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Study of an analytical method for benzo(a)pyrene metabolites in human urine

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ABSTRACT

Title of Thesis: STUDY OF AN ANALYTICAL METHOD FOR BENZO(A)PYRENE METABOLITES IN HUMAN URINE

Zheng Ouyang, Master of Science in Environmental Science, 1989

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A method developed by Becher and Bjorseth for analysis of PAH metabolites in urine was employed to investigate human exposure to benzo(a)pyrene (BaP) - an ubiquitous environmental carcinogen. Preliminary results are presented showing the relationship between exposure to BaP and urinary elimination. Although the correlation between the two variables is not statistically significant, there appears to be a positive association with selected exposure varibles such as smoking.

The identification of an association may establish urinary BaP as a marker of exposure.

However, detailed study of the analytical procedure indicated that recoveries with this method were as low as 3-8% for the total procedure and uncertainties in the urinary BaP determination are likely to be high due to the low recoveries of the method. Iodine formed during the reduction reaction was found to be responsible for the low recovery of BaP and attempts were made to improve recoveries. The chemical treatment of the sample was modified to include red phosphorus which is believed to quench free iodine and prevent its reaction with the parent BaP. Pyrene and pyrene metabolites had acceptable recoveries with the Becher/Bjorseth procedure.

These improved detection techniques were developed to make these compounds usful as biomarkers for assessment of environmental human exposure to polycyclic aromatic hydrocarbons.

STUDY OF AN ANALYTICAL METHOD FOR BENZO(A)PYRENE METABOLITES IN HUMAN URINE

by

ZHENG OUYANG

Thesis submitted to the Faculty of the Graduate School of
the New Jersey Institute of Technology
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of
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CHAPTER 1. INTRODUCTION

Polynuclear Aromatic Hydrocarbons (PAHs) are substances composed of two or more fused benzene rings. The most commonly studied PAH found in the environment include anthracene, benzo(a)pyrene (BaP), benz(a)anthracene, fluoranthene, indeno(1,2,3-cd)pyrene, phenanthrene, perylene, and pyrene. Many other PAH have also been reported[1,2].

PAH are of concern in the environment not only because they are ubiquitous contaminants but also many, particularly BaP, are well-documented carcinogens (Figure 1 shows structures of 12 carcinogenic PAH). Studies of occupational hazards related to coal tar pitch volatiles in general and PAH in particular have a long history. The association of these compounds with the development of cancer has been recognized for more than 200 years[1,2]. Analytical studies show that PAH appear in a large number of industrial processes, mainly due to high temperature treatment of coal tar and pitch as well as incomplete combustion or pyrolysis of organic marterial in general. Furthermore, biological and toxicological studies show that many PAH compounds exhibit carcinogenic effects in experimental animals. By 1976, more than 30 PAH compounds and several hundred PAH derivatives were reported to have carcinogenic effects[1-3], making PAH the largest class of chemical carcinogens known today.

In order to assess the nature of PAH hazards to humans, most analytical investigations have been concentrated on samples from the atmosphere, automobile exhausts, cigarette smoke potable water and human food. Table 1 presents estimates of human exposure to benzo(a)pyrene.

TABLE 1 Estimation of Daily Human Exposure to Benzo(a)pyrene[a]

Food	: 1- 3 ug
	: 2-16 ug
	: <0.5 ug
	: 0.05 ug
Water < 50 ng/l	: 0.05 ug
Air pollution 0-30 ng/m3	: 0.45 ug
Smoking 20 cigarettes	: 0.4 ug
Daily intake	: 0.5-16 ug

a. See reference 4 and sources cited therein.

Air sampling is of limited value since it dose not measure human uptake. Biological monitoring has been successfully used to obtain information on the uptake of hazardous chemicals in the bodies of exposure workers, and to evaluate the relationship to airborne levels[5-7].

The research described in this thesis deals with the study of benzo(a)pyrene in human urine and examination of the method of assay to develop a representive indicator of the burden of exposure population to PAH.

