# THE FATE OF BENZO(A)PYRENE IN TISSUES OF MICE EXPOSED TO DIESEL EXHAUST

THESIS

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By

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Mice were exposed to diesel exhaust for 9 months prior to evaluation for benzo(a)pyrene disposition. On the last day of exposure the mice were instilled intratracheally with tritiated-benzo(a)pyrene ([$^3$H]BP). The mice were sacrificed at intervals of 2, 24, and 168 hours.

Disappearance of radioactivity from lungs and liver was rapid and essentially linear with time. In lungs, liver, and testes; [$^3$H]BP metabolites were found mainly as conjugates, a form readily excretable. Clearance of the hydrocarbon from liver and testes in exposed mice was not markedly different from that in nonexposed mice. However, mice exposed to diesel exhaust had delayed [$^3$H]BP clearance from lungs, possibly due to [$^3$H]BP adsorption to diesel smoke particles.

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# CHAPTER I

## INTRODUCTION

It is estimated that by 1985 up to 25% of passenger cars in the United States will be powered by diesel fuel because diesel engines offer about a 25% improvement in fuel savings compared to gasoline powered vehicles (1). This expected change will greatly increase the automotive contribution to particulate-adsorbed polynuclear aromatic hydrocarbons (PAH) and other organics in the air. Such an increase will occur because a diesel engine emits 30 to 50 times more particulate matter than a comparable gasoline engine with a catalytic converter (2).

The gaseous oxides of nitrogen ($NO_x$) and particulate organic matter are the major pollutants of concern from diesel exhaust emissions. A key constituent of $NO_x$ is nitrogen dioxide, which "is a 'criteria' pollutant per se, and also represents, through its photolysis, the only known anthropogenic source of ozone in photochemical smog" (3). Nitrogen oxides form the largest fraction of the various gases formed by diesel exhaust (4). These gases are similar to those emitted by gasoline exhaust; however, the particles in the diesel exhaust are quite different in quantity and composition (1). The particulate organic matter in diesel exhaust

is basically a carbonaceous material with organic chemicals of high molecular weight adsorbed on them. Approximately 1% of the particulate organic matter is PAH (5). The small size of these particles (0.2-0.3 μm mean diameter) enables them to reach the alveoli (6).

The PAH are among the earliest classes of compounds to be demonstrated as carcinogens in man. In 1775, Percival Pott, a British physician, was the first to attribute the high incidence of scrotal cancer among London chimney sweeps to their prolonged exposure to coal tar and soot (7). The first example of experimental chemical carcinogenesis was in 1915, when Yamagiwa and Ichikawa repeatedly applied coal tar to the ears of rabbits and found malignant tumors (8). Hydrocarbons are naturally present in many forms of vegetation and fossil fuels, and PAH result from incomplete combustion of these hydrocarbons. Therefore many types of PAH are produced as a result of coal and oil combustion for commercial energy generation, refuse burning, power plants, wood burning stoves, use of various types of gasoline and diesel fuels, and many other forms of effluents resulting from commercial and personal energy production (9).

Benzo(a)pyrene (BP) is generally accepted as a model compound for studies of PAH. Since the 1930s, when BP was first isolated from coal tar by Cook, Kennaway, and their collaborators (10), a great deal of research has been performed. BP has been identified as an atmospheric pollutant.

Indeed, 2000 tons of BP are released into the environment of the United States each year (11). BP is one of the major PAH associated with diesel exhaust (12). Estimates of human exposure to PAH usually employ BP as a model compound (13).

The pathway for BP disposition within the body is a multistep, highly complex process. The first step that is amenable to control is at the level of exposure (14). It has been estimated that approximately 85% of all human cancers result indirectly or directly from environmental influences (15, 16) and BP is present in both the aquatic and atmospheric environment (17, 18). It follows, therefore, that a decrease in level of exposure to BP and other PAH should result in a decreased cancer risk.

Exposure levels normally determine uptake and absorption of PAH. BP is chemically inert and lipophilic. If BP is not metabolized, it would remain in the body for a lifetime (19). Specific enzyme systems will metabolize BP to polar end products which are readily excreted (19). This metabolic process also controls the production of intermediates that may be more biologically reactive than their parent compound (20). Figure 1 illustrates several possible pathways of BP metabolism in man. Microsomal mixed-function oxidases catalyze the initial oxygenation of the BP to the 4,5-; 7,8-; or 9,10-arene oxide form (21, 22, 23). BP 4,5-oxide is the most stable of the metabolites (24). These reactive electrophiles undergo additional metabolic transformations to

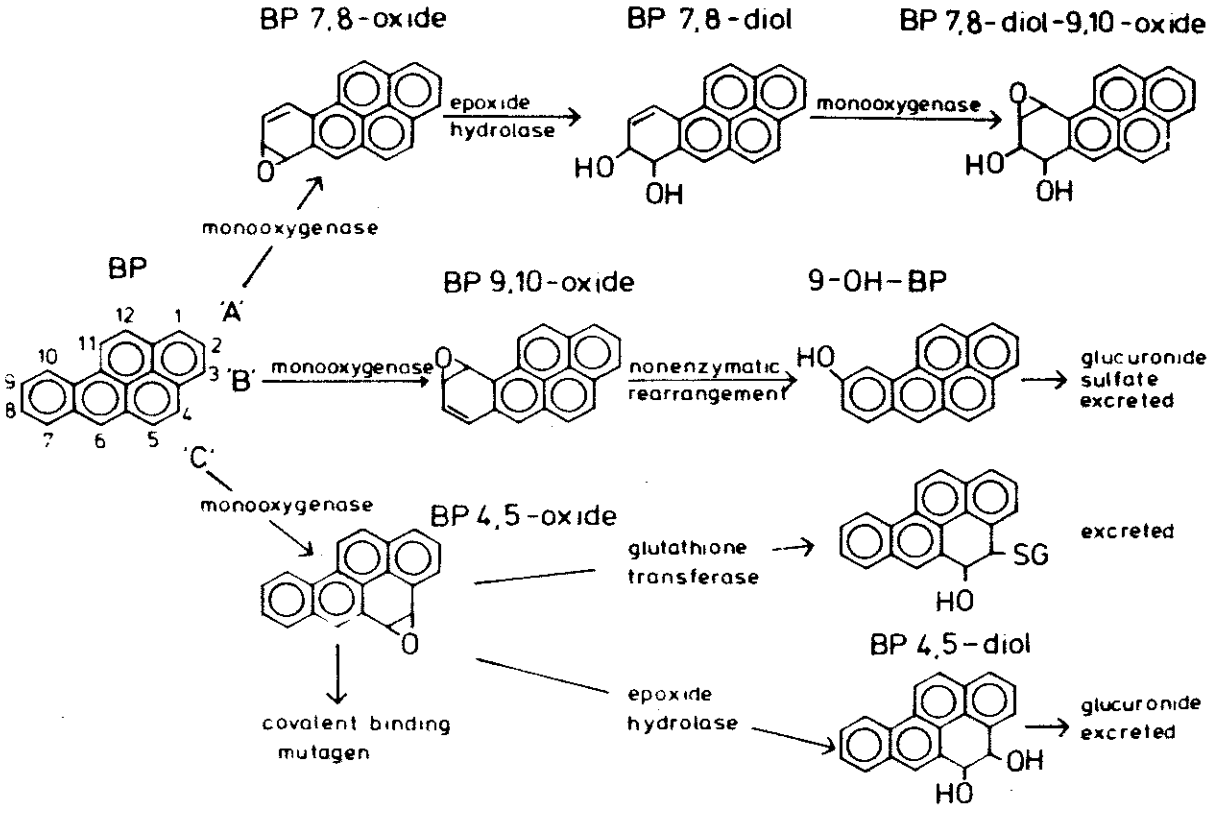



Figure 1: Pathways of Benzo(a)pyrene Activation and Inactivation (taken from Guenthner and Oesch (19)).

forms which are either more toxic or less toxic.

In pathway "A," BP 7,8-oxide is hydrated by epoxide hydratase to form a dihydrodiol (25). Normally this could be considered a detoxification step, however, the dihydrodiol can also serve as substrate for the mixed-function oxidases. This further metabolism results in formation of BP 7,8-diol-9,10-oxide. This metabolite is considered to be the single most potent carcinogenic and mutagenic form of BP (26), and it can readily react covalently with cellular nucleophiles (27, 28). Apparently this metabolite is a poor substrate for epoxide hydratase (19); but in the presence of NADPH or NADH, BP 7,8-diol-9,10-oxide can be reduced nonenzymatically to triols and tetrols (20, 29, 30).

Pathway "B" illustrates that a phenol such as 9-OH-BP can be formed via nonenzymatic rearrangement of BP 9,10-oxide. UDP-glucuronyl transferase and sulfo-transferase react with this metabolite, forming readily excretable compounds (20).

A different pathway is exemplified by pathway "C." BP 4,5-oxide, an intermediate in the pathway, can produce mutagenic effects by binding to DNA (26). However, an epoxide hydratase hydrates BP 4,5-oxide; and a more polar dihydrodiol can be rapidly excreted as such or as a glucuronic acid conjugate (31). Another means of detoxification is via glutathione s-epoxide transferase, which forms a glutathione conjugate. This is also easily excreted (31).

These pathways illustrate a few of the ways in which man can metabolize or activate a PAH to either more or less toxic compounds. At this point man still has several avenues left to deal with the toxic compounds. The reactive intermediates may bind to cellular macromolecules, which leads to cell death and lysis followed by excretion of adducts. PAH adducts may be recognized by cellular repair enzymes and the damaged portion of DNA may be repaired (20, 32). If this does not happen, transformation may occur and the immune system may recognize the tumor cells as foreign to the body and kill them. Unfortunately, some cells may escape the immune surveillance and a tumor results (33). The above description of the carcinogenic pathways of PAH is outlined in Figure 2.

An understanding of the fate of PAH and its metabolites can lead to at least a partial understanding of the carcinogenic potential of diesel exhaust. The present study evaluates the fate of BP given intratracheally to mice which were either exposed or not exposed to diesel exhaust. The time course of clearance and metabolite formation of BP is analyzed in these tissues: lungs, liver, and testes. Also, the extent of DNA binding to BP in the lungs and liver is measured.

