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Vicars, Lynda J., M.A.

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A COMPARATIVE STUDY OF DNA ADDUCTION BY CARCINOGENS IN SOMATIC AND SPERM CELL CHROMATIN

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

San Jose State University

In Partial Fulfillment

of the Requirements for the Degree

Master of Arts

By

Lynda J. Vicars

December 1990

Approved for the Department of Biology

Robert Fowler

Dr. Robert Fowler

B. D. Balhorn

Dr. Rodney Balhorn

Pamela C. Stacks

Dr. Pamela Stacks

APPROVED FOR THE UNIVERSITY

Serena H. Stanford

ABSTRACT

A COMPARATIVE STUDY OF DNA ADDUCTION BY CARCINOGENS IN SOMATIC AND SPERM CELL CHROMATIN

by Lynda J. Vicars

A comparison of DNA adduction to both somatic and sperm chromatin was made to determine if known differences in DNA accessibilities and chromatin structure might affect the extent to which DNA is damaged in liver and sperm cells by similar exposures to carcinogens. Four carcinogens with different chemical reactivities were used: Tryptophan-p-2, an aromatic amine and three polyaromatic hydrocarbons, Benzo-(a)pyrene, 7-Bromomethylbenz(a)anthracene and 7,12-Dimethylbenz(a)anthracene.

The research showed that neither ^{3}H-Benzo(a)pyrene or ^{3}H-Tryptophan-p-2 were able to bind to sperm DNA *in vivo*. *In vitro*, ^{3}H-7-Bromomethylbenz(a)anthracene was able to bind both the liver DNA and sperm DNA in almost equal amounts. The *in vitro* studies which required metabolic activation suggested that no binding occurred in the sperm DNA nuclei, but futher experimentation is needed for verification. This study identifies the proper conditions and techniques necessary for monitoring the binding of carcinogens requiring metabolic activation to free DNA and DNA in somatic and sperm chromatin.

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Introduction

The structure of somatic chromatin and sperm chromatin are very different. Somatic chromatin resides in the cell nucleus, using histones (basic proteins) to aid in the packaging of DNA. In sperm chromatin most of the histones have been replaced by another basic protein known as protamine. The protamine interacts with DNA to inactivate the genes and compact the DNA into a volume much smaller than that accomplished by histone binding. Greater detail on these differences will be provided in the subsequent section.

By comparing the way DNA adducts are formed on these two types of chromatin, implications can be drawn regarding the structural constraints that may be involved with carcinogenic exposure. Some implications to consider would include: the lack of DNA repair found in sperm, DNA availability in the sperm chromatin versus somatic chromatin and gene inactivation.

In vivo and *in vitro* studies were performed on chromatin from both somatic and sperm cells using several known carcinogens. The carcinogens used had different binding affinities to DNA. Tryptophan-p-2 (CAS No. 62450-07-1) forms adducts to C8 in guanine in the major groove. Both benzo(a)pyrene (CAS No. 50-32-8) and 7,12-dibromobenz(a)anthracene (CAS No. 57-97-6) form adducts to N2-guanine in the minor groove and 7-bromomethylbenz(a)anthracene (CAS No. 24961-39-5) forms adducts to the amino groups in adenine and guanine, found in the major and minor groove respectively. Each chemical was tested separately in order to address the following questions:

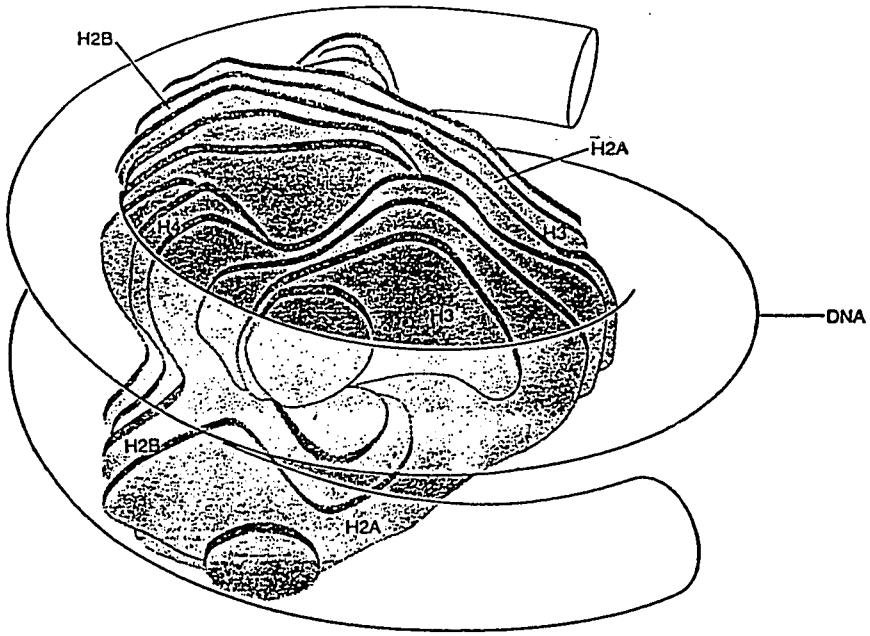
1. Quantitatively, how much damage is observed in the sperm chromatin compared to the somatic chromatin?
2. Does the unusual structure of the sperm chromatin prevent binding of certain chemicals or inhibit the formation of certain adducts?
3. Based on the results from question 1, what toxicological effects might be expected as a result of exposures to these carcinogens?

Literature Review

Comparison of Somatic and Sperm Chromatin

Somatic chromatin resides in the nucleus of higher cells. It is the chromosomal material composed of double stranded DNA and basic proteins. These basic proteins primarily consist of five histones: H1, H2a, H2b, H3 and H4. Histones are abundant in arginine, lysine and histidine amino acids. The amino acid sequences of histones H3 and H4 have been found to be highly conserved, and these two proteins play a significant role in binding to the central part of the core DNA [1]. Evidence shows that histones H2a, H2b, H3 and H4 combine to form an octamer, while the H1 histone remains separate and is thought to aid in supercoiling [1-8]. This supercoiling of DNA is required for the efficient DNA packaging found in chromosomes.

A closer look at the DNA packaging in chromosomes reveals that there are approximately 200 nucleotide base pairs wrapped around each histone octamer, called a nucleosome [1-8]. The double stranded DNA is bound to the histone octamer through both hydrophobic and electrostatic interactions. Since the basic amino acids are positively charged, they associate with the negatively charged groups of the DNA, e.g., the phosphate backbone. Below, the currently accepted model of a nucleosome core is shown [4]:



Model of Nucleosome Core

The double stranded DNA found in somatic chromatin is typically twisted into a right-handed B form helical orientation. This is believed to be the major DNA structure found in solution. There are 10.4 nucleotide pairs per turn in solution, with a 3.4 angstrom rise per pair, [9,10]. The B form of DNA has major and minor grooves, and current research suggests that the histones tend to bind the major groove [1,11]. More specifically, evidence shows that histones are partially buried in the major groove and leave the minor groove of DNA well exposed [1].

Unlike somatic chromatin, the actual structure of sperm chromatin is still unconfirmed. It is well accepted that in vertebrates the histones are replaced during spermatogenesis by another basic protein, protamine. Protamine is a much smaller protein than the core histones previously discussed. It is extremely basic and consist largely (> 50%) of arginine [12,13]. Protamines are synthesized and phosphorylated by ATP in the cytoplasm, and then transferred into the chromatin found in the cell nucleus. Once in chromatin, the protamines replace the histones and bind to the DNA.

The most widely accepted view of sperm chromatin structure consists of protamine wrapped around the DNA helix in an extended polypeptide conformation with inter- and intraprotamine disulfide bonds stabilizing the DNA-protamine complex [12-18]. How the protamine binds the DNA is still under investigation.

One very well documented model on mammalian sperm chromatin [13] presents strong evidence that the protamine binds to the minor groove of the DNA, crosslinking and neutralizing the phosphodiester backbone [14], while the carboxyl and amino-terminal ends of protamine participate in the formation of inter- and intraprotamine bonding. It has been hypothesized that the neutralization of the backbone allows 3.3 pg of DNA to condense into the small volume of sperm nuclei, which could not occur if the DNA was packed with histones.

Another important physical difference between sperm and somatic chromatin includes the inactivation of repair genes during spermatogenesis [19,20]. Basically, once the proteins in the sperm nucleus have been replaced by protamine, the genetic information

in DNA is completely shut off. If any alkylation occurs during this period, serious toxicological effects could result.

Carcinogen Binding Sites on Nuclear DNA

Before describing the process of alkylation, the classes of carcinogens must be described. There are two classes of chemical carcinogens: (A) Genotoxic, which contains the direct-acting (primary carcinogens), procarcinogens (secondary carcinogens) and inorganic carcinogens; and (B) the Epigenetic carcinogen, which includes solid-state carcinogens, hormones, immunosuppressors, cocarcinogens and promotors.

Inherent in the chemical structure of a primary carcinogen is the property of chemical reactivity. These molecules are electrophilic reactants that can interact with nucleophiles, such as DNA or the solvents in which the experiments are taking place. Direct-acting ultimate carcinogens also result from biochemical metabolic activation of precursor compounds, called procarcinogens (secondary carcinogens). Most polycylic or heterocyclic aromatic hydrocarbons, like benzo(a)pyrene or 7,12-dimethyl-benz(a)anthracene, or some aromatic amines, like Trp-p-2, are procarcinogens. This study will focus on the genotoxic class of chemical carcinogens, more specifically the primary and secondary carcinogens found in this class of carcinogens.

There are two ways that carcinogens can interact with nuclear DNA, intercalation and alkylation. Intercalation usually occurs when a planar chemical (heterocyclic) noncovalently inserts between two adjacent bases in DNA [21]. An example of this is when ethidium bromide binds between two base pairs. The binding causes the DNA to

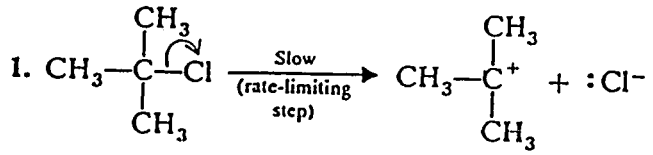
become unwound. As more and more ethidium bromide is bound, the DNA changes from a negative supercoiling (most favorable) to a positive supercoiled [22,23] state.

The type of alkylation which relates to DNA adduction is known as Friedel-Crafts alkylation. In this reaction, the primary halide forms an electrophilic complex by loosing a halide ion and subsequently is nucleophilically attacked by the aromatic ring. Factors which must be considered for this type of reaction include isomerization, temperature and the nucleophilicity of the aromatic ring. Note that in all the following examples, the temperature and pressure are constant.

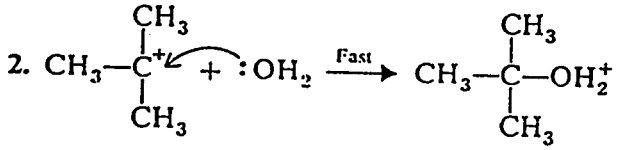
To clearly convey the relationship between chemical reactivity and nucleic acid reactive sites, the different mechanisms by which nucleophilic substitution occurs during the process of alkylation will be discussed. In nucleophilic substitution reactions a nucleophile initiates the reaction with an alkyl halide, resulting with the halogen substituent leaving as a halide ion. In the example below, the alkyl halide, tert-butyl chloride, reacts with hydroxide ions to form tert-butyl hydroxide and a chloride ion.

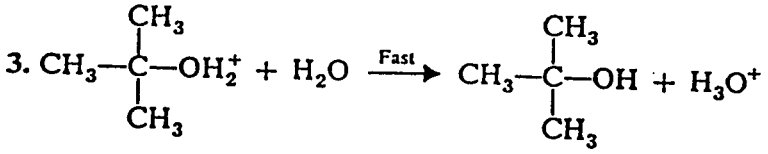
There are two types of nucleophilic substitution reactions of interest for this review, Sn1 and Sn2. Sn1 is defined as substitution, nucleophilic, unimolecular. This indicates that the number of molecules or ions that participate in the transition state of the rate-limiting step of this mechanism is one. (Refer to previous example.) The reaction rate of the above equation is only dependent on the amount of tert-butyl chloride, thus

making it a Sn1 type of mechanism. The mechanism for the Sn1 type of reaction involves three steps. Two distinct intermediates are formed. The first step is the rate-limiting step.



The next two steps are the following:

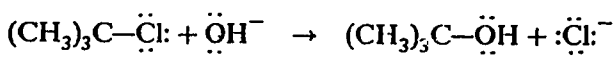




Sn1 Reaction

In the second step, the intermediate tert-butyl cation reacts rapidly with water to produce a tert-butyloxonium ion (another intermediate) which, in the third step rapidly transfers a proton to a molecule of water producing a tert-butyl alcohol. This step is stabilized by solvation [24], a process in which the nucleophile is a molecule of the solvent where the substitution is taking place. It is important to note that the primary factor that determines the reactivity of organic substrates in an Sn1 reaction is an electronic effect that stabilizes an electrical charge on the transition state or intermediate.

Sn2 is defined as substitution, nucleophilic, bimolecular. As implied, these reactions are dependent on the concentration of both substituents. If, using the same example as before,



one doubles the concentration of tert-butyl chloride while keeping the concentration of the hydroxide ion constant, the rate of the reaction doubles. When one doubles the concentration of the hydroxide ion while keeping the concentration of the tert-butyl chloride constant, the rate doubles again. Lastly, when the concentration of both substituents are doubled the rate increases four times, as defined in a bimolecular mechanism.