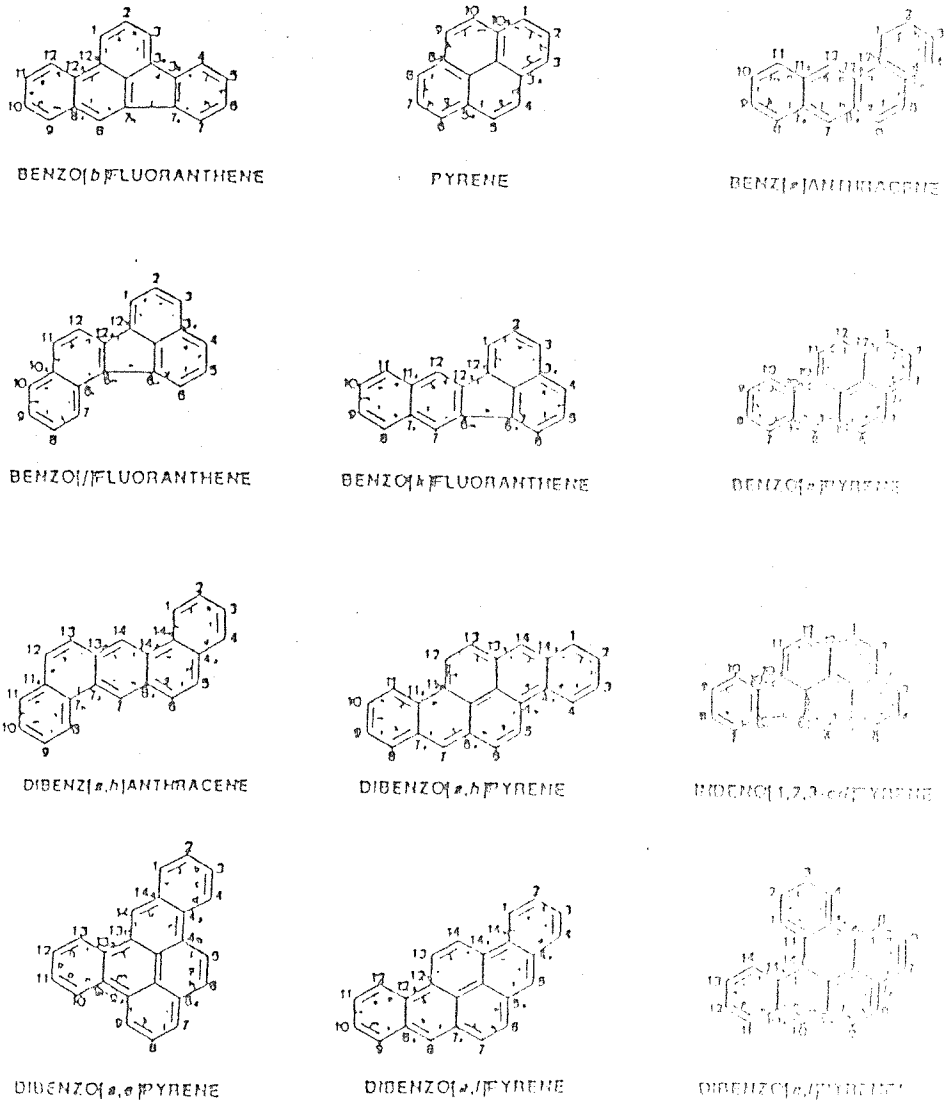

Figure 1 Molecular structure of selected PAH

1.1 OCCURRENCE OF BENZO(a)PYRENE IN THE ENVIRONMENT

PAH are primarily products of the incomplete combustion of organic materials such as forest fires or the burning of fossil fuels. As is to be expected from such commonly available source materials, PAH are ubiquitous in the environment, with higher concentrations reported near urban and industrial areas. Table 2 lists benzo(a)pyrene concentrations in the atmospheres of some cities in the United States.

Table 2 BaP in The Atmosphere of Some Cities in U. S.[a]

CITY	YEAR	CONCENTRATION(ng/m^3)
100 large urban communities	1958-1959	6.6
	1962	5
32 large urban stations	1966-1967	3.3
	1968	2.7
	1969	2.9
	1970	2
Los Angeles	1971-1972	1.1, 0.5, 3.5, 0.03
New York City	1979	3.0-4.4

a. Source: Sawicki(1976)[8].

PAH can be formed by thermal decomposition of any organic materials containing carbon and hydrogen. Important

industrial sources of PAH include coal gasification and liquification processes, combustion of fossil fuels(gasoline, kerosene, coal, natural gas, diesel fuel), waste incineration, automobile emissions as well as production of coke, carbon black, coal tar pitch, asphalt and petroleum cracking. Other sources of PAH which may be important in terms of human exposure are foods and cigarette smoking.

1.2. POTENTIAL ROUTES OF RELEASE TO THE ENVIRONMENT

a. To The Atmosphere

The formation of PAH compounds during the incomplete combustion of organic matter represents the major source of such compounds in the atmosphere. The principal sources of PAH in the atmosphere are: (a) Coal and oil fired power stations. (b) Domestic heating. (c) Miscellaneous industrial processes. (d)Automobile emissions. (e)Cigarette smoke, and (f) Forest fires and volcanic activity. From these sources, PAH will be released directly into the atmosphere.

b. To Aquatic Systems

PAH gain entry to aquatic systems through several routes, including precipitation and fallout from the atmosphere, wastewater discharges (residential and industrial), runoff from asphalt-covered surfaces(e.g. roads), and oil spills.

Waste effluents of certain industrial processes, for example electrolytic reduction of magnesium and ferro alloy smelting, provide a direct route of entry for PAH to aquatic ecosystems.

1.3 HUMAN INTAKE, DISTRIBUTION, AND EXCRETION OF PAH

The intake of PAH, their distribution in the organism, and their excretion are dependent on numerous physiological, physical, and chemical factors which have still not been sufficiently elucidated. Nevertheless, it is possible to make limited assumptions based on the results of animal studies conducted with several PAH, particularly BaP, a well-known animal carcinogen.

PAH, once adsorbed, becomes localized in a wide variety of body tissues . The distribution of radioactivity derived from ^{14}C-BaP in the rat and mouse was determined following subcutaneoous, intravenous, and intratracheal administration.[10]

Table 3 DISTRIBUTION AND ELIMINATION OF RADIOACTIVITY AFTER INTRATRACHEAL INSTILLATION OF ^{14}C-BaP INTO RATS[a]

Site	% Of administered dose after: 1 hr	24 hr
Feces	0	28.0
Urine	0	1.2
Stomach	0	0
Intestine	37.0	11.1
Kidney	0	2.4
Liver	1.3	4.3
lung	43.2	38.6
Other organs	0.08	0.43
% Recovered	81.58	86.03

a. Dose: 25 ug ^{14}C-BaP in 0.3 ml H_2O.