The disappearance of radioactivity from the lungs and livers was rapid and was essentially linear with time. In the lungs, liver, and testes, the [$^3$H]BP metabolites were

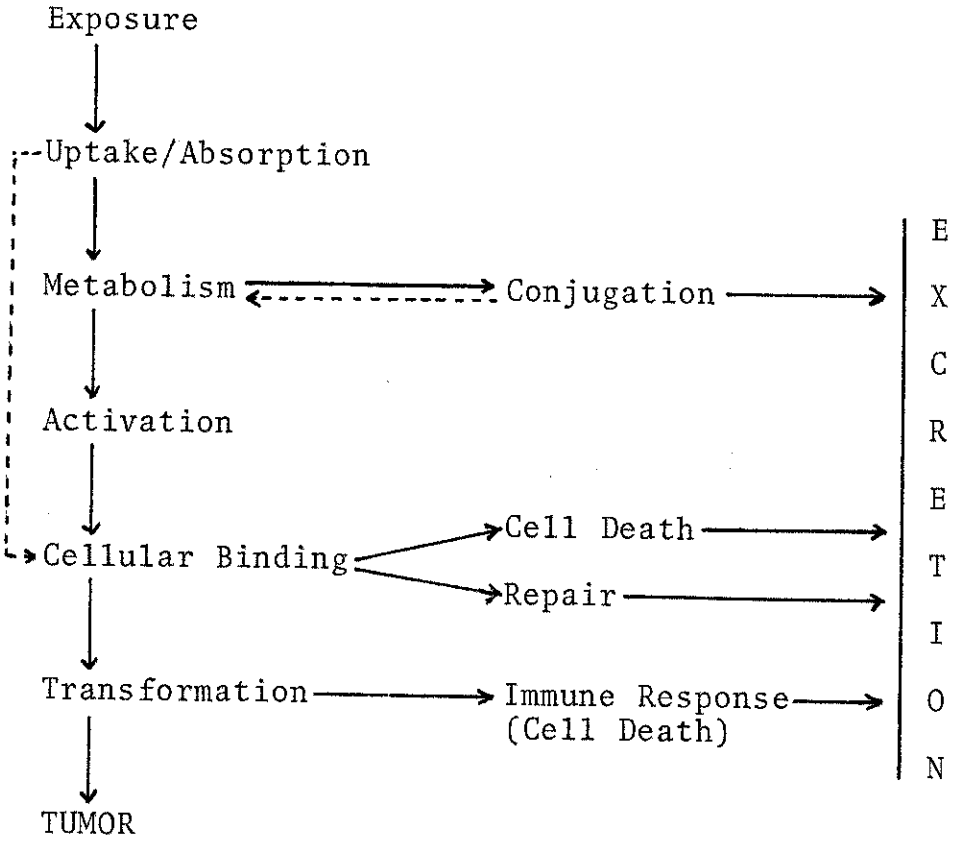



Figure 2: Sequence of Events in Chemical Carcinogenesis.

found mainly as conjugates, a form readily excretable. Clearance of the hydrocarbon from the liver and testes in exposed mice was not markedly different than in nonexposed mice. However, the mice exposed to diesel exhaust had a delay in [$^3$H]BP clearance from the lungs. This is probably due to [$^3$H]BP adsorption to diesel smoke particles.

# CHAPTER II

# MATERIALS AND METHODS

## Materials

### Animals

Young adult (usually 4 to 8 weeks) male strain A mice were purchased by the Environmental Protection Agency from Jackson Laboratories in Bar Harbor, Maine. Animals were maintained on Purina Rat Chow and tap water ad libitum prior to sacrifice (6).

### Equipment

High-speed centrifugation was performed in a Beckman J6B centrifuge (Irvine, California). Ultracentrifugation was performed in a refrigerated OTD 50 Sorvall ultracentrifuge with a T865 rotor head (Newtown, Connecticut).

Separation of compounds was achieved by the use of a Waters 6000-A high performance liquid chromatograph (Milford, Massachusetts). Radioactivity was determined by a Beckman LS-7500 (Irvine, California) or a Packard Tri-Carb 460C liquid scintillation counter (Downers Grove, Illinois). A Hotpack $CO_2$ incubator was used (Philadelphia, Pennsylvania). A Multiple Automated Sample Harvester (MASH) II by Microbiological Associates (Bethesda, Maryland) and a Millipore

vacuum manifold (Bedford, Massachusetts) were employed to filter the mouse tissues. A Sonifier Cell Disrupter 350 by Branson (Danbury, Connecticut) was utilized to sonically disrupt cells.

## Chemicals and Other Supplies

Generally labeled, tritiated benzo(a)pyrene ([$^3$H]BP) with a specific activity of 40 Ci/mM was obtained from Amersham (Arlington Heights, Illinois). Protease from Streto-myces griseus, RNAase from bovine pancrease, DNAase from bovine pancrease, NADPH, and salmon sperm DNA were purchased from Sigma Chemical Company (St. Louis, Missouri). Methanol, ethanol, hexane, formic acid, and phosphate buffer were acquired from Fisher Scientific Company (Pittsburgh, Pennsylvania). The cocktail used for the liquid scintillation counter was Scintiverse fluid from Fisher. The glass fiber filters used in the MASH II and Millipore vacuum manifold were Whatman grade 934 AH (Clifton, New Jersey). The medium used for the lymphocytes was Joklick's modified minimum essential medium from Gibco containing 10% fetal calf serum, 0.5% pokeweed mitogen, and 1% phytohemagglutinin also from Gibco (Grand Island, New York).

## Animal Exposure

The mice were housed and exposed at the United States Environmental Protection Agency, Center Hill Facility in Cincinnati, Ohio by Robert Danner. The mice were maintained

in stainless steel inhalation chambers in groups of 10 per cage for their exposure to purified air (control) or diluted diesel exhaust (test). The exhaust was generated by 2 Nissan CN-6 diesel engines, which were used alternately. The engines repeated cycles of acceleration, deceleration, cruise, and idle modes. The exhaust was diluted with filtered conditioned air at 18:1 dilution ratio in mixing chambers. Exposure time was 8 hours per day, 7 days per week, for 9 months. This exposure is considered to correspond to the amount a worker would receive if he worked in a bus depot or around diesel engines for a living. The inhalation chambers were kept at approximately 72°F and at 50% relative humidity. These chambers were continuously ventilated at a rate of 15 volumes per hour. The engines produced a concentration of particles in the exposure chambers of 6 to 7 $mg/m^3$. The animal quarters were cleaned regularly to minimize ammonia production from animal excreta (6).

After nine months exposure, the mice were randomly sorted into 3 subgroups for separate studies. The first group was employed for whole body autoradiography which was performed by Harry Tyrer in Missouri (34). The second group was for histofluorescence studies which were performed by the Histology department at Texas College of Osteopathic Medicine and Elroy Cantrell (35). The third group was for biochemical studies of [$^3$H]BP uptake by selected tissues, and this group is the subject of the present report.

On the first day after removal from the exposure chambers, the animals were instilled intratracheally with a suspension of 0.2% gelatin and 0.05 mg/ml of [$^3$H]BP (5 μCi). Prior to instillation the mice were injected with 0.1 ml atropine (1 mg/kg) intraperitoneally to reduce mucous secretion in the airway and 0.25 ml pentobarbital (40 mg/kg) for anesthesia. Ten animals were sacrificed by cervical dislocation at intervals of 2 hours, 10 at 24 hours, and 10 at 168 hours after instillation. Half of each group of 10 animals were control and the others were exposed to diesel exhaust. Immediately the lungs, liver, and testes were removed, weighed, and frozen on dry ice. All tissues were kept frozen until assayed (34, 35).

## Biochemical Studies

### Soluble Versus Insoluble

The frozen lungs, liver, and testes were thawed. Half of each organ was weighed. Each organ aliquot was placed into 2 ml of deionized water and homogenized. Next, α-tocopherol (5 mg) was added to each homogenate to serve as an antioxidant followed by 6 ml of ice cold ethanol. The homogenates remained for at least 2 hours in the refrigerator. After this they were sedimented in an ultracentrifuge for 15 minutes at 50,000 x gravity at 5°C. The supernatant of each sample was removed and dried under a nitrogen stream. Each

supernatant residue was redissolved in 1.2 ml of 80% ethanol. The pellet from each sample was resuspended in 2 ml of deionized water. The radioactivity of both the supernatants and pellets was determined in the Beckman liquid scintillation counter. Both internal and external standards were employed for calculation of disintegrations per minute (dpm).

## Chromatography of [$^3$H]BP and Metabolites

A high performance liquid chromatograph was utilized to separate [$^3$H]BP, its metabolites, and conjugates. The supernatant solutions (200 µl) were injected into a 4.6 mm by 100 mm stainless steel column, packed with neutral alumina (80-200 mesh). The [$^3$H]BP and metabolites were eluted stepwise from the column. First, [$^3$H]BP was eluted with 10 ml of hexane; second, 15 ml of ethanol eluted hydroxy-[$^3$H]BP; third, 15 ml of water eluted [$^3$H]BP-sulfate; fourth, 15 ml of 0.5 M ammonium phosphate (pH = 3.0) eluted an unknown material; and fifth, 20 ml of 25% formic acid eluted [$^3$H]BP-glucuronide and [$^3$H]BP-glutathione (36, 37). The identification of the compounds eluted by each solvent was confirmed by internal and external standards monitored by spectrofluorometry. The flow rate was 2 ml/min and fractions were collected at 1 min intervals. The column was conditioned before each sample injection by washing sequentially with 0.5 M ammonium phosphate at pH = 7.0, deionized

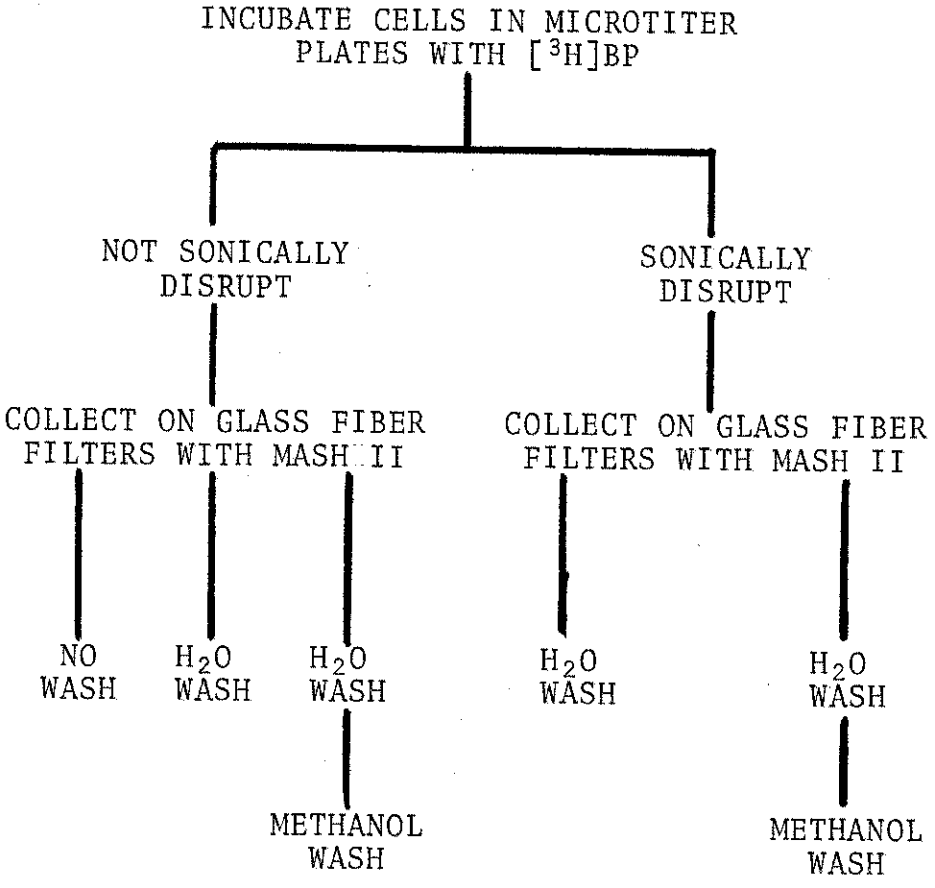
water, ethanol, and hexane. The radioactivity in each fraction was determined by the Beckman liquid scintillation counter.