In the Sn2 mechanism, the nucleophile approaches the carbon bearing the leaving group from the back side. It attacks from the side directly opposite the leaving group. The orbital that contains the electron pair of the nucleophile begins to overlap with the small lobe of an orbital of the carbon bearing the leaving group. As the reaction progresses the lobe between the carbon atom and the leaving group shrinks. As this happens the leaving group is pushed away. The formation of the bond between the nucleophile and the carbon provides most of the energy necessary to break the bond between the carbon atom and the leaving group. This is a one step displacement mechanism and there are no intermediates. Simple alkyl halides show the following general order of reactivity in Sn2 reactions:

Methyl > Primary > Secondary > Tertiary

An important item to note in this order of reactivity is the steric effect. For example, steric hindrance is observed in reactions containing tertiary molecules. The spatial arrangement of the atoms or groups at or near the reacting site of a molecule hinders the reaction. For a Sn2 reaction to take place, their reactive centers must be able to come within bonding distance of each other. Large bulky groups can often hinder the formation of the required transition state altogether. Thus, tertiary halides usually react by the Sn1 mechanism.

Another important area to consider when discussing the binding of carcinogens is the Swain-Scott substrate constant s [25]. In 1953, Swain and Scott published a two-parameter equation which was found to correlate the relative rates of reaction of various nucleophilic reagents with various organic substrates (alkyl halides). In this relationship:

$$\log (Ky/Ko) = sn$$

indicates the susceptibility of the substrate s to the nucleophile of nucleophilic constant **n**, while **Ky** is the rate constant for this reaction and **Ko** is the rate constant for the reaction with water. Alkylating agents with low s values exhibit greater reaction with sites of low nucleophilicity in nucleic acids such as the O6-position of guanine residues [26]. This reaction is thought to occur by an Sn1-type substitution. Likewise, alkylating agents with higher s values will react with sites of higher nucleophilicity in nucleic acids, such as the exocyclic nitrogens, resulting from an Sn2-type substitution [27-30].

The DNA binding sites known to exist are as follows [31-34]:

Nucleic Acid	Major Groove	Minor Groove
Guanine	N7	N2*
	06*	N3
	C8	
Adenine	N7	N3
	N6*	
Cytosine	N4*	O2*
	N3*	
Thymine	O4	O2*

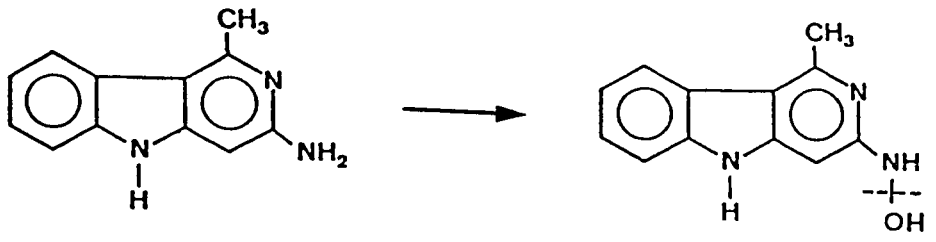
(*) denotes exocylic location in DNA.

Carcinogen Activity

Carcinogenic activity can be best summarized by first assessing the metabolic requirements of the carcinogens used in this review. The carcinogens tested in this study can be broken into two groups of genotoxins, aromatic amines and polycyclic aromatic hydrocarbons (PAHs). Besides the obvious structural differences between the two groups of carcinogens, their mode of action is quite different. For example, very small doses of PAHs administered subcutaneously or topically yield tumors at the site of

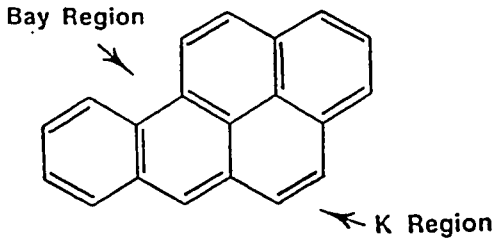
administration. Aromatic amines administered similarly show no local tumor inductions and relatively large doses have to be administered parenterally over a substantial time for the generation of tumors [35].

The aromatic amine used in this project was Trp-p-2. Tryptophan-p-2 requires metabolic activation to produce its most carcinogenic form. Typically, the metabolic activation of aromatic amines results in the formation of an N-hydroxy derivative and this electrophile reacts with the guanine moiety at the C-8 position and to a lesser extent at the N-2 position. It is the metabolic formation of the somewhat unstable N-O bond which is the real source of the carcinogenic activity of the aromatic amines. This bond can be cleaved by mild acid conditions or through various esterification reactions which serve to generate a better leaving group, thus yielding a potent arylamidating agent. In the case with Trp-p-2, the two major microsomal metabolites have been identified as 3-hydroxyamino and nitroso derivatives [36]. When metabolically activated, Trp-p-2 and its sister compound Trp-p-1 are the strongest frame shift mutagens known.



Tryptophan-p-2

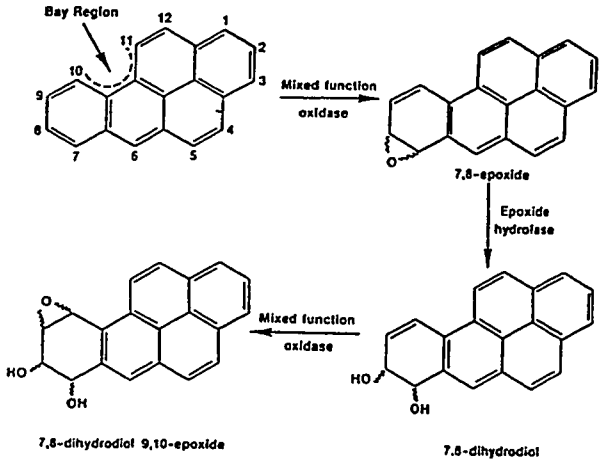
The remaining carcinogens used are all classified as PAHs. The structure of the specific PAH has much to do with its carcinogenic activity. It was found previously that the presence of a favorable bay region which could be metabolized into a dihydrodiol-epoxide would determine the potency of the PAH [37,38]. For example, a straight chain PAH like Anthracene (CAS No. 120-12-7), is non-carcinogenic. In contrast, Benzo(a)pyrene (B(a)P) has a bay region and a K-region (see below) present, which increases its carcinogenicity potential greatly.



Benzo(a)pyrene

As mentioned earlier, the fate of epoxide formation adds weight to predicting the carcinogenic potentials [39]. In contrast to the high carcinogenicity potential of the bay region dihydrodiol epoxide formation, K-region dihydrodiol epoxides have a much lower carcinogenic potential. *In vitro*, K-region epoxides were found to be cytotoxic and mutagenic and very effective in cellular transformation assays [40,41]. However, *in vivo*, the metabolically formed K-region epoxides have been found to be inactive as carcinogens. It seems that these K-region epoxides, *in vivo*, usually react with a glutathione-S-transferase which inactivates the carcinogen and is ultimately excreted.

One of the best studied PAHs is B(a)P. For the reasons mentioned previously, B(a)P has a high carcinogenicity potential. Metabolic activation is required [42] and even though it's known to be metabolized into approximately 20 primary and secondary oxidized metabolites, including a variety of conjugates, currently only the 7,8-diol-9,10-epoxide metabolite is considered to be the ultimate carcinogen [43].

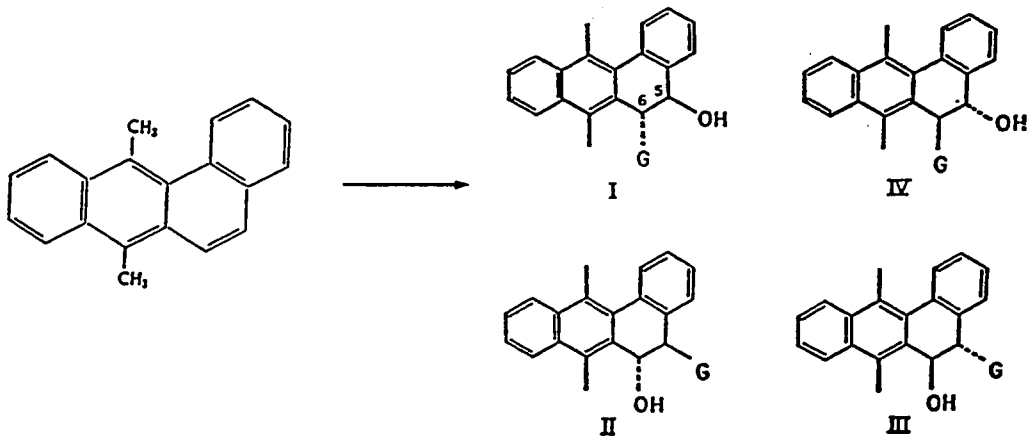


Metabolic Activation of Benzo(a)pyrene

In addition to B(a)P's ability to bind covalently to the N-2 group of guanine [44], its planar structure would allow intercalation to occur. Benzo(a)pyrene occurs ubiquitously in products of incomplete combustion (including barbecued foods and cigarette smoke) plus fossil fuels.

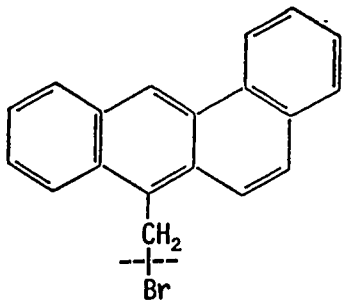
Another PAH tested which required metabolic activation was DMBA [45-47]. It too has a high carcinogenicity potential for the same structural reasons as B(a)P. Like B(a)P, DMBA has both a bay region and a K-region. Unlike B(a)P, the actual *in vivo* ultimate carcinogen has not been identified. *In vitro* DMBA studies show a high yield

of the K-region oxide formation (DMBA-5,6-oxide) reacting preferentially with the N-2 of guanine [46,48]. There are actually four derivatives of DMBA-5,6-oxide which have been isolated and identified [35,48,49]. Of all the PAHs, DMBA is one of the most potent tumor initiators known [50].



7,12-Dimethylbenz(a)anthracene and metabolites

The other PAH tested (BMBA) did not need metabolic activation. This is due to bromine being such a good leaving group, that this compound readily forms a fairly reactive electrophile. 7-Bromomethylbenz(a)anthracene has been found to preferentially bind the exocyclic amino groups of guanine and adenine over the ring nitrogens [33,51,52]. A very small percentage of binding has been found to occur on the N-3 of cytosine.



7-Bromomethylbenz(a)anthracene

The metabolic activation of the PAHs have some similarities to the aromatic amines. Typically, they both require more than one metabolic reaction for activation, and a proximate carcinogen (the N-hydroxy derivatives for the aromatic amines and the dihydrodiol precursors of the dihydrodiol epoxides for the PAHs) can be isolated which exhibits equal to or greater carcinogenic potency than the parent compound. In either case, the source of cell-damaging and tumor initiating activity lies in the carcinogen-oxygen bond, i.e., N-O bond for aromatic amines and the C-O bond for the PAHs.

Metabolic Activation of Carcinogens

The process of metabolic activation is dependent on a series of enzymatic and non-enzymatic processes which may or may not result in a toxic effect [53,54]. As previously described, the first step in metabolic activation typically leads to an epoxide formation [55]. Epoxides are formed by the addition of oxygen across a carbon-carbon unsaturated bond, in a reaction catalyzed by the microsomal mixed function oxidase system enzymes. Epoxides are sufficiently electrophilic at this point to react

with any surrounding nucleophilic material, i.e. nucleic acids, proteins or other molecules present in tissues. Epoxides may also spontaneously rearrange to phenols, which are readily conjugated with sulfate or glucuronide and subsequently excreted. Additionally, the epoxide may be enzymatically converted to dihydrodiols via epoxide hydrolase or glutathione conjugates by glutathione-S-transferases. These too are generally less toxic than the epoxide intermediate and are readily excreted.

Mixed function oxidases are predominantly found in the liver. This enzyme system utilizes both NADPH and molecular oxygen. The principal enzyme is cytochrome P450, a heme protein which absorbs light maximally at 450 nm when in the reduced form. Cytochrome P450 is capable of binding substrate and oxygen, passing one of the oxygen atoms of O_2 to the substrate in the form of an OH group while the other oxygen atom combines with H^+ to form water. The electrons are delivered to P450 mostly from NADPH by an endoplasmic-reticulum-associated electron transport chain involving flavoproteins and in some cases other cytochromes [56]. Cytochrome P450 and other elements of the microsomal electron transport system seem to be membrane-bound proteins present mostly on the cytoplasmic surface of the smooth endoplasmic reticulum [57,58]. Their concentration in liver cells vary with need.

In summary, a number of points need to be considered when binding carcinogens to DNA, both *in vivo* and *in vitro*. These points include: the structural constraints allowing or inhibiting possible DNA binding, the DNA repair mechanism capabilities, and the reactivity of the natural metabolic fates of the carcinogen, i.e., electrophilicity ramifications, K- or bay region dihydrodiol expoxide formation potentials, etc.

Materials and Methods

All work was performed at Lawrence Livermore National Laboratories (LLNL), Livermore, CA. All animal handling protocols were approved by the Institute for Animal Use and Care Committee in the Biomedical Division at LLNL.

Carcinogens

Both the tritiated benzo(a)pyrene (B(a)P) and the tritiated 7,12-dimethylbenz(a)anthracene (DMBA) were purchased from Amersham with a specific activity of 82 Ci/mmol and 41 Ci/mmol respectively. Tritiated tryptophan-p-2 (Trp-p-2) was purchased from Moravek Biochemicals, Inc. at a specific activity of 16 Ci/mmol. The tritiated 7-bromomethylbenz(a)anthracene (BMBA) was prepared as described by Thompson et al. [59] with a resulting specific activity of 194 Ci/mmol.

RNAase A

Pancreatic RNAase A was dissolved at a concentration of 10 mg/ml in 10 mM Tris (pH 7.5) and 15mM sodium chloride, heated to 100 °C for 15 min and slowly allowed to cool to room temperature. Aliquots were dispensed and stored at -20 °C. All procedures were followed as described by the Molecular Cloning Reference Manual [60].