Adapted from Kotin, P., Falk, H.L., and Busser, R., J.Natl. Cancer Inst., 23, 541, 1959.

The pattern of distribution was found to be similar in all cases, except for high local pulmonary concentrations following intratracheal instillation (Table 3). Concentrations of BaP-derived radioactivity in the liver reached a maximum

within only 10 min after injection and represented 12% of the total dose. Radioactivity in the liver was reduced to 1 to 3% of the administered dose within 24 hr. Similarly, maximum blood levels of BaP following i.v. injection were reached very quickly, and radioactivity became barely detectable after 10 min. Animal tissue localization of BaP and/or its metabolites occurred in the spleen, kidney, lung, and stomach; maximum radioactivity derived from labeled BaP was recovered in the bile and feces. Levels of radioactivity in fat, skin, and muscle were not determined, nor was the amount of unchanged BaP measured in any tissue. Bock and Dao (1961)[11] showed that relative to other tissues, unmetabolized BaP was located extensively in the mammary gland and general body fat after a single feeding of the carcinogen(10-30 mg). This accumulation of BaP was greater than that resulting from administration of 3-methylchlol-anthrene, 7,12-dimethylbenz(a)anthracene, or phenanthrene. In all cases, the level of carcinogens detected in the tissue was directly related to the dose administered, and was dependent upon the use of a lipid vehicle.

In summary, the results of the studies indicate the following:

a. Detectable levels of PAH and/or PAH metabolites can be observed in most internal organs from minutes to hours after administration.

b. Mammary and other fatty tissues are significant storage depots where PAH may accumulate and be slowly released.

c. The gut contains relatively high levels of PAH and/or PAH metabolites as a result of hepatobiliary excretion or of the ingestion of particulate PAH following mucociliary clearance after inhalation.

Hepatobiliary excretion and elimination through the feces is the major route by which PAH are removed from the body. Kotin et al. [10] observed that 4 to 12% of the subcutaneously injected dose of BaP was eliminated in the urine of mice within 6 days after injection, while 70% to 75% of the dose was recovered in the feces. Less than 1% of BaP recovered in the bile was unmetabolized. Camus et al [12] determined fecal excretion rates of BaP in two different strains of mice following i.p. injection of BaP. The cumulative excretion of BaP in feces followed an exponential curve with half-lives of 1.2 and 0.6 days for the two different strains. The majority of metabolites in the urine appeared as highly water -soluble conjugates. For example, the relative amount of unmetabolized BaP found in urine from mice following i.p. injection of ^{14}C-labeled BaP was as low as 0.7% of the total amount excreted in urine.[13] After enzymatic deconjugation, various oxidized BaP metabolites could be identified.

1.4 METABOLISM AND ACTIVATION OF PAH

PAH are among the chemical carcinogens which are not chemically highly reactive themselves, but exert their carcinogenic activity through metabolites which are sufficiently reactive to modify cellular macromolecules such as nucleic acids (DNA, RNA) and proteins.[14]

Metabolism is dominated by oxidation through the microsomal mixed-function oxidase (MFO) system, often termed aryl hydrocarbon hydroxylase (AHH), which is most abundant in the liver. This enzyme system has been studied extensively and is the subject of several reviews.[15,16] While it is known that this enzyme complex is involved with detoxification of xenobiotics in conjunction with various P-450-type cytochromes, it is apparent that this system is also responsible for the metabolism of polycyclic hydrocarbons to their active species (Figure 2).

The first step in this metabolic activation of PAH, catalyzed by the cytochrome P-450 monooxygenase system, gives rise to epoxide and phenolic groups in different positions on the polycyclic ring system.[17] A second microsomal enzyme, epoxide hydrolase (EH), converts epoxides into vicinal diols.

Information on EH has been summarized recently,[18] and its importance in the formation of three known dihydrodiols