## Tissue Bound Benzo(a)pyrene

### Tissue Binding Studies

In order to standardize and to evaluate the MASH II, the method of assaying tritiated-thymidine uptake by cultured lymphocytes was modified for this study (38). Lymphocytes were incubated at 37°C in Joklick's modified minimum essential medium containing 10 to 15% fetal calf serum, 1% pokeweed mitogen, 1% phytohemagglutinin, and 50 units/ml heparin. The concentration was 750,000 lymphocytes per microtiter well. Before incubation 0.05 μM [$^3$H]BP was added to each well. After 48 hours half of the cells were sonically disrupted and the remainder were left intact. Both suspensions were trapped on glass fiber filters presoaked in 100 mM pyrophosphate buffer at pH = 7.1 containing 10 mM thymidine. The filters were washed with water, or water and methanol as illustrated in Figure 3. The filters were dried and their radioactivity measured using a Packard liquid scintillation counter. All of the remaining experiments used this instrument. This experiment was performed in triplicate.

Figure 3: Experimental Flow Chart for Various Wash Procedures on Retention of [$^3$H]BP in Human Lymphocytes. The lymphocyte cells were incubated with [$^3$H]BP in microtiter plates for 24 hours. Part of the cells were sonically disrupted and the others were left intact. Both sets were collected on glass fiber filters with the MASH II. Three different methods of washing the cells were used: no wash, water only, or water followed by methanol.


INCUBATE CELLS IN MICROTITER PLATES WITH [3H]BP
NOT SONICALLY DISRUPT
SONICALLY DISRUPT
COLLECT ON GLASS FIBER FILTERS WITH MASH II
COLLECT ON GLASS FIBER FILTERS WITH MASH II
NO WASH
H2O WASH
H2O WASH
METHANOL WASH
H2O WASH
H2O WASH
METHANOL WASH

## DNA-Bound [$^3$H]BP

To test the effectiveness of the cell harvester in quantifying soluble naked DNA covalently bound to [$^3$H]BP, the polynuclear aromatic hydrocarbon (PAH) was enzymatically attached to DNA (39, 40). To accomplish this the following substances were combined on ice in one polycarbonate culture tube: 3.5 ml of Tris buffer (0.01 M) pH = 7.2 with 0.003 M $MgCl_2$, 0.25 mg of mouse microsomes at 20 mg/ml, 0.05 ml NADPH at 20 mg/ml, 0.50 ml DNA at 10 mg/ml, and 125 µCi [$^3$H]BP (0.25 ml). The culture tube was incubated at 37°C for 120 minutes. Samples of 200 µl aliquots were placed in separate polycarbonate tubes along with 800 µl of Tris buffer (0.01 M) pH = 7.2 with 0.003 M $MgCl_2$. These tubes were placed in ice water.

To study the effects of protease, RNAase, and DNAase on the solutions prepared in the previous paragraph, these enzymes were added to the culture tubes. The following amounts of these individual enzymes were added to different tubes: 0 µg, 2 µg, 5 µg, and 10 µg. The tubes were allowed to incubate for 10 minutes at room temperature. Afterwards they were collected with a MASH II and were washed briefly with water, followed by 10% trichloroacetic acid, and then with methanol. The radioactivity of each filter was determined. This experiment was performed in triplicate.

In order to estimate the specificity of DNA labelling, 4 test tubes were prepared as described in the previous

paragraphs, except that 2 did not contain [$^3$H]BP until after incubation. These exceptions were labelled "A" and "B," while tubes "C" and "D" contained [$^3$H]BP before incubation. Samples of 200 µl aliquots of these incubated solutions were placed in separate test tubes in the presence of 800 µl of Tris buffer (pH = 7.2). No enzymes were added to tubes "A" and "C"; however, 20 mg of RNAase were added to tubes "B" and "D." These 2 tubes were placed in ice for 10 minutes, and then 20 mg of protease were added. Again the tubes were placed in ice for 10 minutes. All of the solutions were harvested in identical fashion on the MASH II, using the technique described earlier in this section. This experiment was performed in triplicate.

The previous procedure was repeated, except that only protease was employed for digestion. The trapping efficiency of the filter was estimated by passing the first eluate through fresh filters and measuring the amount of radioactivity trapped during the second pass.

## Mouse Tissue

The ethanol extracted pellet that was prepared in the subsection Soluble Versus Insoluble was used for this experiment. The pellet was resuspended by sonification in 2 ml of water. Quadruplicate 50 µl aliquots were diluted with 450 µl of Tris buffer at pH = 7.2. Half of the tubes received 20 mg of RNAase and were allowed to remain at room

temperature for 15 minutes. Next, these same tubes received 40 mg of protease for 10 minutes at room temperature. After the enzymes were added, all of the tubes were placed in ice water. Both the enzyme treated and non-treated tubes were harvested on the Millipore vacuum manifold with presoaked glass fiber filters. The following solutions were used to wash the filters: water, 10% trichloroacetic acid, and methanol. The filters were dried and placed in Scintiverse cocktail, and the radioactivity was determined. Only plasticware was used during this experiment. This procedure was used on all the lungs and liver from both the control and exposed mice. Each tissue was enzyme or non-enzyme treated in duplicate. Testes were not assayed due to low levels of radioactivity in the samples.

## DNA Purification

Lung tissue (2 mg) and salmon sperm DNA (1 mg) were suspended in 2 ml of solution A (see Table I). The tissue was disrupted by sonic pulses (at 50% duty cycle). Solution B was added dropwise until the solution cleared. Next, 2 volumes of solution C were swirled into the tube. The tube was incubated in a 60°C water bath for 15 minutes and swirled at 1 minute intervals. This caused the protein to precipitate. These tubes were centrifuged at 1200 x gravity for 10 minutes. The top organic layer with nucleic acids was pipetted off and transferred to a clean

polyethylene tube. Radioactivity was measured in aliquots of the aqueous layer. After the top layer was cooled in an

TABLE I

STOCK SOLUTIONS PREPARED FOR DNA PURIFICATION

| Solution | Description | Composition |
|---|---|---|
| A* | Homogenization Buffer | 0.320 g Sucrose<br>0.121 g Tris buffer<br>0.504 g Ethylenediamine tetra-acetic acid |
| B* | Isotonic SDS | 0.800 g Sodium chloride<br>1.680 g Ethylenediamine tetra-acetic acid<br>25.000 g Sodium dodecyl sulfate |
| C | Organic Solvent | 24 volumes Chloroform<br>1 volume Isoamyl alcohol |
| D | Ethanol | 95% Ethanol (ice cold) |
| E* | Citrate Buffer (pH = 7.0) | 0.870 g Sodium chloride<br>0.044 g Sodium citrate |

*For each case, a sufficient volume of water was added to produce 100 ml of solution.

ice bath, an equal volume of cold solution D was added and swirled gently together with the top layer. The DNA was sedimented by centrifugation at 1200 x gravity. The supernatant was removed and radioactivity determined. The DNA was redissolved in 1 ml of solution E and an aliquot was taken in which the radioactivity was measured (41).

# CHAPTER III

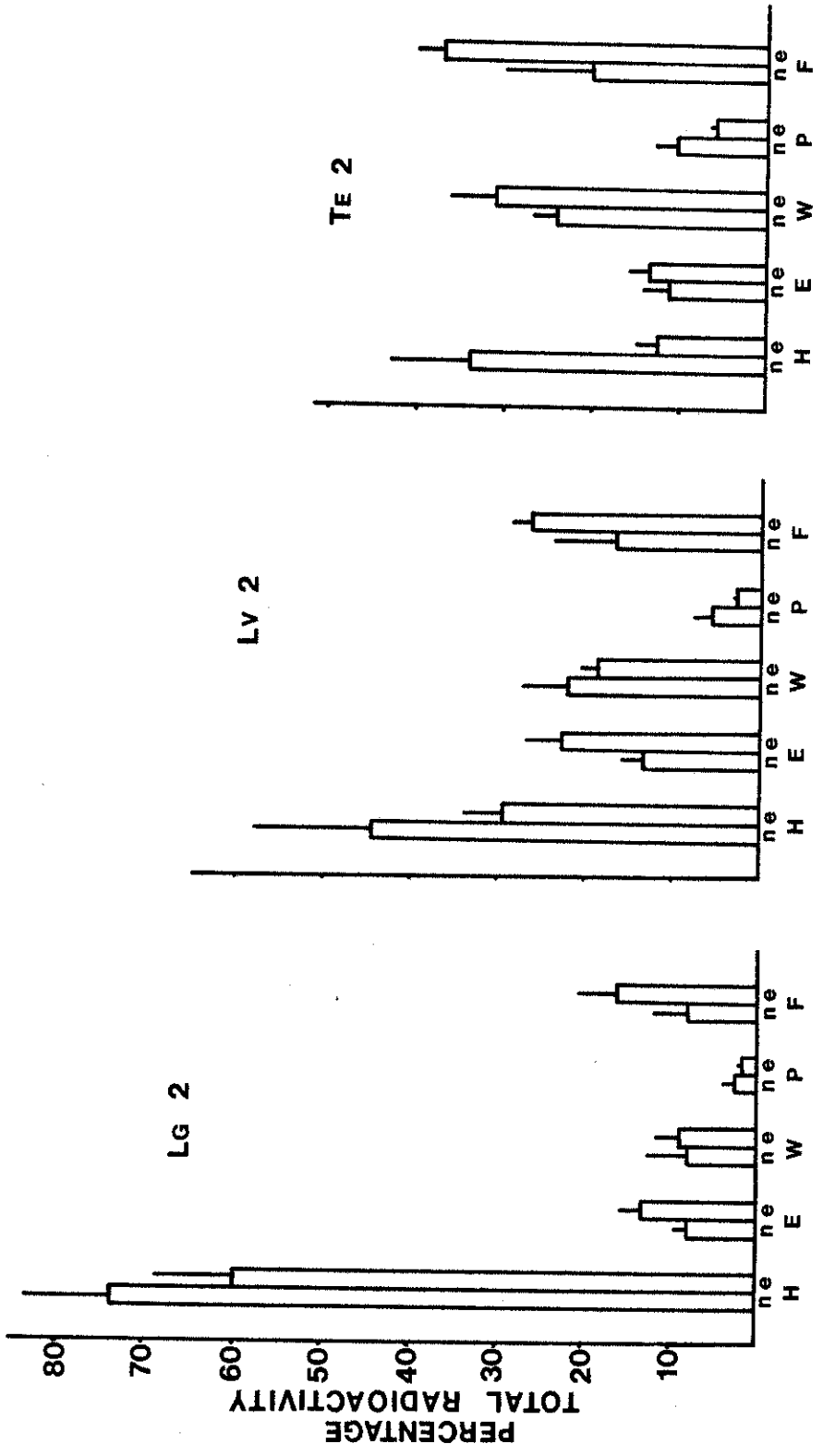
# RESULTS AND DISCUSSION

## Biochemical Studies

### Chromatography of [$^3$H]BP and Metabolites

Metabolism of tritiated benzo(a)pyrene ([$^3$H]BP) occurs in all 3 tissues examined in this study. This notion is supported by the presence of both primary and secondary metabolites in the tissues at the various times after instillation. Figures 4, 5, and 6 present a summary of the recovery of [$^3$H]BP and its metabolites from lungs, liver, and testes. The percentage of total radioactivity in the ethanol extract is expressed on the ordinate. The bars represent the proportional amount of each fraction. Hexane eluted [$^3$H]BP, ethanol eluted hydroxy[$^3$H]BP, water eluted [$^3$H]BP-sulfate, phosphate buffer eluted an unknown material, and formic acid eluted [$^3$H]BP-glucuronide and [$^3$H]BP-glutathione. Figure 4 represents results from the lungs, liver, and testes 2 hours after instillation. In the lungs at 2 hours the majority of radioactivity was from nonmetabolized [$^3$H]BP as identified fluorometrically. In contrast, liver and testes at 2 hours contained more than half of the radioactivity as metabolites, with a substantial degree of