Microsome Preparation

Swiss Webster mice obtained from Simonson Laboratories in Gilroy, CA, were injected interperitoneally with 500 mg/kg Archlor 1254 in vegetable oil. Purina lab chow and water were given to the mice ad libitum until fasting 12 hours before the liver extraction. The mice were sacrificed by cervical dislocation and the livers were carefully removed and immediately placed in cold 0.8% sodium chloride, 0.02% potassium

chloride, 0.14% disodium hydrogen phosphate, 0.02% potassium dihydrogen phosphate (phosphate buffer solution, PBS), 10 ml per gram tissue at 4 °C. All steps were performed at 4 °C with cold and sterile solutions and glassware. The whole livers were rinsed with PBS, minced with scissors, and homogenized with a Brinkmann Polytron at a setting of 10. The homogenate was then centrifuged for 10 min at 13,360 × g. The supernatants were collected and centrifuged at 131,000 × g for 1 h. The resulting microsomal pellets were resuspended in 0.05 M Tris, pH 8, 30% glycerol in a ratio of 1.25 ml/g of liver and stored at -80 °C.

Protein Determination—Microsome preparations were assayed for total protein using the Folin phenol reagent in the presence of copper-treated protein samples [61].

Enzyme Activity Analysis—The aryl hydroxylase activity of the preparation was determined from fluorescence of 3-hydroxybenzo(a)pyrene produced from B(a)P. Fluorescence measurements at excitation and emission wavelengths of 396 nm and 560 nm, respectively, were performed in a Aminco-Bowman spectroflurometer Model J48963 [62].

Calf Thymus DNA

Solid calf thymus (CT) DNA purchased from Worthington Biochemical Corporation was solubilized with 0.01 M potassium chloride, 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 7. To eliminate any protein contamination, the CT DNA was purified by gel permeation high pressure liquid chromatography (HPLC). Solid guanidine hydrochloride (GuCl) was added to the CT DNA to yield a final concentration of 1 M.

Peaks containing the purified CT DNA were collected and dialyzed, against 0.01 KCl, 0.5 mM EDTA solution, then diluted to 0.04 mg/ml with 0.1 M sodium phosphate buffer, pH 6.8, and kept at 5 °C.

Bulk Liver and Sperm Isolation for *In vitro* Analysis

Both the whole liver and the cauda epididymides were excised from mature (9 month old) AC/J male mice obtained from Jackson Laboratories in Bar Harbor, Me. The whole livers were stored at -20 °C. During the extractions the epididymides were stored in 0.01 M Tris, pH 8, 0.9% sodium chloride (Tris-saline buffer) at 4 °C until they could be gently homogenized using a Brinkmann Polytron at a setting between 0.5 and 1, to release the whole sperm. The sperm suspension was filtered through silk gauze (80um), to separate the sperm from the epididymal tissue, then centrifuged at 1875 × g for 3 min, washed three times in Tris-saline buffer, and stored in frozen pellets at -20 °C.

Blood, Liver and Sperm Isolation and Preparation for *In vivo* Analysis

Mature (9 month old) C57BL/6J mice from Jackson Laboratories in Bar Harbor, Me. and C57BL/6 B&K mice from Bantin and Kingman Laboratories in Fremont, Ca. were injected i.p. with tritiated Trp-p-2 and B(a)P respectively. The liver and the cauda epididymides were removed and isolated as previously described. Aliquots of blood were taken at each time point or dose and counts per minute per volume blood were determined by liquid scintillation to establish the carcinogens concentration.

Liver Nuclei Preparation and Isolation of DNA

A known weight of liver was brought up to a 20 ml volume in freshly prepared 0.25 M sucrose, 0.01 M Tris, pH 8, 2.5 mM $MgCl_2$ and 0.05 M sodium bisulfite (BIS) and then homogenized with a Virtis homogenizer at 5000 rpm for 3 min at 4 degrees C. Connective tissue was removed by filtration through cheese cloth. The filtrate was then layered onto 30 ml of 1M sucrose, 2.5 mM $MgCl_2$, 0.01 M Tris, pH 8, 0.05 BIS, 0.01% Trition X-100 solution and centrifuged at 4124 × g for 10 min. The pellet was resuspended in 0.25 M sucrose, 0.01 M Tris, pH 8, and 2.5 mM $MgCl_2$, homogenized with a Wheaton overhead stirrer at a setting of 3, and centrifuged at 659 × g for 3 min to pellet the liver nuclei. To extract the liver DNA, 3 M GuCl was added to the liver nuclei pellet and the solution was sonicated with a W-220 Branson Ultrasonic Processor at a setting of approximately 2 for less than 1 min, to break up the nuclei and release the DNA. This mixture was then centrifuged at 10,588 × g for 10 min. The supernatant containing the liver DNA was kept on ice until high pressure liquid chromatographic analysis.

Sperm DNA Isolation

The sperm DNA was isolated as outlined by Balhorn et al. [63]. To the thawed sperm pellet, 4 ml of 10 mM dithiothreitol (DTT), 0.05 M Tris, pH 8 was added, then sonicated and incubated at 4 °C for 15 min. Mixed cetyltrimethylammonium bromide (MTAB; Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 1% and incubated at 4 °C for 30 min to remove the sperm tails and membranes. The sperm nuclei were pelleted by centrifugation at 4124 × g for 3 min and washed once (centrifuged at 4124 × g for 3 min) with 1% MTAB, 0.01 M Tris, pH 8. Five M GuCl

was added to the purified sperm nuclei, sonicated with a W-220 Branson Ultrasonic Processor at a setting of approximately 4, and then centrifuged at 10,588 × g for 10 min. The supernatant was saved for chromatographic analysis.

Metabolic Activation of *In vitro* Samples

The cofactor solution consisting of 0.03% NADH, 0.03% NADPH, 0.2 M Tris, pH 7.25, 0.1 M $MgCl_2$ plus microsomes, (which were thawed at 4 °C), were added to each of the samples at 4 °C and placed into a water bath shaker at 37 °C (shaker speed 3). Aliquots of tritiated B(a)P were added and the samples were incubated for 25 min under amber lights, to reduce the possibility of B(a)P photodegradation. The DMBA samples were treated the same as B(a)P with the exception of 20 minutes for incubation.

The treated samples were removed from the 37 °C water bath shaker and set on ice. CT DNA samples were immediately transferred to test tubes containing a known quantity of hydroxyapatite, then vortexed and incubated for 30 min at 4 °C. The liver nuclei and sperm samples were centrifuged at 1875 × g for 3 min, three times in 0.25 M sucrose, 0.01 M Tris, pH 8, 2.5 mM $MgCl_2$ solution. The liver nuclei were kept at 4 °C in 3 M GuCl until chromatographic analysis. The sperm were stored at 4 °C in 0.25 M sucrose, 0.01 M Tris, pH 8, 2.5 mM $MgCl_2$ solution followed by DNA isolation and chromatographic determinations. The CT DNA-hydroxyapatite mixture was washed (centrifuged at 1875 × g for 3 min) three times with 0.1 M sodium phosphate buffer, pH 6.8, then stored in 0.5 M sodium phosphate buffer, pH 6.8 until chromatographic analysis. The CT DNA-hydroxyapatite mixture in 0.5 M sodium phosphate buffer, pH 6.8, was centrifuged at 1875 × g for 3 min. The supernatant con-

taining the CT DNA was collected and 5 M GuCl was added to this solution to a final concentration of 1 M.

Quantitation of DNA for *In vitro* Analysis

The combined use of gel permeation chromatography to purify the DNA and the absorption at 260 nm (extinction coefficient of 20 cm^2/mg) to quantitate its concentration were employed to adjust the DNA from CT DNA, liver nuclei and sperm to equal concentrations. The CT DNA is used as the standard. Aliquots with a final total volume of 1 ml per sample were used for each *in vitro* analysis.

HPLC

All DNA samples were purified by gel permeation high pressure liquid chromatography on a Bio-Gel TSK-40 XL column (two 300 mm × 7.5 mm columns connected in tandem) at a flow rate of 1.0 ml/min. The DNA was detected by its absorbance at 260 nm. The DNA samples were carefully collected between the early and midpoint portions of the peak in order avoid any RNA contamination [64].

All HPLC runs were initiated with two injections of unlabeled CT DNA to reduce any residual contamination in the column. One M GuCl was the carrier solvent for CT DNA and liver DNA; 2 M GuCl used for the sperm DNA. Sperm DNA requires the higher salt concentration to prevent the protamine from rebinding to the DNA and causing precipitation. Treated CT DNA was the most labile of the three DNAs and was the initial DNA to be chromatographed.

Characterization of HPLC Fractions

Pooled chromatographic fractions were characterized by absorption measurements at 260 nm on a Gilford Spectrophotometer. The collected fractions (greater than 4 ml) were pooled and vortexed. The amount of radioactivity in an aliquot was determined directly by the addition of 20 ml Universol (ICN Biomed. Div.) and by counting the samples in a Beckman LS8100 Liquid Scintillation Counter to obtain the total counts. The remaining portion was extracted five times with water-saturated butanol, in order to eliminate the non-covalent, non-specific binding of carcinogens [64], followed by liquid scintillation counting.

Results

DNA Isolation and Analysis

DNA adduct formation to DNA packaged by protamine (in sperm nuclei) and histones (in liver nuclei) were compared *in vivo* and *in vitro* using an assay that employed HPLC to isolate and purify the DNA. Concomitantly, DNA adduct formation to free DNA was determined in the *in vitro* studies. With the exception of the BMBA experiments, the *in vitro* chemical reactions were performed in the presence of microsomal enzymes. The sperm, liver nuclei and free DNA were recovered and prepared for chromatographic analysis as outlined in the Materials and Methods section.

Figures 1 and 2 show the DNA peaks which were collected from the HPLC for further analysis by U.V. absorption spectroscopy and liquid scintillation measurements. Calf thymus DNA (Figure 1) has less protein contamination than the liver nuclei DNA (Figure 2).

In vivo Trp-p-2 Studies

The time course for Trp-p-2 adduction to DNA was previously determined by Balhorn, et al. (unpublished data). Three C57BL/6 B&K male mice per dose point were injected interperitoneally with Trp-p-2 in DMSO (100 μl Trp-p-2 per 35 g body weight) at various doses of Trp-p-2 per gram body weight and sacrificed 1.5 hours later. The control mice were injected at the highest dosage with DMSO, and otherwise treated the same. The three livers per dose point were minced together, then divided into three separate samples.

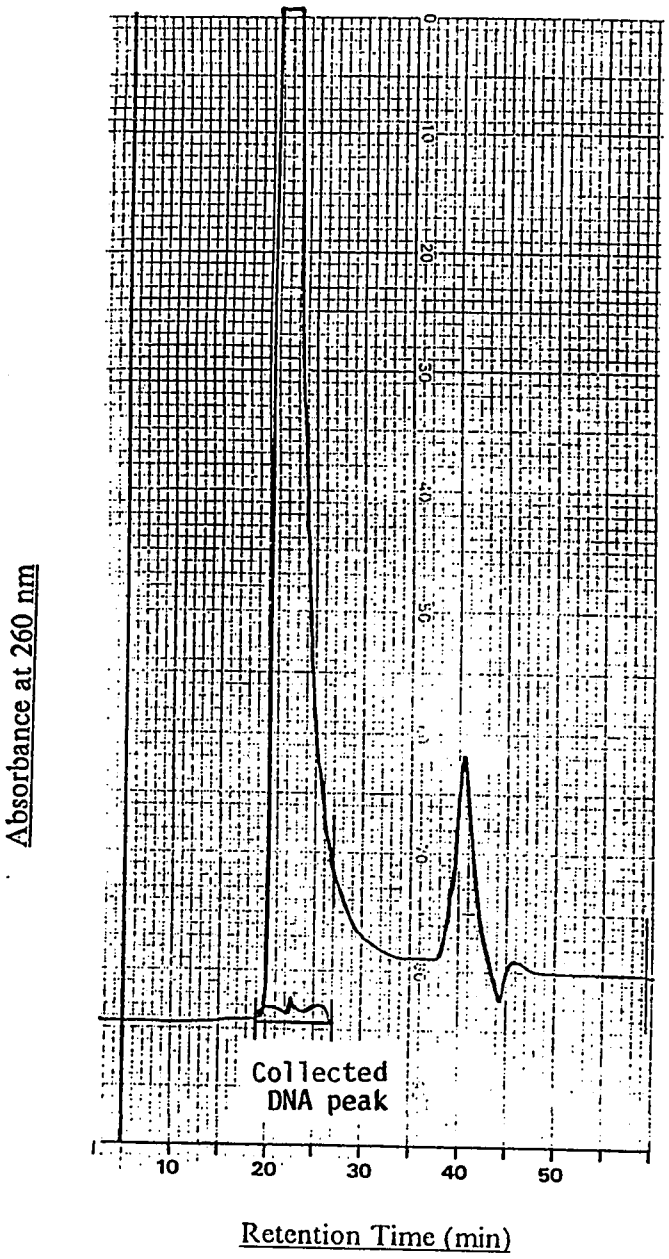


Figure 1. Calf Thymus DNA Chromatogram

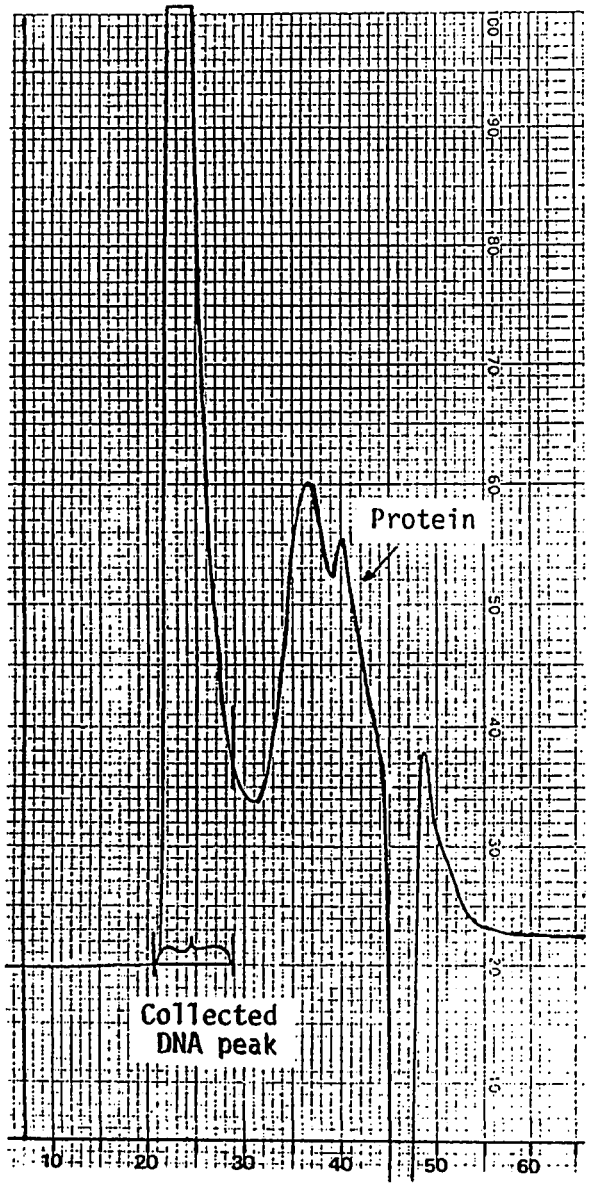


Figure 2. Liver Nuclei DNA Chromatogram

The three samples were analyzed independently and the results were averaged. The three cauda epididymes per dose were pooled to provide enough sperm for a single data point.