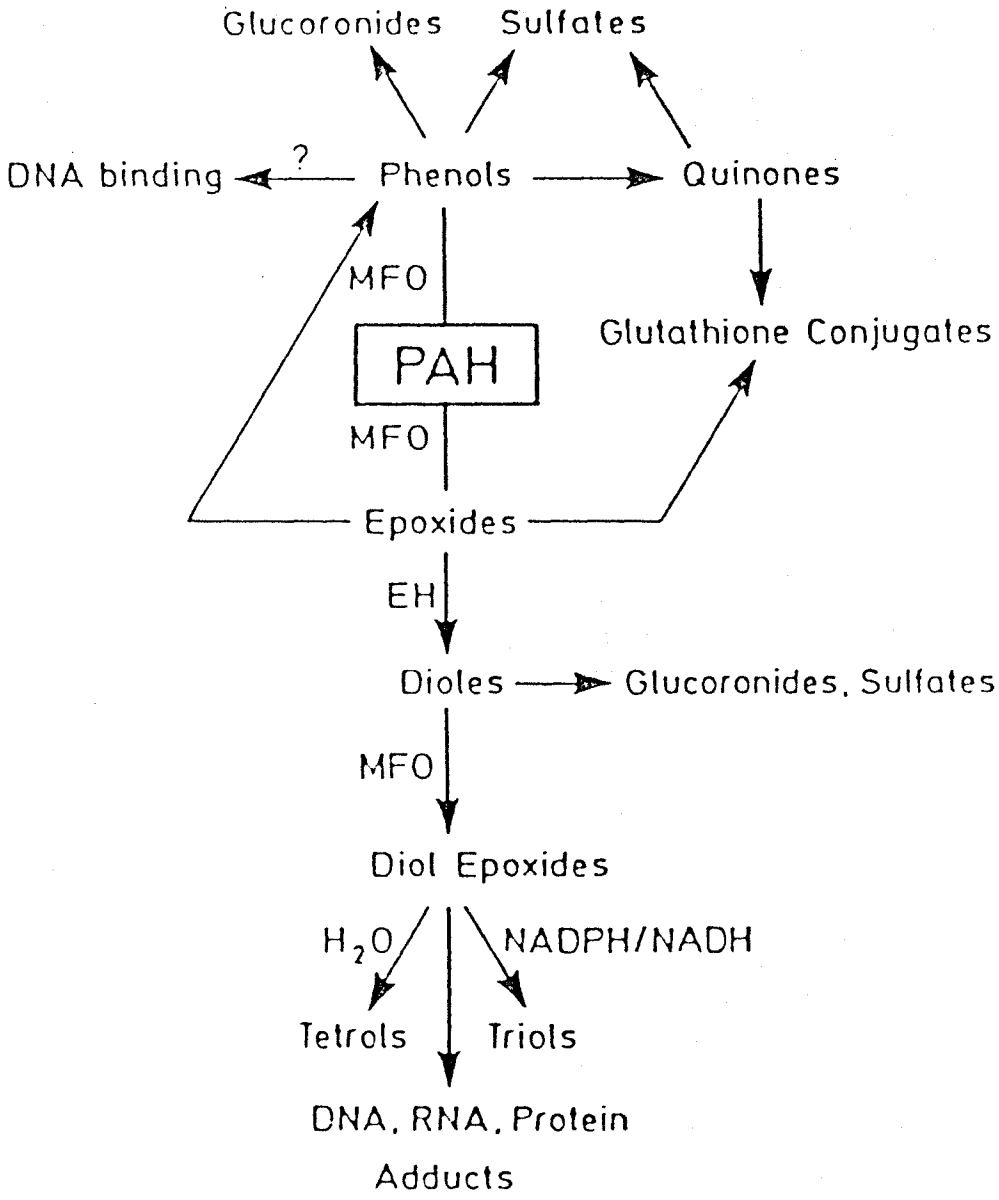

FIGURE 2 Enzymatic pathways involved in the activation and detoxification of PAH.

source: ref. 13

of BaP has been demonstrated.[19] Dihydrodiols may be further oxidized by the MFO system to dihydrodiol epoxides.[20] There is now considerable evidence that a particular structural class of diol-epoxides, namely the bay-region dihydrodiol-epoxides, operate as the ultimate carcinogenic form of PAH, which react easily with cellular macromolecules, in particular DNA.[21,22]

Other reactions involved in the metabolism of PAH are enzymatic conjugations of the oxygenated intermediates to glucoronic acid, sulfate, and glutathione.[23] These water-soluble conjugates are readily removed from the organism through bile, feces, and urine, and have been generally viewed as detoxification products.

Identification of metabolites has been performed for some PAH, with BaP being the compound studied most extensively. The metabolism of BaP is outlined in Figure 3. BaP metabolites found in microsomal incubation are 1-hydroxy-BaP, 3-hydroxy-BaP, 6-hydroxy-BaP, 7-hydroxy-BaP, and 9-hydroxy-BaP. The BaP-4,5-epoxides have been isolated and identified as precursors of the BaP-4,5-diol. Other studies indicate that epoxides are precursors of the 7,8-diol and 9,10-diol as well. There have been no intermediates isolated as phenol precursors, although recent evidence using deuterium labeling suggests that at least a portion of 3-OH-BaP is derived from an intermediate 2,3-