Figure 4: Relative Amounts of Metabolites and Free [$^3$H]BP Found in the Lungs, Liver, and Testes 2 Hours after Instillation. "H" is the radioactivity eluted with 10 ml of hexane, i.e. [$^3$H]BP; "E" is the radioactivity eluted with 15 ml of ethanol, i.e. hydroxy-[$^3$H]BP; "W" is the radioactivity eluted with 15 ml of water, i.e. [$^3$H]BP-sulfate conjugates; "P" is the radioactivity eluted with 15 ml of phosphate buffer; and "F" is the radioactivity eluted with 20 ml of formic acid, i.e. [$^3$H]BP-glucuronide and [$^3$H]BP-glutathione conjugates. The ordinate represents the percentage of total radioactivity in the tissues. Each bar represents the mean of 5 mice, "n" represents nonexposed and "e" represents diesel exhaust exposed. The vertical lines illustrate the standard error of the mean. "Lg" lungs, "Lv" liver, "Te" testes.


PERCENTAGE
TOTAL RADIOACTIVITY
80
70
60
50
40
30
20
10
Lg 2
Lv 2
Te 2
H
E
W
P
F
ne

secondary metabolism to conjugates. The diesel exposed animals appeared to have less free [$^3$H]BP in tissues at 2 hours and this may reflect on induction of the enzymes for primary metabolism of polynuclear aromatic hydrocarbons (PAH). Within 24 hours after instillation, the [$^3$H]BP in these tissues was principally in the form of metabolites, with a substantial degree of conjugation, as shown in Figure 5. At 24 hours, the percentage of total radioactivity due to metabolites of [$^3$H]BP was 65% to 75% in the lungs, 80% to 90% in the liver, and 75% to 80% in the testes. Figure 6 shows that the small amount of [$^3$H]BP found was present mainly as conjugated metabolite by 168 hours after instillation in the liver and testes. The relatively high proportion of unmetabolized [$^3$H]BP in the diesel exposed lungs at 168 hours suggests that a fraction of [$^3$H]BP is adsorbed onto smoke particles and is unavailable to the enzymes. These smoke particles are cleared very slowly from the lungs and may provide a means whereby carcinogenic hydrocarbons may be sequestered for long periods of time.

## Soluble Versus Insoluble

This experiment measured the time course of clearance of label from 3 tissues. They were the lungs (the portal of entry), the liver (the organ which is responsible for most of the metabolism), and the testes (a tissue that receives relatively little blood flow compared to the first

Figure 5: Relative Amounts of Metabolites and Free [$^3$H]BP Found in the Lungs, Liver, and Testes 24 Hours after Instillation. "H" is the radioactivity eluted with 10 ml of hexane, i.e. [$^3$H]BP; "E" is the radioactivity eluted with 15 ml of ethanol, i.e. hydroxy-[$^3$H]BP; "W" is the radioactivity eluted with 15 ml of water, i.e. [$^3$H]BP-sulfate conjugates; "P" is the radioactivity eluted with 15 ml of phosphate buffer; and "F" is the radioactivity eluted with 20 ml of formic acid, i.e. [$^3$H]BP-glucuronide and [$^3$H]BP-glutathione conjugates. The ordinate represents the percentage of total radioactivity in the tissues. Each bar represents the mean of 5 mice, "n" represents nonexposed and "e" represents diesel exhaust exposed. The vertical lines illustrate the standard error of the mean. "Lg" lungs, "Lv" liver, "Te" testes.

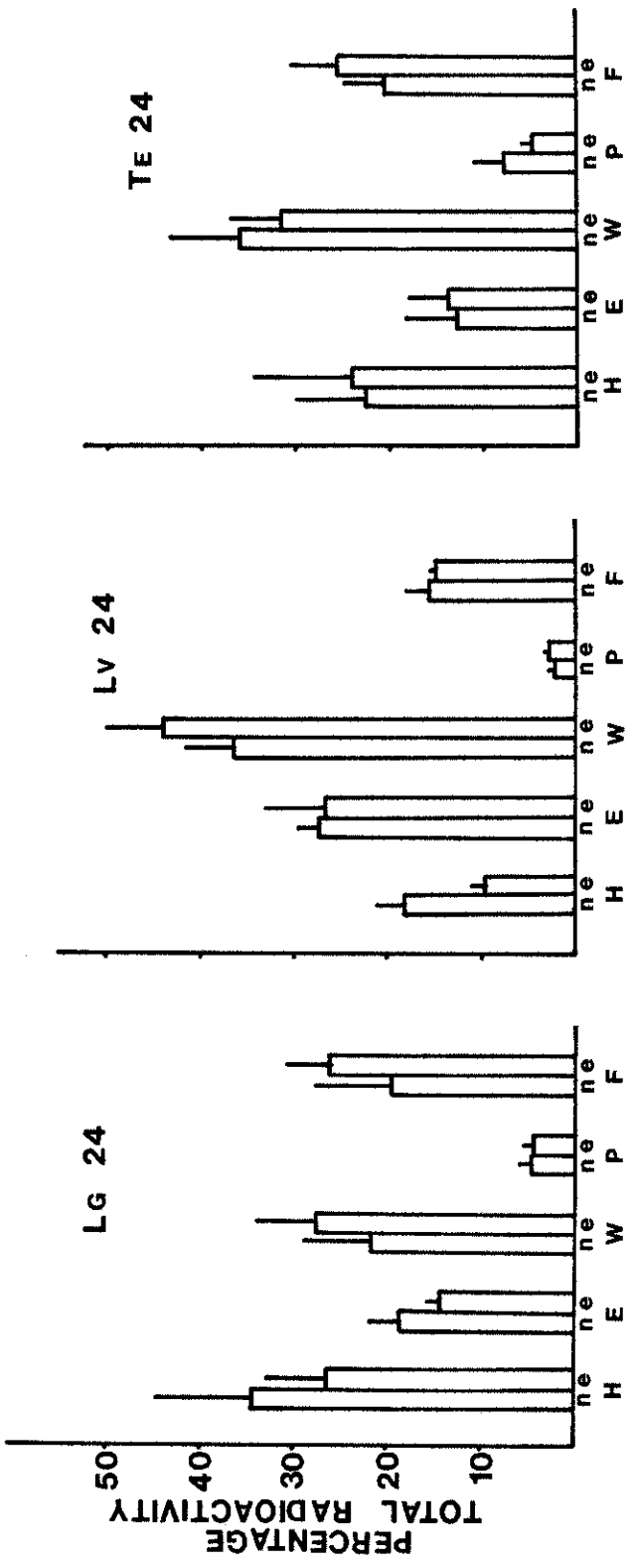
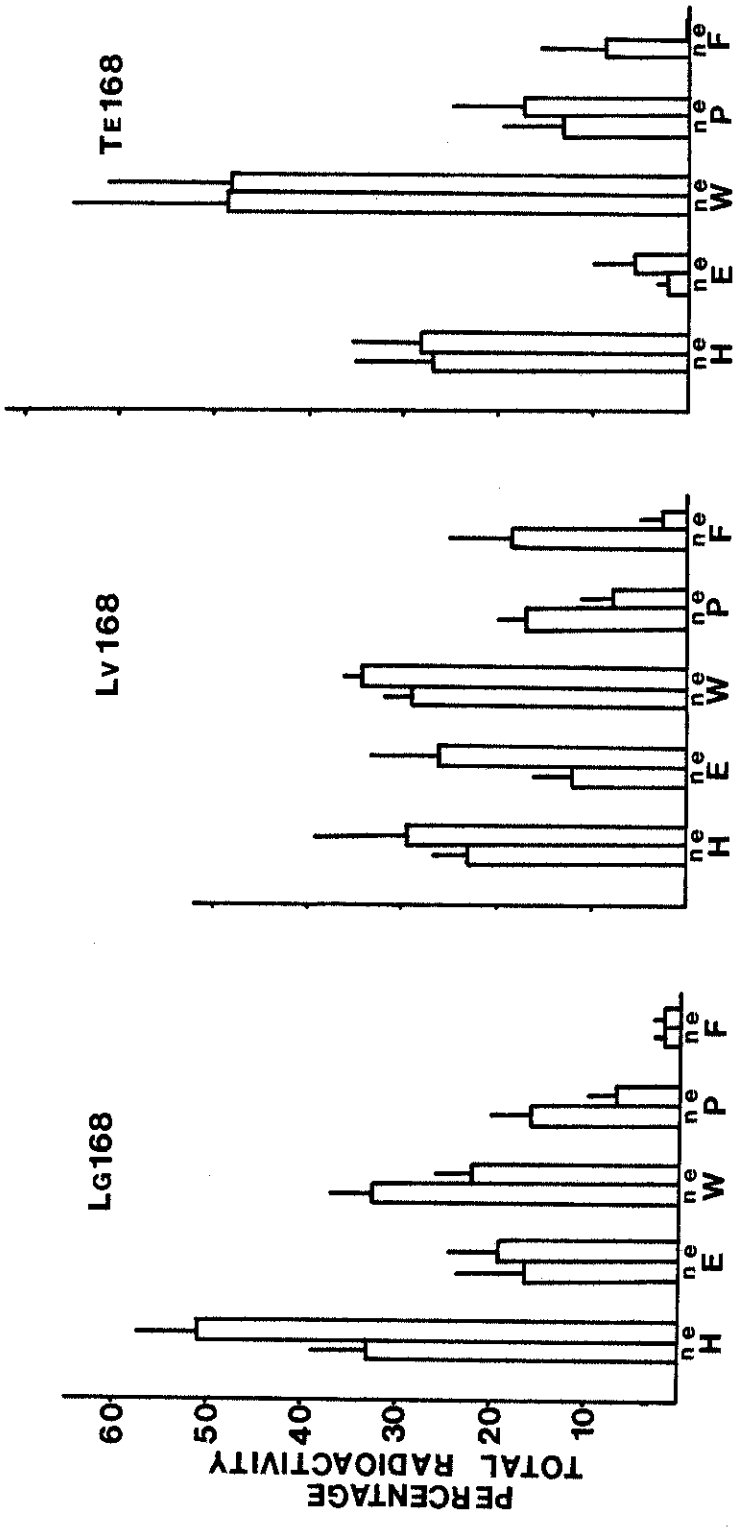

PERCENTAGE
TOTAL RADIOACTIVITY
50
40
30
20
10
LG 24
LV 24
TE 24
H
E
W
P
F
ne

Figure 6: Relative Amounts of Metabolites and Free [$^3$H]BP Found in the Lungs, Liver, and Testes 168 Hours after Instillation. "H" is the radioactivity eluted with 10 ml of hexane, i.e. [$^3$H]BP; "E" is the radioactivity eluted with 15 ml of ethanol, i.e. hydroxy-[$^3$H]BP; "W" is the radioactivity eluted with 15 ml of water, i.e. [$^3$H]BP-sulfate conjugates; "P" is the radioactivity eluted with 15 ml of phosphate buffer; and "F" is the radioactivity eluted with 20 ml of formic acid, i.e. [$^3$H]BP-glucuronide and [$^3$H]BP-glutathione conjugates. The ordinate represents the percentage of total radioactivity in the tissues. Each bar represents the mean of 5 mice, "n" represents nonexposed and "e" represents diesel exhaust exposed. The vertical lines illustrate the standard error of the mean. "Lg" lungs, "Lv" liver, "Te" testes.