One hundred μl of each freshly homogenized liver (in 0.25 M sucrose, 0.01 M Tris, pH8, 2.5 mM MgCl2, 0.05 BIS) and 100 μl of polytroned sperm (in tris-saline buffer) were analyzed by liquid scintillation counting to determine the total amount of tritiated Trp-p-2 retained in the liver and sperm tissue respectively. The data in Figure 3 show that the dose of Trp-p-2 received by the sperm and liver cell differ by almost seven fold.

The DNA from the liver and sperm nuclei were isolated and the amount of Trp-p-2 DNA adducts were determined. The sperm DNA data points were not above the sperm control (unexposed) DNA, thus no binding occurred (Figure 4).

In vivo Benzo(a)pyrene Studies

A. Time Course

Three C57BL/6J male mice per time point were injected interperitoneally with 100 μl/40 gram body weight of tritiated B(a)P in DMSO (5 mCi/ml). The animals were sacrificed and the organs removed after 1, 2, 4, 8, 16, 24 and 48 h. The control mice were injected with DMSO and sacrificed 5 h later. Fifty μl of the blood-citrate mixture (100 μl blood added to 1 ml of 1% sodium citrate) was taken at each time to determine the amount of tritiated B(a)P in the blood (Figure 5).

Figure 3. Trp-p-2 Dose Curve: Tissue Binding

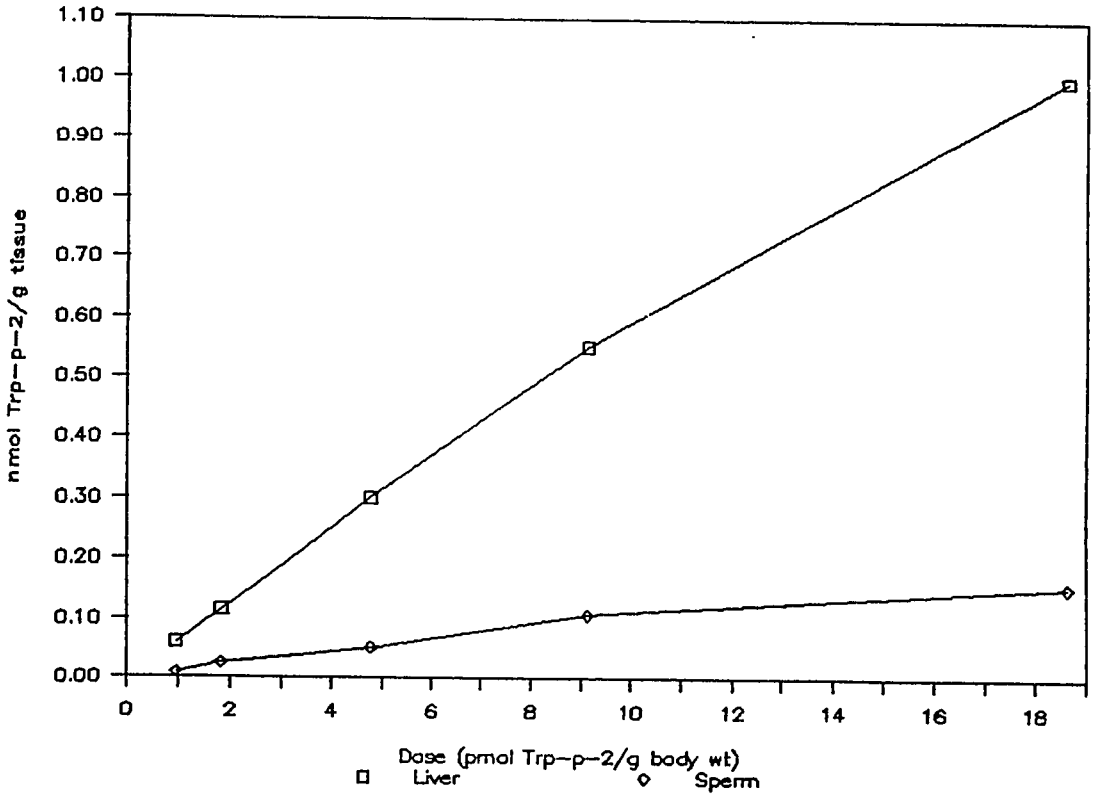


Figure 4. Trp-p-2 Dose Curve: DNA Binding

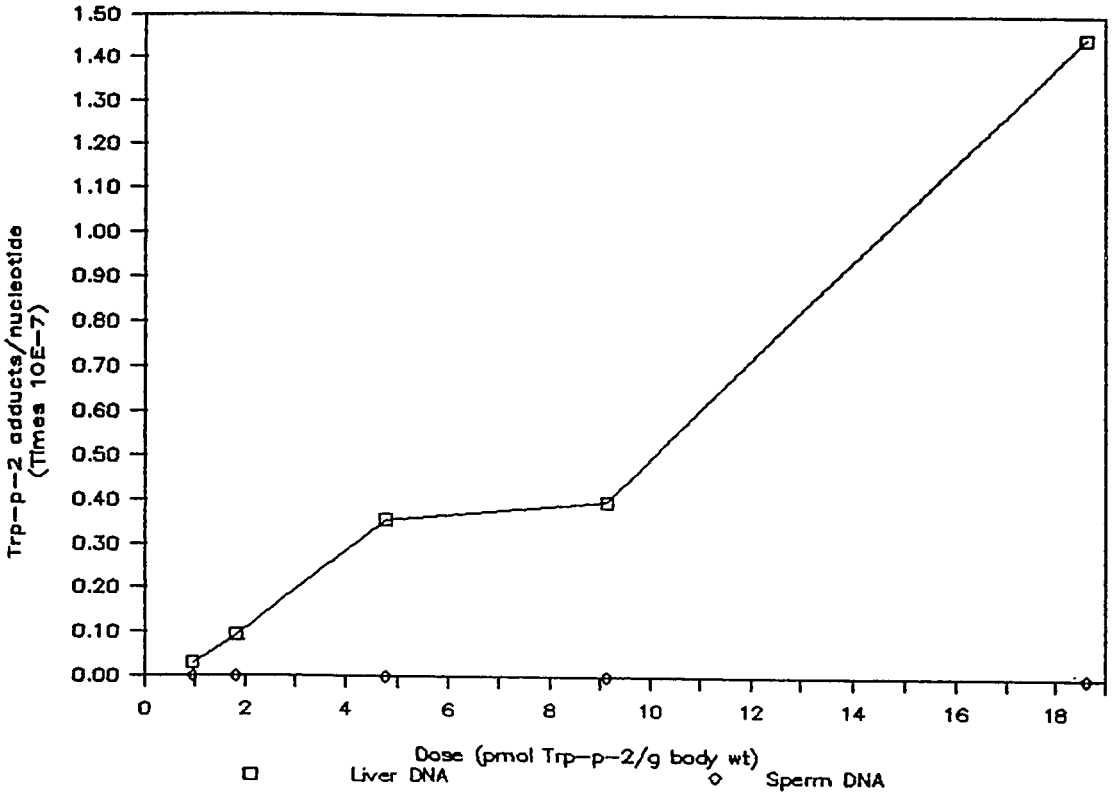
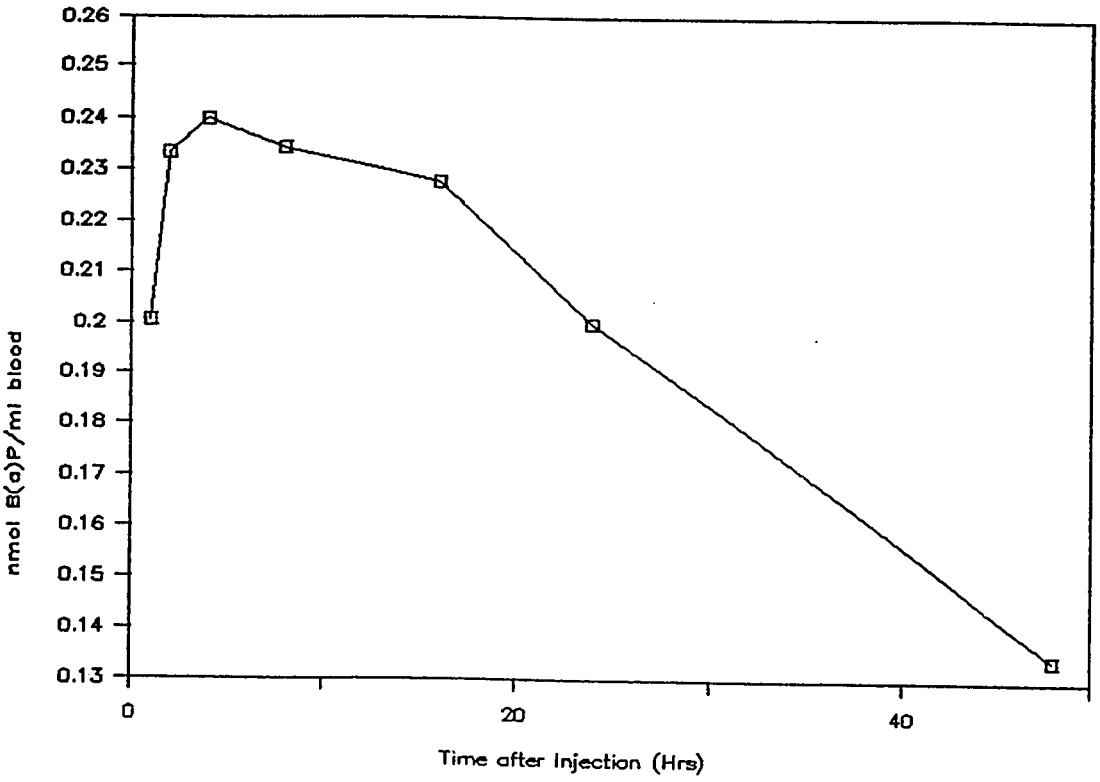


Figure 5. B(a)P Time Course: Blood Results



One hundred μl of freshly homogenized liver (in 0.25 M sucrose, 0.01 M Tris, pH8, 2.5 mM MgCl2, 0.05 BIS) and 100 μl of polytroned sperm in tris-saline buffer were analyzed to determine the total amount of tritiated B(a)P present in the liver and sperm tissue, respectively (Figure 6).

To determine the number of B(a)P adducts produced in liver and sperm DNA, the tritiated DNA was isolated and the number of adducts determined. Note that the amount of B(a)P binding to sperm DNA was so low that after subtracting the control data for the sperm (background), only two points were greater than zero. Based on these results, one hour exposures to tritiated B(a)P is sufficient time to obtain maximum binding in the liver DNA prior to the removal of adducts by cellular repair (Figure 7).

B. Dose Curve

Three C57BL/6J male mice were injected interperitoneally with 100 μl B(a)P/40 gram body weight of tritiated B(a)P in DMSO (5 mCi/ml). Fifty μl of the blood-citrate mixture (100 μl blood added to 1 ml of 1% sodium citrate) was taken for each sample to determine the amount of tritiated B(a)P in the blood. The dose range was between 0.05 and 14.4 nanomoles B(a)P per gram body weight. The control mice were injected at the highest dosage point with DMSO and otherwise treated equally. The relationship between the injected dose of B(a)P and concentration of B(a)P in the blood were found to be linear (Figure 8).

