To the Graduate Council:

I am submitting herewith a dissertation written by Evelyn A. Settle entitled "An Investigation of the Relationship Between Sulfur Nutritional Status and Lung Neoplasms Induced by Benzo(a)pyrene in Rats." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Home Economics.

John T. Smith

John T. Smith, Major Professor

We have read this dissertation
and recommend its acceptance:

Marjorie P. Penfield

Frances E. Andrews

Gordon Carlson

Accepted for the Council:

L. Evans Roth

Vice Chancellor
Graduate Studies and Research

Thesis
76
.S447
cop. 2

AN INVESTIGATION OF THE RELATIONSHIP BETWEEN SULFUR NUTRITIONAL STATUS AND LUNG NEOPLASMS INDUCED BY BENZO(A)PYRENE IN RATS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Evelyn A. Settle

December 1977

1347946

ACKNOWLEDGMENTS

The author wishes to express her sincere appreciation to the many people who helped direct her during her years in graduate school. A special thanks is extended to:

Dr. John T. Smith who provided guidance, assistance, understanding, and encouragement during the planning and execution of this project;

Dr. Frances Andrews, Dr. Marjorie Penfield, and Dr. J. G. Carlson who provided assistance and suggestions during the preparation of this manuscript;

her parents and grandmother who were a source of never-ending love, understanding, and support;

graduate students and friends who shared knowledge, ideas, concern, and companionship; especially Michelle Laborde, Dale Terry, Chris Anderson, Raymond McCord, Sharon Patton, and Jerry Lehman.

The author is grateful to the Department of Food Science, Nutrition, and Food Systems Administration for the financial support she received through a National Institutes of Health Fellowship, a General Foods Fund Fellowship, and a Graduate Teaching Assistantship.

ABSTRACT

In order to determine if sulfur nutritional status was related to lung lesions produced by benzo(a)pyrene intratracheal instillations, rats were fed diets with the following sulfur contents: 0.0002% inorganic sulfate (Diet A), 0.42% inorganic sulfate (Diet B), 0.0002% inorganic sulfate plus 0.5% cysteine (Diet C), 0.02% inorganic sulfate plus 0.5% cysteine (Diet D), and 0.42% inorganic sulfate plus 0.5% cysteine (Diet E). One week after 10 weekly instillations of 3 mg benzo(a)pyrene-3 mg ferric oxide in 0.2 ml 0.9% NaCl, the animals were sacrificed; the lungs removed and cleared for lesion counting; and the lung and liver homogenized for glutathione measures. In the first experiment, the highest number of lesions occurred in animals consuming Diet B, intermediate for Diet D animals, and lowest for Diet A animals. There was a significant ($P \leq 0.05$) difference among the mean lesion counts. In the second experiment, animals consuming Diet E had lesion counts significantly ($P < 0.05$) lower than Diet B animals. Diet E animals also had a lower number of lesions than animals consuming Diet C or D. Availability of cysteine for glutathione biosynthesis is suggested as an explanation for the observed differences in lesion counts.

Liver glutathione levels showed no significant differences due to diet. Lung glutathione levels of animals consuming Diet B were significantly ($P < 0.05$) lower than Diet D levels. After addition of 0.5% cysteine

to the diets no differences in lung glutathione levels were noted. In a group of rats fed Diet D but untreated with benzo(a)pyrene, a significantly ($P<0.05$) higher lung glutathione level was found compared to treated rats fed Diet D. These results indicated utilization of glutathione for detoxication of benzo(a)pyrene. Glutathione biosynthesis seems to explain the susceptability of rats to benzo(a)pyrene induced lesions for all animals except those fed Diet E. Dietary alteration of enzyme activity is proposed as an explanation of the latter finding.

TABLE OF CONTENTS

CHAPTER		PAGE
I.	INTRODUCTION	1
II.	REVIEW OF LITERATURE	4
	Sulfur Nutrition	4
	Glutathione	7
	Experimental Lung Cancer	12
	Benzo(a)pyrene	16
III.	EXPERIMENTAL PROCEDURE	27
	General Plan	27
	Intratracheal Instillation of Benzo(a)pyrene	31
	Counting of Lesions	33
	Determination of Tissue Glutathione	35
	Statistical Analysis	40
IV.	RESULTS	41
V.	DISCUSSION	52
VI.	SUMMARY	59
LITERATURE CITED		61
VITA		71

LIST OF TABLES

TABLE		PAGE
1.	Composition of Basal Diet	29
2.	Additions to Basal Dietary Mixture	30
3.	Number of Lesions Counted on Lungs of Rats After 10 Intratracheal Instillations of 3 mg Benzo(a)pyrene	42
4.	Comparison of the Number of Lesions Counted on Lungs of Rats After 10 Intratracheal Instillations of 3 mg Benzo(a)pyrene to Two Different Diet Groups	44
5.	Lesion Counts for Lungs Minus the Left Lobe After 10 Intratracheal Instillations of 3 mg Benzo(a)pyrene	46
6.	Determination of Glutathione Tissue Levels in Rat Liver	47
7.	Determinations of Glutathione Tissue Levels in Rat Lungs	49
8.	Determination of Glutathione Tissue Levels in Rat Lungs After Addition of Cysteine to the Diet	50

CHAPTER I

INTRODUCTION

Cancer is the number two killer of humans in the United States today (1). Malignant neoplasm of the trachea, bronchus and lung was listed as the cause of death for 74,933 people in 1973 (2). The death rate from lung cancer has risen from about 2.5 per 100,000 population in 1930 (1) to 32.7 per 100,000 population in 1969 (3). This increase in lung cancer incidence has been attributed to cigarette smoking and air pollution (1). Automobile exhaust gases, tobacco smoke, and other air pollutants all contain benzo(a)pyrene, a known carcinogen (4).

Because of the inherent problems of using human subjects to study the genesis and incidence of lung cancer, animal models have been used to study the different aspects of the disease (5). Bronchogenic cancer similar to that seen in humans has been produced in rats by intratracheal instillations of benzo(a)pyrene attached to a carrier dust (4,6,7). These studies have shown that rats are an acceptable model for the study of inhalation carcinogenesis.

The mechanism by which benzo(a)pyrene produces cancer is not yet completely elucidated. There is evidence that the compound is activated by the mixed-function oxidase system (8,9). This activated form then may induce cellular changes by combining with DNA, RNA, or proteins or it may be detoxified by conjugation with glutathione (10,11,12,13).

Availability of glutathione and the level of glutathione S-transferase in the target tissue could be crucial to detoxication of the activated benzo(a)pyrene. Administration of polycyclic hydrocarbons like benzo-(a)pyrene has been shown to induce both the mixed-function oxidase and glutathione S-transferase enzymes (12). The presence and inducibility of these enzymes has been demonstrated in respiratory tract tissue from rats, hamsters, and humans (13).

In numerous studies, changes in the nutrition of test animals resulted in alterations in rates of tumor and/or cancer production. Whether or not the alterations in diet or the exposure to carcinogens resulted in the changes observed has not been resolved, although in many studies diet has been shown to be a significant factor (14).

Studies in this laboratory have shown rats require both inorganic (0.02% of the diet) and organic sulfur (15,16). Evidence has been gathered to indicate that for glutathione synthesis to proceed at an optimum rate cysteine is required in the diet of a rat (0.4% of cysteine in a fibrin based diet has been suggested).[1,2] This requirement is in addition to the established methionine requirement (the values vary from 0.26-0.67 depending on literature source) (17). Rats fed diets adequate in inorganic

---

[1]Smith, J. T. (1977) Manuscript in preparation.

[2]Carruth, K. B. (1975) Model investigations of improved amino acid mixtures for total parenteral nutrition. Unpublished PhD dissertation. The University of Tennessee, Knoxville.

sulfate and methionine had lower liver and kidney glutathione levels than rats fed the same level of inorganic sulfate and the molar equivalent of cysteine.[3] If a methyl demand is created by including cod liver oil in the diet, methionine leaves the remethylation pathway as homocysteine and enters the pathway leading to cysteine biosynthesis.[4] Cysteine then is available for glutathione biosynthesis.

The present study was designed to investigate the relationship of sulfur nutritional status to the number of lung lesions produced in rats after intratracheal instillations of a benzo(a)pyrene-ferric oxide mixture. In addition, glutathione levels were measured in an attempt to determine if the metabolic fate of the carcinogen was related to the sulfur composition of the diet.

---

[3]Carruth, K. B. (1975) Model investigations of improved amino acid mixtures for total parenteral nutrition. Unpublished PhD dissertation. The University of Tennessee, Knoxville.

[4]Smith, J. T. (1977) Manuscript in preparation.

CHAPTER II

REVIEW OF LITERATURE

Sulfur Nutrition

Sulfur is an essential nutrient for both humans and rats. There are two main sources of this nutrient, organic and inorganic. The sulfur amino acids, methionine and cysteine, are the sources of organic sulfur (18). Organic sulfur, especially that found in methionine, is an essential nutrient for man. Inorganic sulfate has been shown to be utilized by the rat for synthesis of mucopolysaccharides (19).

In this laboratory an optimum dietary level of inorganic sulfate has been identified for rats (15). Based on the hypothesis that inorganic sulfate would spare catabolism of methionine, the optimal level of inorganic sulfate supplementation was defined as the point at which the least $^{14}CO_2$ expiration was measured from 1-^{14}C-methionine treated rats. The optimum level was shown to be 0.02% of inorganic sulfate (16).

Alteration of the inorganic sulfate level in the diet of a rat has been shown to measurably affect several metabolic processes. Brown et al. (20) showed that a decreasing level of inorganic sulfate in the diet resulted in altered collagen in rat skin. The collagen from skins of rats fed 0.0002% inorganic sulfate contained less neutral salt-soluble and total collagen than skins of rats fed 0.02% inorganic sulfate. Furthermore, this collagen did not form thermal gels.

Feland et al. (21) fed rats diets containing different levels of total sulfate and different ratios of neutral to inorganic sulfate. They showed that the ratio of glycocholic to taurocholic acid (G:T) in bile salts was altered by these diets. Furthermore, the G:T ratio affected blood levels of cholesterol. A low G:T ratio and low blood cholesterol were associated with a 0.1% inorganic sulfate plus 0.4% cysteine diet while a diet with 0.42% inorganic sulfate plus no added cysteine was associated with a high G:T ratio and high blood cholesterol. Diets low in inorganic sulfate (0.0002% inorganic sulfate) were shown by Whittle and Smith (22) to increase the amount of ^{35}S-cysteine excreted as ^{35}S-taurine in rats while diets higher in inorganic sulfate (0.1% inorganic sulfate) caused less ^{35}S-cysteine excretion as ^{35}S-taurine. These data indicated that inorganic sulfate could spare cysteine.

Cysteine currently is considered a dispensable amino acid in most mammals. Human subjects maintained nitrogen balance when fed cysteine-free diets if adequate methionine was present in the diet to allow synthesis of cysteine from methionine. Furthermore, cysteine can replace about 80% of the methionine requirement (17). In another study, human subjects receiving a low protein diet showed a fall in plasma cystine levels while methionine levels remained within normal limits indicating methionine conservation takes place at the expense of cystine (23). Studies showed a marked drop in cysteine plasma levels when healthy human subjects were administered cysteine-free preparations parenterally while nasogastric feedings of the same preparation showed a rise in blood

cysteine, but not to baseline values. These data indicated limited synthesis of cysteine from methionine in parenterally fed adults (24).

In rats, cysteine has been shown to have a unique role not entirely filled by synthesis from methionine, indicating a specific requirement for this amino acid.[5] Casein-based diets supplemented with molar equivalents of either cysteine or methionine showed statistically significant ($P<0.05$) differences in kidney tissue glutathione levels. The highest kidney and liver glutathione levels were measured in the cysteine-supplemented rats.

In rats fed protein-free diets or diets with gelatin as the only source of protein, addition of dietary cysteine was shown to increase liver glutathione in direct proportion to the level of added cysteine. A smaller increase was measured with the gelatin-based than with the protein-free diets. Addition of tryptophan to the gelatin diets decreased the observed increase of glutathione further (25). If the diets used by Smith[5] contained cod liver oil as a source of polyunsaturated fat, an increased rate of metabolism of methionine was demonstrated.

The long chain polyunsaturated fatty acids, like those found in cod liver oil, are preferentially shunted into the phosphatidylethanolamine pathway of lecithin synthesis (26). Synthesis of phosphatidyl choline from phosphatidylethanolamine requires the methyl groups from three S-adenosylmethionines. This utilization of methionine provides homocysteine, the substrate for cystathionine synthetase, and leads to cysteine

---

[5]Smith, J. T. (1977) Manuscript in preparation.

biosynthesis. Once synthesized, cysteine then contributes to production of proteins, glutathione, coenzyme-A, mercapturic acids, hydrogen sulfide, cysteine sulfinic acid, thiazoladine derivatives, cysteic acid, taurine, and sulfate (27).