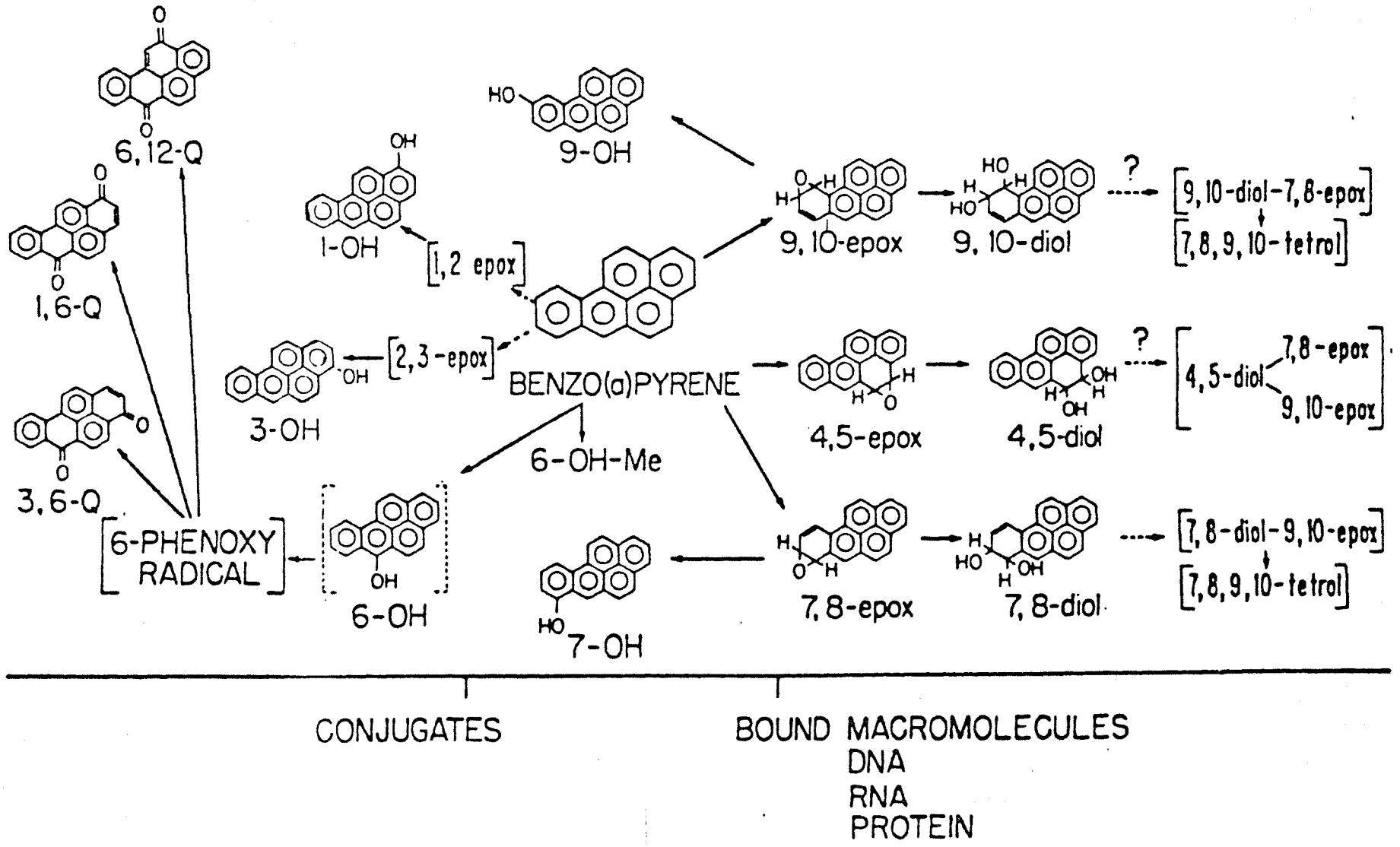

FIGURE 3 Composite of metabolic products of benzo[a]pyrene.

Source: ref. 13

epoxide.[24] In addition to the hydroxylated metabolites, 1,6-, 3,6-, and 6,12-BaP-quinones have been identified.[25] These are produced enzymatically by microsomes and nonenzymatically by air oxidation of phenols.[26]

Further oxidation of 7,8-dihydroxy-7,8-dihydrobenzo(a)pyrene by the MFO system leads to the formation of the highly reactive and probably ultimate carcinogenic metabolites, the isomeric 7,8-dihydroxy-9,10-oxy-7,8,9,10-tetrahydro-benzo(a)pyrene in which the epoxy ring is adjacent to the "bay" of the hydrocarbon.[27] These diol-epoxides react rapidly by electrophilic attack with cellular macromolecules. Adduct formation has been observed with DNA, RNA, and proteins.[22,28]

The relative proportions of metabolites have been estimated by many authors. This is usually done using tritium-labelled BaP, although there is some error due to detritiation during metabolism. The proportions vary according to whether and how the enzyme system was induced, and consequently which form of cytochrome P450 is present. The products of metabolism of the known forms of P450 in rat liver were determined by Wilson et al (1984);[29] some data are given (% of total metabolites) in Table 4. P450PCN is a form of low activity, induced by pregnenolone-16a-carbonitrile. Note that this and the phenobarbitone-induced forms produced mainly BaP-4,5-dihydrodiol in

comparable proportions.

Table 4 Products of metabolism of P450 in rat liver[24]

	Dihydrodiols			Diones		Phenols	
Form	4,5-	7,8-	9,10-	1,6-	3,6-	3-	9-
Uninduced	18	9	11	8	24	22	8
P450b	52	1	2	6	7	30	3
P450c	17	15	33	8	12	13	2
P450PCN	55	0	0	0	0	45	0

1.5 CANCER IN WORKERS EXPOSED TO PAH.

Since it has been known for many years that coal tar, as well as some isolated PAH are carcinogens in experimental animals, many investigators have studied the cancer frequency of workers known to be exposed to coal tar or coal tar pitch volatiles (CTPV) in relatively high concentrations. The International Agency for Research on Cancer (IARC, 1983) stated that there is sufficient evidence to indicate that 11 PAH are carcinogenic to experimental animals[30] (Table 5).

TABLE 5. PAH For Which Sufficient Evidence Is Known That These Compounds Are Carcinogenic To Experimental Animals

Chemical Name	Chem. Abstr. Services Reg No.
benz(a)anthracene	56-55-3
benzo(b)fluoranthene	205-99-2
benzo(j)fluoranthene	205-82-3
benzo(k)fluoranthene	207-08-9
benzo(a)pyrene	50-32-8
dibenzo(a,h)pyrene	53-70-3
dibenzo(a,e)pyrene	192-65-4
dibenzo(a,h)pyrene	189-64-0
dibenzo(a,i)pyrene	189-55-9
dibenzo(a,l)pyrene	191-30-0
indeno(1,2,3-cd)pyrene	193-39-5

Source: IARC(1983)[30]

1.6 BACKGROUND AND OBJECTIVE OF THE THESIS

Since 1987, a Total Human Environmental Exposure Study (THEES) has been conducted to investigate multimedia exposure to the ubiquitous environmental carcinogen, benzo(a)pyrene. This study represents the State of New Jersey's primary research effort in the rapidly growing field of total human exposure monitoring.[31]