The total amount of tritiated B(a)P was determined in 100 μl of freshly homogenized B(a)P treated liver in 0.25 M sucrose, 0.01 M Tris, pH8, 2.5 mM $MgCl_2$, 0.05 BIS and

Figure 6. B(a)P Time Course: Tissue Binding

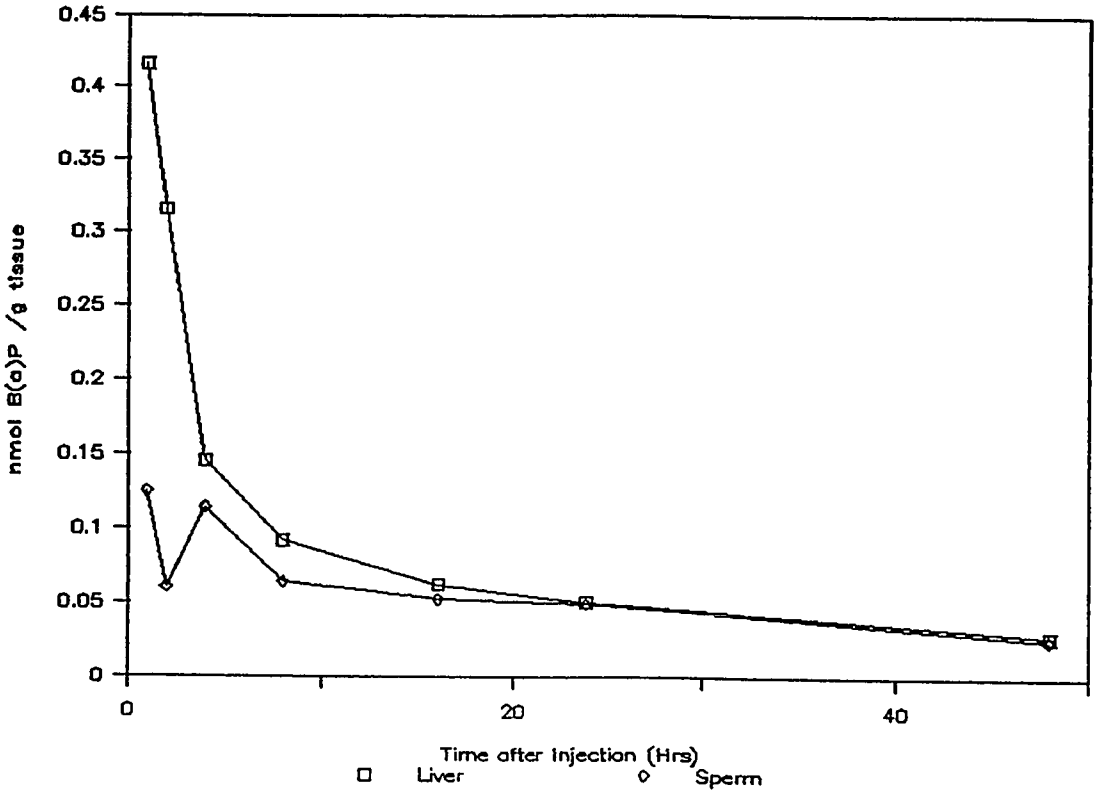


Figure 7. B(a)P Time Course: DNA Binding

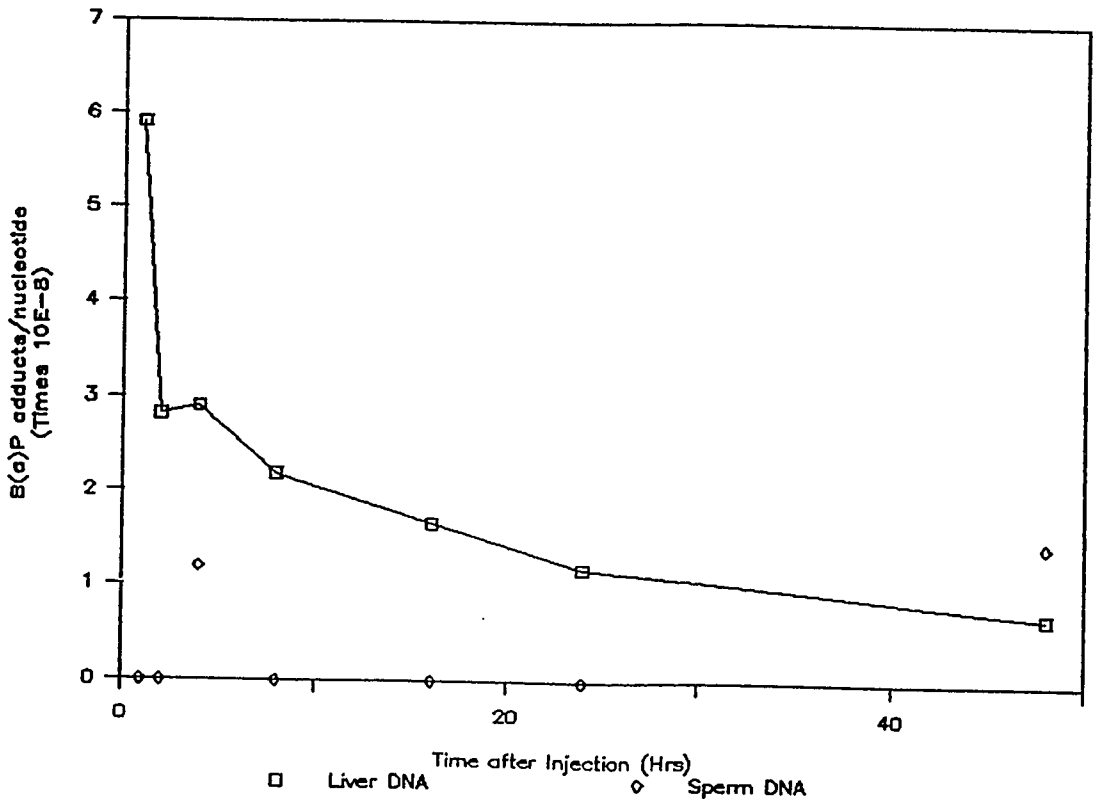
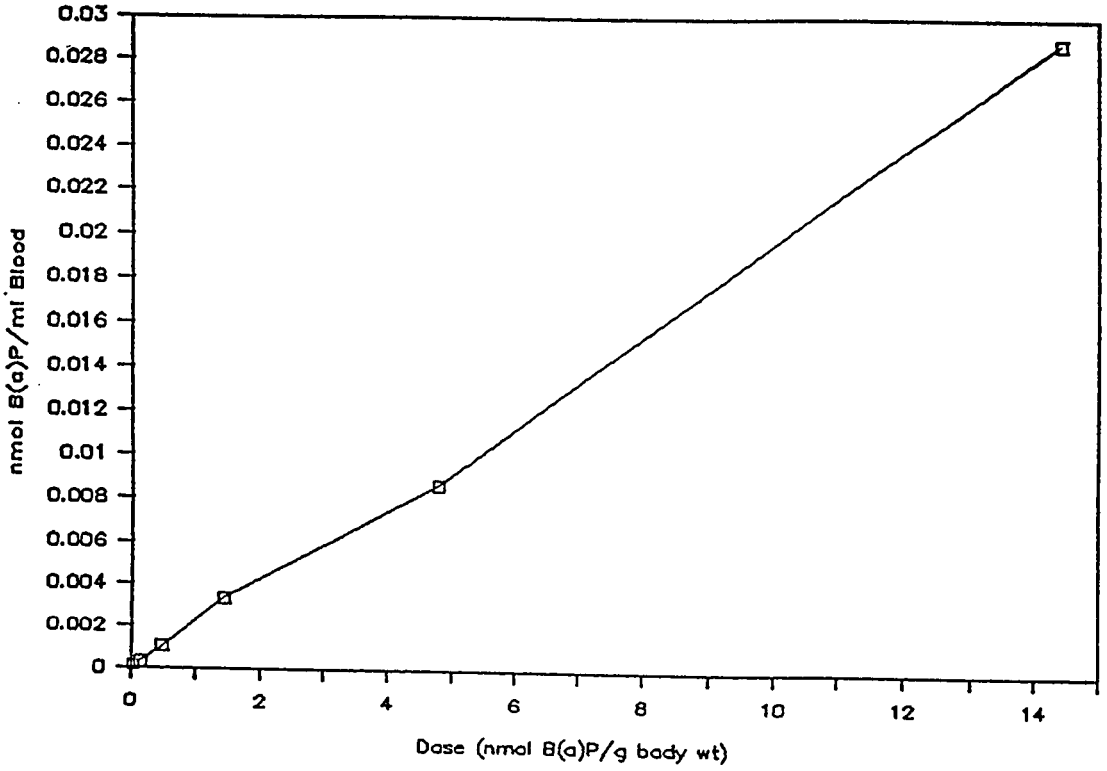


Figure 8. B(a)P Dose Curve: Blood Results



100 microliters of polytroned epididymides in tris-saline buffer were analyzed by liquid scintillation counting to determine the total amount of tritiated B(a)P present in the liver and epididymal tissue respectively. The concentration of B(a)P was observed to be two to three fold higher in the liver than the epididymis (Figure 9). In both cases, the concentration increased linearly with the dose.

The DNA from the liver and sperm were isolated and the amount of B(a)P adducts present determined. *In vivo*, B(a)P binds to the DNA in the liver and not the sperm (Figure 10).

In vitro 7-Bromomethylbenz(a)anthracene Studies

7-Bromomethylbenz(a)anthracene is a chemical mutagen that does not require metabolic activation to bind to DNA. It has been shown previously that it forms adducts to the amino groups of adenine and guanine found in the major and minor grooves respectively.

Equivalent quantities of liver and sperm nuclei (containing 0.355 mg DNA per ml) were mixed with freshly prepared 0.25 M sucrose, 0.01 M Tris, pH8, 2.5 mM $MgCl_2$, 0.05 BIS for a maximum total volume of 1 ml. Calculated doses of 17, 140, 273, 338, 845, 1550, 1830, 2350, 3100 and 8170 nanomoles of ^{3}H-BMBA per mg DNA were added to the sperm and liver nuclei *in vitro* for 30 min. Sperm and liver nuclei controls were treated with 140 μl of DMSO. When the results of 7-BMBA binding to sperm and liver DNA were compared, the BMBA binding to the liver and sperm DNA were found to be nearly identical (Figure 11).