Glutathione

The synthesis of glutathione is of primary concern because of its role in detoxication of compounds. Glutathione is a tripeptide of glutamine, cysteine, and glycine. Synthesis of glutathione requires two enzymes, γ-glutamyl-cysteine synthetase and glutathione synthetase (28). Adequate levels of glutamine, cysteine, and glycine are required for synthesis of glutathione. Data have been presented that indicate dietary cysteine (29) and methionine (30) are the sources of cysteine for glutathione synthesis. Rats fed a semi-purified casein diet containing corn oil and supplemented with cysteine showed higher kidney and liver tissue levels of reduced glutathione than rats fed the same diet supplemented with methionine.[6]

The suggestion has been made that either the level of cysteine or γ-glutamyl-cysteine regulates the synthesis of glutathione since the concentrations of these two compounds are normally low in most tissues. Studies dealing with γ-glutamyl-cysteine as a possible regulator of glutathione synthesis have been contradictory. Cysteine is present in tissues

---

[6]Carruth, K. B. (1975) Model investigations of improved amino acid mixtures for total parenteral nutrition. Unpublished PhD dissertation. The University of Tennessee, Knoxville.

at a low level and γ-glutamyl-cysteine is the substrate for three enzymes: γ-glutamyltranspeptidase, γ-glutamylcyclotransferase, and glutathione synthetase. Competition for substrate by these three enzymes could allow cysteine and γ-glutamyl-cysteine to regulate glutathione synthesis (31). Richman and Meister (32) demonstrated that γ-glutamyl-cysteine synthetase was regulated through nonallosteric feedback inhibition by glutathione. This inhibition occurred when physiological conditions were maintained in vivo and appeared to involve the binding of glutathione to the glutamate site of the enzyme and one other sulfhydryl site on the enzyme.

Tateishi et al. (33) showed that the rapid change in rat liver glutathione between the fed and fasted state was due to availability of substrate rather than changes in activity or level of the two glutathione synthesizing enzymes. Furthermore, the limiting substrate was thought to be cysteine since liver cell concentrations are so low (2×10^{-4}M) and the K_m for the reaction is high (2.5×10^{-3}M). Degradation rates of glutathione seemed to contribute somewhat to the great fluctuation in tissue glutathione concentrations. Jaeger et al. (34) demonstrated a diurnal variation in liver glutathione levels in both the fed and fasted states, although the magnitude of glutathione levels differed between the two states. Minimum glutathione levels were measured between 7 PM and 1 AM while maximum levels were measured between 7 AM and 1 PM. During the period when glutathione levels were low, fed animals were found to be more susceptible to the toxic effects of 1,1-dichloroethylene than during periods when glutathione levels were high.

The synthesis and degradation of glutathione is closely associated with amino acid transport in the kidney (28). Further information on other functions of glutathione and its role in amino acid transport may be found in several review articles (28,35,36).

Glutathione is an important compound in the metabolic detoxication of many foreign compounds. The detoxication involves conjugation of foreign compounds to glutathione, utilizing a class of enzymes called glutathione S-transferases. The conjugation renders non-polar compounds more hydrophilic and leads to excretion of mercapturic acids (35).

Early studies have shown that iodobenzene (a model compound for study of aromatic hydrocarbon interaction with tissue sulfur compounds) conjugates with tissue bound cysteine (37). Conjugation to glutathione reduces the possibility of foreign compounds reacting with thiol-containing cellular proteins and enzymes. The rate of mercapturic acid formation in rat liver was shown to parallel the decrease in liver glutathione when mercapturic acid precusors were administered (31). Glutathione is abundant in the liver and provides one of the sources of the cysteine moiety for mercapturic acid synthesis (35).

Glutathione S-transferases are a class of enzymes widely distributed in vertebrate tissues (31). The enzymes are specific for glutathione but exhibit much less specificity toward a second substrate. The second substrate may be aryls, alkyls, epoxides, or aralkyls (35). Purification of glutathione S-transferase to homogeneity yielded four components-A, B, C, and E--with overlapping specificity for the second substrate. All

four transferases had molecular weights of approximately 45,000 daltons and were made up of two subunits of approximately 25,000 daltons (38).

Kaplowitz et al. (12) studied the induction of glutathione S-transferase in rat liver following phenobarbital or polycyclic hydrocarbon treatment. They found induction of all four glutathione S-transferase enzymes following treatment with phenobarbital, benzo(a)pyrene, and 3-methylcholanthrene. Drug induction increased the V_{max} of the reaction. No change in K_m was noted. Addition of a known competitive inhibitor did not change the K_i of the reaction. All evidence points to enzyme induction by increased levels of the enzyme rather than increased activity. Furthermore, the induction data presented indicate that two pairs of the enzymes are affected similarly. Parallel induction of S-epoxide and S-alkyl-transferase activities (30-40%) took place after induction of S-aryl and S-aralkyl transferase activities (60-80%) by phenobarbital. Both 3-methylcholanthrene and benzo(a)pyrene induced all forms of the enzyme to the same degree. Female rats had lower levels of the enzymes than males. Drug induction of the enzymes in the female was proportional to that in males for all forms of the enzyme except the S-alkyl transferase, which was essentially unchanged by the inducers.

Studies of these enzymes in rat kidney by Clifton et al. (39) showed that the glutathione S-aryl- and S-aralkyl-transferase activities were induced in male rats treated with benzo(a)pyrene, 3-methylcholanthrene and phenobarbital. Both the aryl- and aralkyl-transferases were induced by benzo(a)pyrene and 3-methylcholanthrene while only the aralkyl-transferase was induced by phenobarbital. Female to male ratios of enzyme

activities in rat kidney were: aryl 0.74, aralkyl 2.37, epoxide 1.52, and alkyl 1.33 (39).

Mukhtar and Bresnick (40) showed that the specific activity of glutathione S-epoxide transferase (crude cytosol preparation) in rat liver and lung at 18 days of gestation was 20% of that measured in the same adult tissue. At 21 days the activity had risen to the level measured in adult rats. Hepatectomy reduced the activity by 50% but the activity returned to normal 48 hours following surgery. The enzyme activity in lung is approximately 30% of that in liver throughout life. The enzyme did not exhibit diurnal variation.

Pabst et al. (41) demonstrated that the kinetic mechanism of glutathione S-transferase was dependent on substrate concentration. At high (0.15-5 mM) glutathione concentrations the reaction followed an ordered sequential mechanism in which glutathione binds to the enzyme first. At low (0.1 mM or less) glutathione concentrations a ping-pong mechanism is predominant with the electrophilic substrate binding to the enzyme first. As the concentration of electrophilic substrate is lowered the saturation concentration for glutathione also is lowered. The conditions found in normal rat liver (high glutathione concentrations) would favor the ordered sequential mechanism.

Nemoto et al. (11), using purified glutathione S-transferases from rat and human liver, showed glutathione conjugation was a significant pathway for detoxication of polycyclic aromatic hydrocarbon epoxides. The polycyclic aromatic hydrocarbon epoxide substrate used was benzopyrene-4, 5-oxide. From this study they suggested that the activity of the glutathione

S-transferase enzymes in target tissues may be related to susceptibility of that tissue to carcinogenesis.

Once the glutathione conjugate is formed it may be excreted in the bile or may be further metabolized to mercapturic acids for excretion in urine. More glutathione conjugates are excreted in urine than bile. Mercapturic acid formation follows the pathway of glutathione breakdown after the conjugate is formed (31). Transpeptidation by γ-glutamyl-transferase to give the cysteinylglycine derivative by removal of the glutamate is the next step. This reaction is followed by removal of glycine by the peptidase (cysteinylglycinase), to form the cysteine derivative. The final step in mercapturic acid formation is the acetylation of the cysteine residue by an acetylase and acetyl-coenzyme A (35).

Experimental Lung Cancer

Models of human lung cancer are necessary for the study of the genesis of this disease. Early attempts to develop an acceptable model yielded variable results. Some of the early methods of introducing the test substance into the lungs were: inhalation, intratracheal introduction by tracheal puncture, suturing the lungs with carcinogen impregnated threads, and surgical insertion of pills containing carcinogens. Currently the intratracheal instillation method is used most often.

Various strains of mice, hamsters, and rats have been used as test animals. Some differences between lung tumor type and location have been noted between rats and hamsters. Rats tend to develop peripheral

airway lesions of the adeno and squamous cell type while hamsters develop tumors in all parts of the respiratory tract especially the trachea and stem bronchi. A high incidence of tumors is found in rats after small doses of polycyclic hydrocarbons, while in hamsters a larger dose is required. Hamsters are more resistant to respiratory infections than rats. Both hamsters and rats are considered better animal models than mice because rats and hamsters develop fewer spontaneous respiratory tract tumors than mice (42). For most experimental lung cancer studies either hamsters or rats have been used as the animal model. In many cases rats have been chosen as the experimental animal because the lesions produced closely resemble those seen in humans (4). The subject of lung cancer induction was discussed in current reviews (42,43,44).

Rats treated by intratracheal intubation with the carcinogens 9,10-dimethyl-1,2-benzanthracene or 3,4-benzpyrene and India ink powder developed pulmonary changes which resemble those seen in man. The sequence of development was described by four stages. The first stage consisted of diffuse, irregular, and inadequate hyperplasia. This was followed by a second stage of focal nodular proliferations and a third stage of adenoma or papilloma tumors. The fourth stage was appearance of the malignant tumors (4).

In dose-response studies, Shabad (6) showed formation of cancerous and precancerous lesions in rat lungs, following intratracheal intubation with 3,4-benzpyrene, was dependent on dose. Furthermore, several doses were more effective in producing lesions than a single dose. After

10 doses (1 per week) of 2.5 mg benzopyrene each, 40% of the rats had survived and 80% of the surviving rats had developed lung tumors. Malignant tumors represented 42.5% of the total tumors. A minimal permissible dose of this carcinogen was not set because even at very small doses precancerous states developed in the rats. The development of lesions seemed to be dependent on deposition of the carcinogen in the lung. The deposition varies greatly and is not easily controlled.

Several studies have been conducted to determine what factors affect the development of lung lesions when the intratracheal instillation method of lung tumor induction is used. Kennedy and Little (45) studied the deposition patterns in the hamster lung of benzo(a)pyrene suspended in a gelatin-0.9% NaCl solution. They found that the disperse small particles were deposited and taken up mainly by alveolar cells. After 2 hours only a few particles of benzo(a)pyrene remained in the airways. This pattern of deposition was in contrast to benzo(a)pyrene-ferric oxide-0.9% NaCl preparations which were deposited in the larger airways and entered the epithelial cells after removal of the benzo(a)pyrene from the ferric oxide. These larger particles promoted tumor development in the tracheobronchial region. Henry et al. (46) compared the tumor induction properties of three benzo(a)pyrene-ferric oxide mixtures administered intratracheally to hamsters. A preparation of benzo(a)pyrene physically attached to ferric oxide resulted in larger particles and a higher incidence of lung tumors than a benzo(a)pyrene-ferric oxide mixture of smaller particles produced by hand grinding the two compounds together. In an

earlier study (47) the authors had shown that the physically attached carcinogen particles in the smaller size range (0.5 to 5 μm) produced a higher tumor incidence than larger particles (5 to 30 μm). Sacrifice of the animals immediately after intratracheal instillation showed that about one half of the calculated dose was present in the respiratory tract after treatment, regardless of the method of preparation. Different incidence patterns were attributed to the clearance rates of the carcinogen. In general the larger particles remained in the respiratory tract longer than the smaller particles. The removal rate changed the effective dose present in the target tissue, therefore the tumor incidence. Physical attachment of the carcinogen to the dust slowed removal of the carcinogen.

Ferric oxide alone has not been found to produce lung cancer or increase the incidence of lung cancer. Ferric oxide has been shown to augment lung tumor response when administered with a known carcinogen. When given with diethylnitrosamine, ferric oxide displayed a synergistic effect on tumor production. This effect is not entirely explained by delay of carcinogen removal (42). Creasea and Nettesheim (48) suggested ferric oxide has a cytopathological effect on the cells at the site of deposition which promotes carcinogenesis. The cytopathological effect would enhance the already described retardation of removal. (This effect would implicate a role for particulate air contaminants in development of lung cancer in man.) The rate of carcinogen release from the carrier dust at the target tissue was a factor further noted in tumor induction. The faster the release, the higher the tumor incidence.

Benzo(a)pyrene

Plott in 1775 described a disease of English Chimney Sweeps as "soot wart." Prolonged skin contact with soot was associated with the development of scrotal skin cancer. In the early 1900's coal tar was shown to induce skin cancer in both rabbits and mice. The tar portion of coal tar rather than the dust was shown to produce both skin and lung cancer (49). The polycyclic hydrocarbon, benzo(a)pyrene, was isolated from coal tar in 1933 (50) and shown to be carcinogenic. The isolation was made on the basis of two properties of coal tar which had been associated with carcinogenesis: 1) the tars became carcinogenic at the same temperature tars show an increase in aromatic hydrocarbons and 2) carcinogenic tars are fluorescent under ultraviolet light (50).

Environmental sources of benzo(a)pyrene are many and varied. Tobacco smoke, exhaust fumes of gasoline and diesel engines, charcoal broiled steaks, paraffin wax of food containers, and smoked foodstuffs are just a few of the many sources of this compound (51). Recommendations for a maximum permissible dose of this carcinogen have not been made even though benzo(a)pyrene produced lesions have been shown to be dose dependent. Repeated dosage showed a greater effect than a single dose and at small dosages (too small to cause cancer) precancerous lesions were found (6).

Polycyclic hydrocarbons, in general, and benzo(a)pyrene in particular, are thought to be inert. Following metabolic activation, they become carcinogenic. Most polycyclic hydrocarbons undergo addition,

oxidation,and reduction reactions at analogous sites referred to as "K-regions." The "K-regions" have similar high electron densities and can donate electrons to a cellular receptor. Other regions of the molecule are designated "L-regions" or "M-regions" based on reactive properties. Two theories of carcinogenesis are based on this KLM theory of polycyclic hydrocarbon bond reactivity. The first is that DNA and the carcinogen interact or bind and the second is that a protein and the carcinogen interact and in some way alter gene expression. Both theories require activation of the polycyclic hydrocarbon by metabolism through the mixed-function oxidase enzyme system (52).