PERCENTAGE
TOTAL RADIOACTIVITY
60
50
40
30
20
10
LG168
LV168
TE168
H
E
W
P
F
ne

2 organs, but is significant from the standpoint of genetic considerations).

Figure 7 is a log-log plot of the time course of clearance of [$^3$H]BP from the tissues. In the ethanol extracts

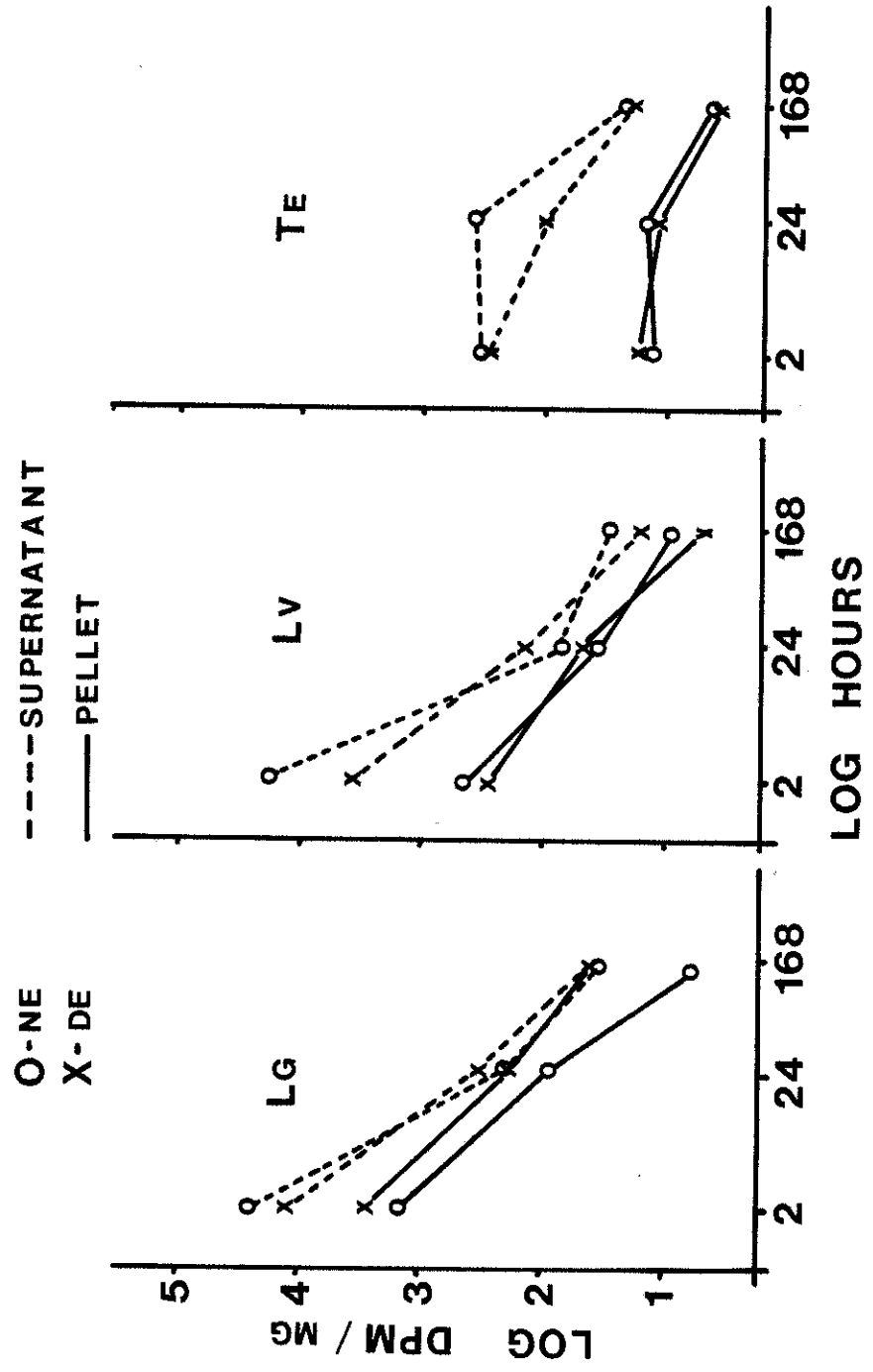
TABLE II

MEANS AND STANDARD ERRORS OF DPM/MG MEASUREMENTS FROM LUNGS, LIVER, AND TESTES

| Time/ Exposure/ Fraction* | Lungs | | Liver | | Testes | |
|---|---|---|---|---|---|---|
| | Mean | Standard Error | Mean | Standard Error | Mean | Standard Error |
| 2/E/P | 2697 | 727 | 290 | 128 | 16 | 18 |
| 2/N/P | 1679 | 710 | 446 | 240 | 14 | 6 |
| 2/E/S | 12564 | 4287 | 3658 | 1859 | 267 | 89 |
| 2/N/S | 29988 | 18138 | 21384 | 17690 | 367 | 135 |
| 24/E/P | 185 | 67 | 50 | 22 | 13 | 2 |
| 24/N/P | 84 | 27 | 35 | 1 | 15 | 9 |
| 24/E/S | 332 | 152 | 140 | 21 | 101 | 61 |
| 24/N/S | 228 | 50 | 73 | 19 | 380 | 253 |
| 168/E/P | 40 | 8 | 5 | 1 | 4 | 2 |
| 168/N/P | 6 | 1 | 9 | 2 | 4 | 1 |
| 168/E/S | 46 | 8 | 16 | 2 | 19 | 6 |
| 168/N/S | 38 | 12 | 30 | 13 | 20 | 7 |

*"2"--2 hours after instillation, "24"--24 hours after instillation, "168"--168 hours after instillation, "E"--exposed to diesel exhaust, "N"--not exposed to diesel exhaust, "P"--pellet fraction, "S"--supernatant fraction.

Figure 7: Disappearance of Radioactivity after Intratracheal Instillation of [$^3$H]BP. Each point represents the mean of 5 mice. Radioactivity is expressed as dpm/mg of wet tissue. "NE" nonexposed, "DE" diesel exhaust exposed, "Lg" lungs, "Lv" liver, and "Te" testes.


O-NE
X-DE
----SUPERNATANT
——PELLET
LG
LV
TE
LOG DPM / MG
5
4
3
2
1
2
24
168
LOG HOURS

from the tissue, there was no significant difference in the clearance of soluble metabolites by either the nonexposed or diesel exposed animals from the lungs, liver, or testes. The values for the means and standard errors of the measurements of the disintegrations per minute per milligram (dpm/mg) of wet tissue weight are shown in Table II.

The amount of radioactivity remaining in the ethanol insoluble pellet is also shown in Figure 7. These insoluble fractions probably contain [$^3$H]BP bound to cellular components. Both groups were capable of clearing the bound [$^3$H]BP, but there was a significantly higher amount of residual [$^3$H]BP in the lungs of diesel exposed mice 168 hours after instillation.

## Tissue Bound Benzo(a)pyrene

### Tissue Binding Studies

These experiments evaluate the nature of [$^3$H]BP binding to tissue components and suggest a possible cause of the delay in clearance from the lungs of the exposed mice. This is important since the lungs are a target tissue for many airborne carcinogenic hydrocarbons (42, 43, 44).

Lest all the mouse tissues be consumed in development of the assay, the initial experiments employed other tissues which were readily available. Cultured human lymphocytes were employed since the lymphocytes were the cell type for which the Multiple Automated Sample Harvester (MASH) II was

designed (38). After confirming that these intact cells could be assayed by this technique, the procedure was adapted for analysis of the [$^3$H]BP bound to tissue homogenates from the mice. Figure 8 shows the effects of different wash procedures on retention of [$^3$H]BP in human lymphocytes. The unstippled bars represent the cells which were not sonically disrupted. Radioactivity in the intact cells was greater than that in the sonically disrupted cells. These results may be due to trapping of unbound [$^3$H]BP within the intact cells, thus the hydrocarbon was unable to pass through the filter. The water washed cells retained more radioactivity than the water plus methanol washed cells, suggesting that methanol solubilized some of the adsorbed non-covalently bound metabolites.

## DNA-Bound [$^3$H]BP

In an effort to estimate the relative amount of [$^3$H]BP binding to protein and RNA, varied amounts of each individual enzyme were added to tubes containing the bound DNA. Figures 9, 10, and 11 compare the amount of radioactivity retained by the filter on the ordinate scale, with varying amounts of enzymes represented on the abscissas. Figures 9 and 10 indicate at approximately 5 µg that the enzymes are no longer a limiting factor in solubilizing RNA and protein. Figure 11 shows the failure of DNAase treatment to alter the trapping of radioactivity on the filter. This suggests the

Figure 8: Effects of Different Wash Procedures on Retention of [$^3$H]BP in Human Lymphocytes. The bars represent the amount of radioactivity found with the various washings after a 24 hour incubation period. The unstippled bars were not sonically disrupted and were either not washed or washed with water or water followed by methanol (ME OH). The sonically disrupted cells (stippled bars) were washed with water or water followed by methanol. The vertical lines represent standard error.