As a substudy of THEES, the relationship between exposure to benzo(a)pyrene and biological levels, as measured BaP metabolites in human urine, is being modeled. As mentioned earlier, BaP was eliminated mainly through feces (the excreted amount of BaP via feces is five to seven fold of that of BaP via urine). However, urine was chosen as a biomonitoring specimen in this study due to the reasons of (a) non-invasive (urine was much easier to collect from volunteers). (b) feces was more difficult to assay and (c) there are a lot of data about BaP metabolites in urine. The results of BaP metabolites analysis will be used to verify a human compartmental model that was developed based on animal pharmacokinetic studies in the literature.[10-12]

The method employed to analyze BaP in urine samples is based on a procedure developed by Becher and Bjorseth.[32] The procedure was investigated. Discoveries show that it was necessary to modify the procedure in order to obtain resonable and consistent yields.

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CHAPTER 2. PAH BIOLOGICAL MONITORING PROFILES

Air monitoring of PAH only quantifies the respiratory intake (external dose). PAH may be absorbed not only in the lungs, but also in the gastrointestinal tract and through the skin. The monitoring of PAH or their metabolites in body fluids reflects the total uptake (internal dose). Biological monitoring of PAH may thus be useful in order to make an accurate estimation of the individual dose. It can help industrial hygeinists to identify highly exposed workers. Moreover, unbiased estimations of exposure to PAH are needed to increase the power of epidemiologic studies.

2.1 Conditions For The Development Of A Method Of Biological Monitoring.

Knowledge of Toxicokinetics of PAH. - Knowledge of the metabolism of benzo(a)pyrene, one of the PAH, is extensive. In many studies on chemical carcinogenesis, BaP has been used as a model substrate. Excellent metabolism reviews have been presented.[1,2] The intermediary epoxide-PAH and dihydrodiol-epoxide-PAH are supposed to be crucial metabolites in the carcinogenesis of PAH. The interindividual variation of the metabolism of PAH in cultured human tissues and cells is great.[3] The excretion of PAH-metabolites has been studied in experimental animals. After inhalation of BaP by rats most

of the excreted metabolites (85-95%) were found in the feces.[4,5] A minor part was excreted in urine.

Validation Of A Biological Test - When the basic toxicological investigations have suggested a useful biological parameter, other demands must be met before applying this test in preventive industrial health care.

The demands of a valid biological parameter are:[6]

- sufficiently sensitive
  (low detection limit, high accuracy and high reproducibility)
- Sufficiently specific
  (few interfering factors)
- Sufficiently stable to allow storage of samples
- Non-time consuming analysis by a not overly sophisticated technique
- When these demands are met a normal value among non-exposure referents must be determined.

2.2 View of Biological Monitoring

Biological monitoring is primarily an activity where repetitive measurements of toxic chemicals in biological specimens are used to assess the exposure levels of individual workers and of groups of workers. Up to now three

ways of biological monitoring of PAH have been proposed: (a) the urinary mutagenicity assay, (b) determination of the unmetabolized and metabolized BaP in body fluids and (c) reduction of urinary PAH-metabolites to their parent compounds.

a. The urinary mutagenicity assay has been applied for the detection of exposure to PAH. Volunteers and dermatological patients excreted mutagenic urine after application of crude coal tar to the skin.[7,8] The determination of mutagenic metabolites in urine with the aid of the Ames-assay was proposed to detect occupational exposure to mutagenic PAH. Kriebel et al.[9] reported a higher mutagenic activity in urine of non-smoking, coke plant workers than in the non-smoking control group. Several other studies have failed to detect enhanced excretion of mutagens of the urine of workers exposed to PAH.[10-12] However, a dose-dependent excretion of mutagens in the urine of rats after exposure to benzo(a)pyrene BaP was shown.[13] The poor sensitivity of the urinary mutagenicity assay to detect exposure to PAH seems to be the cause of the negative results in human urine.

b. Other reports deal with the measurement of unmetabolized BaP as an indicator of PAH. Hutcheon et al,[14] using a radio-immunoassay (RIA), found a higher level of BaP in the plasmas of occupants of an urban-industrial area compared to an outer suburban area (1.62 vs. 0.04 ug/l). Low

concentrations of fluorescent material, presumably unmetabolized PAH, have been observed in urine samples from tobacco smokers and nonsmokers (Table 6). Maly[15] used acid hydrolysis of the urine samples petroleum extraction, and paper chromatography for the semiquantitative determination of dibenzo(a,l)pyrene. Repetto and Martinez[16] separated BaP from methylenechloride extracts of urine samples by preparative colunm chromatography on silica, and quantitated BaP by spectrofluorimetry. Szyja found high amounts of unmetabolized BaP in urine samples of topside coke oven workers, collected after 6 hr of work (Table 6).