Cavalieri and Calvin (8) related the chemical reactivity of benzo(a)pyrene to its ability to bind to nucleic acids. Electrophilic attack at the 6 position (fig. 1) by a positive oxygen atom produced by action of the mixed-function oxidase system forms electrophilic centers at positions 1 and 3. These electrophilic centers then may react with nucleophilic cellular components. Jerina and Daly (9), in a recent review, related the role of arene oxide (epoxide) formation from aromatic hydrocarbons to carcinogenesis. Arene oxides are intermediates formed by the action of the mixed-function oxidase enzymes on aromatic hydrocarbons. These reactive intermediates may lead to the formation of one of several products: phenols, glutathione conjugates, *trans*-dihydrodiols, or covalent bonds to DNA, RNA, or cellular proteins (fig. 2). (The latter is correlated with malignant cell changes.) Factors which control the formation of the final product are not known but there is some evidence

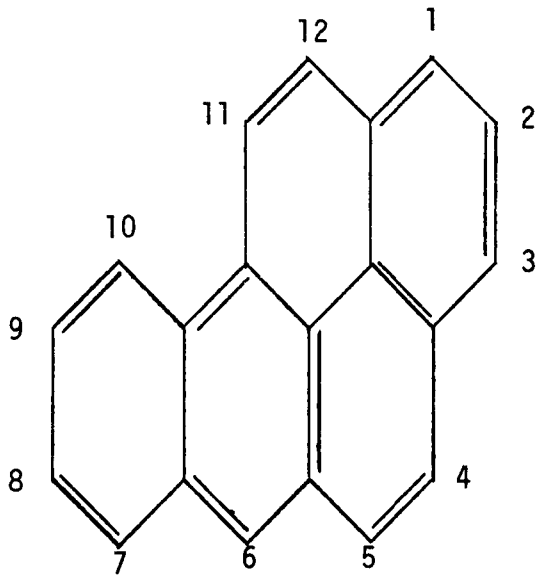


Fig. 1--Structure and numbering of benzo(a)pyrene.

Source: Grover, P. L., Hewer, A. & Sims, P. (1974) Metabolism of polycyclic hydrocarbons by rat-lung preparations. Biochem. Parmacol, 23, 324.

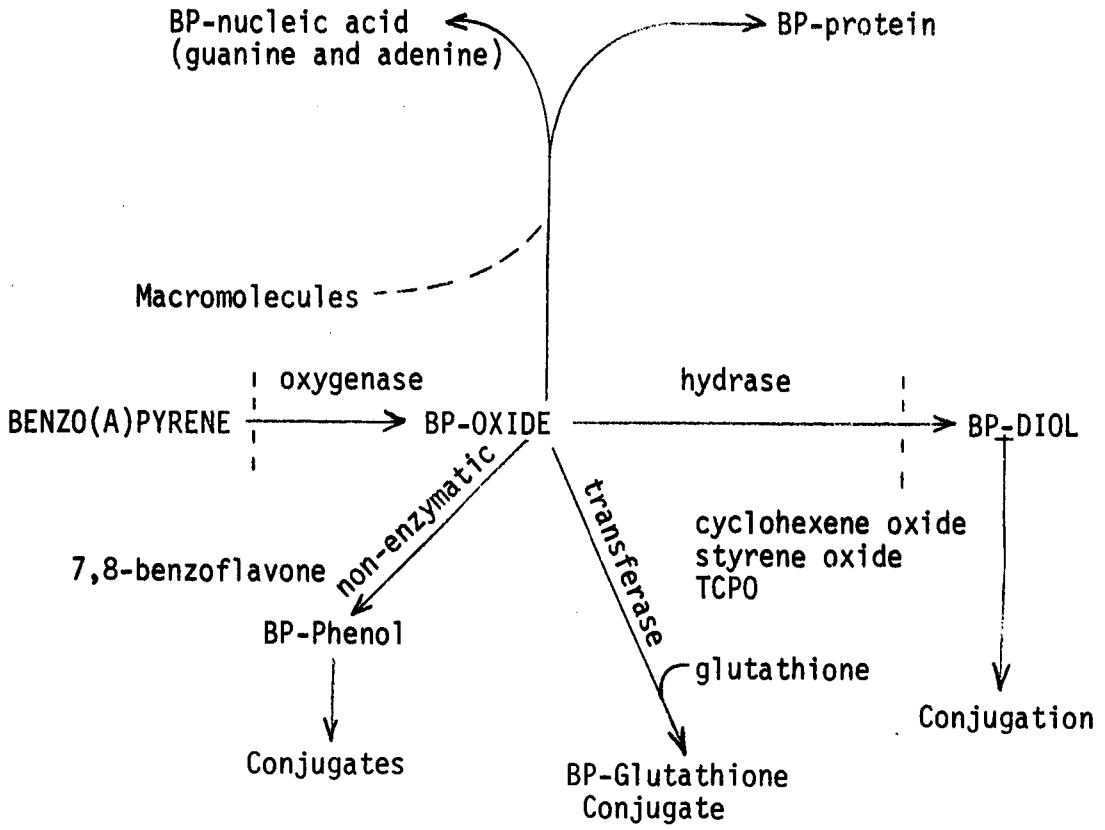


Fig. 2--Proposed scheme of microsomal metabolism of benzo(a)-pyrene, leading to various detoxification products and to nucleic acid binding. BP-protein and BP-nucleic acid designate the reaction products of BP with protein and nucleic acid, respectively, ---- apparent sites of action of the various inhibitors, . . .TCPO, tricholropylene oxide.

Source: Pietropaolo, C. & Weinstein, T. B. (1975) Binding of [^{3}H]-benzo(a)pyrene to natural and synthetic nucleic acids in a subcellular microsomal system. Cancer Res. 35, 2192.

that either presence or activity of epoxide hydrase, glutathione S-epoxide transferase, or amount of glutathione present in the tissue influences which final product is formed.

The mixed-function oxidase system of enzymes may be induced or inhibited. Induction requires protein synthesis. RNA synthesis is required during the first 30 to 60 minutes only. The presence and activity of this enzyme system in tissues is related to the susceptibility of that tissue to carcinogenesis. Inhibitors of this enzyme system, like 7,8-benzoflavone, decreased the toxicity of polycyclic hydrocarbons to cells; while 3-hydroxybenzo(a)pyrene, a product of the enzyme system, produced toxic effects in cells which contained low levels of the enzyme system and were resistant to benzo(a)pyrene (53,54). The enzyme system is induced by many of its substrates, especially benzo(a)-pyrene and phenobarbital, in both rat lung and liver. The induced enzymes exhibit different specificities toward the two substrates, benzo-(a)pyrene and phenobarbital. Products of the induced enzymes also depend on the inducer. Phenobarbital and 3-methylcholanthrene induced liver enzymes which activated benzo(a)pyrene bacterial mutagen. Phenobarbital required less enzyme protein than 3-methylcholanthrene for activation (55). Mannering (56) showed polycyclic hydrocarbons induce production of a cytochrome P-450 which is different from cytochrome P-450 normally present and induced by phenobarbital. This polycyclic hydrocarbon induced cytochrome P-450 is referred to as P-448 or P_1450. Evidence has been presented for only two forms of cytochrome P-450 in hamster lung and several forms

in liver (57). Corn oil pretreatment of the hamsters followed by treatment with either benzo(a)pyrene or biphenyl showed biphenyl-4-hydroxylation in both liver and lung tissue. Induction of the enzyme with 3-methylcholanthrene revealed induction of biphenyl-2-hydroxylation in both tissues, benzo(a)pyrene hydroxylation in lung and biphenyl-4-hydroxylation in liver. Prough et al. (58) suggested attributing these differences in substrate specificity to membrane location of the enzyme.

Inhibitors of the mixed-function oxidase system have been identified as 7,8-benzoflavone (59), organophosphate insecticides (60), and adjuvant-disease (models of chronic inflammatory diseases) (61).

Evidence that the "K-region" epoxide is the carcinogenic form of benzo(a)pyrene has not been conclusive. Flaks and Sims (62) tested both benzo(a)pyrene and benzo(a)pyrene-4,5-oxide in mice using treated implanted isologus tissue. They found no differences in proliferative growth response. Rasmussen and Wang (55) showed that in vitro inhibition of epoxide hydrase in rat liver homogenates resulted in production and accumulation of a bacterial mutagen, probably benzo(a)pyrene-4,5-epoxide. Chromatographic studies of rat lung and liver metabolites of benzo(a)-pyrene revealed production of 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene; 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene; 9,10-dihydro-9,10-dihydroxybenzo-(a)pyrene; and 3-hydroxybenzo(a)pyrene (63).

The most recent studies of benzo(a)pyrene metabolism (64,65) indicate benzo(a)pyrene is converted by the mixed-function oxidase system and epoxide hydrase to one enantiomer, (-)r-7,t-8-dihydroxy-7,8-dihydro-benzo(a)pyrene. This enantiomer is stereospecifically metabolized by

the mixed-function oxidase system to r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene (diol-epoxide 1). This diol-epoxide 1 is unstable in aqueous medium, highly mutagenic, optically active, and thought to be the carcinogenic form of benzo(a)pyrene (66,67). Another author has presented evidence that diol-epoxide 1 is further converted, in the presence of NADPH, non-enzymatically to (7/8,9)-trihydroxy-7,8,9,10,10-pentahydrobenzo(a)pyrene. If diol-epoxide 2, the *cis* isomer, is the substrate a (7,9/8)-trihydroxy-7,8,9,10,10-pentahydrobenzo(a)pyrene is formed (68).

The metabolites separated after benzo(a)pyrene treatment seem to vary with the test system: K-region epoxides are identified as the active form of benzo(a)pyrene if the study is carried out in vitro. The K-region epoxides are found to be less carcinogenic than the parent polycyclic hydrocarbon if administered subcutaneously or in vivo (69).

When liver and lung were compared on the basis of tissue weight, liver showed the more active metabolism of polycyclic hydrocarbons but when compared on the basis of tissue protein the two tissues were similar. Ring-hydroxylated products were found most often indicating epoxide formation especially in the lung. Metabolism of isolated microsomes was compared to the tissue homogenates. Lung microsomes formed K-region dihydrodiols twice as fast as liver microsomes, while the corresponding lung and liver homogenates contained fewer dihydrodiols (63). Using microsomal preparations of rat lung, Grover (70) demonstrated formation of the K-region epoxide, benzo(a)pyrene-4,5-oxide, from benzo(a)pyrene.

In vitro glutathione conjugates, which could be formed from the epoxide through the cytoplasmic glutathione S-epoxide transferase, have been detected. Therefore, if these conjugates are formed the epoxide must be formed in and released from the endoplasmic reticulum for conjugation to take place. The glutathione S-epoxide transferase is present in human lung and liver at similar activities to those found in the corresponding rat tissue. Since the epoxide hydrase enzyme, which is coupled to the mixed-function oxidase system in the microsomes, is less active in lung, skin, and intestines of rodents than it is in liver or kidney, release of the epoxide to cytosol and conjugation to glutathione may be the major detoxication mechanism of these tissues. Furthermore, epoxides may accumulate and have more of an opportunity to bind to DNA in these tissues (71). Vainio et al. (72) measured metabolites of ^{3}H-benzo(a)pyrene after instillation into a perfused rat lung which had been pretreated with a mixed-function oxidase inducer. A decrease in ^{3}H-benzo(a)pyrene and an increase in phenols (7 fold) and 9,10-diols (5 fold) were found in the perfusion medium. A comparison of benzo(a)pyrene metabolites produced in cultured trachea and bronchi vs. perfused organ revealed production of more dihydrodiols in the trachea and bronchi than in other areas (73). These results correspond to the observation that trachea and bronchi are more susceptible than other areas of the lung to carcinogenesis and that dihydrodiols are most likely the precusor forms of the active carcinogen.

The ability of benzo(a)pyrene to cause mutations in cultured cells has been demonstrated (55,74,75). Cultured cells from various strains of

bacteria and hamster V79 cells were tested for mutagenic response to benzo(a)pyrene metabolites. Benzo(a)pyrene-4,5-oxide was the most mutagenic to all types of cells. Benzo(a)pyrene-11,12-oxide was as mutagenic as the 4,5-oxide in the V79 cells (76). Furthermore, this ability to cause mutations has been correlated with carcinogenesis (74). Mutation seems to follow covalent binding to DNA (75), which has been demonstrated to occur in vitro (10,59). The evidence for benzo(a)pyrene metabolites being bound to DNA, RNA, and proteins is fairly concrete, but the metabolite which is bound and the position(s) to which it is bound on the cellular component are not known. Raha et al. (77) presented evidence that benzo(a)pyrene-4,5-oxide is covalently bound at the 4 or 5 position to microsomal or cytosolic proteins. More recent evidence has indicated that both diol-epoxide 1 and diol-epoxide 2 are bound to DNA in cell cultures, in vivo (78), in short term organ culture, and on mouse skin (65). The majority of binding involved the diol-epoxide 1 (78). In cultured bovine bronchial mucosa the diol-epoxide 2 was identified as an intermediate in RNA binding (79). The alkylation of the diol-epoxide to the 2 amino group of polyguanylic acid has been demonstrated (80). Position 10 of the diol-epoxide was identified as the position of binding (79,81). Binding of a benzo(a)-pyrene metabolite to guanine and adenine (to a lesser extent) (59) is thought to result in a distortion of the DNA, which causes mispairing during replication. This could shift the reading frame of mRNA and result in synthesis of a different peptide (75).

