mechanism, physical absorption, could occur with some PAH since these have been shown to be solubilized in aqueous solutions of DNA in vitro (7, 96) and to bind covalently to proteins in vivo. The in vivo binding is likely to occur via metabolites of PAH involving an electron dense area of the molecule known as the K region (36, 37, 38).

There is no present experimental basis for selecting any one of the above mechanisms (viral oncogenesis, immune response, somatic mutation) as the sole mechanism of chemically induced carcinogenesis. It is probable that each mechanism may rationally explain the chemical induction of

tumors in specific instances or may interact in several combinations during either chemically or non-chemically induced carcinogenesis.

The Relationship of AHH to Tumorigenesis

There are currently two bodies of evidence suggesting opposing action of the mixed function oxygenase, AHH, on carcinogenic or potential carcinogenic compounds.

The enzyme system, AHH, oxidizes a large number of PAH to various mono and dihydroxy derivatives and quinones (17, 43, 87). These hydroxylated metabolites are judged to be inactive or, at least, less active carcinogens and for this reason, the enzyme complex is considered by some to be a detoxification system (1, 38, 44). Recent studies by Wattenberg, et al. (95) demonstrated that pretreatment of mouse skin with B-napthaflavone stimulated AHH activity and inhibited the carcinogenicity of topically applied benzo(α)pyrene. Pretreatment of animals with certain flavones or other compounds that induce AHH has been reported to inhibit the formation of lung cancer by benzo(α)-pyrene or by 7,12 dimethylbenz(α)anthracene (DMBA) (93, 94). For example, Gelboin, et al. (29, 30) showed that application of 7,8 benzoflavone with DMBA on mouse skin inhibited the tumorigenetic activity of DMBA.

Conversely, pretreatment of animals with certain inhibitors of AHH activity prior to the administration of

DMBA increases the yield of mammary cancers in rats (97). Falk and Kotin (26) showed that PAH administered to rats were excreted through the liver as metabolites in bile soon after application. This suggests that the hydroxylase activity in liver is important in clearing PAH from the systemic circulation, thus limiting the amount of PAH that reaches non-hepatic target cells. Kodama et al. (51) working in vivo with mice suggested that AHH appears to play an important role in protecting mammals from the carcinogenic effects of PAH which are encountered in the environment. These views would seem to be logical from an evolutionary standpoint since the products of incomplete carbohydrate combustion and other foreign chemicals have been a part of the earth's environment for millions of years. Thus, a moderately polluted environment could well be selective for those organisms that have developed enzyme systems capable of removing or detoxifying noxious chemicals normally present in their environment, while a heavily polluted environment might be selective against AHH due to the enzymatic potentiation of environmental carcinogens. The fact that the AHH system is inducible and represents a response of an organism to a transient condition in the environment lends further credence to the view that the enzyme system serves mainly to detoxify or alter

otherwise endogenous or exogenous chemicals novel to or intermittently present in the internal or external environment.

An opposite hypothesis, that the AHH system favors the carcinogenic process by activation of the PAH molecules, is also supported by a large body of evidence. Many carcinogens, especially the PAH, are not carcinogenic per se, but must be metabolically activated to an ultimate carcinogenic form (36, 37, 39, 60). AHH is one of the enzyme systems that metabolizes PAH through a stage of epoxide intermediates (36, 85). Wang, et al. (88) found the 4,5-epoxide of benzo(α)pyrene, a K-region metabolite, to bind DNA more efficiently than the parent molecule or any other of the metabolites of benzo(α)pyrene. This 4,5-epoxide may, in turn, be metabolized to 4,5-dihydrodiol by an epoxide hydrase (47). Burki and Bresnick (13) showed an increased carcinogenicity of 3-methylcholanthrene to be directly associated with blockage of the epoxide hydrase system in experimental animals. Inhibition of AHH activity <u>in vivo</u> and <u>in vitro</u> is correlated to a decrease in PAH-induced cellular transformation (5, 29, 30). Kouri et al. (52), found a close relationship between susceptibility to 3-methylcholanthrene (3 MC)-induced subcutaneous tumors and the presence of AHH induction. Mice that were found

to be inducible for AHH were observed to be 5 to 10 times more susceptible to 3MC tumorigenesis than their non-inducible littermates.

These results agree with findings of Schmidt et al. (84) who reported that the C57Bl/6J strain of mice is more sensitive to DMBA-induced subcutaneous tumors than the noninducible AKR/J strain.

In light of these opposing bodies of evidence it appears that the carginogenic effect of a hydrocarbon in a given tissue may be determined by the relative amounts of epoxide produced by the AHH system. Inconsistencies in this paradox may be reconciled by the fact that the epoxide needs to be present for a definite length of time (18, 53), a time that evidently is directly influenced by another of the inducible microsomal enzymes, epoxide hydrase (13, 76). It is likely that this paradox is also influenced by the fact that the AHH system may be quite complex and capable of metabolic activation by epoxide formation as well as metabolic deactivation in PAH tumorigenicity by dihydrodiol, glutathione and hydroxylated metabolite formation in varying ratios (39).

The Genetics of AHH Induction

Nebert et al. (74), Gielen et al. (31), and Thomas et al. (89) attempted to establish the pattern of inheritance of AHH induction in response to PAH applications.

From their experiments with genetic crosses representing extremes of susceptibility and resistance to 3MC induction of AHH, they originally concluded that the enzyme induction followed simple Mendelian genetics in mouse tissues and was inherited as a simple autosomal dominant trait. Additional work by Nebert et al. (72) indicates the genetics of AHH induction to be less clear-cut in certain mouse strains. These authors also concluded that, in the individual mouse genetically responsive to PAH, the hydroxylase activity is induced as an all-or-none phenomenon in all tissues which regularly contain the PAH-inducible enzyme (31, 74, 89). However, Burki et al. (12) found that this proposed all-or-none phenomenon is not a general concept. His results showed a tissue-specific variability of enzyme induction in experimental animals.

Nebert et al. (67, 69, 74) proposed that the locus of the genes controlling this autosomal dominant trait be termed Ah and ah. Therefore, in any individual mouse homozygous or heterozygous for Ah, the AHH activity is fully inducible by PAH. In any inbred or hybrid mouse homozygous for the allele, ah, there is little or no hydroyxlase induction following PAH administration (31, 73). In man, variations in magnitude of AHH induction have been found (47, 48). Significant differences in extent of hydroxylase induction were found in cultured lymphocytes

from a sample of several hundred people and the genetic expression of AHH induction was found to segregate as two alleles at a single locus (14, 15, 47, 48). A significant correlation between lung cancer of probable chemical etiology and AHH induction has been reported for a population of human cancer patients (48).

Animals living in a heavily polluted pond have been found to have six types of abnormal tissue. One of these types has been found to be an invasive, malignant melanoma. Statistical studies have shown that the first year animals have no melanomas and comprise about 90% of the total population. Twenty per cent of the second year animals have melanomas and comprise about 10% of the total population giving this population a 2% cancer index (80).

Statement of Problem

In this thesis, the author proposes to examine animals from a pond receiving treated human sewage. The experimental animals, Ambystoma tigrinum, come from a population whose first year animals are reported to be essentially tumor free (80). The second year animals have a high incidence of tumors. The aim of this study is to determine: (a) if the affected animals are competent for AHH induction, (b) the ratios of PAH metabolites that may be produced in these animals, and (c) if this enzyme system may be compared with that of mammals, including man.