Table 6 CONCENTRATION OF PAH IN HUMAN URINE SAMPLES

PAH	Subjects	No. of samples	Concentration (mean in ug/l)	Ref.
Dibenzo-(a,l)pyrene	Active smoker	1	1.1	15
	Passive smoker	1	0.3	15
BaP	Active smoker (morning)	8	0.17	16
	Active smoker (evening)	7	0.34	16
	Passive smoker (morning)	1	0.15	16
	Passive smoker (evening)	1	0.23	16
	Nonexposed (morning)	1	0.01	16
	Nonexposed (evening)	1	0.06	16
BaP	Topside coke oven worker			
	After 6 hr work	19	4.67	17
	After 18 hr rest	13	2.1	17
	After 48 hr rest	8	1.6	17
BaP	Persons from urban/industrial area	451	0.690	18
	Persons from rural area	35	0.445	18

(continues)

PAH	Subjects	No. Samples	Concentration (Mean in ug/l)	ref.
Benz(a)-anthracene	Persons from urban/industrial area	437	0.701	18
	Persons from rural area	34	0.489	18
Sum of 11 prominent PAH[a]	Aluminum workers			
	Smokers	7	68.2[b]	28
	Nonsmokers	4	79.3[b]	28
	Control Smokers	6	45.4[b]	28
	Nonsmokers	4	13.2[b]	28

a. Sum of PAH and PAH metabolites determined by reversed-metabolism method.

b. Results converted from ug PAH/mmol creatinine using an average excretion of 12 mmol creatinine per 1 urine.

The urinary PAH levels were significantly lower after 18- and 48-hr rests. Michels and Einbrodt[18] determined the concentrations of BaP and benz(a)anthracene (BaA) in more than 480 urine samples of randomly selected persons from a highly industrialized area, and from a rural area as reference. The excretion of both BaP and BaA was found to be significantly higher in the polluted area compared to the reference area (Table 6). No differences, however, were observed between urines from smokers and nonsmokers. Song et al [19] reported a method for the determination of BaP in urine. Urinary BaP levels in operators working on the top of the coke oven were higher than those in operators working at the side of the coke-oven (AM=0.063 and 0.026 ug/l respectively). BaP was not found in urine samples of non-exposed workers (< 0.010 ug/l).[20] The presence of BaP in the body as reported by Hutcheon,[14] Song,[19] Szyja[17] and Michels & Einbrodt[18] may be overestimated by interferences of hydroxylated metabolites, due either to the known cross-reactivity of the RIA for 3-OH-BaP[21] or to separation techniques with poor discrimination.

In the studies described above, only the unmetabolized part of the PAH excreted in urine was determined. However, as mentioned earlier, PAH are metabolized to a great extent to polar, water-soluble metabolites both in vitro and in vivo. Thus, an alternative approach has been suggested by Keimig et al[22] to analyze urine for metabolites of specific

PAH compounds that are consistently prominent in environmental samples. They have identified 1-hydroxypyrene as a major metabolite in the urine of pigs and propose this metabolite for monitoring PAH-exposed workers. Later, Jongeneelen et al[23] isolated 1-hydroxypyrene from human urine by enzymatic hydrolysis, HPLC separation with fluorimetric detection. A similar method was used again by Jongeneelen et al[23] to analysis 3-hydroxybenz(a)anthracene and 3-hydroxybenzo(a)pyrene in human urine. An HPLC method for measuring BaP and phenolic BaP-metabolites in urine is proposed by Mathieu et al.[25] They did not report measurements in urine samples from exposed individuals. Grimmer et al.[26] separated phenanthrene and five isomeric hydroxyphenanthrenes in urines of man and rat by gas chromatography after acidic hydrolysis.

Although the methods mentioned above are sensitive and specific, their practical applications for estimation of human exposure to BaP are still limited. Moreover, as mentioned early, BaP is transformed in the body to more than twenty metabolites (IARC 1983)[27] and the proportions of BaP metabolites in human urine are still unclear. Thus, there are still many uncertainties in using any of these metabolites to indicate the burden of the body.

c. An interesting monitoring method was developed by Becher and Bjorseth.[28] They have described a method to

determine multiple PAH compounds in urine specimens based on the reduction of excreted, oxidized PAH metabolites back to the parent hydrocarbons. The analytical procedure included extraction of PAH and PAH metabolites from urine using cartridges containing C18 modified silica, reduction of metabolites to PAH by refluxing hydriodic acid ("reversed metabolism"), and subsequent analysis of 11 prominent PAH by HPLC with fluorimetric detection. This analytical procedure has been the subject of our detailed investigation as described later in this thesis.

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CHAPTER 3 SAMPLE COLLECTION

3.1 Field Study-Location

The THEES project is being conducted in Phillipsburg, a municipality of approximately 16,500, in a rural section of New Jersey. A major smoke stack industry in the center of town, a grey iron pipe manufacturing company, has existed since 1856. The other major local outdoor air pollution sources are space heating and motor vehicle traffic. The northern portion of the city is situated adjacent to primary truck routes. The center of Phillipsburg and the pipe manufacturing plant are located in a Delaware River valley.

A previous two year air sampling study for airborne BaP in New Jersey showed that, of twenty seven monitoring sites, Phillipsburg had the highest BaP concentration for a twenty-four hour sample (7.8 ng/m^3) and the second highest mean concentration (0.8 ng/m^3)[1]. In contrast, Phillipsburg ranked only fourteenth in maximum non-polar extractable organics concentration, suggestion enrichment of BaP in the area.

Central heating for homes is primarily #2 oil while room space heating includes kerosene and coal burning stoves. The major water purveyor for the area in the Garden State Water Company. The water supply comes from three wells in

town and two wells that are located approximately 1.6 km away[2].

3.2 Population and Home Characteristics[2]

The population for this total exposure study was not meant to be a statistically selected subgroup or representative sample of the types of people living in the Phillipsburg area. The selection of a limited number of participants was based upon the desire to examine the daily contributions of indoor and local outdoor air pollution and other media to human exposure in detail over the course of two weeks. The study participants were selected from respondents to a mail solicitation made to individuals living in the area surrounding the plant, and control areas to the north and south of the plant.