Detoxication of the activated benzo(a)pyrene is by conjugation of the active arene oxide or epoxide to glutathione by the glutathione S-epoxide transferase of cytosol, rearrangement to phenols followed by conjugation, or formation of *trans*-dihydrodihydroxy derivatives followed by glucuronic acid or sulfate conjugation (fig. 2, page 19 and fig. 3). An in vitro study of benzo(a)pyrene-phenols and benzo(a)pyrene-7,8-diol metabolism showed that glutathione inhibited the binding of benzo(a)pyrene metabolites to DNA (82). Addition of liver cytosol and glutathione to a microsomal preparation caused an 18 fold increase in conjugation of benzo(a)pyrene metabolites (83).

The glutathione conjugates are excreted in bile or converted to mercapturic acids for excretion in urine (the latter occurs most often) (84). Inhibitors of epoxide hydrase or glutathione S-transferase increase mutagenesis of benzo(a)pyrene (75). Binding of ^{3}H-benzo(a)pyrene to nucleic acids is decreased by glutathione, 7,8-benzoflavone, or magnesium in reaction mixtures containing rat liver microsomes and mixed-function oxidase components (59). Rat lung tissue levels of glutathione S-epoxide transferase are higher than liver levels while epoxide hydrase levels are lower in lung (70). Conjugation of benzo(a)pyrene-4,5-oxide to glutathione via the glutathione S-epoxide transferase enzyme is thought to be a significant reaction, since these enzymes represent 2% of extractable human liver protein and 10% of rat liver protein (11).

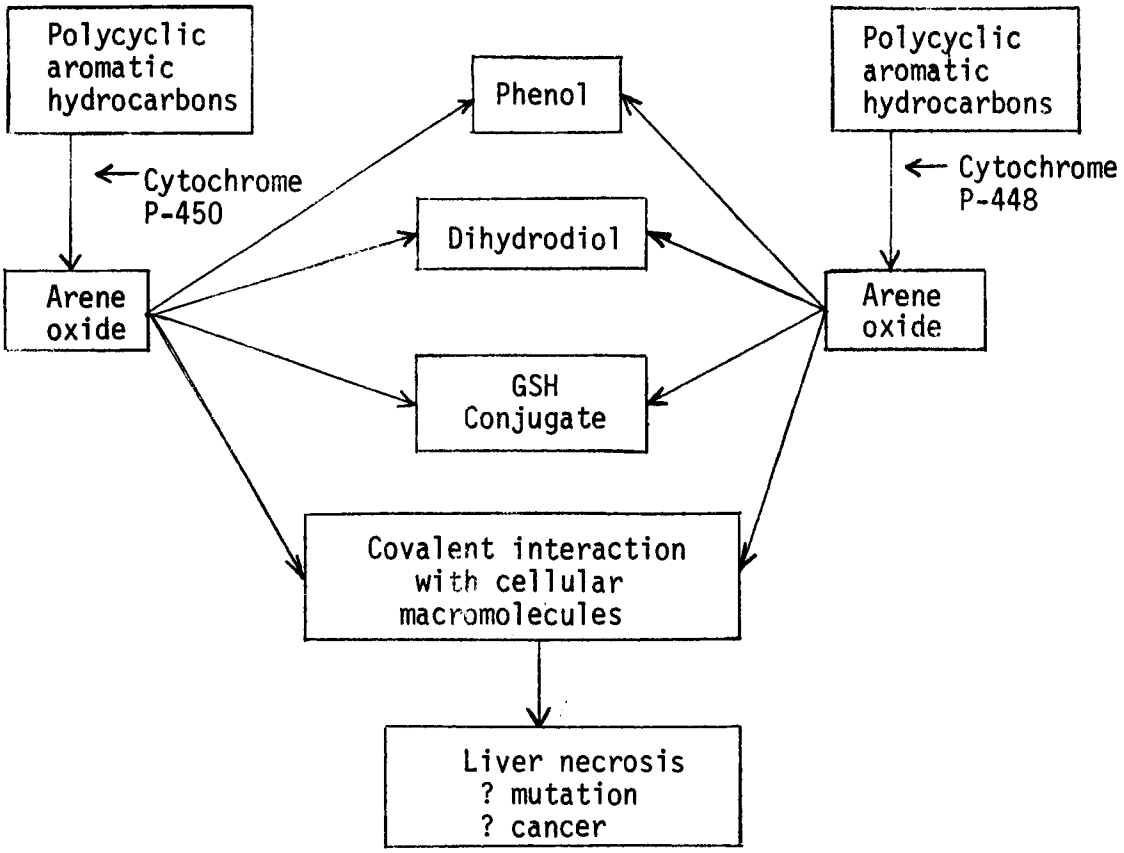


Fig. 3--Possible metabolic pathways for the formation and disappearance of oxides of polycyclic aromatic hydrocarbons.

Source: Nebert, D. W. (1974) Metabolic activity as a factor in carcinogenic response. In: Carcinogenesis Testing of Chemicals. (Goldberg, L., ed.), pp. 76, CRC Press, Inc., Cleveland.

CHAPTER III

EXPERIMENTAL PROCEDURE

General Plan

Female adult rats of a Wistar, Sprague-Dawley, Long Evans crossed strain were used for the study. Rats weighing 150-200 g were randomly assigned to one of the five experimental diets. The animals consumed the experimental diets for 17 days before administration of the carcinogen. The rats continued to receive the diet until they were sacrificed. For the entire study the rats were housed in wire bottom galvanized cages, 5 to a cage. Diet and distilled water were provided ad libitum.

Rats were fed diets containing different levels of inorganic sulfate to determine if this dietary component would affect the number of lung neoplasms produced by a known environmental carcinogen, benzo(a)pyrene. The conjugation of glutathione to the activated benzo(a)pyrene is one route of detoxication for the carcinogen. Since earlier studies in this laboratory had shown liver and kidney levels of glutathione could be altered by diet,[7] measures of lung and liver glutathione were made to determine the possible effect diet might have on this key compound of detoxication when challenged by a carcinogen.

---

[7] Carruth, K. B. (1975) Model investigations of improved amino acid mixtures for total parenteral nutrition. Unpublished PhD dissertation. The University of Tennessee, Knoxville.

The semi-synthetic diets used in this study (shown in tables 1 and 2) were diets previously used in this laboratory.[8,9,10] A modification of the salt mixture of Hubbell et al. (85) was used. The variables in the diet were inorganic sulfate and/or cysteine as shown in table 2. Diets contained 0.0002% inorganic sulfate (Diet A), 0.42% inorganic sulfate (Diet B), 0.0002% inorganic sulfate with added cysteine (Diet C), 0.02% inorganic sulfate with added cysteine (Diet D), and 0.42% inorganic sulfate with added cysteine (Diet E). All diets without added cysteine were marginal with respect to sulfur amino acids. The 0.02% inorganic sulfate with added cysteine diet was the control diet. Previous studies had shown this diet promoted optimum kidney and liver tissue levels of glutathione.[8,9] All diets contained casein as the source of protein.

The study was carried out in two parts. The first part of this study was concerned with the number of lesions produced after the intratracheal instillation of benzo(a)pyrene in relation to the diets consumed by the rats. Three diets were used for this experiment: Diets A, B, and D (table 2). The lesion counts were collected from a total of 68 animals surviving after treatment. There were 21-24 animals per diet. These animals were treated on different dates and the information on lesion counts pooled.

---

[8]Carruth, K. B. (1975) Model investigations of improved amino acid mixtures for total parenteral nutrition. Unpublished PhD dissertation. The University of Tennessee, Knoxville.

[9]Smith, J. T. (1977) Manuscript in preparation.

[10]Kurzynske, J. S. (1975) Investigation of phenolic hydroxyl chemicals as model compounds for determining sulfur status. Unpublished PhD dissertation. The University of Tennessee, Knoxville.

TABLE 1

Composition of Basal Diet

Component	g/100 g diet
Casein[1]	15.00
Sucrose	30.00
Cornstarch	30.00
Cod liver oil	2.00
Vegetable shortening[2]	6.00
Vitamin mixture[3]	2.00
Basal salt mixture[4]	1.34
Variable components	see Table 2

[1]Nutritional Biochemical Corporation, Cleveland, Ohio 44128.

[2]Crisco, Procter and Gamble, Cincinnati, Ohio 45202.

[3]Nutritional Biochemical Corporation, Cleveland, Ohio 44128. Vitamin Diet Fortification Mixture formulated to supply the following amounts of vitamins (g/kg vitamin premix): thiamin hydrochloride 1.0, riboflavin 1.0, niacin 4.5, p-aminobenzoic acid 5.0, calcium pantothenate 3.0, pyridoxine hydrochloride 1.0, ascorbic acid 45.0, inositol 5.0, choline chloride 75.0, menadione 2.25, biotin 0.02, folic acid 0.09, Vitamin B_{12} 0.00135, α-topcopherol 5.0, Vitamin A 9 x 10^5 units, Vitamin D 1 x 10^5 units, and sufficient glucose to make 1 kg.

[4]Formulated to supply the following amounts of minerals (g/kg salt mixture): magnesium carbonate 30.6, sodium chloride 69.0, potassium chloride 112, potassium monobasic phosphate 212.0, ferric phosphate 20.5, potassium iodide 0.08, sodium flouride 0.1, manganese chloride 0.4, aluminum sulfate 0.1 and cupric acetate 0.72.

TABLE 2

Additions to Basal Dietary Mixture

Diet	Inorganic SO_4	$CaSO_4 \cdot 2H_2O$	$CaCO_3$	L-cysteine[1] free base	Non-nutritive bulk[2]
	----%----	----------g/100 g diet----------			
A	0.0002	0.0	1.34	0.0	12.32
B	0.42	0.72	0.92	0.0	12.02
C	0.0002	0.0	1.34	0.50	11.82
D	0.02	0.04	1.32	0.50	11.80
E	0.42	0.72	0.92	0.50	11.52

[1]Nutritional Biochemical Corporation, Cleveland, Ohio 44128.

[2]Alphacel, Nutritional Biochemical Corporation, Cleveland, Ohio 44128.

The second part of this study involved both counting lesions and measuring tissue glutathione levels in lung and liver. The data from this experiment were divided into Groups 1 and 2. In the Group 1 experiment Diets A, B, and D were used and in Group 2 Diets C, D, and E were used. Diets C and E were identical to Diets A and B except that 0.5% cysteine was added to each diet. This set of data were collected on 45 animals with 6-9 animals per diet.

In addition, the control diet was fed to a group of 5 animals that were not treated with benzo(a)pyrene. Lung and liver glutathione levels were measured in these animals and compared to the treated group.

Intratracheal Instillation of Benzo(a)pyrene

The intratracheal instillations of the carcinogen followed the method described by Saffiotti et al. (86). The benzo(a)pyrene-ferric oxide mixture for intratracheal instillation was prepared by weighing equal amounts (1.000 g) of benzo(a)pyrene[11] and ferric oxide.[12] The two compounds were ground together with a mortar and pestle for about one half hour. The powder then was suspended in an autoclaved 0.9% NaCl solution. A concentration of 3 mg benzo(a)pyrene in 0.2 ml of solution was administered to the rat at each treatment. This concentration was selected on

---

[11]Sigma Chemical Company, St. Louis, Mo. 63178 or Eastman Kodak Company, Rochester, N. J. 14650.

[12]Fisher Scientific Company, Fair Lawn, New Jersey 07410.

the basis of a dose-response study (6). The mixture was kept dispersed during the instillation procedure by stirring with a magnetic stirrer. Between treatments the benzo(a)pyrene was stored in a tightly covered flask in a dark cabinet.

Brevital Sodium[13] was chosen as the anesthetic because of its fast action which eliminated problems with lung edema and restoration of respiratory action (87). A 1% solution of the anesthetic was used. Before each treatment the animal was weighed and given an interperitoneal injection of Brevital Sodium after the abdomen had been disinfected with 70% ethanol. An 0.8 ml/g of body weight dose of anesthetic was given at first. This dose was later reduced to 0.6 ml/g of body weight because a number of animals died during treatment.

After being anesthetized the animal was placed on a slanted block with its back next to the block and its mouth held open by a copper wire upon which the lower incisors were hooked and with a rubberband holding the upper incisors. The tongue was pulled aside with forceps and by using a headlamp, a clear view of the pharynx was obtained. A syringe equipped with a blunt 17-gauge needle bent at a 135^{o} angle was used to deliver a measured (0.2 ml) amount of the benzo(a)pyrene-ferric oxide solution directly into the trachea. The animal was left in position for 2 minutes to allow partial recovery from the treatment and help prevent

---

[13]Brevital Sodium (methohexital sodium) Eli Lilly and Company, Indianapolis, Indiana 46206.

regurgitation of the solution. All equipment used for the instillation procedure was sterilized by autoclaving before use.

Ten weekly treatments were administered. This time period was based on results from reports in the literature (6).

Counting of Lesions

One week after the tenth intratracheal instillation of the benzo(a)pyrene-ferric oxide mixture, the animals were sacrificed in the morning. Each animal was stunned by a sharp blow to the head followed by decapitation. The lungs and livers of the animals were quickly removed to a tared beaker containing cold 2% sulfosalicylic acid. The weight of each organ was recorded. If both lung lesion counts and glutathione measures were to be made on the lung, the lung was removed en bloc and inflated in excess of capacity through the trachea using a blunt needle syringe filled with the fixative[14] (90 ml 95% ethanol, 5 ml glacial acetic acid, and 5 ml 40% formaldehyde) and then the large left lung lobe was removed to the 2% sulfosalicylic acid for glutathione measurement. The remainder of the lung was stored in a bottle of fixative.