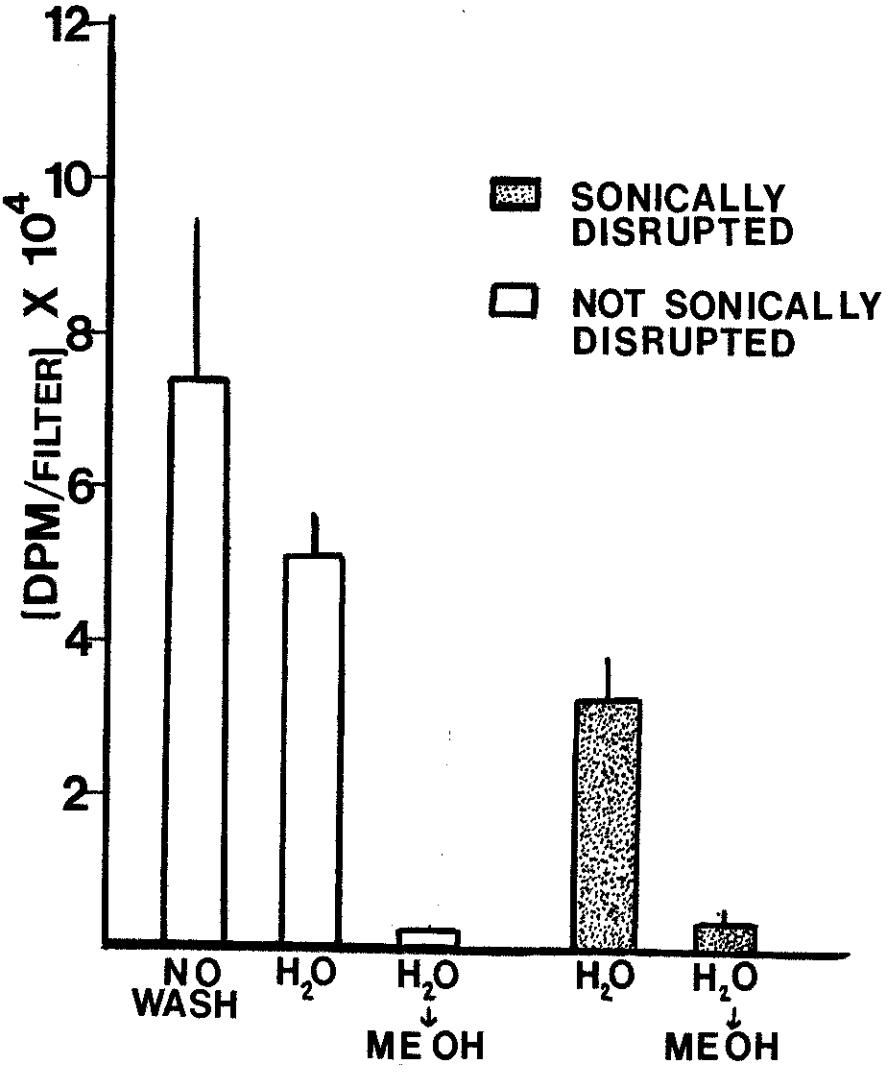

12
10
8
6
4
2
[DPM/FILTER] X $10^4$
SONICALLY DISRUPTED
NOT SONICALLY DISRUPTED
NO WASH
$H_2O$
$H_2O$
MEOH
$H_2O$
$H_2O$
MEOH

Figure 9: The Effects of Protease on [$^3$H]BP Retention by Filters. Various amounts of protease were added to 800 µl Tris buffer and 200 µl of the following 120 minute incubated solution: 3.5 ml of Tris buffer (0.01 M), 0.25 mg of mouse microsomes (20 mg/ml), 0.05 ml NADPH (20 mg/ml), 0.5 ml DNA (10 mg/ml), and 125 µCi [$^3$H]BP (0.25 ml). The abscissa scale shows the different amounts of protease. Each point represents the mean of 3 experiments. The vertical lines represent the standard error.

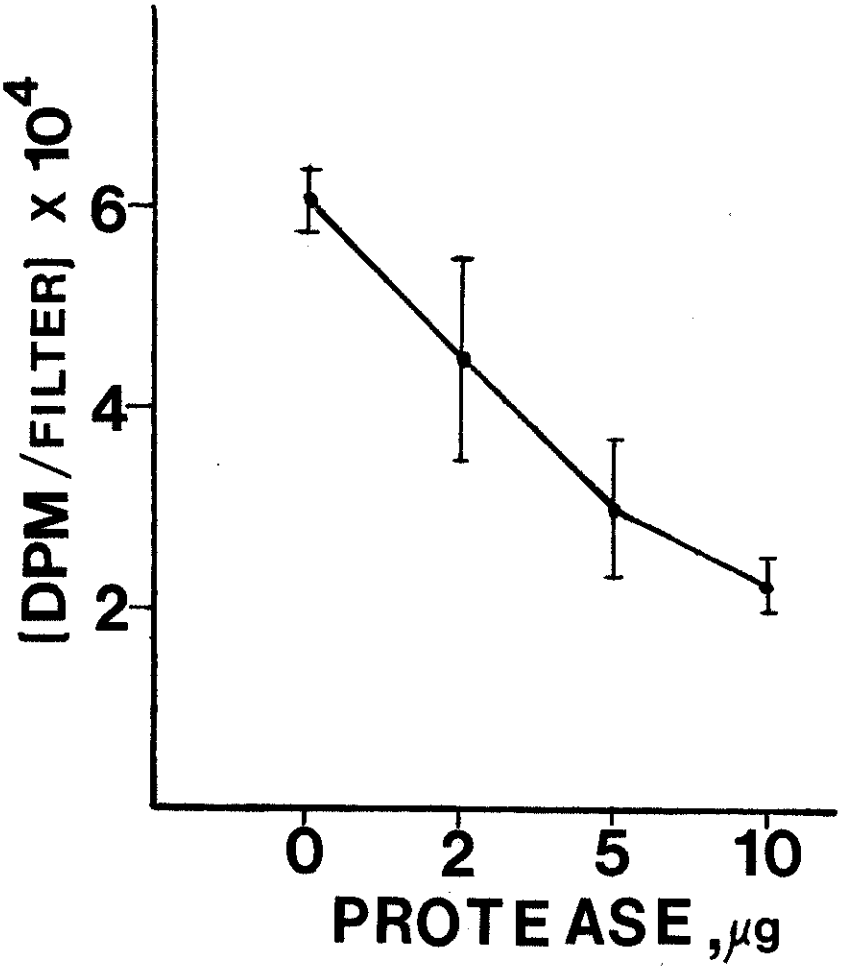

[DPM/FILTER] X $10^4$
6
4
2
0
2
5
10
PROTEASE, μg

Figure 10: The Effects of RNAase on [$^3$H]BP Retention by Filters. Various amounts of RNAase were added to 800 µl Tris buffer and 200 µl of the following 120 minute incubated solution: 3.5 ml of Tris buffer (0.01 M), 0.25 mg of mouse microsomes (20 mg/ml), 0.05 ml NADPH (20 mg/ml), 0.5 ml DNA (10 mg/ml), and 125 µCi [$^3$H]BP (0.25 ml). The abscissa scale shows the different amounts of RNAase. Each point represents the mean of 3 experiments. The vertical lines represent the standard error.

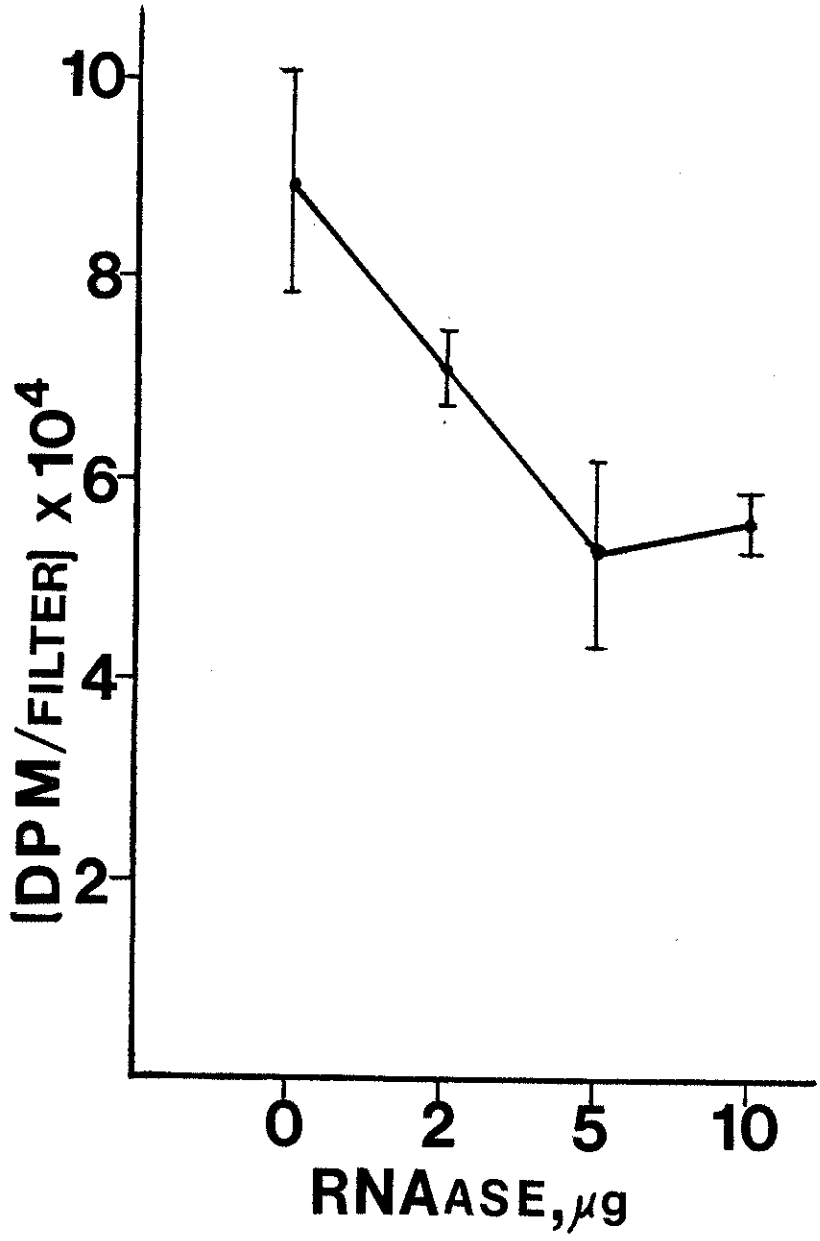
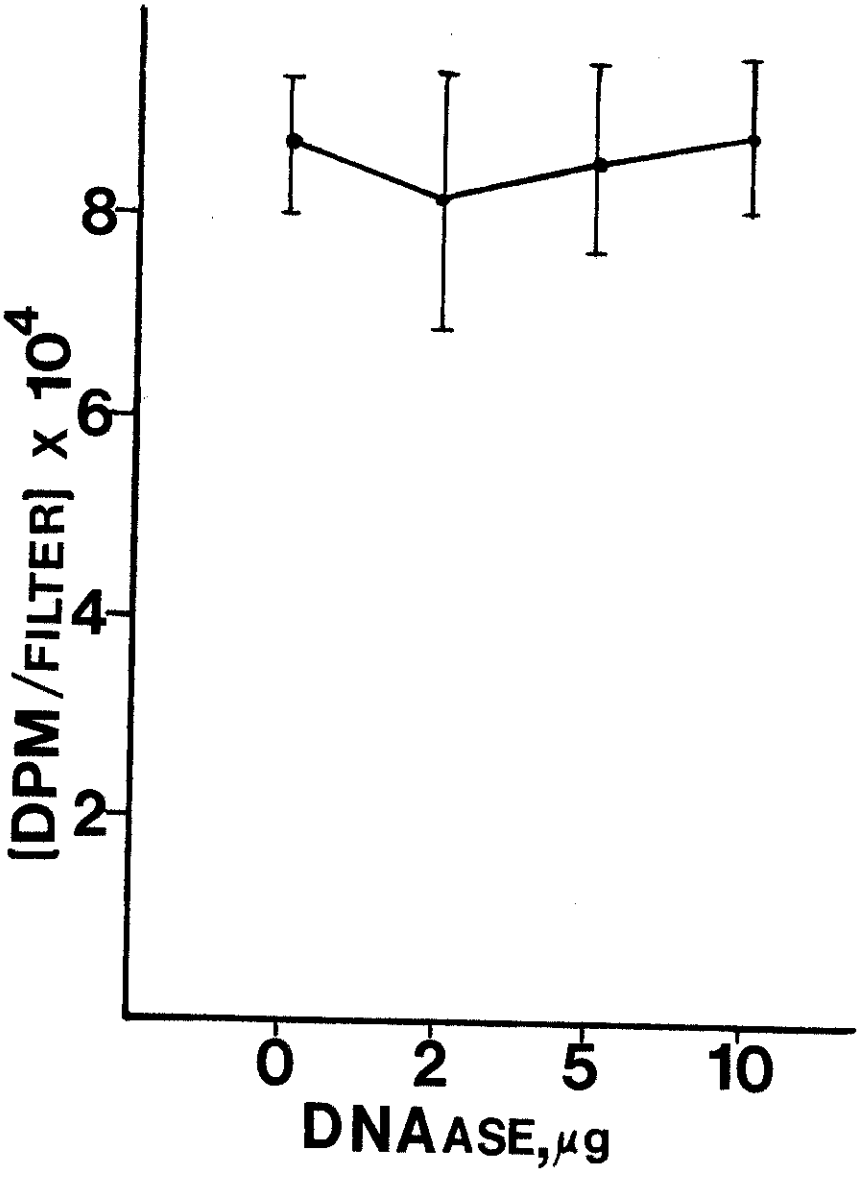