CHAPTER II

MATERIALS AND METHODS

Experimental Animals

Salamanders were collected from Playa Lake by seining and were held in 10 liter aquaria in aerated water. Their diet consisted of earthworms obtained from Sargents Worm Gardens, Lake Dallas, Texas.

C57Bl/6J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine, and maintained in the Animal Colony of The Biology Department of North Texas State University.

Tissue Preparation and AHH Assay

AHH was assayed using a modification of the method of Nebert and Gelboin (70). Tissues were homogenized in TMS buffer (pH 7.5) consisting of 0.05 M Tris-HCl buffer, 0.25 M sucrose, and 3mM MgCl. Cellular debris was removed from tissue homogenates by centrifugation at 5000 x g and the supernatant was assayed for total protein using the Lowry method (58). Supernatant preparations containing approximately 1 mg protein per ml were incubated at specified temperatures for 30 minutes in a final volume of 1.1 ml containing 1.0 mg NADPH and 25 µg of benzo(α)pyrene. The enzymatic reaction was stopped by

addition of 2 ml of an acetone/hexane mixture (1:3) and vortex mixing for a period of 1 minute.

Aqueous and organic phases were separated by centrifugation at 500 x g. The organic phase was removed and extracted by vortex mixing with 1.2 ml of 1 N NaOH for 1 minute. The phases from this mixture were separated by centrifugation at 500 x g. Flourescence determinations were made on the lower, aqueous, phase using an Aminco-Bowman 4-8203 Spectrophotofluorometer with monochromatic settings of 396 nm excitation and 522 nm emission. These readings were compared with prepared standards of authentic 3-hydroxybenzo(α)pyrene obtained from Dr. H. V. Gelboin, National Institutes of Health. The unit of enzyme activity was defined as the fluorescence produced per minute per mg of protein equivalent to a picomole of 3-hydroxybenzo(α)pyrene. The extraction efficiency was calculated to be 60% and final fluorescence values were corrected to reflect this efficiency rating.

Microsomal Preparations

Minced livers were washed in cold 0.15 M KCl to remove blood (heme). The tissue was then mixed with cold TMS buffer (pH 7.5) and disrupted with a ground glass-teflon homogenizer.

Cellular debris, including mitochondria, nuclei, and intact cells, was removed from the homogenates by

centrifugation at 10,000 x g for 10 minutes. Microsomes were sedimented from this supernatant by centrifugation at 80,000 x g for 90 minutes using a Beckman Model L Preparative Ultracentrifuge equipped with a TI-65 rotor. Pellets were washed in 0.15 M KCl using an 18 ga. needle and 10 ml syringe to disperse the microsomal button and were resedimented at 80,000 x g for 90 minutes.

AHH Induction

In vivo induction of AHH was accomplished using two methods. In the first instance, 3MC (10mg/Kg body weight) in corn oil was given by intraperitoneal (IP) injection. Secondly, by the addition of 3MC (75 µg/ml final 3MC concentration) dissolved in acetone to the water of experimental holding tanks.

In vitro induction of AHH was accomplished in short-term organ culture by maintaining diced liver sections (1 mm^2) for 24 to 48 hours in Amphibian Culture Medium (Gibco) containing 3MC at specified concentrations. The optimum temperature for induction was determined by maintaining cultures containing 3MC at 15^0, 20^0, 25^0, and 37^0.

Measurement of Properties of Salamander AHH

A. Cytochrome P-450 determination

To determine the presence of cytochrome P-450 associated with AHH, CO was bubbled through one of two aliquots of a salamander microsome preparation that had been reduced

with dithionite. These reduced microsomes were scanned in a Beckmann DB-GT double-beam grating spectrophotometer using unreduced microsomes as a reference, and a dithionite-reduced cytochrome difference spectrum was determined. This spectrum was the difference in reduced cytochrome absorbance readings at 450 nm and 490 nm and it was used to calculate the cytochrome P-450 content with an extinction coefficient of 91 nM^{-1} (97).

B. CO Inhibition

CO inhibition of AHH activity was determined by bubbling CO, or N_2 for the control, through TMS tissue preparations before the addition of benzo(α)pyrene (BP) or NADPH to the tissue preparations. Samples were then flushed with air, BP and NADPH were added and the tissue preparations were incubated at 20^0 C and assayed.

Metabolite Assay

Salamander liver microsomes in TMS buffer (pH 7.5) were incubated with ^{3}H-BP containing 1 mg NADPH to a total volume of 1.1 ml for a period of 30 minutes. Following incubation the mixture was extracted with 2 ml of ethyl acetate. The organic layer was removed and evaporated to dryness under nitrogen. Acetone (0.10 ml) was then added to pooled, dry samples. The acetone-metabolite solution was spotted onto Eastman Kodak silica-gel thin-layer chromatographic (TLC) sheets. The initial solvent

development system of the two-dimensional chromatographic procedure was benzene. The second solvent consisted of 5% ethanol in benzene. For each dimension the solvent front ran to approximately 10 cm. Fluorescing spots were marked visually under UV light. Radioactive spots were identified by the following autoradiographic method. Those spots found to be radioactive were cut out and placed into scintillation vials containing 1 ml of PPO in 10 ml of toluene, and were counted in a Beckmann LS-100 liquid scintillation counter. The metabolites were tentatively identified by characteristic fluorescing color or by comparing their chromatographic mobilites with known literature values (91).

CHAPTER III

RESULTS

Preface

This study was accomplished in three phases:

1. A characterization of salamander AHH activity.
   a. Induction characteristics
   b. Sensitivity to CO
   c. Association with cytochrome P-450
   d. Temperature of enzyme induction and activity
2. Comparative study of metabolites between a representative mammal (C57Bl/6J mice) and the salamander, Ambystoma tigrinum.
3. A test of naturally occurring animals for environmentally induced AHH activity.

To achieve these studies, 3MC was used as an inducer of the AHH enzyme with benzo(α)pyrene (BP) serving as the enzymatic substrate. As an indication of AHH activity the metabolite 3-hydroxybenzo(α)pyrene was monitored utilizing fluorescence measured spectrophotofluorometrically at the indicated wavelengths. The metabolite study was carried out using the previously mentioned TLC methods. To determine if AHH was being environmentally induced in

animals from the experimental population of Playa Lake, animals taken directly from the lake were tested for AHH activity.

Induction of AHH Activity: Characteristics of the Salamander AHH Enzyme

Figure 1 shows the induction of AHH activity in salamander liver tissue upon IP injection of 3MC in corn oil. There was an approximate 15 fold increase in AHH activity in the liver tissue. This activity peaked at about 48 hours and declined in a nearly linear fashion to the basal level over a period of two weeks. Control animals received corn oil injections and showed no increase in AHH activity.

Figure 2 shows the cytochrome CO-dithionite difference spectrum of liver microsomes from salamanders induced by IP injections of 3MC. This difference spectrum exhibits a soret maximum of 450 nm. Calculations using the absorbance difference between the 450 nm and 490 nm peaks indicate the microsome preparation contained 0.034 mg of cytochrome P-450 per 5 mg of microsomal protein. There is no indication that salamander cytochrome P-450 differs from that of mammals reported elsewhere (65).