Lungs were cleared using a modification of the technique described by Yuhas.[15] The lung was fixed overnight or longer, after which it was removed from the fixative and the lobes split along the major bronchus. Each piece of lung was identified by a colored string. Any lesions visible on the surface of the lungs were noted. The characteristics of

---

[14]Yuhas, J. M. (1975) Personal communication.

[15]Yuhas, J. M. (1975) Personal communication.

these lesions have been described as firm, white multiple confluent nodules visible on the peripheral areas of the lung (88,89).

The lung sections were rinsed once in distilled water and then allowed to stand in fresh distilled water for 25 minutes. The lungs were allowed to stain for 1 hour. The stain was prepared from a stock stain, modified from Jones (90), which consisted of 2 g Wright's stain, 187.5 ml methyl alcohol, and 62.5 ml glycerol. This stock stain was mixed 13 ml stain to 2 ml "phosphate buffer" and diluted with 85 ml 50% alcohol. The "phosphate buffer" (91) consisted of 7.09 g Na_2HPO_4 in 25% absolute methanol to make 250 ml. After staining, the lungs were rinsed once in distilled water and destained in 70% alcohol for at least 15 minutes. The tissue was then dehydrated in 3 one-hour rinses of *tert*-butyl alcohol. During the dehydration process the tissue was held in a 35-37^{0} water bath. The lungs were then allowed to clear for at least one week in oil of cedarwood, then stored and cleared further in methyl salicylate (2 days or more). The stained and cleared tissue was protected from light.

The lesions were counted and the approximate size noted by looking at the cleared tissue under low magnification with a source of light under the tissue. A lesion was identified as a small dark nodule in the cleared tissue.

Determination of Tissue Glutathione

Determinations of glutathione concentration in liver and lung were made using the glyoxalase method described by Patterson and Lazarow (92) as modified by Carruth.[16]

Sulfosalicylic acid solutions of 2% and 1% were prepared from a 1 M solution. The 1 M solution was prepared from 25 g sulfosalicylic acid which then was diluted with distilled water to a final volume of 250 ml. The 2% solution was prepared using 23 ml of the 1 M solution and diluting it with distilled water to a final volume of 250 ml. The 1% solution was prepared using 11.5 ml of the 1 M solution and diluting it with distilled water to a final volume of 250 ml. All solutions were stored in a refrigerator at 4^{o}.

An aqueous solution of 0.2 M sodium bicarbonate was prepared by weighing 1.68 g of $NaHCO_3$ and diluting it with distilled water to a final volume of 100 ml.

Livers and lungs of the sacrificed animals were weighed and then homogenized in four volumes of 2% sulfosalicylic acid per gram of tissue with a motor-driven Thomas homogenizer equipped with a Teflon pestle and glass homogenizing tube. The homogenized tissue then was diluted with 1% sulfosalicylic acid to a final concentration of 1 part wet weight of tissue to 10 parts of solution. Approximately 2-3 g of liver tissue

---

[16]Carruth, K. B. (1975) Model investigations of improved amino acid mixtures for total parenteral nutrition. Unpublished PhD dissertation. The University of Tennessee, Knoxville.

were used and the entire large left lobe of the lung was used (approximately 0.5-1 g tissue). After homogenization with the sulfosalicylic acid (which precipitates the protein), the tissue was covered and stored in a refrigerator until the glutathione determinations were made. Before the determinations were carried out the tissue homogenates were centrifuged at 896 x *g* for 2 minutes to obtain the glutathione containing supernatant.

The glyoxalase method for determination of tissue glutathione concentrations is a specific measure for glutathione. The glyoxalase enzyme requires catalytic amounts of glutathione as a cofactor in the conversion of methyl glyoxal to lactic acid. The lactic acid liberates CO_2 from added bicarbonate. The liberation of CO_2, measured manometrically using a Warburg respirometer, is directly related to the concentration of glutathione when glutathione is the limiting factor in the reaction. With a set of glutathione standards, a standard glutathione curve was constructed and the tissue concentration of glutathione was calculated (93).

The glyoxylase enzyme preparation was obtained from baker's yeast as described by Patterson and Lazarow (92). Approximately 180 g (12 cakes) of fresh pressed baker's yeast were crumbled, weighed, and mixed by stirring with 720 ml of cold (4^0) acetone for 10 minutes. The acetone then was removed by suction filtration. The above procedure was repeated by extracting the residue with an additional 240 ml of cold acetone. To remove the glutathione from the glyoxylase preparation the mixture was

washed with cold water. The residue was suspended and stirred for 10 minutes with 288 ml distilled water cooled to 4^{o}. The solid enzyme containing portion then was separated by centrifugation at 4100 x *g* in a refrigerated centrifuge. The water was removed by decanting and the process repeated 5 times.

Finally, the residue was dried by suspending it in 240 ml of cold acetone and blending in a blender. The acetone then was removed by suction filtration. This process was repeated 2 times. The last time the acetone was removed the residue was washed immediately with 144 ml of cold (4^{o}) ether by pouring the ether over the residue in the suction filter. The residue was spread immediately on paper and worked with the hands until dry. The glyoxylase preparation was weighed, then stored at -20^{o} in a dark tinted glass bottle.

To carry out the measurement of glutathione the water bath of the Warburg apparatus was preheated to 25^{o} and the one side arm, without center well flasks were placed in an ice bath. A 1.5 ml portion of glyoxylase reaction mixture (4.0 g yeast glyoxylase preparation, 15.0 ml distilled water, 11.9 ml 0.2 M $NaHCO_3$, 0.6 ml 40% methyl glyoxal, and 0.2 ml 2-mercaptoethanol) was pipetted into the main compartment of the flask while 0.5 ml of the tissue homogenate or glutathione standard was pipetted into the side arm. A thermobarometer was prepared by pipetting 2 ml of distilled water into a flask. One sample from each diet group was measured each time the determinations were made so that comparisons could be made. Duplicates of each sample were run and glutathione

concentrations from the duplicates averaged to get one value for each sample.

After the flasks were connected to the manometers, they were placed in the water bath and allowed to equilibrate by shaking for 5 minutes with the system open. Before the contents of the flasks were mixed, the Brodie's solution in the manometers was adjusted to read 150 mm on the right hand scale. The left hand scale reading was the zero reading. The contents of the flasks then were mixed. The system was closed 4 minutes after mixing and readings were taken every 5 minutes for 15 or 20 minutes. The manometers were read, while shaking, by adjusting the right hand scale to 150 mm and reading the left hand scale.

After the readings were completed, the systems were opened, the Brodie's solution in the manometers was lowered, and the flasks removed from the manometers. The flasks were degreased with acetone and cleaned by the permanganate method of Ryan[17] rather than the usual dichromate method. The permanganate solution was prepared by dissolving 20.0 g of potassium permanganate and 50.0 g of sodium hydroxide in 1 liter of water. Following the degreasing, the flasks were washed in distilled water and soaked 30 minutes in the permanganate cleaning solution. The flasks then were rinsed in distilled water and soaked in a saturated oxalic acid solution until bleached. The flasks were rinsed again in distilled water,

---

[17]Ryan, W. L. (1953) A metabolic study of cold shock in mammalian spermatoza. Unpublished PhD dissertation. The University of Missouri, Columbia.

placed in a pyrex dish, covered with distilled water, and boiled on a hot plate at approximately 260°. The flasks were allowed to cool; rinsed in distilled water and demineralized water; and dried in a convection oven.

The data from the tissue glutathione determinations were expressed as µg glutathione per gram tissue. Calculations were based on relating the µl of CO_2 released from the sample reaction to the µl of CO_2 released from the reaction of standards of known concentration. The change in the thermobarometer was first calculated by subtraction of the zero reading from the final reading and multiplication by the flask and manometer constant of the apparatus. (The flask and manometer constants had been determined for the equipment by the method described by Umbreit et al. (93).) The µl CO_2 produced by the standard glutathione sample was calculated by substraction of the zero reading from the final reading and multiplication by the flask and manometer constants. The change in the thermobarometer then was subtracted and the µl glutathione per µl CO_2 calculated by division of the µl CO_2 produced by the glutathione concentration in the flask. The µl CO_2 produced by each sample was calculated by subtraction of the zero reading from the final reading and multiplication by the flask and manometer constant. The change in the thermobarometer was subtracted. The µl CO_2 then was related to the µg glutathione in the sample by division of the µl CO_2 by µg glutathione per µl CO_2 in the standard.

Statistical Analysis

Whenever statistical treatment was used data were analyzed by the specific method appropriate for the data. The methods were those described by Sokal and Rohlf (94,95). All data computations were performed using the Decsystem-10 computer and Stat-Pack-V4.[18]

[18]Houchard, R. (1974) Western Michigan University.

CHAPTER IV

RESULTS

The carcinogen, benzo(a)pyrene, causes lung cancer when administered intratracheally to rats, mice, and hamsters. Benzo(a)pyrene conjugation to glutathione for detoxication is a well documented step in the metabolic pathway of this carcinogen (11,59,70,82,83,84). Considerable evidence has been gathered to support the hypothesis that glutathione tissue levels can be altered by dietary manipulation of sulfur intake (25,31,33) especially through the sulfur amino acids methionine and cysteine.[19,20] Therefore, it seems reasonable that animals fed different levels of sulfur would respond differently to benzo(a)pyrene stress.

Lesion counts were made on the lungs of rats that survived the entire test period of the first experiment. A summary of the results of those lesion counts are recorded in table 3. The 0.0002% inorganic sulfate without added cysteine diet (Diet A) group had the in the smallest mean number (34±21 SD) of counted lesions. The 0.02% inorganic sulfate plus 0.5% cysteine diet (Diet D) group had the intermediate mean (41±39 SD)

---

[19]Smith, J. T. (1977) Manuscript in preparation.

[20]Carruth, K. B. (1975) Model investigations of improved amino acid mixtures for total parenteral nutrition. Unpublished PhD dissertation. The University of Tennessee, Knoxville.

TABLE 3

Number of Lesions Counted on Lungs of Rats After 10 Intratracheal Instillations of 3 mg Benzo(a)pyrene[1]

Diet	No. of animals	Lung lesions[2] Total	Lung lesions[2] Minus left lobe
A. 0.0002% $SO_4^=$	24	34 ± 21	21 ± 14[3]
D. 0.02% $SO_4^=$ + 0.5% cysteine	23	41 ± 39	30 ± 22
B. 0.42% $SO_4^=$	21	49 ± 36	36 ± 28

[1]For instillation, equal parts of benzo(a)pyrene and ferric oxide were ground together and suspended in sufficient 0.9% NaCl to make a concentration of 3 mg benzo(a)pyrene in 0.2 ml solution.

[2]Values are the mean ± standard deviation.

[3]A 2-way analysis of variance was performed on the square roots of the lesion counts for the lung minus the left lobe. With the variance due to date of treatment held constant, a significant ($P<0.05$) difference among means was measured.

number of lesions. The 0.42% inorganic sulfate without added cysteine diet (Diet B) group had the highest mean (49±35SD) number of lesions. The means were not significantly different when tested by a 1-way analysis of variance.

Since later counts of lung lesions were made on lungs without the left lobe and since counts on the total lung compared to counts on lungs with the left lobe missing were highly correlated ($P<0.01$), the counts from the left lobe were subtracted from the total count and recorded in table 3. The group of animals consuming Diet A had the smallest mean (21±14 SD) lesion count, the animals consuming Diet D the intermediate mean (30±22 SD) lesion count, and the animals consuming Diet B the largest mean (36±28 SD) lesion count. A 2-way analysis of variance was performed on the square roots of the lesion counts for the lung minus the left lobe. With variance due to date of treatment held constant, a significant ($P<0.05$) difference among means was found.

In the second experiment lesion counts and glutathione tissue levels were measured in animals after benzo(a)pyrene treatment (table 4). The rats in Group 1 consumed the same three test diets used for the previous experiment. In Group 1, animals consuming Diet D showed the lowest (21±14 SD) number of lesions, animals consuming Diet A showed the intermediate (24±26 SD) number of lesions, and animals consuming Diet B showed the highest (43±31 SD) number of lesions. A significant ($P<0.05$) difference in mean lesion counts minus the left lobe was found between animals consuming Diet B and Diet D using a one-tailed t-test on log transformed data.

TABLE 4

Comparison of the Number of Lesions Counted on Lungs of Rats After 10 Intratracheal Instillations of 3 mg Benzo(a)pyrene[1] to Two Different Diet Groups

Diet	No. of animals	Lung lesions Lung minus the left lobe[2]
Group 1		
A. 0.0002% $SO_4^=$	6	24 ± 26
D. 0.02% $SO_4^=$ + 0.5% cysteine	7	21 ± 14[a3]
B. 0.42% $SO_4^=$	7	43 ± 31[ab4]
Group 2		
C. 0.0002% $SO_4^=$ + 0.5% cysteine	9	22 ± 18
D. 0.02% $SO_4^=$ + 0.5% cysteine	8	22 ± 17
E. 0.42% $SO_4^=$ + 0.5% cysteine	8	13 ± 9[b]

[1]For instillation, equal parts of benzo(a)pyrene and ferric oxide were ground together and suspended in sufficient 0.9% NaCl to make a concentration of 3 mg benzo(a)pyrene in 0.2 ml solution.

[2]Values are means ± standard deviations.

[3]Means followed by the same superscript letter (a) are significantly ($P<0.05$) different by a one-tailed t-test on log transformed data.

[4]Means followed by the same superscript letter (b) are significantly ($P<0.05$) different by a one-tailed t-test.