Figure 9. B(a)P Dose Curve: Tissue Binding

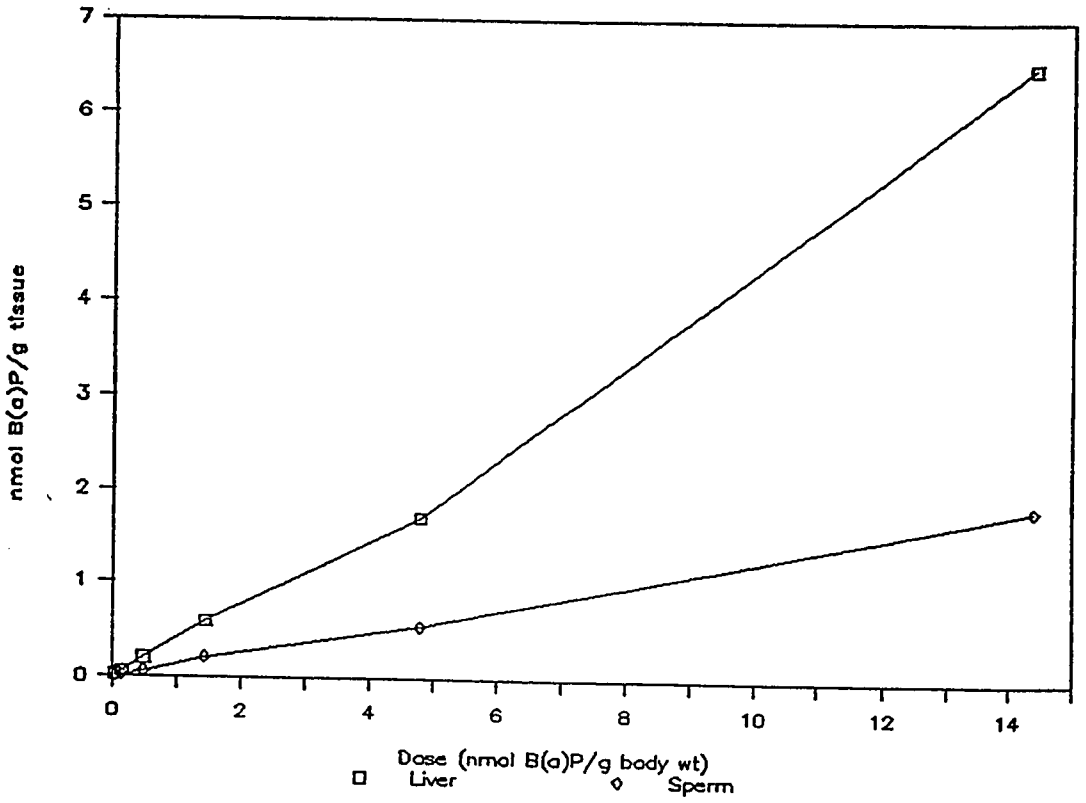


Figure 10. B(a)P Dose Curve: DNA Binding

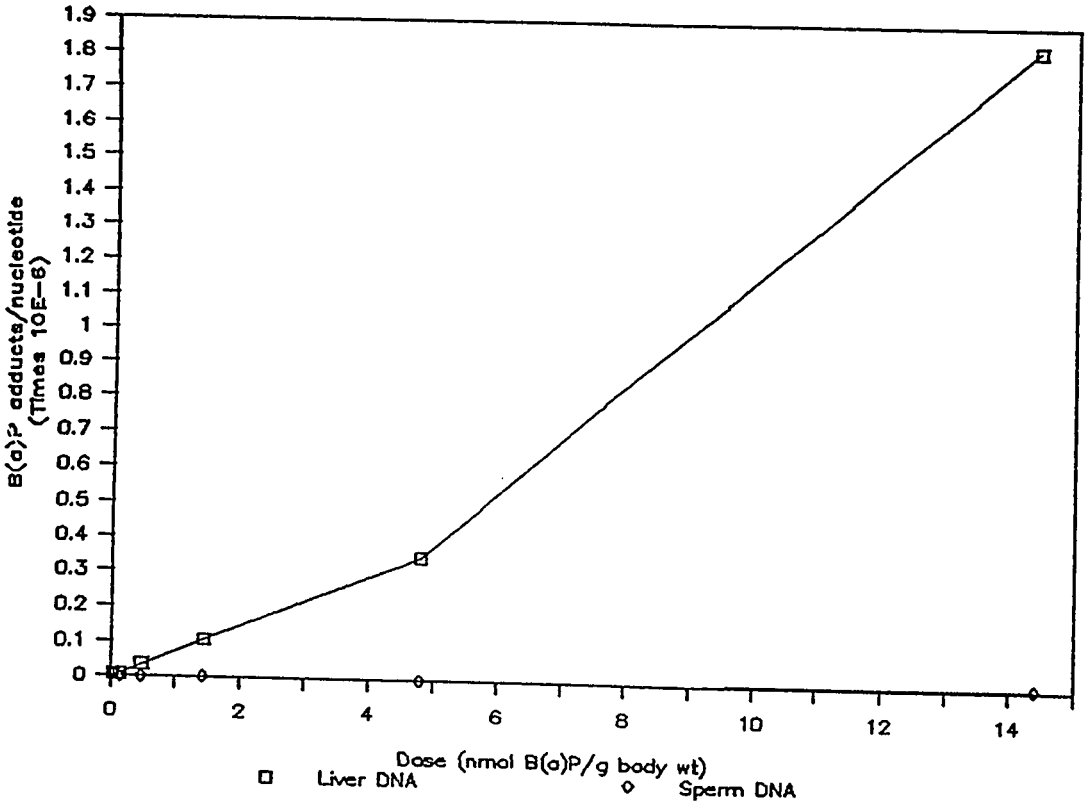
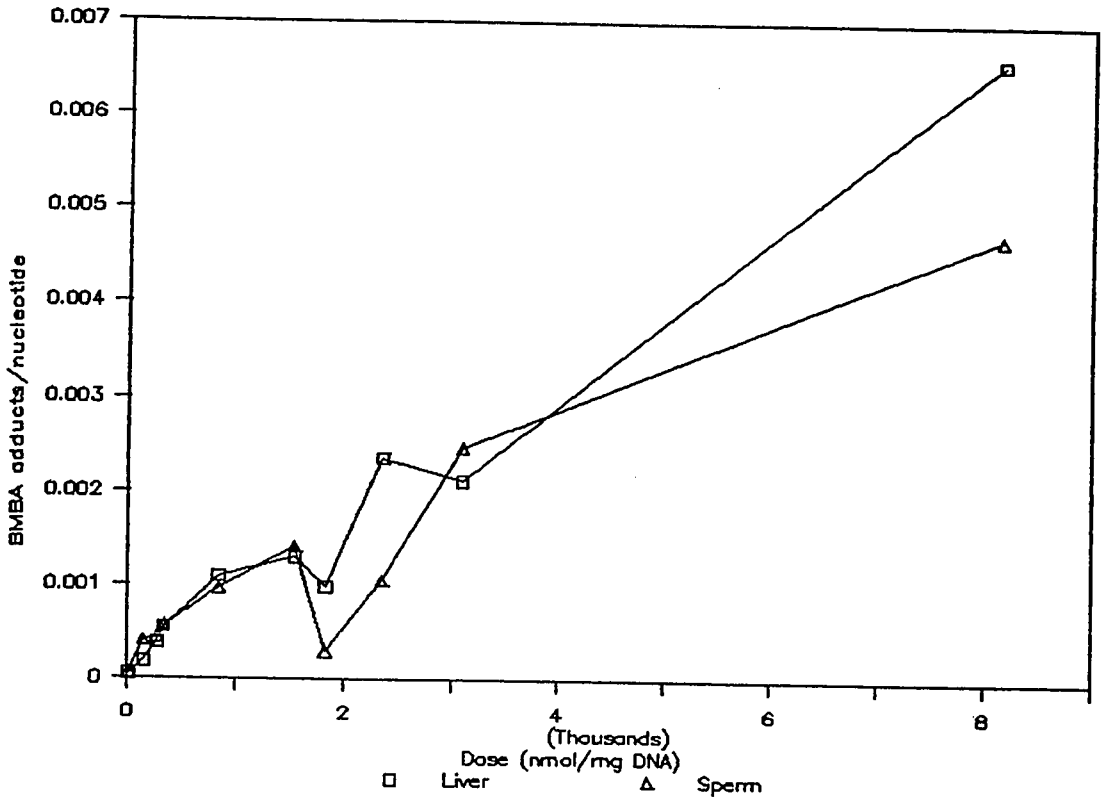


Figure 11. BMBA Dose Curve: DNA Binding



Microsome Activity Determinations

Each of the remaining chemicals tested in this study requires metabolic activation to form reactive intermediates capable of binding to DNA. This activation was accomplished by using microsomal enzymes isolated from livers of mice pre-induced with Arochlor 1254.

The Arochlor 1254 induced microsomes were compared to 3-methylcholanthrene induced microsomal standards to ensure the presence of aryl hydroxylase activity. The enzymes present in the microsomes are temperature sensitive and lose activity with time. Thus, the microsomes were tested on a regular basis to confirm that they were still active. Three different batches of microsomes (A, B and C) were prepared and used during the course of this study (Table 1).

TABLE 1

Microsome Determinations

Microsome	Standard	Protein (mg protein/ml)	Activity (pmole ^{3}0H-B(a)P/ min/mg protein)
A	—	36.49	32,757
—	A	13.37	12,830
A*	—	—	30,229
B	—	27.49	4,469
—	B	13.57	18,805
B*	—	—	3,260
—	B*	—	3,840
C	—	21.09	2,782
—	C	19.18	2,874

* Re-tested microsomes

Microsome Preparation

A series of experiments were performed to determine the appropriate amount of microsomes to be used in the *in vitro* experiments and to resolve problems encountered in using them with free DNA and nuclei. The primary problem was that the microsomes often precipitated during the incubation step, preventing the quantitative recovery of DNA. First, the albumin was removed from the cofactor solution to reduce the overall protein content. Next, the number of ethyl acetate extractions were increased to improve the separation of the DNA from the microsomes. The step of heating the DNA after the ethyl acetate extraction to increase the removal of any residual microsomal proteins and concentrate the DNA was tested and found to be unessential; this step was removed from the protocol. When the experiment was repeated with the new protocol to identify the optimal amount of microsome-A, the results indicated that the amount of DNA recovered decreased with increasing amounts of microsomes and showed (Figure 12) that the greatest amount of B(a)P binding occurred using 36 μl of microsome-A in the reaction mixture with CT DNA.

In subsequent experiments, hydroxyapatite was substituted for the excess ethyl acetate extractions to increase the recovery of CT DNA. Once the correct amount of hydroxyapatite was determined for isolating the amount of CT DNA present in the reaction mixture, additional tests were run to refine the *in vitro* protocol. These tests included: performing the experiment without CT DNA to ensure that only DNA was being recovered; comparing the results with an experiment only using "standard" (see Table 1) microsomes; adding a second Bio-Gel TSK-40 XL column in tandem with the existing one to increase the separation of peaks; and repeating the experiment to deter-

mine the appropriate amount of microsome-A required for the maximal B(a)P binding without reducing the recovery of CT DNA.

The results showed that a greater amount of CT DNA was recovered when using the hydroxyapatite to isolate DNA and any contaminating microsomal protein could be successfully removed from the DNA. Additionally, it was determined that only 15 μl of microsome-A were required for the greatest amount of B(a)P binding for the amount of CT DNA used in the reaction, and the presence of the protein did not seem to hinder the CT DNA recovery.

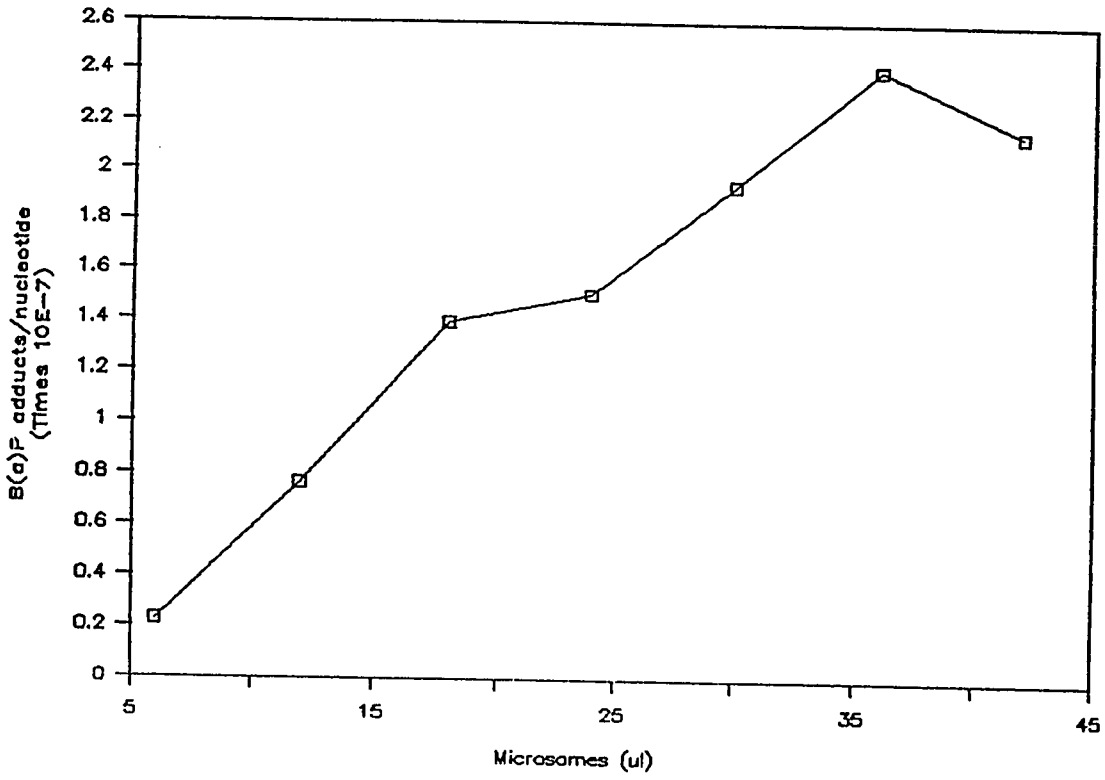
In vitro Benzo(a)pyrene Time Course

Calf Thymus DNA (0.04 mg/ml) was incubated with 15 μl of microsome-A, 0.9 ml of cofactor (without albumin), and 40 μl of tritiated B(a)P in methanol for 5, 10, 15, 20 and 25 min to determine the length of time necessary for maximum B(a)P binding to DNA. Controls were prepared without microsomes. The results, shown in Figure 13, indicate that maximal binding was reached after 25 minutes.

Refining the *In vitro* Protocol

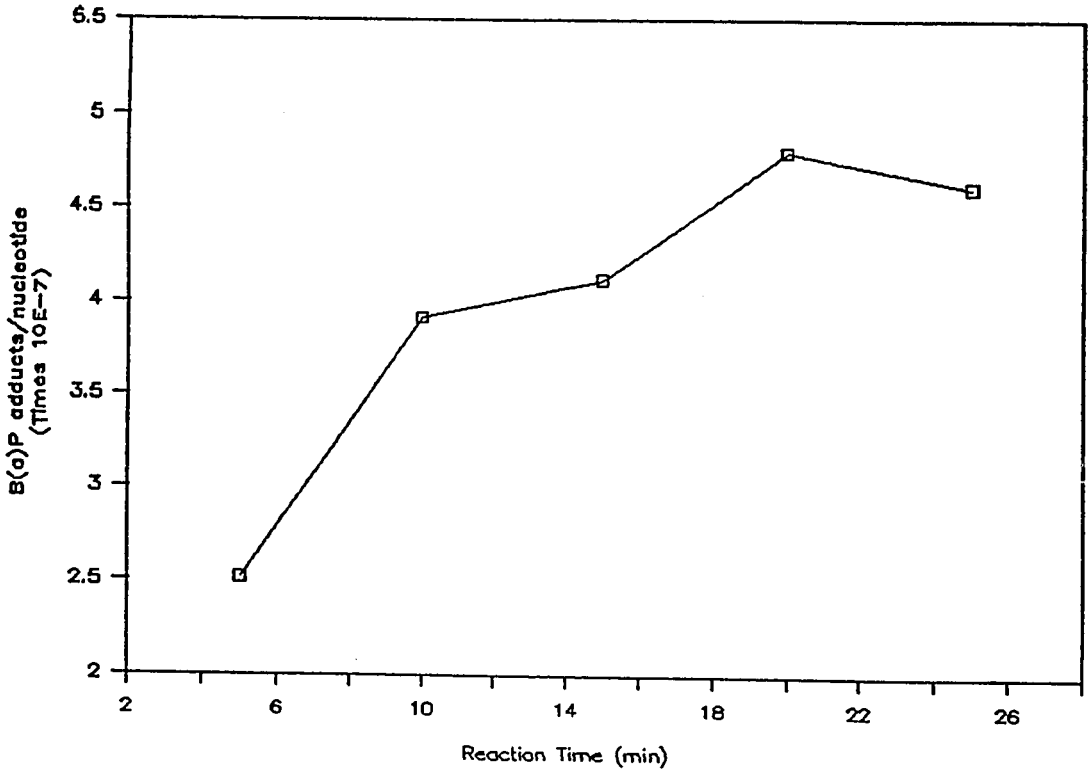
As the research continued there were still problems in the protocol that had to be resolved before the free DNA, sperm and liver nuclei could be isolated simultaneously. The microsomal precipitation problem reappeared and interfered with the recovery of DNA. Tests were run both on different stock solutions used in the reaction mixture (which methodically had different components withheld) and with the enzyme, Protease K, to determine if it could be used to digest the microsomal precipitate. None of the

Figure 12. Microsome Activity Determination



Ten samples were tested including two controls. All samples contained 0.18 mg/ml of CT DNA, 40 μl of tritiated B(a)P and 0.9 ml cofactor (without albumin). The samples contained the following aliquots of microsome—A: 6, 12, 18, 24, 30, 36 and 42 μl. Only the two controls did not contain any microsomes.