Figure 3 shows the results of three separate experiments that were performed in order to further characterize the salamander enzyme. Enzymatic activity was tested in

oxygen, CO, and N_2 atmospheres. Enzymatic activity was shown to proceed in a nearly linear fashion over about 20 minutes of the incubation period in an oxygen environment. AHH activity was diminished severely when tissue preparations were saturated with CO or N_2. Such interference is reported in AHH preparations from mice and humans and is considered to be a property of the mammalian system (4, 17, 47).

The temperature optimum for salamander AHH activity was found to be different from that of mammals. Figure 4 shows the results of assaying liver preparations from animals maintained in water containing 75 µg of 3MC per ml at temperatures of 15°, 20°, 25°, and 37°. It is evident that 20° is the temperature at which optimum activity is seen.

Figure 5 shows induction of AHH in organ culture to have a temperature optimum of 25° C. Although induction at both 15° and 37° was less than half that seen at 25°, less 3MC was required for AHH induction at both 15° and 37° than was required at 25°. In the case of AHH induction at 37°, only 4 µg of 3MC/ml of culture medium was required.

Metabolite Assay

A comparison between ^{3}H-BP metabolites of mouse liver and salamander liver microsomes is shown in Table I. Two

important trends are seen. There was decreased formation of 3-hydroxybenzo(α)pryene in salamanders (13.5%) when compared to that of the mouse (69.9%). Also, the salamander is shown to produce more of the 4,5-epoxide (AM) than does the mouse, and the 4,5-epoxide to 4,5-dihydrodiol ratio is higher in the salamander (9:2) than in the mice (1:1). Results of Figure 5 were obtained using TLC silica gel sheets. The chromotographic separation of ^{3}H-BP metabolites produced by liver microsomes is shown in Figure 6 for the salamander and in Figure 7 for the mouse. The shaded spots represent metabolites that fluoresced but showed no radioactive counts. A visual comparison between Figures 6 and 7 shows the same basic distributions of metabolites in salamanders and in mice.

Extent of AHH Induction from Environmental Conditions of Playa Lake

As seen in Figure 8, the animals taken directly from Playa Lake do exhibit high levels of enzyme activity. Around a 12 fold increase over control levels is seen.

Figure 1. Aryl hydrocarbon hydroxylase induction and decay in salamander liver preparations following intraperitoneal injection of 3MC (1mg/kg. body weight) dissolved in corn oil: (■) 3MC injected animals, (●) control animals receiving only corn oil.

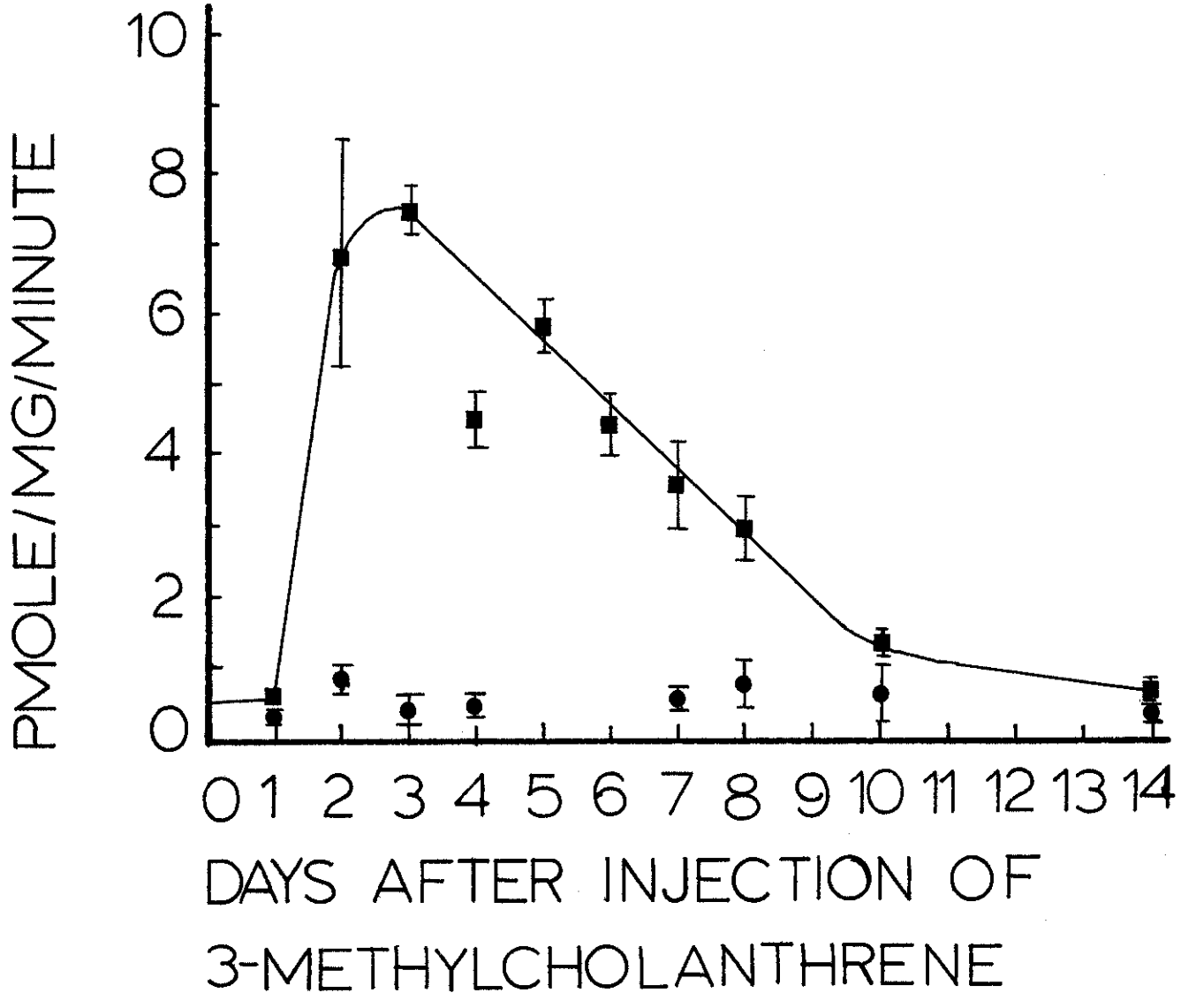
PMOLE/MG/MINUTE
0
2
4
6
8
10
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
DAYS AFTER INJECTION OF
3-METHYLCHOLANTHRENE

FIGURE 1

Figure 2. The CO difference spectrum of liver microsomes of the Salamander, Ambystoma tigrinum reduced with dithionite.