The data from all lesion counts for animals consuming Diets A, B, and D were combined in table 5. Combination of the data showed rats consuming Diet A lowest (22±17 SD) in the number of counted lesions, rats consuming Diet D intermediate (26±19 SD) in the number of counted lesions, and rats consuming Diet B highest (39±28 SD) in the number of counted lesions. These data showed the same relationship as was shown in table 3, page 42, for the first experiment. The data in table 5 were not tested to determine statistical significance.

Cysteine was added at the 0.5% level to all diets in Group 2 (table 4). The lungs of animals consuming the 0.0002% inorganic sulfate plus 0.5% syteine diet (Diet C) and the 0.02% inorganic sulfate plus 0.5% cysteine diet (Diet D) had the same mean number of lesions. The addition of 0.5% cysteine to the diet containing 0.42% inorganic sulfate (Diet E) animals resulted in a reduction of lung lesion counts. The numbers were significantly ($P<0.05$) different as determined by a one-tailed t-test.

Pathological evaluation of slides prepared from the lungs revealed peribronchial lymphoid proliferation, alveolar wall thickening, edema, and iron deposits in peripheral areas of the lung. No evidence of murine pneumonia or carcinoma was noted with the tissue evaluated, although mild atypia was present.

The glutathione tissue levels measured in the livers of the rats in Group 1, Group 2, and a control group not treated with benzo(a)-pyrene are recorded in table 6. No significant differences were found

TABLE 5

Lesion Counts for Lungs Minus the Left Lobe
After 10 Intratracheal Instillations
of 3 mg Benzo(a)pyrene[1]

	Diet	No. of animals	No. of lesions lung minus left lobe[2]
A.	0.0002% $SO_4^=$	30	22 ± 17
D.	0.02% $SO_4^=$ + 0.5% cysteine	30	26 ± 19
B.	0.42% $SO_4^=$	29	39 ± 28

[1]For instillation, equal parts of benzo(a)pyrene and ferric oxide were ground together and suspended in sufficient 0.9% NaCl to make a concentration of 3 mg benzo(a)pyrene in 0.2 ml solution.

[2]Values are means ± standard deviations.

TABLE 6

Determination of Glutathione Tissue Levels in Rat Liver

Diets	No. of animals	Glutathione per g wet wt. tissue[1]
Benzo(a)pyrene treated		µg x 10^{-3}
Group 1		
A. 0.0002% $SO_4^=$	6	1.75 ± 0.03
D. 0.02% $SO_4^=$ + 0.5% cysteine	7	1.74 ± 0.02
B. 0.42% $SO_4^=$	8	1.77 ± 0.08
Group 2		
C. 0.0002% $SO_4^=$ + 0.5% cysteine	9	1.85 ± 0.08
D. 0.02% $SO_4^=$ + 0.5% cysteine	8	1.74 ± 0.09
E. 0.42% $SO_4^=$ + 0.5% cysteine	8	1.84 ± 0.08
Untreated		
D. 0.02% $SO_4^=$ + 0.5% cysteine	5	1.79 ± 0.16

[1]Values are means ± standard error of the mean.

among the means by a 1-way analysis of variance. However, a slight increase in glutathione concentration was noted in livers of animals consuming Diets C and E, which were supplemented with cysteine, compared with animals consuming Diets A and B not supplemented with cysteine. Liver glutathione concentrations for untreated rats consuming Diet D were higher than for those treated rats consuming the same diet.

Measurements of lung glutathione were made on the left lobe of each rat in Group 1, Group 2, and the control group untreated with benzo(a)pyrene. The results of the glutathione determinations made on Group 1 and the control, untreated group are recorded in table 7. Rats consuming Diet A had the highest tissue glutathione, 4.38 μg x 10^{-2}/g wet weight tissue, measured in this group. An intermediate level of glutathione (4.05 μg x 10^{-2}/g wet weight tissue) was found in Diet D animals. The lowest tissue glutathione measured in this group was 3.73 μg x 10^{-2}/g wet weight tissue in animals consuming Diet B. The differences between means were not significant for Group 1 using *t*-tests.

The mean glutathione tissue level of 5.62 μg x 10^{-2}/g wet weight tissue for Diet D animals not treated with benzo(a)pyrene was higher than the measures recorded for animals consuming any diet in Group 1. The difference was significant ($P<0.05$) when the latter group was compared with animals consuming Diet D using a one-tailed *t*-test.

Measurements of glutathione were made on the left lung lobe of rats treated with benzo(a)pyrene and fed diets to which 0.5% cysteine had been added. The data from these measurements are recorded in table 8. Although

TABLE 7

Determinations of Glutathione Tissue Levels in Rat Lungs

Diets	No. of animals	Glutathione per wet wt. tissue[1]
Benzo(a)pyrene treated		$\mu g \times 10^{-2}$
Group 1		
A. 0.0002% $SO_4^=$	6	4.38 ± 0.69
D. 0.02% $SO_4^=$ + 0.5% cysteine	7	4.05 ± 0.14[a2]
B. 0.42% $SO_4^=$	7	3.73 ± 0.52
Untreated		
D. 0.02% $SO_4^=$ + 0.5% cysteine	5	5.62 ± 0.87[a]

[1]Values are means ± standard error of the mean.

[2]Means followed the same superscript letter are significantly ($P<0.05$) different by a one-tailed *t*-test.

TABLE 8

Determination of Glutathione Tissue Levels in Rat Lungs
After Addition of Cysteine to the Diet

Diets	No. of animals	Glutathione per g wet wt. tissue[1]
Benzo(a)pyrene treated		$\mu g \times 10^{-2}$
Group 2		
C. 0.0002% $SO_4^=$ + 0.5% cysteine	9	3.51 ± 0.53
D. 0.02% $SO_4^=$ + 0.5% cysteine	8	3.88 ± 0.41
E. 0.42% $SO_4^=$ + 0.5% cysteine	6	3.64 ± 0.83

[1]Values are means ± standard error of the mean.

the highest mean glutathione level (3.88 μg x 10^{-2}/g wet weight tissue) was recorded for animals consuming Diet D, this mean was not significantly different from that of Diet C (3.51 μg x 10^{-2}/g wet weight tissue) or Diet E (3.64 μg x 10^{-2}/g wet weight tissue) animals as determined by a *t*-test. Yet, the glutathione value for Diet D animals was significantly ($P<0.05$) less than the glutathione value for Diet D with untreated rats (table 7, page 49) when compared using a one-tailed *t*-test.

CHAPTER V

DISCUSSION

The data in table 3, page 42 show rats fed 0.42% inorganic sulfate without added cysteine (Diet B) were more susceptible to benzo(a)-pyrene induced lesions than rats fed either the 0.0002% inorganic sulfate without added cysteine diet (Diet A) or the 0.02% inorganic sulfate plus 0.5% cysteine diet (Diet D). Although these data suggest that the number of lesions counted were directly proportional to the inorganic sulfate in the diet, repetition of the experiment (Group 1, table 4, page 44) did not duplicate these results. In this experiment the fewest number of lesions was associated with Diet D and the intermediate number of lesions was associated with Diet A. The lesion counts for Diet B remained the highest number in both studies. When the lung lesion data were considered together (table 5, page 46) the lung lesion counts again were proportional to the inorganic sulfate in the diet (table 3, page 42). These results suggest a relationship between diet and the production of lung lesions.

Work performed in this laboratory[20] and other laboratories (25,33) has shown that availability of cysteine in the diet of a rat is a determining factor in glutathione biosynthesis. Since glutathione conjugation

---

[20]Smith, J. T. (1977) Manuscript in preparation.

to benzo(a)pyrene is at least one documented pathway for detoxication of this carcinogen and since alteration of sulfur nutritional status had been shown to change tissue levels of glutathione, the availability of cysteine for glutathione biosynthesis in the lung is presented as a possible explanation for these results.

Additional evidence that availability of cysteine for glutathione biosynthesis was a factor in production of lung lesions is presented in table 4, page 44. All the diets in Group 2 were supplemented with 0.5% cysteine for this experiment. The addition of 0.5% cysteine to the diet resulted in a lower lesion count. The differences in lesion count in animals consuming the 0.42% inorganic sulfate plus 0.5% cysteine diet (Diet E) and the 0.42% inorganic sulfate diet without cysteine were significant ($P<0.05$) as determined using a one-tailed t-test. Addition of cysteine to the diet provided for increased biosynthesis of glutathione. Tateishi et al. (25) showed addition of cysteine to the protein-free diet of a rat for 12 hours following a 40 hour fast resulted in higher liver glutathione levels. The liver glutathione level was proportional to the quantity of added cysteine. Feeding an 18% gelatin-based diet with addition of cysteine also resulted in an increase in liver glutathione, although the increase was relatively smaller than the increase with the protein-free diet.

The increase in tissue glutathione could result in an increase in detoxication by glutathione conjugation. The increase in detoxication of benzo(a)pyrene would account for the decrease in counted lesions. This

idea is supported by previous work. Pietropaolo and Weinstein (59) showed, in vitro, addition of glutathione to rat liver microsomes resulted in a reduction of ^{3}H-benzo(a)pyrene binding to DNA, RNA, and synthetic polyribonucleotides. Liver necrosis, as induced by radioactive bromobenzene, was increased when liver glutathione was depleted with diethyl maleate and was decreased when cysteine was administered before bromobenzene (9).

The finding of mildly atypical cells in the lung tissue eleven weeks after the first benzo(a)pyrene treatment in this investigation agreed with the findings of other investigators (7,42,62,96) who showed progressive changes leading to carcinoma. Following intratracheal instillation with 3-methylcholanthrene, rats were found to have small tumor nodules as early as two weeks after five weekly instillations of 5 mg each. These nodules were macroscopically visible. Histologically, as early as four weeks after 5 weekly instillations of 5 mg each, "microgranulomas" of fibroblasts, histiocytes, and multinucleated giant cells were identified. The lesions were located in the bronchiolalveolar area where deposition of ferric oxide had taken place. At this time, although proliferative, the lesions were benign. After 22 weeks huge tumor masses and metastasis to lymph nodes of the diaphragm or thorax walls were found (97). A sequential study of cytological changes produced in hamsters treated with benzo(a)pyrene-ferric oxide for 14 weeks revealed only mild, moderate, or marked atypical cells up to 15 weeks. At 15 weeks and thereafter, conclusive diagnosis of malignancy was made (96).

Furthermore, pathologists vary considerably in their classification of lung cancer cellular types (2-42%). Classification of lung cancer cellular types on two successive readings of the same slide by the same pathologist varied from 2 to 20% (98). Although it is not therefore an irrefutable assumption, these observations make it reasonable to assume that these lesions were precancerous.

The data from measurement of liver glutathione levels are presented in table 6, page 47. No differences in mean glutathione tissue levels were found with the diets in Group 1. Group 2 tissue glutathione concentrations did not show differences in means. Comparison of Group 1 with Group 2 tissue glutathione concentrations show addition of 0.5% cysteine to the diet resulted in a slight increase in liver glutathione. When the rats were not treated with benzo(a)pyrene, liver glutathione levels were higher than for the treated rats fed the same diet. Although none of the liver glutathione data showed statistical significance, the results seem to indicate that dietary cysteine increases glutathione biosynthesis and that benzo(a)pyrene utilizes glutathione for detoxication.

Reports in the literature support these findings. Tateishi et al. (33) found maintenance of liver glutathione was not related to levels or activities of glutathione synthesizing enzymes. After a period of fasting to deplete glutathione, food intake (especially as it affected liver concentrations of cysteine) determined glutathione biosynthesis in refed rats. Administration of compounds which are

excreted as mercapturic acids also have been shown to lower liver glutathione in proportion to the quantity of mercapturic acid excreted (31).

Since glutathione is synthesized within the cells of all tissues and since evidence for transport of glutathione from one tissue to another is not conclusive (33), measurements of glutathione tissue levels were made on lung, the target tissue. The data are recorded in table 7, page 49. Although the means were not significantly different using *t*-tests, the highest level of glutathione was recorded for animals consuming Diet A, the medium level of glutathione for animals consuming Diet D, and the lowest level of glutathione for animals consuming Diet B. These data indicate limited availability of glutathione for detoxication reactions in rats fed a diet high in inorganic sulfate (Diet B). These same rats were more susceptible to benzo(a)pyrene stress than rats with higher tissue glutathione levels in the lung as indicated by the higher lesion count (table 4, page 44) for these same rats. Again an indication that availability of cysteine for glutathione synthesis is a factor in development of lung lesions after benzo(a)pyrene treatment was shown.

Comparison of the glutathione tissue levels of treated rats with untreated rats revealed a reduction of glutathione associated with benzo(a)pyrene treatment. The reduction was significant ($P<0.05$) for animals consuming Diet D (table 7, page 49) as determined using a one-tailed *t*-test. This reduction indicates glutathione is utilized in the detoxication of benzo(a)pyrene in the lung.

The glutathione tissue levels of lungs were measured in rats that had been fed diets supplemented with 0.5% cysteine, Group 2 (table 8, page 50).

Cysteine supplementation resulted in tissue glutathione concentrations which were approximately the same for animals fed all these diets. These results indicate biosynthesis of glutathione is dependent on availability of cysteine rather than inorganic sulfate in the diet. Utilization of glutathione for detoxication also is shown by the lower tissue levels in treated rats compared to untreated rats (table 7, page 49). The glutathione measured for treated Diet D fed rats is significantly ($P<0.05$) lower than the glutathione measured for the untreated rats fed the same diet (table 7, page 49) as determined by a one-tailed t-test.