10
8
6
4
2
[DPM/FILTER] X 10^4
0
2
5
10
RNAASE, μg

Figure 11: The Effects of DNAase on [$^3$H]BP Retention by Filters. Various amounts of DNAase were added to 800 µl Tris buffer and 200 µl of the following 120 minute incubated solution: 3.5 ml of Tris buffer (0.01 M), 0.25 mg of mouse microsomes (20 mg/ml), 0.05 ml NADPH (20 mg/ml), 0.5 ml DNA (10 mg/ml), and 125 µCi [$^3$H]BP (0.25 ml). The abscissa scale shows the different amounts of DNAase. Each point represents the mean of 3 experiments. The vertical lines represent the standard error.

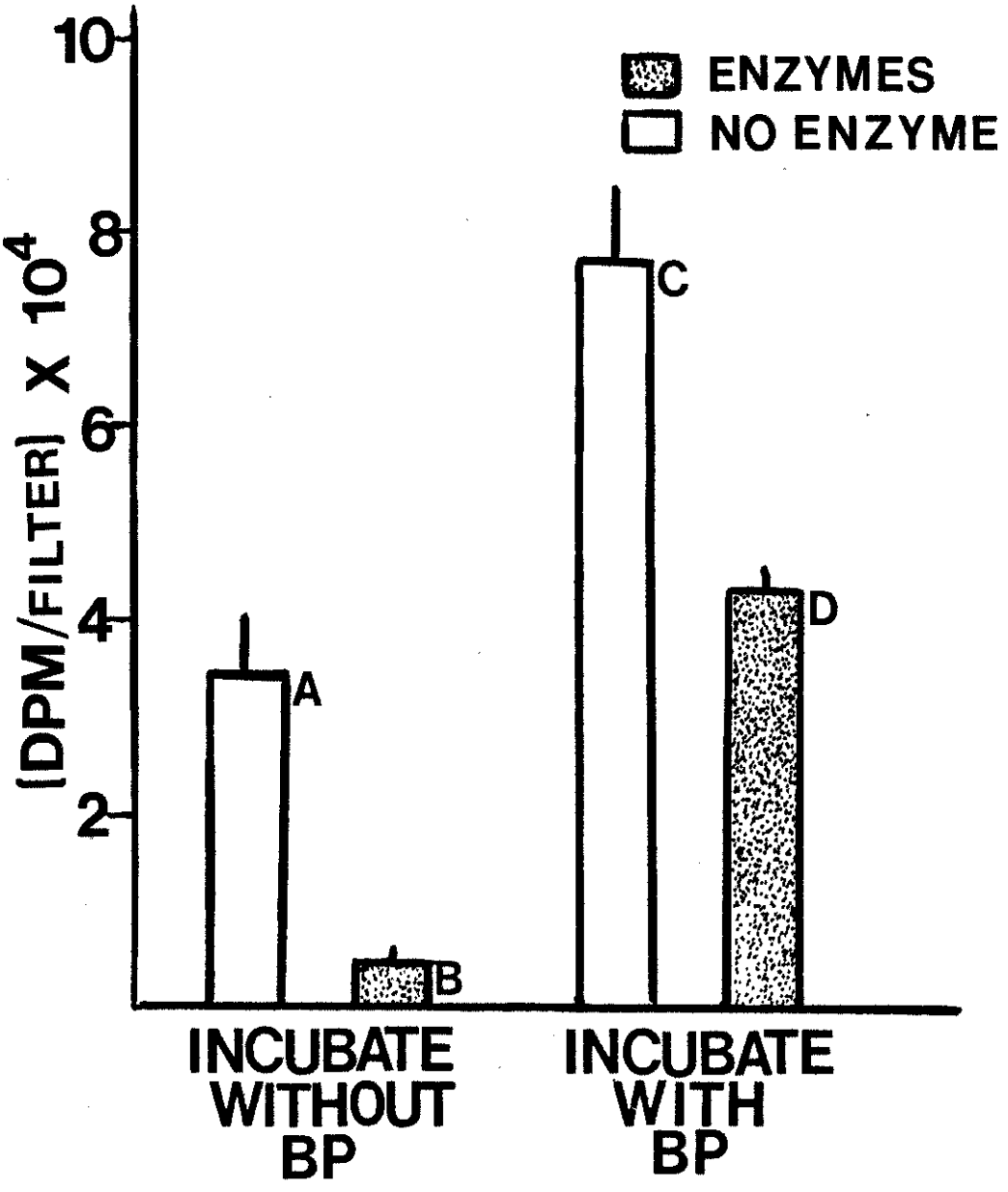

[DPM/FILTER] x 10^4
8
6
4
2
0
2
5
10
DNAASE, μg

possibility that the nucleotides adhere to glass regardless of whether they exist as large polymers, or oligomers, or monomer nucleotide/adducts.

In order to estimate the relative amounts of [$^3$H]BP bound to DNA, 4 tubes containing the following constituents were prepared and incubated for 120 minutes: Tris buffer, mouse microsomes, NADPH, DNA, and [$^3$H]BP (all in quantities specified in the previous chapter). [$^3$H]BP was added to 2 tubes designated "A" and "B" following incubation, and was added to 2 tubes designated "C" and "D" prior to incubation. After incubation, tubes "B" and "D" were further treated with RNAase and protease to solubilize the RNA and protein, so that these cellular components could pass through glass fiber filters. Figure 12 illustrates the results of this experiment, showing the dpm/filter associated with each of the 4 tubes. Bar "B" reflects nonspecific binding to DNA (4000 dpm/filter), while the difference in heights of bars "A" and "B" (30,000 dpm/filter) reflects nonspecific binding to protein and RNA. The difference in heights of bars "C" and "A" (42,000 dpm/filter) reflects total binding due to enzymatic activation of [$^3$H]BP. Finally, the difference in heights of bars "D" and "B" (38,000 dpm/filter) represents enzymatic binding to DNA.

To estimate the DNA trapping efficiency of the glass fiber filters, the eluate was passed through clean filters for a second time. It was found that the average amount of

Figure 12: Enzyme Catalyzed Binding of [$^3$H]BP to DNA. A solution of 3.5 ml Tris buffer (0.01 M), 0.25 mg of mouse microsomes (20 mg/ml), 0.05 ml NADPH (20 mg/ml), 0.5 ml DNA (10 mg/ml), and 125 μCi [$^3$H]BP (0.25 ml) was incubated for 120 minutes (bars "C" and "D"). An identical solution was incubated at the same time, except the [$^3$H]BP was added after the incubation (bars "A" and "B"). The enzymes, RNAase and protease, were added to tubes "B" and "D." The bars represent the mean of 3 experiments. The vertical lines represent standard error.


10
8
6
4
2
[DPM/FILTER] X $10^4$
ENZYMES
NO ENZYME
A
B
C
D
INCUBATE WITHOUT BP
INCUBATE WITH BP

radioactivity collected on the second filter was 2.5 ± 1.0% of the radioactivity collected on the first filter. Therefore, the trapping efficiency of the glass filter was approximately 97%.

## Mouse Tissue

Using the techniques and experience acquired in the previous experiments, the liver and lungs of the mice were further analyzed to discover if DNA was actually bound to

TABLE III

MEANS, STANDARD ERRORS, AND P VALUES OF DPM/MG MEASUREMENTS FROM LIVER

| Time After Instillation, Hours | Diesel Exposed | Enzyme Treated | Mean, dpm/mg | Standard Error | P Value |
|---|---|---|---|---|---|
| 2 | Yes | No | 51 | 18 | >0.200 |
| 2 | No | No | 111 | 49 | |
| 2 | Yes | Yes | 26 | 8 | >0.300 |
| 2 | No | Yes | 43 | 15 | |
| 24 | Yes | No | 13 | 3 | >0.900 |
| 24 | No | No | 12 | 2 | |
| 24 | Yes | Yes | 9 | 2 | >0.500 |
| 24 | No | Yes | 10 | 2 | |
| 168 | Yes | No | 3 | 1 | >0.500 |
| 168 | No | No | 4 | 1 | |
| 168 | Yes | Yes | 3 | 0.4 | >0.100 |
| 168 | No | Yes | 4 | 1 | |