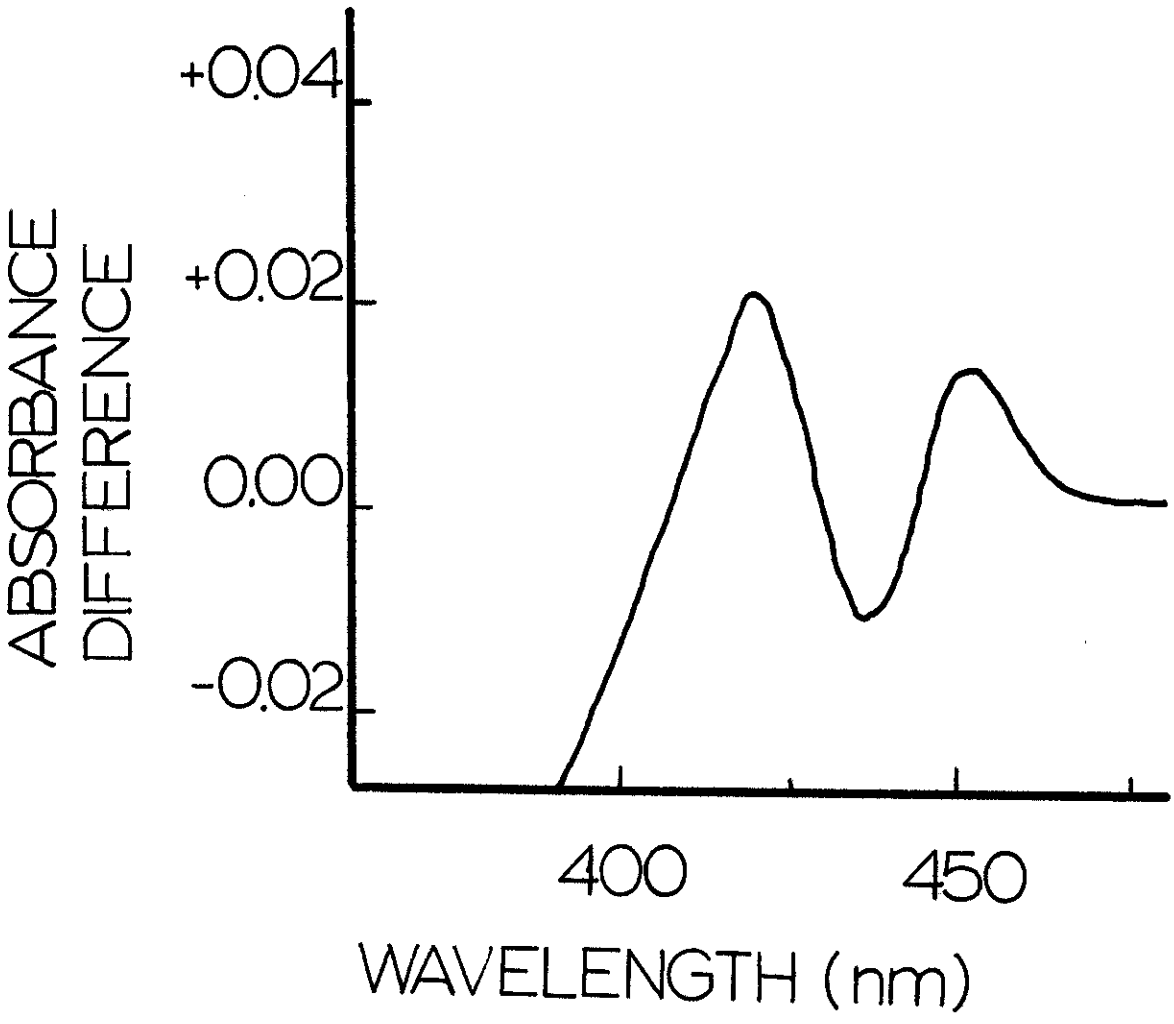
+0.04
+0.02
0.00
-0.02
ABSORBANCE DIFFERENCE
400
450
WAVELENGTH (nm)

FIGURE 2

Figure 3. Enzymatic activity of AHH during incubation at 20° C in the presence of O_2 (●), after saturation with CO (▲), and after saturation with N_2 (■). A unit of AHH is defined as that quantity producing fluorescing metabolites equivalent to 1 picomole of authentic 3-hydroxybenzo(α)-pyrene/mg of protein/min.

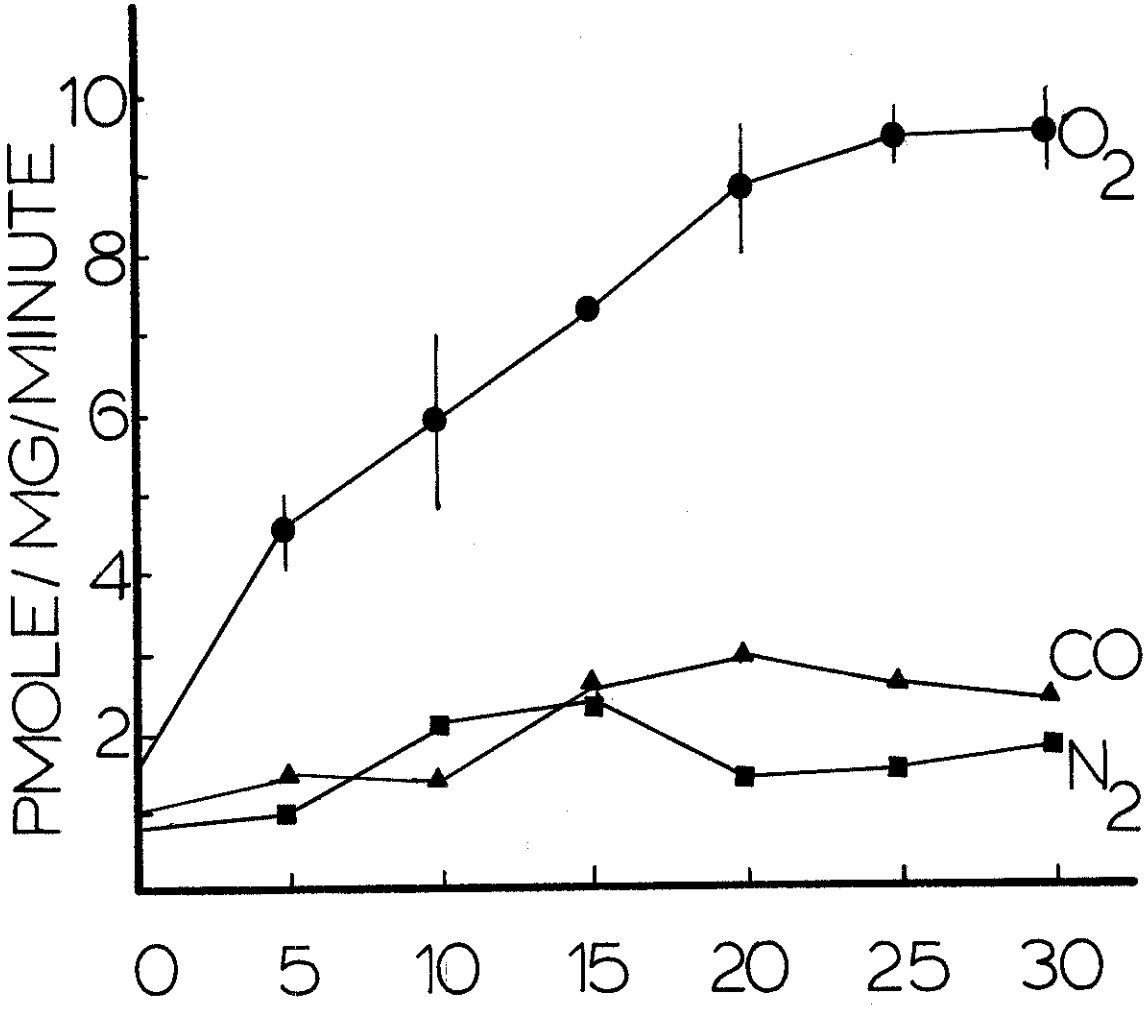
PMOLE/MG/MINUTE
10
8
6
4
2
0
5
10
15
20
25
30
O_2
CO
N_2
MINUTES OF INCUBATION
AT 20° C

FIGURE 3

Figure 4. AHH activity at different incubation temperatures of salamander liver induced *in vivo* by addition of 3MC (75 μg/ml final concentration) to the aqueous environment of the animal.