Although rats consuming Diet E had significantly fewer ($P<0.05$) counted lesions than rats consuming Diet B (table 4, page 44), measurements of lung glutathione concentrations of animals fed the same diets did not show statistically significant differences when compared using a t-test. Enhanced utilization of glutathione for detoxication reactions may explain the lower than expected lung glutathione concentrations when lesion production had been significantly decreased. The glutathione S-transferase enzyme, which catalyzed the conjugation reaction, has been shown to exhibit a different kinetic mechanism in response to either low or high glutathione levels (41). Further study is needed to determine if the activity or mechanism of action of this enzyme is significantly altered by diet.

It is conceivable that the activity or level of any or all of the enzymes in the metabolic pathways of benzo(a)pyrene could be affected by diet. Dietary alteration of the activity of enzymes in the mixed-function oxidase system has been documented (99). Changes in the sulfur

nutritional status of a rat have been shown to alter the ratio of glucronic acid to sulfate conjugates of salicylamide in urine and alter activity of UDP-glucuronyltransferase.[21] The possibility that part of the detoxication of benzo(a)pyrene metabolites could take place through either of these mechanisms has been suggested (100). Falk et al. (101) presented evidence for benzo(a)pyrene conjugation with glucuronic acid and sulfate. Rats given ^{14}C-benzo(a)pyrene intravenously excreted benzo(a)pyrene metabolites in the bile as glucuronic or sulfate conjugates or as labile metabolites collected after mild acid treatment.

Further study is indicated to determine the effect these diets have on the activity of key enzymes in the benzo(a)pyrene metabolic pathway which may also be a factor in the animals response to carcinogenic insult.

---

[21] Kurzynske, J. S. (1975) Investigation of phenolic hydroxyl chemicals as model compounds for determining sulfur status. Unpublished PhD dissertation. The University of Tennessee, Knoxville.

CHAPTER VI

SUMMARY

Rats were fed diets differing in sulfur content and were treated by intratracheal instillations of a benzo(a)pyrene-ferric oxide mixture in a 0.9% NaCl solution. One week after 10 weekly instillations the animals were sacrificed. Lesions visible on the lungs after the lungs had been cleared were counted under low magnification. Lesion counts in animals which had received a diet low in inorganic sulfate (0.0002%) without added cysteine (Diet A) were not significantly different from those animals fed adequate inorganic sulfate (0.02%) with 0.5% cysteine added (Diet D). Animals which had been fed a diet high in inorganic sulfate (0.42%) without added cysteine (Diet B) showed higher lesion counts than those fed Diet A or D, but significant ($P<0.05$) for B compared to D only. Supplementation of all the diets with 0.5% cysteine reduced the lesion counts significantly ($P<0.05$) for the high inorganic sulfate diet (Diet E). Availability of cysteine for glutathione synthesis allowing detoxication of benzo(a)pyrene, is suggested as an explanation of these findings.

The glutathione levels of lung and liver then were measured for these animals. Although not statistically significant, liver glutathione measures were higher after addition of 0.5% cysteine to both the low and high inorganic sulfate diets. Lung glutathione levels for animals consuming Diets A, B, and D were not significantly different. Animals fed

Diet B did have the largest number of counted lung lesions and the lowest lung glutathione level. These data seemed to show that availability of cysteine for glutathione biosynthesis was a factor in lung lesion susceptibility of rats. After addition of 0.5% cysteine to all the diets no differences in lung glutathione levels were noted, although the animals fed Diet E had shown a significantly lower lesion count. This diet may have altered the activity of key enzymes in the metabolic pathway of benzo(a)pyrene and enhanced the utilization of glutathione for detoxication reactions.

Since glutathione conjugation to benzo(a)pyrene metabolites is a documented pathway for detoxication and since alteration of sulfur nutritional status, especially availability of cysteine, has been shown to alter tissue levels of glutathione, these results suggest that availability of cysteine in the diet of a rat for glutathione synthesis is a factor in the susceptibility of a rat to lung lesions produced by intratracheal instillation of benzo(a)pyrene. The diet high in inorganic sulfate with added cysteine was an exception. This diet may protect the animal by alteration of enzyme activity.

LITERATURE CITED

LITERATURE CITED

1. _____ (1971) Facts on the major killing and crippling diseases in the United States today. pp. 1-22, The National Health Education Committee, Inc., N. Y.

2. _____ (1976) World Health Statistics Annual 1973-6, Vol. 1, Vital Statistics and Causes of Death. pp. 340, WHO, Genéve.

3. _____ (1974) Mortality trends for leading causes of death. United States 1950-69. In: DHEW Pub. No. (HRA) 74-1853 Series 20, No. 16. pp. 12-13, U. S. DHEW Public Health Service, Rockville, Md.

4. Shabad, L. M. (1962) Experimental cancer of the lungs. Fed. Proc. 22, T331-T335.

5. Hegsted, D. M. (1975) Relevance of animal studies to human disease. Cancer Res. 35, 2537-2539.

6. Shabad, L. M. (1971) Dose-response studies in experimentally induced lung tumors. Environmental Res. 4, 305-315.

7. Schreiber, H., Martin, D. H. & Pazmiño, N. (1975) Species differences in the effect of benzo(a)pyrene-ferric oxide on the respiratory tract of rats and hamsters. Cancer Res. 35, 1654-1661.

8. Cavalieri, E. & Calvin, M. (1971) Molecular characteristics of some carcinogenic hydrocarbons. Proc. Nat. Acad. Sci. USA 68, 1251-1253.

9. Jerina, D. M. & Daly, J. W. (1974) Arene oxides: A new aspect of drug metabolism. Science 185, 573-582.

10. Umans, R. S., Lesko, S. A. & Ts'o, P. O. P. (1969) Chemical linkage of carcinogenic 3,4-benzpyrene to DNA in aqueous solution induced by peroxide and iodine. Nature 221, 763-764.

11. Nemoto, N., Gelboin, H. V., Habig, W. H., Ketley, J. N. & Jakoby, W. B. (1975) K-region benzo(a)pyrene-4,5-oxide is conjugated by homogeneous glutathione S-transferases. Nature 255, 512.

12. Kaplowitz, N., Kuhlenkamp, J. & Clifton, G. (1975) Drug induction of hepatic glutathione S-transferases in male and female rats. Biochem. J. 146, 351-356.

13. Pal, K., Grover, P. L. & Sims, P. (1975) The metabolism of carcinogenic polycyclic hydrocarbons by tissues of the respiratory tract. Biochemical Society Transactions 3, 174-175.

14. Tannenbaum, A. & Silverstone, H. (1953) Nutrition in relation to cancer. In: Advances in Cancer Research, Vol. 1 (Greenstein, J. P. & Haddow, A., eds.), pp. 474-476, Academic Press Inc., Pub., N. Y.

15. Michels, F. G. & Smith, J. T. (1965) A comparison of the utilization of organic and inorganic sulfur by the rat. J. Nutr. 87, 217-220.

16. Smith, J. T. (1973) An optimal level of inorganic sulfate for the diet of a rat. J. Nutr. 103, 1008-1011.

17. Finkelstein, J. D. (1970) Control of sulfur metabolism in mammals. In: Symposium: Sulfur in Nutrition. (Muth, O. H. & Oldefield, J. E., eds.), pp. 46-58, Avi Pub. Co., Inc., Wesport, Conn.

18. Kun, E. (1961) The metabolism of sulfur-containing compounds. In: Metabolic Pathways. 2nd Ed. Vol. II. (Greenberg, D. M., ed.), pp. 237-261, Academic Press, N. Y.

19. Button, G. M., Brown, R. G., Michels, F. G. & Smith, J. T. (1965) Utilization of calcium and sodium sulfate by the rat. J. Nutr. 87, 211-216.

20. Brown, R. G., Button, G. M. & Smith, J. T. (1965) Changes in collagen metabolism caused by feeding diets low in inorganic sulfur. J. Nutr. 87, 228-232.

21. Feland, B., Fuqua, E. G. & Smith, J. T. (1973) Effect of dietary sulfur on serum cholesterol and the glycocholic:taurocholic acid ratio of the rat. J. Nutr. 103, 1561-1565.

22. Whittle, B. & Smith, J. T. (1974) Effect of dietary sulfur on taurine excretion by the rat. J. Nutr. 104, 666-670.

23. Swendseid, M. E. & Wang, M. (1970) Sulfur requirements of humans. In: Symposium: Sulfur in Nutrition. (Muth, O. H. & Oldfield, J. E., eds.), pp. 209-221, Avi Pub. Co., Inc., Wesport, Conn.

24. Stegink, L. D. & Besten, L. D. (1972) Synthesis of cysteine from methionine in normal adult subjects: effect of route of alimentation. Science 178, 514-516.

25. Tateishi, N., Higashi, T., Naruse, A., Nakashima, K., Shiozaki, H. & Sakamoto, Y. (1977) Rat liver glutathione: possible role as a reservoir of cysteine. J. Nutr. 107, 51-60.

26. Lombardi, B., Pani, P., Schlunk, F. F. & Shi-Hua, C. (1969) Labeling of liver and plasma lecithins after injection of 1-2^{14}C-2-dimethylaminoethanol and ^{14}C-methionine-methyl to choline deficient rats. Lipids 4, 67-75.

27. Young, L. & Maw, G. A. (1958) Thiols and disulphides. In: The Metabolism of Sulfur Compounds. pp. 24, John Wiley & Sons, Inc., N. Y.

28. Meister, A. (1974) Biosynthesis and utilization of glutathione: γ-glutamyl cycle and its function in amino acid transport. In: Glutathione, Proc. Conf. Ger. Soc. Biol. Chem. 16th 1973. (Flohé, L., ed.), pp. 56-67, Theime, Stuttgard, Ger.

29. Waetsch, H. & Rittenberg, D. (1942) Glutathione: II. The metabolism of glutathione studied with isotopic ammonia and glutamic acid. J. Biol. Chem. 144, 53-58.

30. Tabachnick, M. & Tarver, H. (1955) The conversion of methionine-^{35}S to cystathionine-^{35}S and taurine-^{35}S in the rat. Arch. Biochem. Biophys. 56, 115-122.

31. Meister, A. (1975) Biochemistry of glutathione. In: Metabolic Pathways, 3rd. (Greenberg, D. M., ed.), pp. 7, 101-188, Academic Press, N. Y.

32. Richman, P. G. & Meister, A. (1975) Regulation of γ-glutamyl cysteine synthetase by nonallosteric feedback inhibition by glutathione. J. Biol. Chem. 250, 1422-1426.

33. Tateishi, N., Higashi, T., Shinya, S., Naruse, A., & Sakamoto, Y. (1974) Studies on the regulation of glutathione level in rat liver. J. Biochem. 75, 93-103.

34. Jaeger, R. J., Conolly, R. B. & Murphy, S. D. (1973) Diurnal variation of hepatic glutathione concentration and its correlation with 1,1-dichloroethylene inhalation toxicity in rats. Res. Commun. Chem. Pathol. Pharmaco. 6, 465-471.

35. Boyland, E. & Chasseaud, L. F. (1969) The role of glutathione and glutathione S-transfereases in mercapturic acid biosynthesis. In: Adv. in Enzymology Vol. 32. (Nord, F. F., ed.), pp. 173-219, Interscience Pub., N. Y.

36. Meister, A. (1974) Glutathione; metabolism and function via the γ-glutamyl cycle. Life Sci. 15, 177-190.

37. Smith, J. T. & Wood, J. L. (1959) Mercapturic acids from tissue proteins. J. Biol. Chem. 234, 3192-3195.

38. Habig, W. H., Pabst, M. J. & Jakoby, W. B. (1974) Glutathione S-transferases. J. Biol. Chem. 249, 7130-7139.

39. Clifton, G., Kaplowitz, N., Wallin, J. D. & Kuhlenkamp, J. (1975) Drug induction and sex differences of renal glutathione S-transferases in the rat. Biochem. J. 150, 259-262.

40. Mukhtar, H. & Bresnick, E. (1976) Glutathione-S-epoxide transferase activity during development and the effect of partial hepatectomy. Cancer Res. 36, 937-940.

41. Pabst, M. J., Habig, W. H. & Jakoby, W. B. (1974) Glutathione S-transferase A. J. Biol. Chem. 249, 7140-7150.

42. Nettesheim, P. & Schreiber, H. (1975) Advances in experimental lung cancer research. In: Handbuch der Allgemeinen Pathologie VI/7 Geschwrelste Tumors III. Models of Experimental Carcinogenesis. (Grundmann, E., ed.), pp. 603-691, Springer-Verlag, N. Y.

43. Karbe, E. & Park, J., eds. (1974) Experimental Lung Cancer: Carcinogenesis and Bioassays. pp. 1-611, Springer-Verlag, N. Y.

44. Saffiotti, U. (1969) Experimental respiratory tract carcinogenesis. In: Prog. in Experi. Tumor Research, Vol. 11. (Homburger, F., ed.), pp. 306-311, Skarger, N. Y.

45. Kennedy, A. R. & Little, J. B. (1975) Localization of polycyclic hydrocarbon carcinogens in the lung following intratracheal instillation in gelation solution. Cancer Res. 35, 1563-1567.

46. Henry, M. C., Port, C. D. & Kaufman, D. G. (1975) Importance of physical properties of benzo(a)pyrene-ferric oxide mixtures in lung tumor induction. Cancer Res. 35, 207-217.

47. Henry, M. C., Port, C. D. & Kaufman, D. G. (1974) Role of particles in respiratory carcinogenesis bioassay. In: Experimental Lung Cancer: Carcinogenesis and Bioassays. (Karbe, E. & Park, J., eds.), pp. 173-185, Springer, N. Y.