Figure 13. *In vitro* B(a)P Time Course

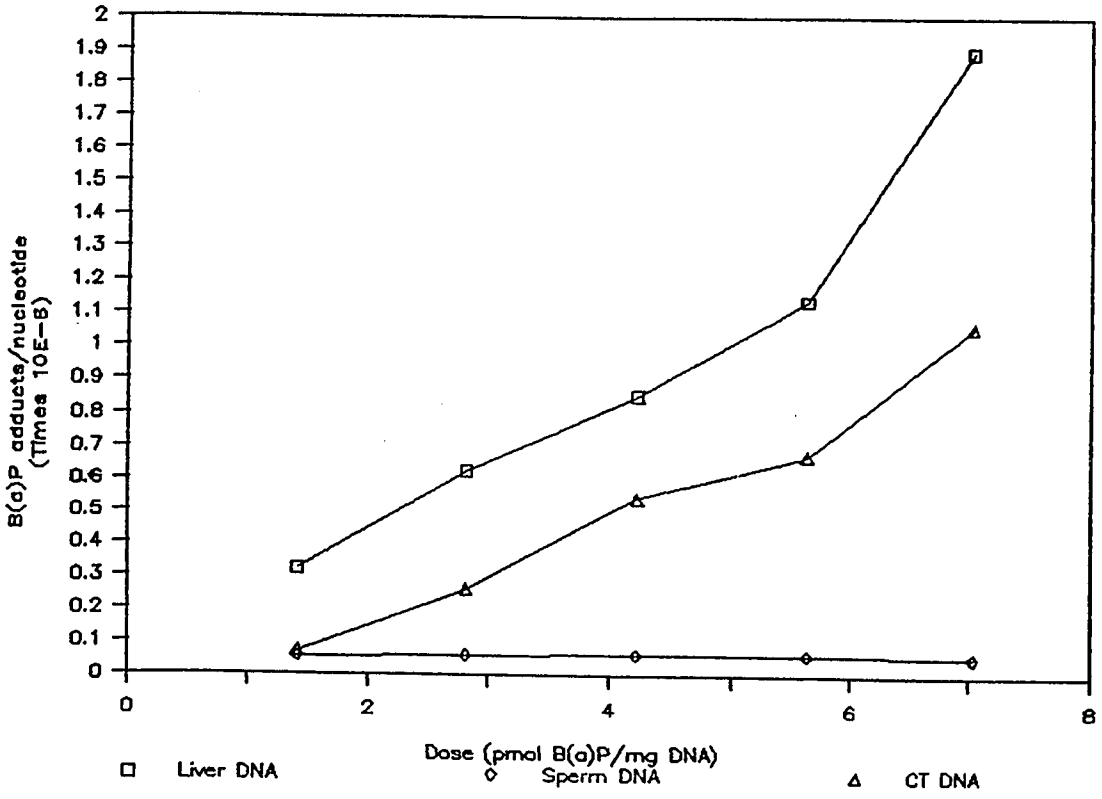


experiments were successful in eliminating the precipitation. The problem was finally resolved when it was determined that the precipitation may have occurred because the samples were not kept on ice (at 4 °C) prior to the incubation step at 37 °C. Careful analysis of the protocol also disclosed that the low sperm DNA recovery was due in part to the inclusion of sodium bisulfite in the grinding media used to isolate the sperm nuclei. Sodium bisulfite (BIS) absorbs strongly at 260 nm, the same wavelength used to quantitate DNA and unless the BIS was accounted for, a larger than actual amount of DNA (as determined by U.V. absorption at 260 nm) would seem to be present in the sample. Once the sperm DNA content was accurately determined in grinding media without BIS, the greatest amount of ^{3}H-B(a)P-DNA binding activity was obtained using 30 μl of the new microsome-B preparation.

Using the refined protocol, the ^{3}H-B(a)P dose curve was repeated with all three different types of DNA. All samples were adjusted to contain 0.04 mg/ml of DNA. Calculated doses of 1.4, 2.8, 4.2, 5.6 and 7.0 pmol ^{3}H-B(a)P per mg DNA were added to the test samples with 0.9 ml cofactor (without albumin) and 30 μl of microsome-B. The DNA was isolated and analyzed. The results showed that the sperm DNA and CT DNA were recovered with reasonable efficiency, but the yields of liver nuclei DNA were very low. Although the results shown in Figure 14 appear to be reasonable, the amount of liver nuclei DNA recovered was too low for accurate spectrophotometric analysis, thus suggesting greater B(a)P binding in liver nuclei than to CT DNA.

Another series of experiments were conducted to determine how to optimize the recovery of DNA from liver nuclei. Two steps in the protocol were found to be important. One involved assaying the amount of DNA present in the sample by chromato-

Figure 14. *In vitro* B(a)P Dose Curve (I): DNA Binding



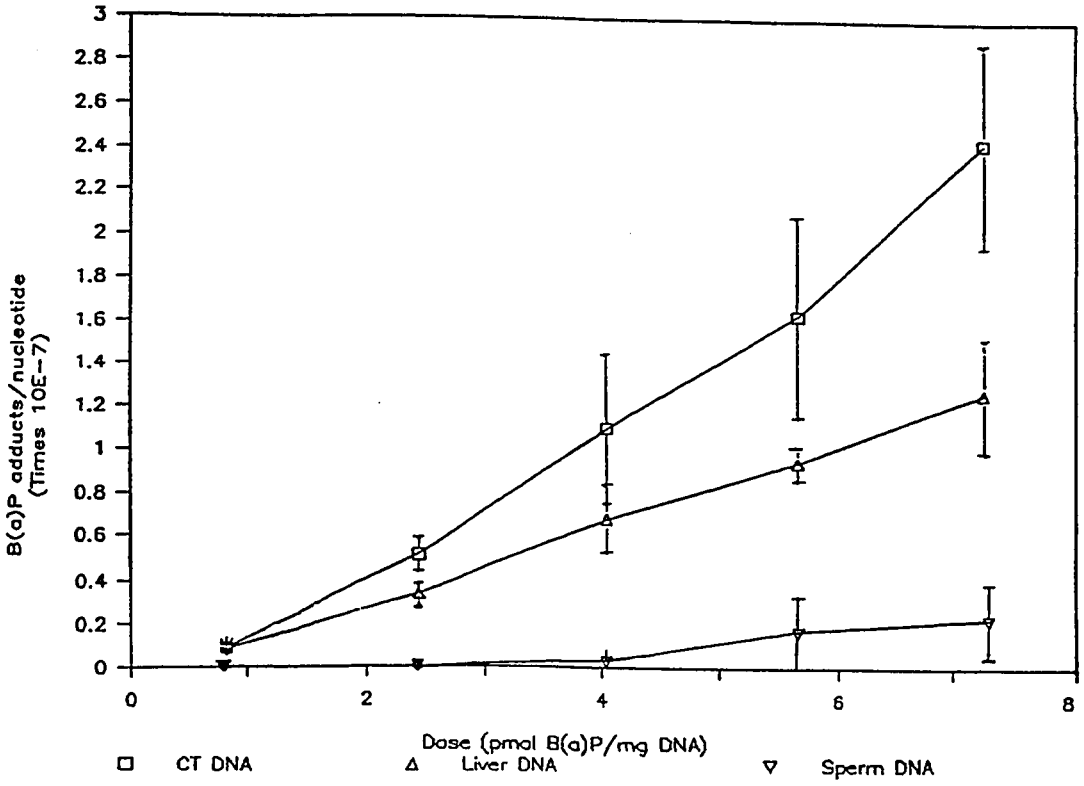
graphy rather than A_{260} measurements. Dissolving nuclei directly in GuCl gave A_{260} readings that were 52% higher in DNA content than those obtained after recovery of the DNA from the HPLC. The most important observation, however, was that the liver nuclei required magnesium to remain stable during isolation and the subsequent incubation. Hence, the $MgCl_2$ concentration in the grinding media was increased to 10 mM.

Experiments were performed to identify the level of endogenous microsomal activity in control liver nuclei. Liver nuclei were found to contain substantial activity and bound B(a)P even in the presence of high concentrations of inhibitors like 50mM 2-methyl-1,2-di-3-pyridyl-1-propanone or 0.1% sodium azide. Attempts were also made to limit the endogenous microsomal activity in these nuclei in absence of microsomes. The best controls which could be obtained were those in which the cofactor and microsomes were both left out of the sample. In addition, the efficiency of extracting noncovalently bound B(a)P was improved by increasing the final number of butanol extractions to five.

In vitro Benzo(a)pyrene Dose Curve

Using these conditions, the B(a)P dose curve shown in Figure 15 was obtained. The samples of calf thymus, liver nuclei and sperm were determined by chromatographic analysis and absorbance at 260 nm to contain 0.04 mg/ml DNA. In addition to the DNA, liver nuclei and sperm, all test samples contained 30 μl microsome-C and 0.9 ml cofactor (without albumin). Calculated doses of 0.8, 2.4, 4.0, 5.7 and 7.3 picomoles of ^{3}H-B(a)P per ml DNA were added to each test sample. All samples were incubated for

Figure 15. *In vitro* B(a)P Dose Curve (II): DNA Binding



25 min at 37 °C. Controls were prepared without cofactor or microsomes. The DNA isolation and characterization were performed as described in Materials and Methods. This procedure was performed three times. The results were averaged and graphed.

In vitro 7,12-Dibromobenz(a)anthracene Time Course

The time course of DNA modification by DMBA was determined using these new conditions. Calf thymus DNA (0.04 mg/ml) was incubated in the presence of 30 μl of microsome-C, 0.9 ml of cofactor (without albumin), and 90 μl of tritiated DMBA in methanol for 5, 10, 15, 20, 25 and 30 min to determine the length of time necessary for maximum binding. Controls were prepared without microsomes. The results, shown in Figure 16, indicate that binding did not increase substantially after 20 min.

Further Refinement of the *In vitro* Experiment

As the DMBA dose curve experiments were performed for the first time, it was noted that the DNA recovered from the reactions with DMBA had a higher absorbance at 260 nm (A_{260}) than the untreated CT DNA controls. In addition, the binding results obtained between the different dose curve experiments were not reproducible. Two experiments were performed to determine why the absorbance values were abnormally high.

The first experiment tested a set of samples using the same DMBA dosage with microsomes alone, eliminating the CT DNA from all samples. The results in Figure 17 show that A_{260} absorbing material (probably RNA) was being recovered from the microsomes and the ^{3}H-DMBA was reacting with this nucleic acid.