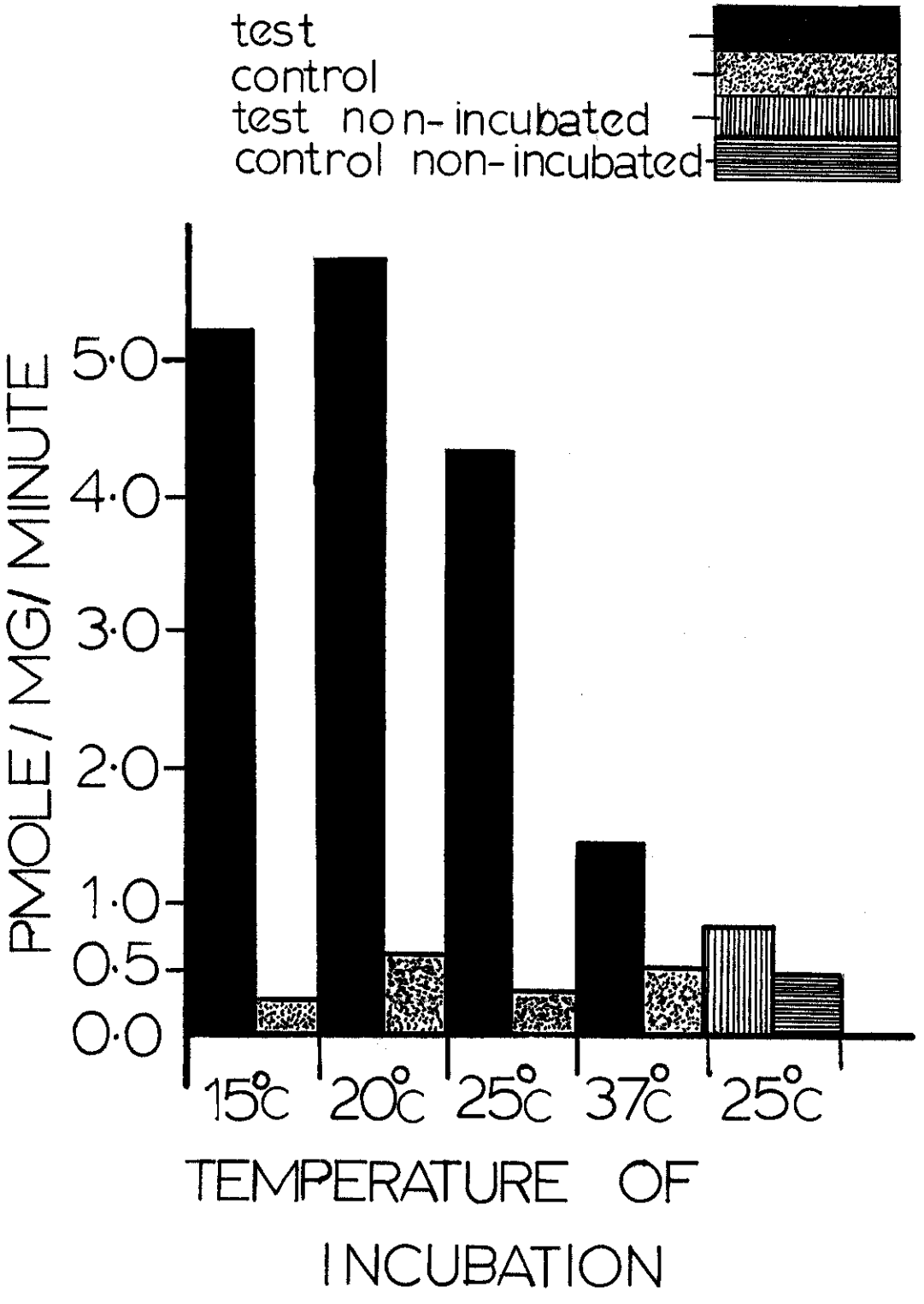
test
control
test non-incubated
control non-incubated
PMOLE/MG/MINUTE
5·0
4·0
3·0
2·0
1·0
0·5
0·0
15°C
20°C
25°C
37°C
25°C
TEMPERATURE OF INCUBATION

FIGURE 4

Figure 5. Differential temperatures and differential 3MC concentrations required for AHH induction in salamander organ cultures maintained in Amphibian Culture Medium (Gibco).

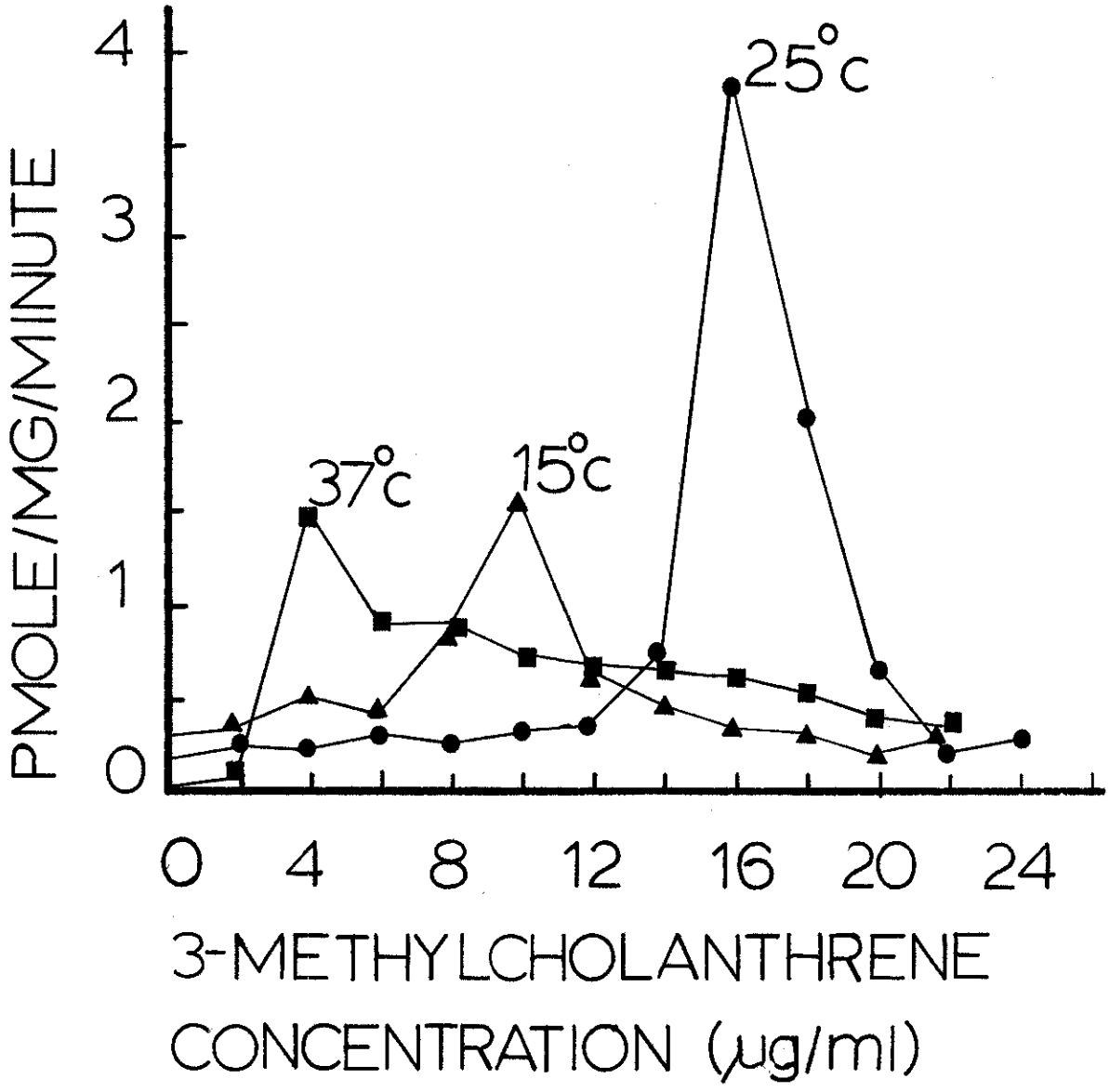
PMOLE/MG/MINUTE
0
1
2
3
4
37°c
15°c
25°c
0
4
8
12
16
20
24
3-METHYLCHOLANTHRENE
CONCENTRATION (µg/ml)