48. Creasia, D. A. & Nettesheim, P. (1974) Respiratory cocarcinogenesis studies with ferric oxide: a test case of current experimental models. In: Experi. Lung Cancer: Carcinogenesis and Bioassays. (Karbe, E. & Park, J., eds.), pp. 234-245, Springer, N. Y.

49. Campbell, J. A. (1937) The effects of road dust "freed" from tar products upon the incidence of primary lung-tumors of mice. Brit. J. Experi. Pathology 18, 215-223.

50. Miller, J. A. (1970) Carcinogenesis by chemicals: an overview---G.H.A. Clowes memorial lecture. Cancer Res. 30, 559-576.

51. Arcos, J. C. & Argus, M. F. (1974) Chemical Induction of Cancer. Vol. IIA. Academic Press, N. Y.

52. Jones, D. W. & Matthews, R. S. (1974) Carcinogenicity and structure in polycyclic hydrocarbons. In: Progress in Medicinal Chemistry 10. (Ellis, G. P. & West, G. B., eds.), pp. 159-203, Amer. Elsevier Pub. Co., Inc., N. Y.

53. Gelboin, H. V., Kinoshita, N. & Wiebel, F. J. (1972) Microsomal hydroxylases: induction and role in polycyclic hydrocarbon carcinogenesis and toxicity. Fed. Proc. 31, 1298-1309.

54. Wiebel, F. J. & Gelboin, H. V. (1974) Enzyme induction and polycyclic hydrocarbon metabolism in cell culture, experimental animals and man. In: Chemical Carcinogenesis Essays, Proc. Workshop 1973. pp. 57-82, IARC Sci. Publ. 10.

55. Rasmussen, R. E. & Wang, I. Y. (1974) Dependence of specific metabolism of benzo(a)pyrene on the inducer of hydroxylase activity. Cancer Res. 34, 2290-2295.

56. Mannering, G. J. (1971) Properties of cytochrome P-450 as affected by environmental factors: qualitative changes due to administration of polycyclic hydrocarbons. Metabolism 20, 228-245.

57. Burke, M. D. & Prough, R. A. (1976) Some characteristics of hamster liver and lung microsomal aryl hydrocarbon (biphenyl and benzo(a)pyrene) hydroxylation reactions. Biochem. Pharmacol. 25, 2187-2195.

58. Prough, R. A., Patrizi, V. M. & Estabrook, R. W. (1976) The direct spectrophotometric observation of benzo(a)pyrene phenol formation by liver microsomes. Cancer Res. 36, 4439-4443.

59. Pietropaolo, C. & Weinstein, I. B. (1975) Binding of [^{3}H]-benzo(a)-pyrene to natural and synthetic nucleic acids in a subcellular microsomal system. Cancer Res. 35, 2191-2198.

60. Weber, R. P., Coon, J. M. & Triolo, A. J. (1974) Effect of the organophosphate insecticide parathion and its active metabolite paraoxon on the metabolism of benzo(a)pyrene in the rat. Cancer Res. 34, 947-952.

61. Carlson, R. P. & Ciaccio, E. (1975) Effect of benzo(a)pyrene induction of liver and lung metabolism in adjuvant-diseased rats. Biochemical Pharmacol. 24, 1893-1895.

62. Flaks, A. & Sims, P. (1975) The activities of some polycyclic hydrocarbons and their "K-region" epoxides in an in vitro - in vivo carcinogenicity test system. Brit. J. Cancer 32, 604-609.

63. Grover, P. L., Hewer, A. & Sims, P. (1974) Metabolism of polycyclic hydrocarbons by rat-lung preparations. Biochem. Pharmacol. 23, 323-332.

64. Yang, S. K., McCourt, D. W., Roller, P. P. & Gelboin, H. V. (1976) Enzyme conversion of benzo(a)pyrene leading predominantly to the diol-epoxide r-7,t-8-dihydroxy-t-9,10-oxy-7,8,9,10-tetrahydrobenzo(a)pyrene through a single enantioner of r-7,t-8-dihydroxy-7,8-dihydrobenzo(a)pyrene. Proc. Natl. Acad. Sci. USA 73, 2594-2598.

65. Grover, P. L., Hewer, A., Pal, K. & Sims, P. (1976) The involvement of a diol-epoxide in the metabolic activation of benzo(a)pyrene in human bronchial and mouse skin. Int. J. Cancer 18, 1-6.

66. Yang, S. D. & Gelboin, H. V. (1976) Microsomal mixed-function oxidases and epoxide hydratase convert benzo(a)pyrene stero-specifically to optically active dihydroxydihydrobenzo(a)pyrenes. Biochem. Pharmacol. 25, 2221-2225.

67. Wood, A. W., Wislocki, P. G., Chang, R. L., Levin, W., Lee, A. Y. H., Yagi, H., Hernandez, O., Jerina, D. M. & Conney, A. H. (1976) Mutagenicity and cytotoxicity of benzo(a)pyrene benzo-ring epoxides. Cancer Res. 36, 3358-3366.

68. Yang, S. K. & Gelboin, H. V. (1976) Nonenzymatic reduction of benzo(a)pyrene diol-epoxides to trihydroxypentahydrobenzo(a)pyrenes by reduced NADPH. Cancer Res. 36, 4185-4189.

69. Flesher, J. W., Harvey, R. G. & Sydnor, K. L. (1976) Oncogenicity of K-region epoxides of benzo(a)pyrene and 7,12-dimethylbenz(a)-anthracene. Int. J. Cancer 18, 351-353.

70. Grover, P. L. (1974) K-region epoxides of polycyclic hydrocarbons: formation and further metabolism by rat-lung preparations. Biochem. Pharmacol. 23, 333-343.

71. Grover, P. L. (1974) Polycyclic hydrocarbon epoxides formation and further metabolism by animal and human tissues. In: Chemical Carcinogenesis Essays, Proc. Workshops, 1973. pp. 83-107, IARC Sci. Publ. 10.

72. Vainio, H., Uotila, P., Hartiala, J. & Pelkonen, O. (1976) The fate of intratracheally installed benzo(a)pyrene in the isolated perfused rat lung of both control and 20-methylcholanthrene pretreated rats. Res. Commun. Chem. Pathol. Pharmacol. 13, 259-271.

73. Cohen, G. M. & Moore, B. P. (1976) Metabolism of [^{3}H]- benzo(a)-pyrene by different portions of the respiratory tract. Biochem. Pharmacol. 25, 1623-1629.

74. Huberman, E. (1974) Cell-mediated mutagenesis of mammalian cells with carcinogenic polycyclic hydrocarbons. In: Chemical Carcinogenesis Essays, Proc. Workshop, 1973. pp. 137-146. IARC Sci. Publ. 10.

75. Felton, J. S. & Nebert, D. W. (1975) Mutagenesis of certain activated carcinogens in vitro associated with genetically mediated decreases in monooxygenase activity and cytochrome P_1-450. J. Biol. Chem. 250, 6769-6778.

76. Wislocki, P. G., Wood, A. W., Chang, R. L., Levin, W., Yagi, H., Hernandez, O., Dansette, P. M., Jerina, D. M. & Conney, A. H. (1976) Mutagenicity and cytotoxicity of benzo(a)pyrene arene oxides, phenols, quinones and dihydrodiols in bacterial and mammalian cells. Cancer Res. 36, 3350-3357.

77. Raha, C. R., Gallagher, C. H., Shubik, P. & Peratt, S. (1976) Covalent binding to protein of the K-region oxide of benzo(a)-pyrene formed by microsomal incubation. J. Natl. Cancer Inst. 57, 33-38.

78. King, H. W., Osborne, M. R., Beland, F. A., Harvey, R. G. & Brookes, P. (1976) (±)7α, 8β-dihydroxy-9β, 10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene is an intermediate in the metabolism and binding to DNA of benzo(a)pyrene. Proc. Natl. Acad. Sci. USA 73, 2679-2681.

79. Weinstein, I. B., Jeffrey, A. M., Jennette, K. W., Blobstein, S. H., Harvey, R. G., Harris, C., Autrup, H., Kasai, H. & Nakanishi, K. (1976) Benzo(a)pyrene diol epoxides as intermediates in nucleic acid binding in vitro and in vivo. Science 193, 592-595.

80. Koreida, M., Moore, P. D., Yagi, H., Yeh, H. J. C. & Jerina, D. M. (1976) Alkylation of polyguanylic acid at the 2-amino group and phosphate by the potent mutagen (±)-7α, 8β- dihydroxy-9β, 10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene. J. Am. Chem. Soc. 98, 6720-6722.

81. Meehan, T., Straub, K. & Calvin, M. (1976) Elucidation of hydrocarbon structure in enzyme-catalyzed benzo(a)pyrene-poly(G) covalent complex. Proc. Natl. Acad. Sci. USA 73, 1437-1441.

82. King, H. W. S., Thompson, M. H. & Brookes, P. (1976) The role of 9-hydroxybenzo(a)pyrene in microsomal mediated binding of benzo(a)pyrene to DNA. Int. J. Cancer 18, 339-344.

83. Booth, J. & Sims, P. (1976) Different paths involved in the metabolism of the 7,8- & 9,10-dihydrodiols of benzo(a)pyrene. Biochem. Pharmacol. 25, 979-980.

84. Boyland, E. (1974) Detoxication of reactive metabolites. Biochemical Society Transactions 2, 167-173.

85. Hubbell, R. G., Mendel, L. B. & Wakeman, A. J. (1937) A new salt mixture for use in experimental diets. J. Nutr. 14, 273-285.

86. Saffiotti, U., Cefis, F. & Klob, L. H. (1968) A method for the experimental induction of bronchogenic carcinoma. Cancer Res. 28, 104-124.

87. Adriani, J. (1970) The Pharmacology of Anesthetic Drugs: A Syllabus for Students and Clinicians, 5th ed. pp. 102, 104, Charles C. Thomas Pub., Springfield, Ill.

88. Schreiber, H., Nettesheim, P., Lijinsky, W., Richter, C. B. & Walburg, H. E. (1972) Induction of lung cancer in germfree, specific-pathogen-free and infected rats by N-nitrosoheptamethyleneimine: enhancement by respiratory infection. J. Natl. Cancer Inst. 49, 1107-1114.

89. Wattenberg, L. W. (1973) Inhibition of chemical carcinogen-induced pulmonary neoplasia by butylatedhydroxyanisole. J. Natl. Cancer Inst. 50, 1541-1544.

90. Jones, R. M. (1966) Basic Microscopic Techniques. pp. 272-273, University of Chicago Press, Chicago.

91. Gude, W. D. (1968) Autoradiographic Techniques Localization of Radioisotopes in Biological Material. pp. 26, Prentice-Hall Inc., Englewood Cliffs, N. Y.

92. Patterson, J. W. & Lazarow, A. (1955) Determination of glutathione. In: Methods of Biochemical Analysis Vol. 2 (Glick, D., ed.), pp. 270-273, Interscience Publishers, Inc., N. Y.

93. Umbreith, W. W., Burris, R. H. & Stauffer, J. F. (1972) Manometric Techniques and Tissue Metabolism. Burgess Publishing Co., N. Y.

94. Sokal, R. R. & Rohlf, F.J. (1969) Biometry: The Principles and Practice of Statistics in Biological Research. W. H. Freeman and Co., San Francisco.

95. Rohlf, F. J. & Sokal, R. R. (1969) Statistical Tables. W. H. Freeman and Co., San Francisco.

96. Stenback, F. (1974) Morphogenesis of experimental lung tumors in hamsters: The effects of carrier dust. In: Experimental Lung Cancer. Carcinogenesis and Bioassay. (Karbe, E. & Park, J., eds.), pp. 161-172, Springer-Verlag, N. Y.

97. Schreiber, H., Nettesheim, P. & Martin, D. H. (1972) Rapid development of bronchiolo-alveolar squamous cell tumors in rats after intratracheal injection of 3-methylcholantrene. J. Natl. Cancer Inst. 49. 541-554.

98. Feinstein, A. R., Gelfman, N. A. & Yesner, R. (1970) Observer variability in the histopathologic diagnosis of lung cancer. Amer. Review of Resp. Disease 101, 671-684.

99. Wattenberg, L. W. (1975) Effects of dietary constituents on the metabolism of chemical carcinogens. Cancer Res. 35, 3326-3331.

100. Sims, P. & Grover, P. L. (1974) Epoxides in polycyclic aromatic hydrocarbon metabolism and carcinogenesis. In: Advances in Cancer Research Vol. 20. (Klein, G., Weinhouse, S. & Haddow, A., eds.), pp. 166-274, Academic Press, N. Y.

101. Falk, H. L., Kotin, P., Lee, S. S. & Nathan, A. (1962) Intermediary metabolism of benzo(a)pyrene in the rat. J. Natl. Cancer Inst. 28, 699-724.

VITA

Evelyn Ann Settle, daughter of Mary Avis and George F. Settle, was born in the city of Corinth, Mississippi, September 20, 1946. She was graduated from Biggersville High School in 1964. She enrolled in Northeast Mississippi Junior College, Booneville, Mississippi, in 1964. In the fall of 1965 she enrolled in Mississippi State College for Women. She received a Bachelor of Science degree in Home Economics from Mississippi State College for Women in 1968. She completed a dietetic internship at Vanderbilt University Hospital, Nashville, Tennessee, in August 1969, at which time she became a member of the American Dietetic Association and a registered dietitian. From September 1969 to May 1974 she was employed as a therapeutic dietitian at Baptist Memorial Hospital, Memphis, Tennessee. In June 1974 she enrolled as a graduate student in the College of Home Economics, University of Tennessee, Knoxville. She received a Doctor of Philosophy degree from the University of Tennessee, Knoxville, December 1977.