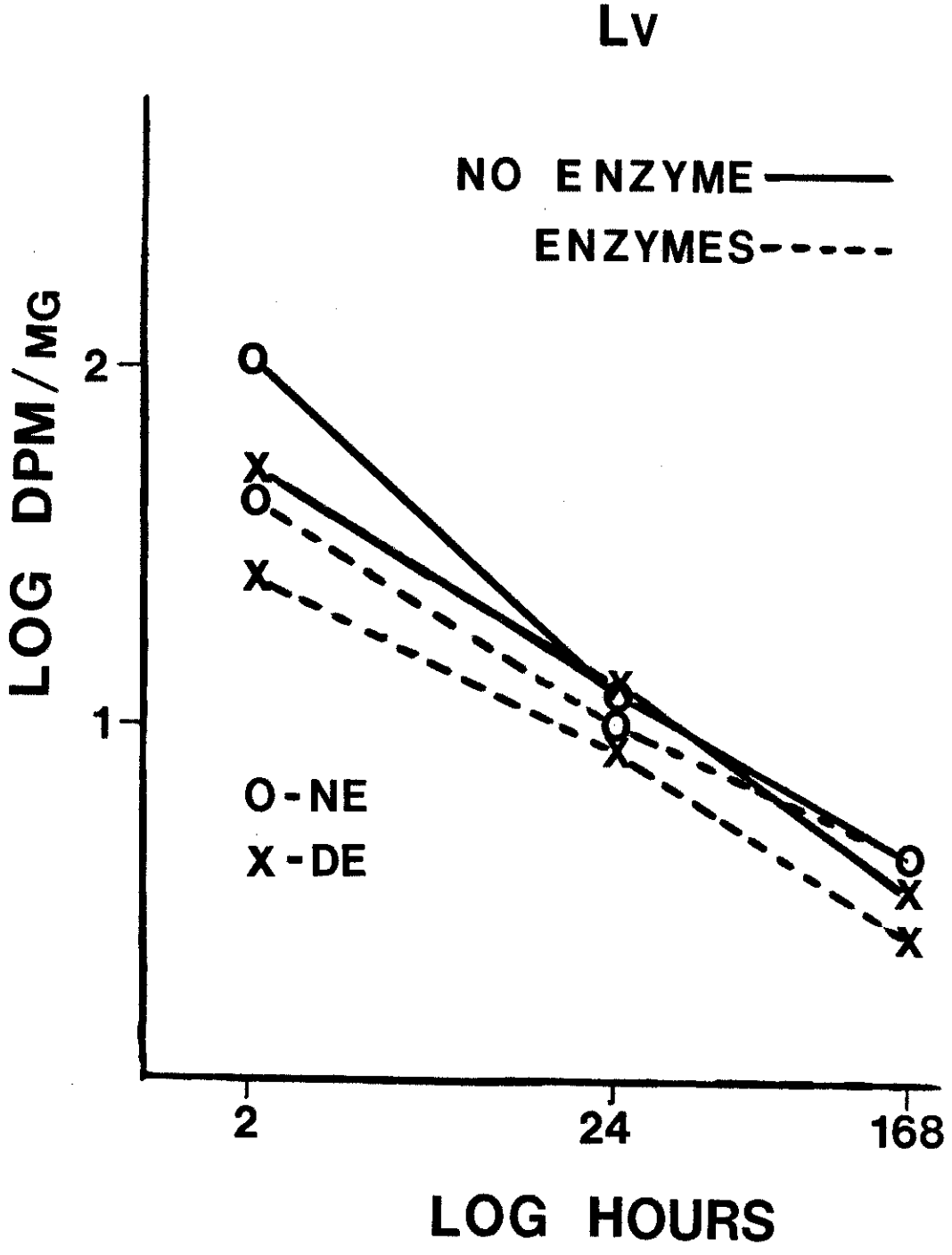
[$^3$H]BP. The Millipore vacuum manifold was utilized in place of the MASH II. Their functions are the same, but the Millipore vacuum manifold's filter area per sample is larger. Figure 13 shows the liver clearance. The time course is on the abscissa and the dpm/mg tissue is on the ordinate. No significant difference was found in the clearance of the bound [$^3$H]BP by the exposed versus nonexposed animals (see Table III). However, in the insoluble lung tissue, a significant difference exists between exposed and

TABLE IV

MEANS, STANDARD ERRORS, AND P VALUES OF DPM/MG MEASUREMENTS FROM LUNGS

| Time After Instillation, Hours | Diesel Exposed | Enzyme Treated | Mean, dpm/mg | Standard Error | P Value |
|---|---|---|---|---|---|
| 2 | Yes | No | 761 | 159 | <0.010 |
| 2 | No | No | 187 | 46 | |
| 2 | Yes | Yes | 766 | 164 | <0.001 |
| 2 | No | Yes | 97 | 23 | |
| 24 | Yes | No | 92 | 22 | <0.010 |
| 24 | No | No | 22 | 3 | |
| 24 | Yes | Yes | 88 | 19 | <0.010 |
| 24 | No | Yes | 19 | 3 | |
| 168 | Yes | No | 35 | 5 | <0.001 |
| 168 | No | No | 6 | 1 | |
| 168 | Yes | Yes | 39 | 5 | <0.010 |
| 168 | No | Yes | 14 | 5 | |

Figure 13: Disappearance of Radioactivity from the Liver after Intratracheal Instillation of [$^3$H]BP. The fractions were either treated with RNAase and protease or not treated with these enzymes. Each point represents the mean of 5 mice. Radioactivity is expressed as dpm/mg of wet liver tissue. "NE" nonexposed, "DE" diesel exhaust exposed.


LV
NO ENZYME
ENZYMES
LOG DPM/MG
2
1
O-NE
X-DE
2
24
168
LOG HOURS

nonexposed animals, as shown in Figure 14 and Table IV. Diesel exposed mice have a significantly higher amount of [$^3$H]BP in the lungs at all 3 time points following intratracheal instillation. These liver and lung data agree with the earlier findings gathered by the less precise method as described in the section entitled Soluble Versus Insoluble and illustrated in Figure 7.

## DNA Purification

The analysis of data in Figures 7 and 14 indicate that the radioactivity was more slowly cleared from smoke exposed animals. The lungs from the exposed mice were gray to black in color, indicating that smoke particles were present even at 168 hours after removal of diesel exhaust. The Marmur method (41) was employed to effect a complete

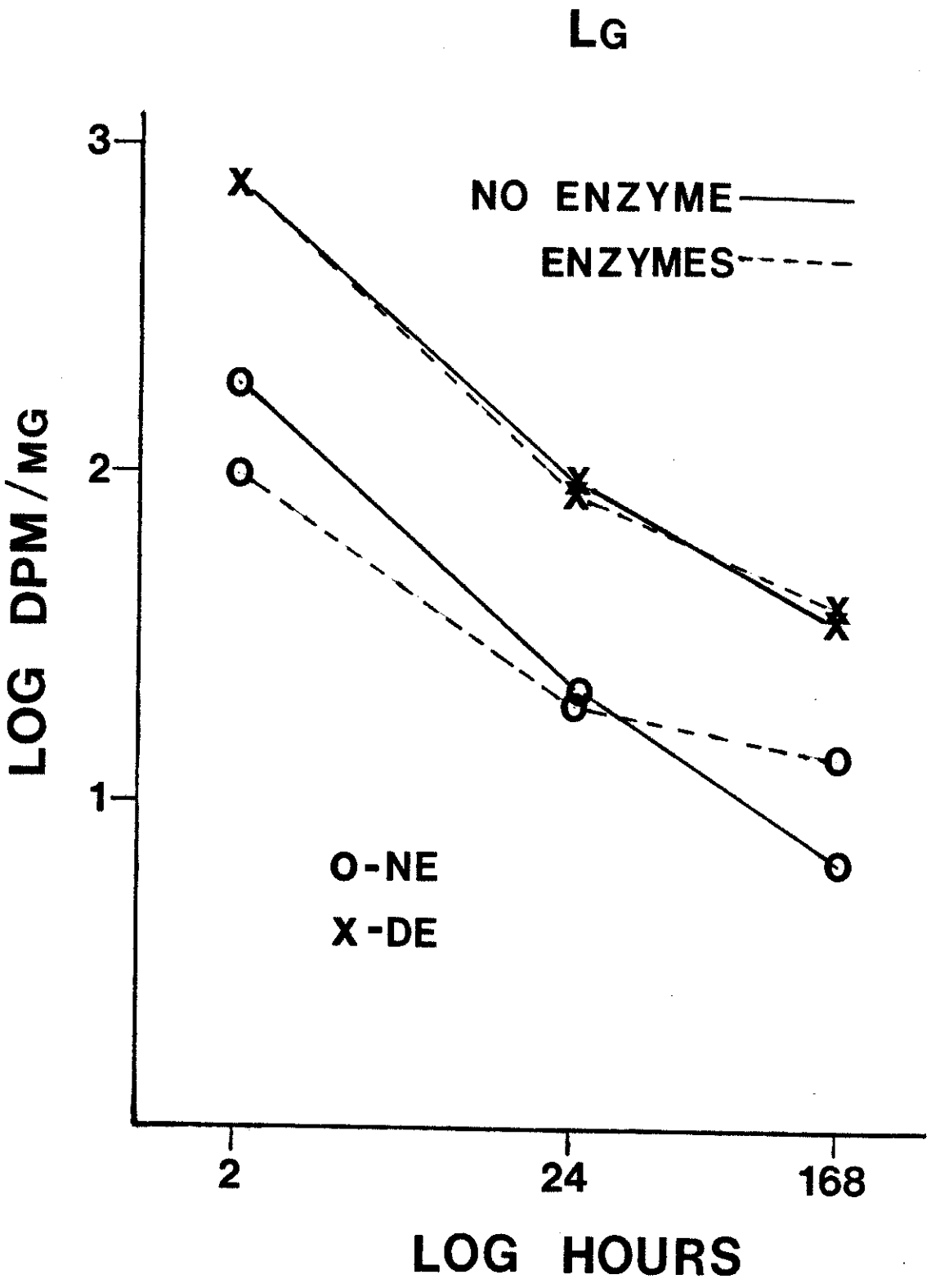
TABLE V

LUNG RADIOACTIVITY

| Diesel Exposed | Total Tissue dpm | DNA dpm |
|---|---|---|
| No | $1.7 \times 10^6$ | $4.8 \times 10^3$ |
| No | $3.0 \times 10^5$ | $4.3 \times 10^3$ |
| Yes | $2.2 \times 10^5$ | $5.3 \times 10^3$ |
| Yes | $3.1 \times 10^5$ | $5.3 \times 10^3$ |

separation of the DNA from the smoke particles. The DNA radioactivity versus total radioactivity is compared in Table V. Although total radioactivity was quite variable,

Figure 14: Disappearance of Radioactivity from the Lungs after Intratracheal Instillation of [$^3$H]BP. The fractions were either treated with RNAase and protease or not treated with these enzymes. Each point represents the mean of 5 mice. Radioactivity is expressed as dpm/mg of wet lung tissue. "NE" nonexposed, "DE" diesel exhaust exposed.


LG
3
2
1
LOG DPM/MG
NO ENZYME
ENZYMES
O-NE
X-DE
2
24
168
LOG HOURS

the DNA associated radioactivity was about the same in control and exposed mice.

# CHAPTER IV

## CONCLUSION

This study evaluated the fate of tritiated benzo(a)pyrene ([$^3$H]BP) given intratracheally to mice which were either exposed or not exposed to diesel exhaust. The time course of clearance and metabolite formation of [$^3$H]BP was analyzed in 3 tissues: lungs, liver, and testes. Within 2 hours after instillation 25% to 40% of the [$^3$H]BP in the lungs was found to exist as metabolites. In liver 55% to 70% existed as metabolites, and in the testes 65% to 87% existed as metabolites. A substantial part of the metabolites were conjugates of sulfate, glucuronic acid, and glutathione. By 24 hours after instillation, the percentage of total radioactivity due to metabolites of [$^3$H]BP was 65% to 75% in the lungs, 80% to 90% in the liver, and 75% to 80% in the testes. By 168 hours after instillation, the total amount of [$^3$H]BP and metabolites had decreased substantially. In all 3 tissues the metabolites were mainly found as conjugates, a form readily excretable.

Exposure to the diesel exhaust had little effect on the capacity to conjugate the [$^3$H]BP metabolites, but there was a reduction in clearance of [$^3$H]BP from the lungs. The carbon and other components in the diesel exhaust possibly

absorb [$^3$H]BP and other hydrocarbons and retard the otherwise rapid clearance of carcinogens from the lungs. There is no apparent hindrance of the DNA repair mechanism in lung tissue by diesel exhaust, but the longer sojourn of [$^3$H]BP may have practical importance in promoting lung carcinogenesis.

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