FIGURE 5

Table I. A comparison between ^{3}H-benzo(α)pyrene metabolites produced by mouse liver and salamander liver microsomes incubated at 20°. AM is tentatively identified as the 4,5 epoxide.

TABLE I

A COMPARISON BETWEEN ^{3}H-BENZO(α)PYRENE METABOLITES OF MOUSE LIVER AND SALAMANDER LIVER MICROSOMES IN VITRO

Metabolites		C57Bl/6J cpm	% of Total Metabolites	Salamander cpm	% of Total Metabolites
	Origin	2140	0.0068	725	0.0213
1	9,10 di	1469	0.0046	486	0.0142
2	7,8 di	1269	0.0041	368	0.0110
3	4,5 di	4052	→0.0129	580	0.0169←
4	3-OHBP	218682	0.6992	4384	0.1350
5	unknown	943	0.0030	1170	0.0348
6	unknown	2676	0.856	3289	0.0957
7	3,6 qui	32020	0.1014	10083	0.2938
8	1.6 qui	46765	0.1495	10098	0.2550
9	AM	3651	→0.0116	3094	0.0893←

Figure 6. An autoradiographic determination of the thin layer chromatographic separation of ^{3}H-benzo(α)pyrene metabolites produced by salamander liver microsomes. Shaded spots represent fluorescing metabolites with no detectable counts.

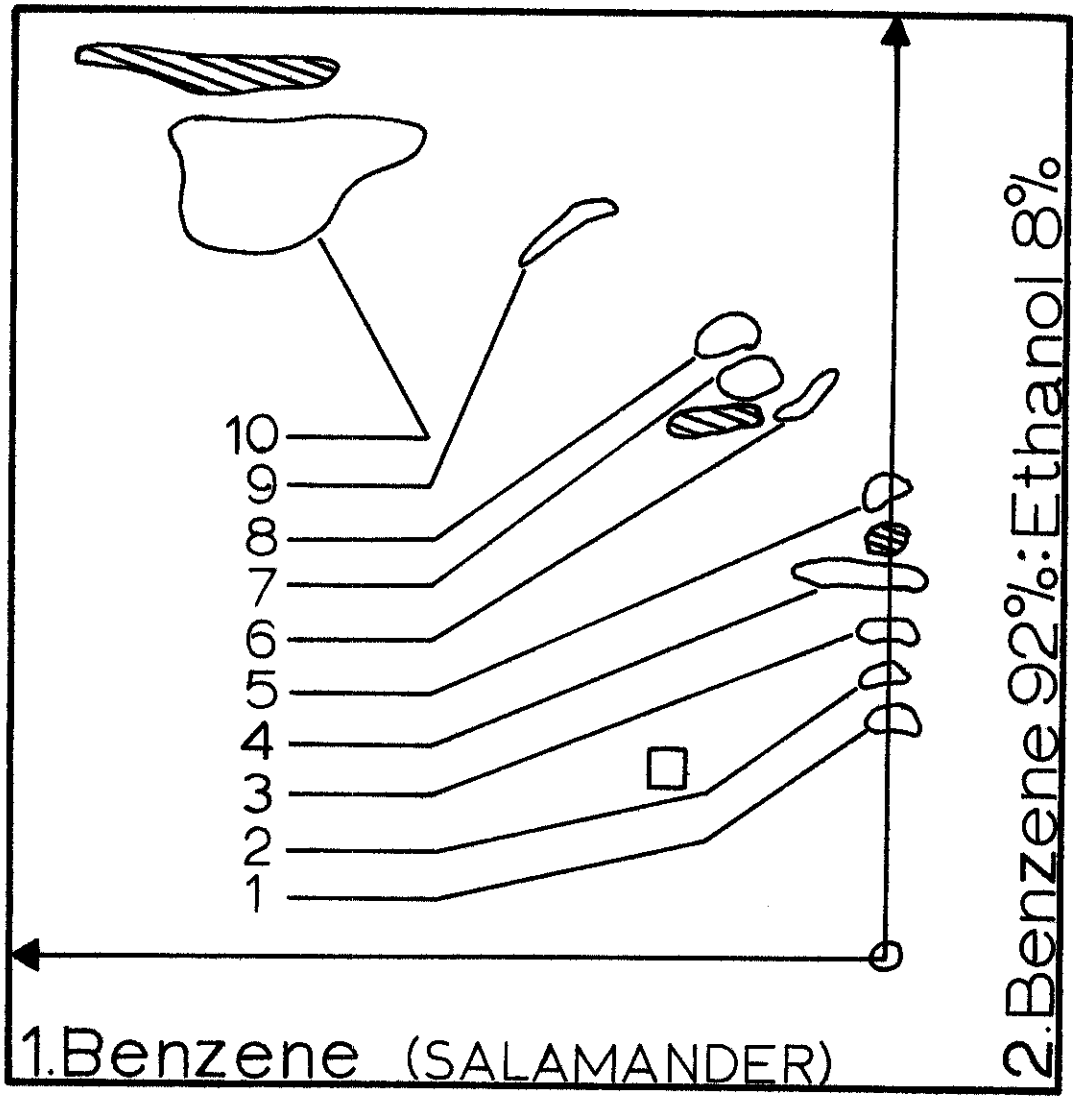

FIGURE 6

Figure 7. An autoradiographic determination of the thin layer chromatographic separation of ^{3}H-benzo(α)pyrene metabolites produced by C57B1/6J mouse liver microsomes. Shaded spots represent fluorescing metabolites with no detectable counts.