Figure 16. DMBA Time Course

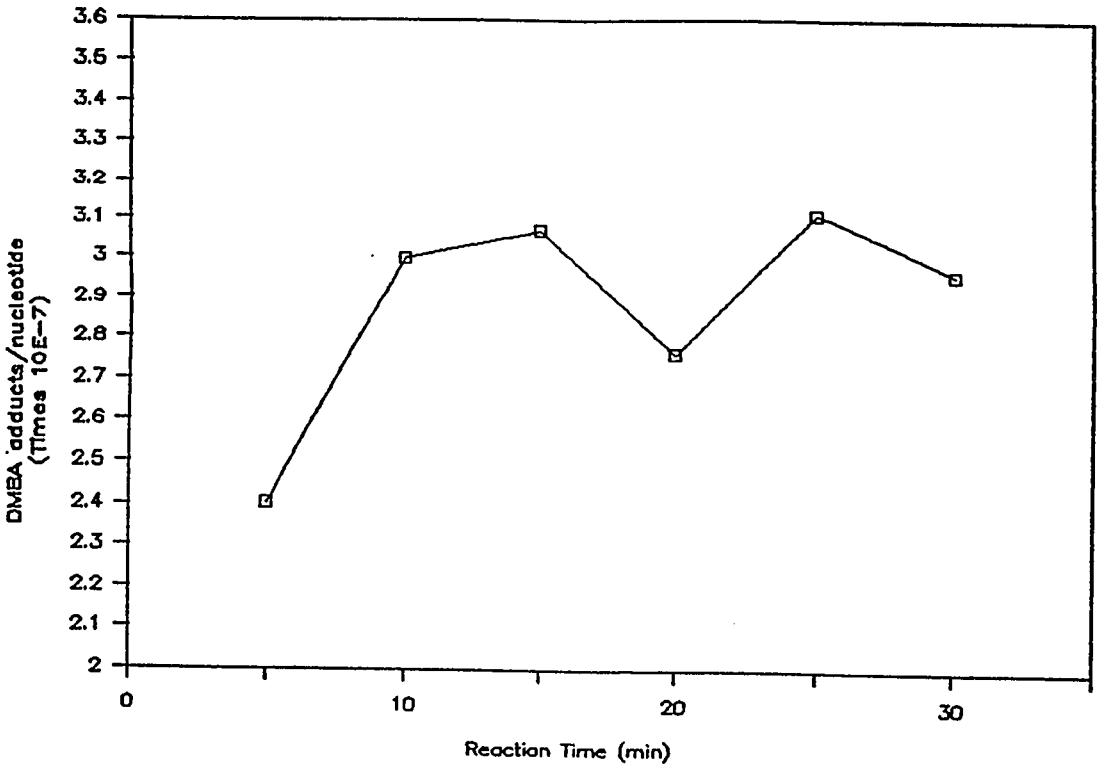
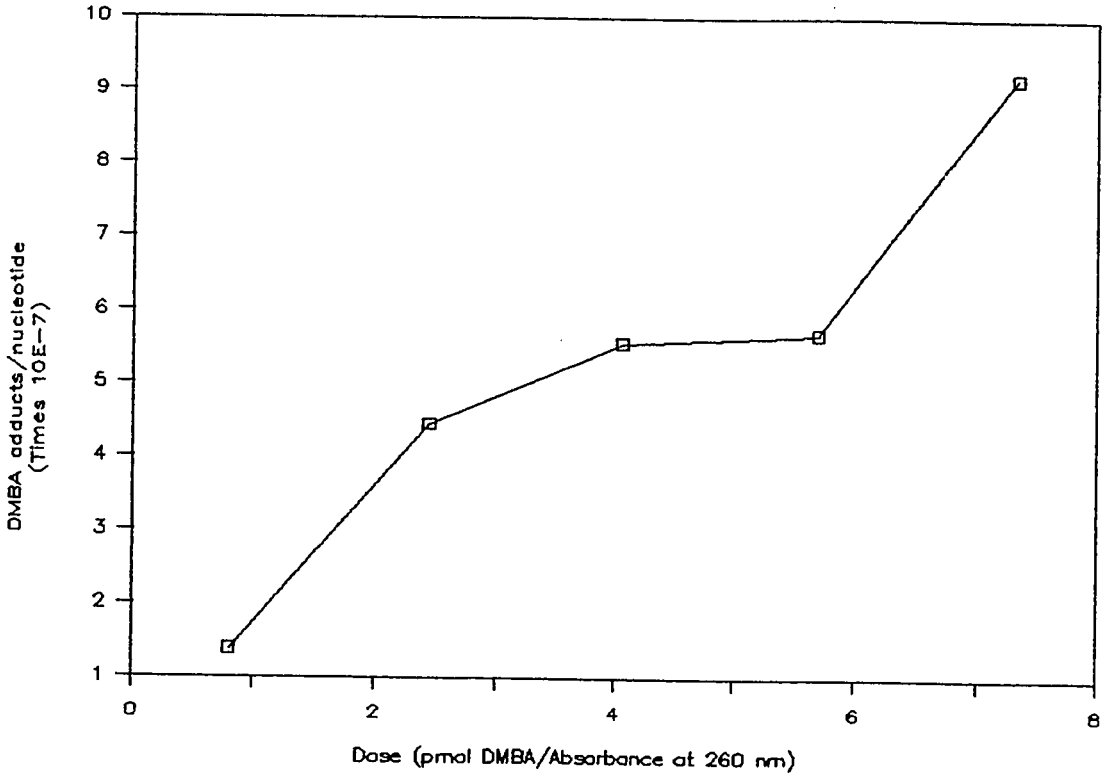


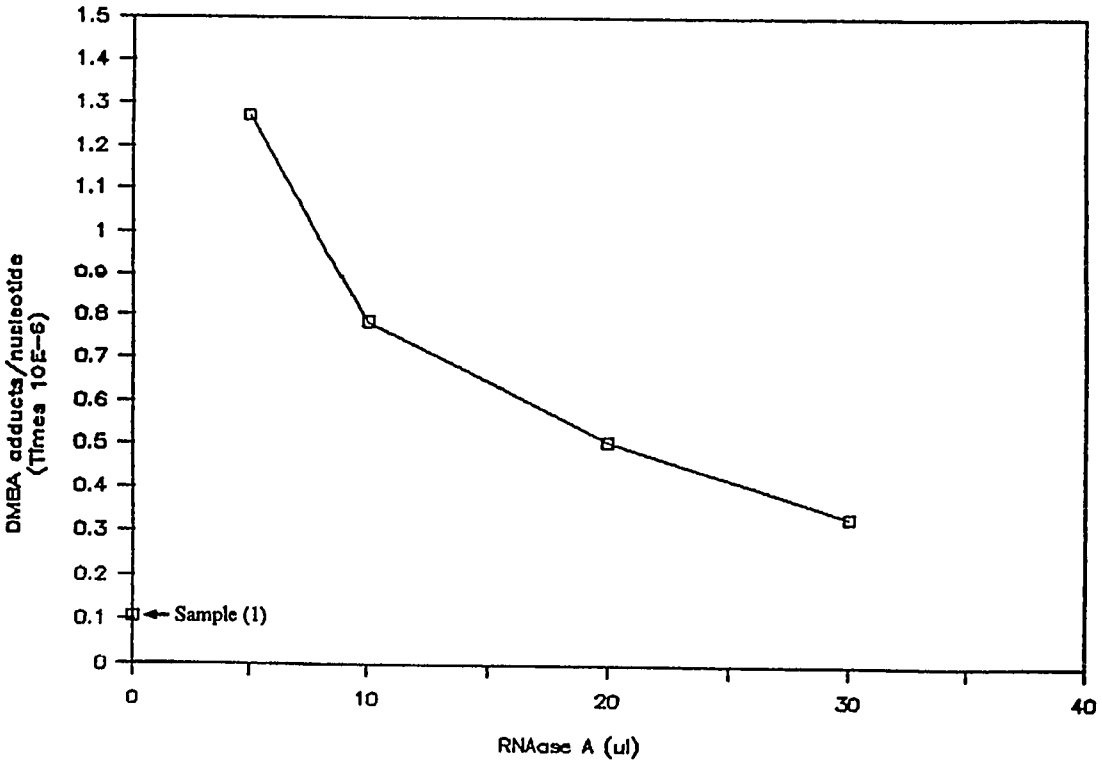
Figure 17. Microsome Binding Determination



Five samples were tested in addition to two controls. The test samples all contained 1 ml of 0.1 M sodium phosphate buffer, pH 6.8, 30 μl of microsome-C, and 0.9 ml cofactor. Calculated doses of 0.8, 2.4, 4.0, 5.7 and 7.3 pmol ^{3}H-DMBA per mg of DNA (even though DNA was not included) were added to the test samples. The samples incubated for 20 min at 37 °C. Hydroxyapatite was added to each sample, as previously described. The samples were then analyzed by chromatography followed by spectrophotometry.

In the second experiment RNAase A was added to the test samples containing microsomes to determine if the enzyme could be used to eliminate the RNA contaminant. The results shown in Figure 18 indicate that the RNAase A digested and successfully removed the the RNA from the samples.

Figure 18. RNAase A Degradation Determination



Six samples were tested in addition to two controls. The test samples contained the following constituents: Sample (1) CT DNA only; Sample (2) No CT DNA, 30 μl of microsome-C, 0.9 ml cofactor and 20 μl of RNAase A; Samples (3-6) contained CT DNA, 30 μl microsome-C, 0.9 ml cofactor and 5, 10, 20, and 30 μl of RNAase A respectively. All samples contained 90 μl of ^{3}H-DMBA and were incubated for 20 min at 37 °C. The DNA was isolated and characterized. Sample (1) was used as a control for comparison. The decrease shown in samples (3-6) correspond to a decrease in contaminant microsomal RNA. Sample (2) resulted with 0.0 absorbance at A_{260} and is not shown.

Discussion

In vivo Studies

As the *in vivo* studies with Trp-p-2 and B(a)P have shown, both the liver and epididymal tissue received significant doses of carcinogens. The dose per gram tissue received by the epididymis, however, is consistently lower (3-7 fold) than that received by the liver. While both compounds were found to actively bind to liver DNA, neither of these carcinogens were able to bind the sperm DNA. Factors which may allow for this difference in binding include the role of the liver as the major site of metabolic activation, tissue specificity, the electrophilic nature of the carcinogen, or the structural constraints either from the packaging of the DNA, nuclear membrane permeability or the physical size of the carcinogen.

The lack of sperm DNA binding detected from both of these carcinogens *in vivo* could be attributed to either a large decrease of metabolic activation found in this location, or the structural constraints resulting from the chromatin packaging in the sperm nuclei disallowing either of these carcingens to bind covalently or by intercalation. Since these carcinogens preferentially bind both the major (Trp-p-2) or minor (B(a)P) groove, the former possibility seems more likely.

In vitro Studies

BMBA Binding to Free DNA and DNA in Liver and Sperm Nuclei

7-Bromomethylbenz(a)anthracene does not require metabolic activation for binding. Thus, it seemed possible that in an *in vitro* setting binding would occur to both the liver and sperm DNA. What is of interest, is that the quantity of binding which

occurred in both DNA environments (sperm and liver nuclei) is almost equal. Quantitative differences would be expected *in vivo*, as found in the experiments described above, due to naturally occurring physical barriers. These differences might also be expected *in vitro* due to the structural hindrances imposed by the sperm nuclei or the preferential binding site in DNA being blocked by the protamine, although this was not the case.

A few limitations were found in the BMBA experimental protocols after the development of the *in vitro* exposures which required metabolic activation. First, free DNA (naked CT DNA) was not tested along with the nuclei as it was in subsequent experiments. Although it would be expected that the free DNA binding results would be similar to or greater than the nuclei results, the quantitative difference would have been of value. Second, the initial analysis steps required to determine equal amounts of nuclear liver and sperm DNA were developed after the BMBA experiments were performed. This would have reduced the amount of error found in the "quick and dirty" spectrophotometric analysis for those two types of nuclear DNA. The liver nuclei were especially dirty and gave abnormally high absorbance readings, with analogous errors associated with the nuclear sperm DNA. Finally, the concentration of DNA used in the these experiments was much higher (0.355 mg DNA/ml versus 0.04 mg DNA/ml) than the subsequent experiments requiring metabolic activation, which made quantitative comparison difficult.

B(a)P Binding to Free DNA and DNA in Liver and Sperm Nuclei

As implied in the results section, the development of this portion of the protocol was the most difficult. Each aspect of the experiment had to be tested separately prior to performing the final series of experiments exposing the liver nuclei, sperm nuclei and free DNA together under identical conditions. In part, most of the problems were caused by the addition of microsomes to the experimental system. Other factors, such as temperature or replacing ethyl acetate extractions with a hydroxyapatite purification step were separate issues that were also resolved. The microsomes had their own physical attributes which made recovering the free DNA or nuclei complicated. Once these problems were resolved, the liver nuclei, sperm nuclei and free DNA were exposed to B(a)P under identical conditions.

As expected, the B(a)P exhibited the greatest amount of binding to the free DNA in the *in vitro* experiments; the DNA in liver nuclei bound 50-60% of the B(a)P bound by free DNA. Only a small amount of binding (less than 10% that observed for free DNA) was observed at the higher dose points in the sperm nuclei DNA. The low level of B(a)P binding to the DNA in sperm nuclei is of interest because it suggests that the minor groove of DNA was not completely accessible.

Non-covalent Binding in the B(a)P Liver Nuclei Controls

Controls samples were run with each form of DNA tested. These samples only contained the tritiated carcinogen of interest and the DNA or nuclei; no microsomes or cofactor were included. Although no enzymes or cofactors were added, the liver nuclei controls seemed to metabolize the B(a)P and form adducts to DNA as observed by the resulting high scintillation counts. A butanol extraction study was performed to make

certain that the B(a)P bound to the DNA was actually covalently attached to DNA. The liver nuclei control test samples were exposed to the highest dose of B(a)P, then systematically extracted with water saturated butanol to determine the amount of extractions required to remove all of the non-covalently bound B(a)P. The results suggested two possibilities for the observed B(a)P binding to the controls: (1) an intercalative environment was enhanced in liver chromatin, or (2) some residual microsomal enzymes in the liver nuclei was enough to allow binding to the liver DNA without addition of more microsomes or cofactor.

Studies with DMBA

When repeating the protocol with DMBA, a new problem associated with the microsomes surfaced. In these experiments, the results obtained after spectrophotometric analysis indicated that there was more DNA present after the HPLC fractionation than at the beginning. The subsequent experimentation determined definitively that the RNA present in microsomes was being recovered with the DNA and that the DMBA was binding to this RNA as well as the DNA. Once this discovery was confirmed, the enzyme RNAase A was used to digest and eliminate the RNA from the samples.

Future Experimentation

Based on this last discovery during the DMBA experimentation, a number of questions may be resolved with future testing. Knowing now that RNA was being recovered with the DNA, it is very probable that the small amount of binding seen in the *in vitro* B(a)P sperm nuclei DNA results was not DNA adduction, but B(a)P bound to the RNA contaminate provided by the microsomes. This seems likely, since this was most

often observed in the high DMBA doses where the largest amount of contaminate was found. (Note that no B(a)P binding was observed at the lower dosages).

The B(a)P binding observed in the liver nuclei controls may be unaffected by the latter discovery, since no microsomes or cofactor were ever added to the control samples. This may add weight to the favorability of enhancing the environment for intercalation or may suggest that more RNA is present in the liver nuclei than previously thought. The present experiments do not, however, rule out the possibility that the liver nuclei retain some residual microsomal activity that metabolize the B(a)P.

One other factor may have contributed to the limited reactivity of sperm DNA to either B(a)P or Trp-p-2. The experiments performed in this study used intact sperm, and the presence of the nuclear membrane and acrosome might severely restrict the entrance of the activated carcinogen into the nucleus. This is significant, since binding would definitely occur to both the tail and membrane proteins. Consequently, the results obtained in these studies do not reflect only the accessibility of the DNA as imposed by the histones or protamines that bind to it in liver and sperm nuclei. One important result of this work is that through the experiments, performed and described here, the proper conditions and techniques have been identified for use in future experiments to compare mutagen binding to free DNA and DNA in liver and sperm.

In summary, additional experiments are required to clearly distinguish the binding possibilities. Even though *in vitro* studies will never recreate the very complex *in vivo* systems, important conclusions can be drawn from such studies about relationships

between the physical characteristics of cellular material and exposures to exogenous carcinogens.

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