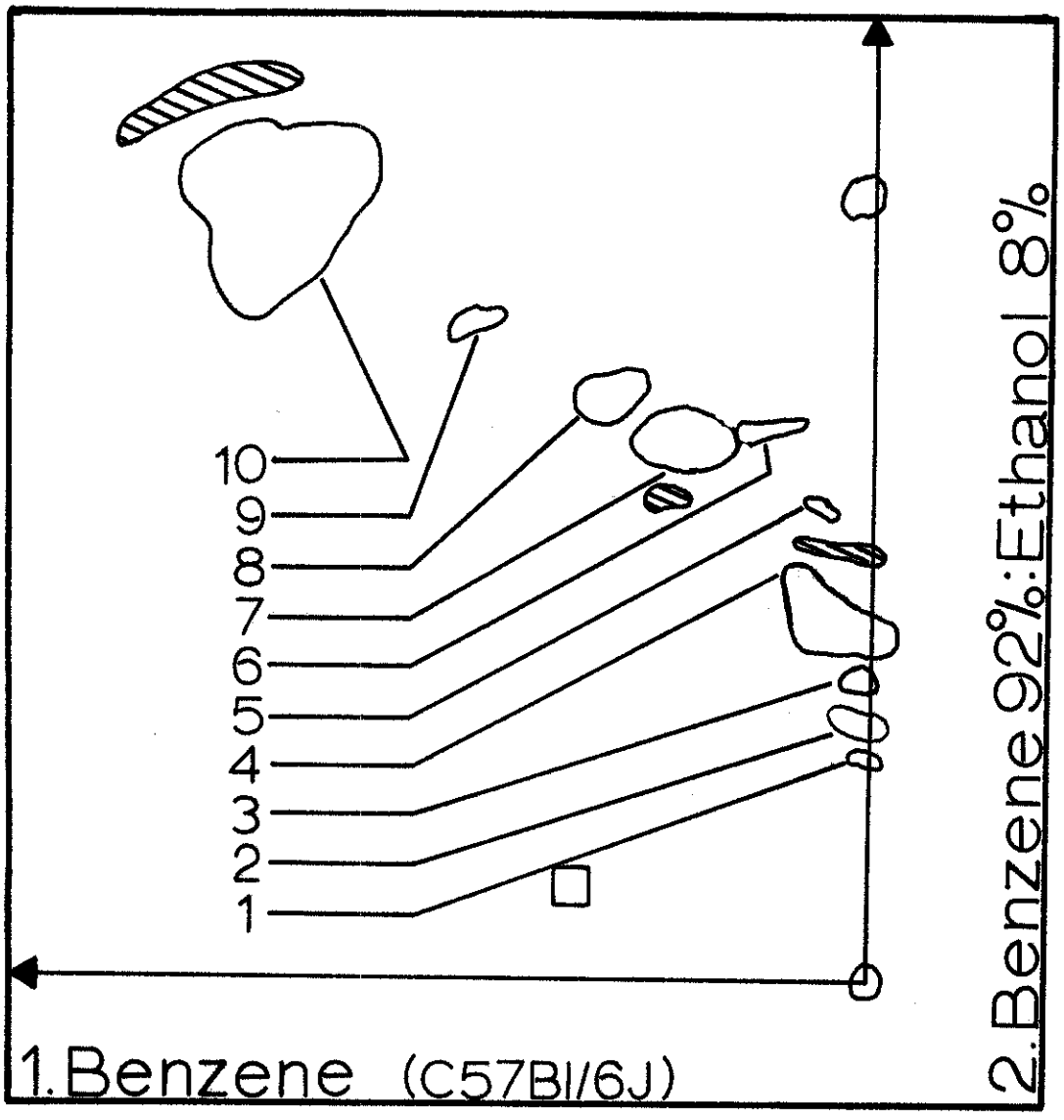

FIGURE 7

Figure 8. A comparison of AHH activity levels between animals induced by intraperitoneal injections of methylcholanthrene in corn oil (10 mg 3MC/kg. of body weight), and test animals taken directly from Playa Lake. Control animals received intraperitoneal injections of corn oil. Control animals were salamanders kept in clean water for a six month period prior to experimentation.

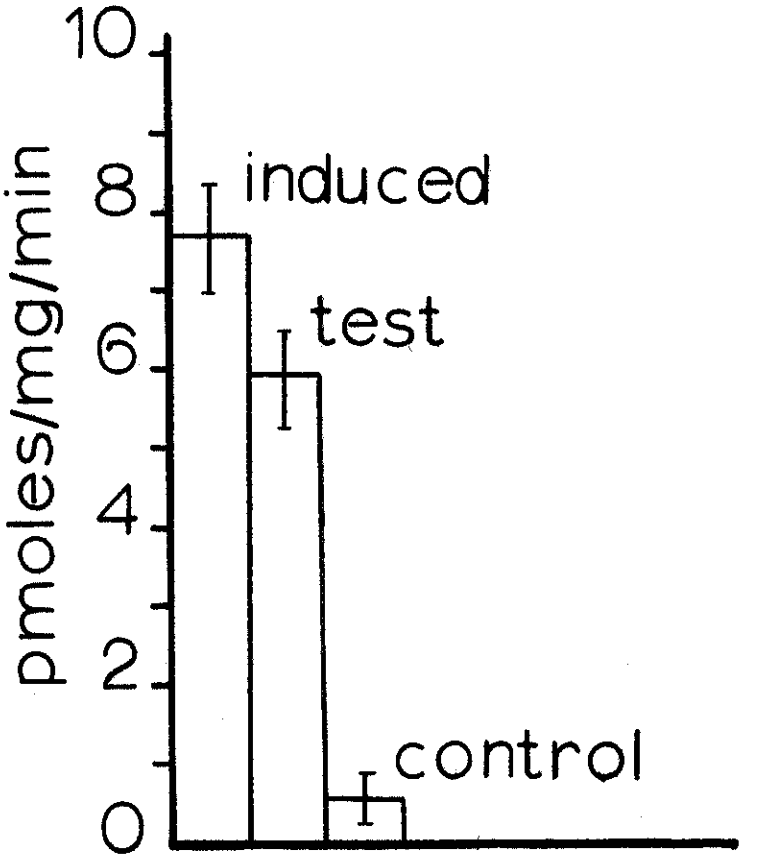

FIGURE 8

CHAPTER IV

DISCUSSION

A long standing objective in the field of cancer research has been to delineate the cellular and molecular mechanisms of PAH carcinogenesis. In the late 1950's, Miller et al. (17) reported that injections of benzo(α)-pyrene and certain other PAH induced the synthesis of a microsomal enzyme, AHH, in rat liver microsomes. The enzyme system was found to metabolize the BP to 3-hydroxy-benzo(α)pyrene and several other hydroxylated products. The induction of AHH in rats by pretreatment with suitable enzyme inducers seemed to provide protection against the carcinogenic effect of BP, 9,10-DMBA,N-methylated aminoazo dyes, 2-acetylaminofluorene, and 4-dimethylaminostilbene (40, 41, 42, 56, 64, 96). These early works indicated that microsomal enzyme inducers inhibit the carcinogenicity of BP and several unrelated chemicals. Current research reports indicate that metabolism of PAH is probably required for initiation of their carcinogenic effects (36, 37, 39, 60).

This is especially true in the case of the highly unstable epoxide intermediates which are capable of binding

with DNA or other macromolecules and are thought to be integral in initiating cellular transformation to a neoplastic form (6, 18, 90). Existing disparities in the mounting body of evidence concerning the responsiveness of AHH to certain chemicals suggest that more than one AHH enzyme system metabolizing PAH may exist. Studies by Mannering and others (2, 3, 4, 59) led to the conclusion that two such AHH systems may exist: (1) P-450 associated AHH found in livers of rats untreated with PAH, and (2) P-448 associated AHH found in livers of rats treated with PAH. These slight differences may reflect basal and induced levels of PAH metabolizing AHH systems and could partially account for the different roles AHH has been reported to pay in PAH metabolite formation and tumorigenesis. It is also probable that AHH induction mechanisms may differ among animal species (16, 19). Another explanation for certain metabolite disparities was suggested by the work of Oesch et al. (75) when they purified and described the properties of an epoxide hydrase, another inducible, microsomal enzyme, perhaps important in the metabolism and detoxification of PAH.

Epoxide hydrase converts intermediate oxidation products, the arene oxides and epoxides, to trans-dihydrodiols (45, 46, 55). Epoxide hydrase activity was demonstrated by Oesch and Daly (76, 77) to be closely associated with

liver microsomes in a variety of species and to be elevated by pretreatment of rats with 3MC or phenobarbital. Kellerman et al. (49) found that this microsomal enzyme to be basally present and inducible in cultured human lymphocytes. They further suggested the mechanism for induction of the two enzymes, AHH and epoxide hydrase, to be the same (49). Oesch et al. (76) working with the epoxide hydrase system from guinea pig microsomes, found evidence suggesting that the AHH system and the epoxide hydrase system form a closely coupled enzyme complex in the microsomal membrane. Oesch (78), contrary to the suggestion by Kellerman et al. (49), further reported that these two functionally closely coupled enzymes are not regulated by the same genetic mechanism. Burki and Bresnick (13) painted the skin of experimental animals with a mixture of carcinogenic PAH and an epoxide hydrase inhibitor and found the incidence of PAH-induced cancer to be significantly elevated. Using these observations as a basis for discussion, the results of the following study may be reviewed.

The salamander, Ambystoma tigrinum, was found to be inducible for AHH activity by IP injections of PAH as well as by addition of PAH to the aqueous environment (Figure 1, Figure 8). Characterization studies showed this AHH enzyme to be cytochrome P-450-associated

(Figure 2) in that the CO-dithionite difference spectrum exhibited a soret maximum of 450 nm. This is a characteristic feature of the mammalian P-450 associated with AHH. Another feature of the salamander AHH system found to be similar to that of mammals was the production of similar types of BP metabolites as shown in Figures 6 and 7.

Several features of AHH enzymatic activity were found to be unique to the salamander system. Enzymatic activity of AHH induced by Ip 3MC injection decreased linerally over a very short period of time (Figure 1). This was particularly evident when compared to AHH activity decay in human alveolar macrophages (15). The optimal temperature of AHH incubation in cultured salamander tissue differed from that of mouse and man, which was characteristically 37^0 (Figure 4) (69). Figure 5 shows salamander enzymatic activity and indicates that the optimal temperature for induction differs from that of mammals (69).

This lack of similarity can be explained in terms of differences of animal body temperature. The salamander, a poikilotherm, has a body temperature lower than that of the mammals studied and the lower temperatures of incubation (Figure 4) and induction (Figure 5) are probably reflections of this fact. Table I shows differences in the relative amounts of certain mouse and salamander liver

microsome ^{3}H-BP metabolites. Specifically the 3-hydroxy-(α)pyrene and the 4,5-epoxide (labeled AM). The 3-hydroxybenzo(α)pyrene, which is non-carcinogenic, is the major metabolite in mice but represents a much smaller per cent of total salamander metabolites. The observation of import in this case is the ratio of 4,5-epoxide (AM) to the 4,5-dihydrodiol produced by each animal group. The ratio is approximately 1:1 (epoxide:4,5-dihydrodiol) in mice, whereas the ratio is higher in the salamander group (9:2). This low ratio in mice plus the larger amount of non-carcinogenic 3-hydroxybenzo(α)pyrene formed by mice shows a definite capacity for more efficient disposition of active metabolites by mice when compared to salamanders.

Accepting that epoxide hydrases convert the 4,5-epoxide (AM) to the 4,5-dihydrodiol (45, 46, 55), there exists the possibility that mice exhibit higher relative levels of the enzyme, epoxide hydrase, which converts epoxides formed in PAH metabolism by AHH to non-carcinogenic dihydrodiols, than do salamanders. This was not determined in this study. The large amount of 4,5-epoxide produced by salamander microsomal AHH and the relative absence of conversion to 4,5-dihydrodiol seem to suggest the possible lack of an epoxide hydrase system or the depression of its efficiency by the lower body temperature of

poikilothermic animals such as salamanders. There is currently no direct evidence to support this suggestion.

Few AHH studies have been made utilizing nonmammalian subjects and no previous AHH studies of amphibians have been reported. Lee et al. (54) studied the metabolism of ^{3}H-BP in three species of marine fish. His data corroborate this study in suggesting the optimum temperature for BP metabolism in poikilotherms from that of mammals. However, his report indicates the major BP metabolite by the fish to be tentatively identified as 7,8-dihydro-7,8-dihydroxybenzo(α)pyrene excreted in the urine.

The major metabolite in mice has been tentatively identified as 3-hydroxybenzo(α)pyrene (Table I). The results of Lee et al. (54) indicate the presence of an efficient detoxification mechanism in these marine fish for the removal of PAH from tissues. Their examinations revealed no tumors in subject marine animals but the animals were observed for only a short period of time.

Ambystoma tigrinum taken directly from Playa Lake were found to be induced for high levels of AHH activity as seen in Figure 8. The ratio of 4,5-epoxide, the active metabolite (AM) to 4,5-dihydrodiol is high (9:2) in these test animals. It would seem probable that the AHH system of this group of animals is either not coupled with an epoxide hydrase system to further metabolize reactive

intermediates, or the epoxide hydrase system is not efficient in these animals. The absence of 4,5-epoxide removal from salamander tissues might explain the high incidence of cancer in this population of animals receiving a steady input of polluted waters into their environment. This suggestion is supported by the increased PAH carcinogenicity reported by Burki and Bresnick (13) when epoxide hydrase inhibitors were applied to experimental animals with the PAH.

CHAPTER V

SUMMARY

The findings of this study indicate a similarity between the AHH enzyme system of the salamander, Ambystoma tigrinum, of Playa Lake and that of mice and men in that it is associated with cytochrome P-450 and is CO-sensitive. The salamander AHH system is either not coupled with an epoxide hydrase system or epoxide hydrase activity is not as efficient in the detoxification of active metabolites in these animals. This offers a tentative explanation for the high incidence of cancer in this natural population of animals found in heavily polluted water.

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