The study design called for a total of ten homes as the indoor sites, eight near the foundry and two outdoor air controls. The location of each home is shown in figure 4. Homes 1-3 are located in the valley near the plant Homes 4, 5 and 6 are in an area near the plant, but up a slight grade across the railroad tracks. Fugitive emissions from the plant may be funneled to these homes through a train trestle underpass. Homes 7 and 8 are located near outdoor site #2. Homes 9 and 10 are located across Route 22 approximately 2 km to the north of the foundry, near

outdoor site #4, on a ridge a kilometer away from the valley floor.

The homes and the study population had some characteristics which were typical to all, and others which were unique to individual homes. Examples of major similarities are: 1) all homes are over fifty years old; 2) nine homes are of wood construction; 3) no homes have an attached garage; 4) no homes have a fireplace; and 5) all homes are less than 5m from the street. Three of the homes had at least one smoker in residence, and six of the homes had children living at home. The ages of the adult participants ranged from 26 through 78, and ages for the children ranged from 6 months through 17 yrs.

Seven homes used oil, two used gas and one used a coal stove for central heating. Home 10 (with the coal stove), was one of the two selected away from the foundry as a control for the outdoor BaP sources. For the winter, however, the coal stove created an indoor source environment which would be used to examine the influence of indoor coal combustion on indoor air quality. Indoor combution source at each THEES home was listed at table 7.

Figure 4 THE PHILLIPSBURG AREA AND SITE LOCATIONS

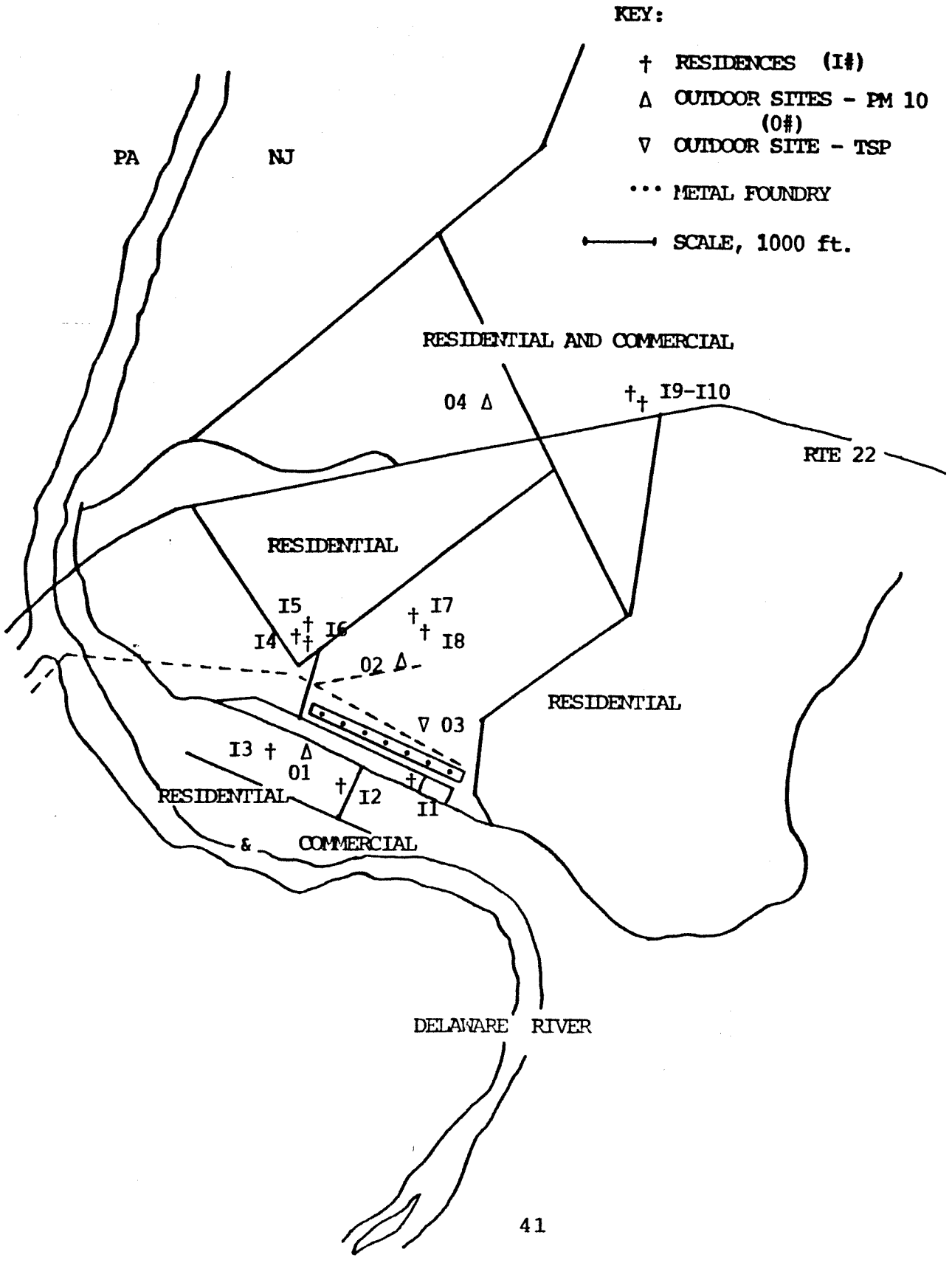

Table 7 INDOOR COMBUSTION SOURCES LOCATED EACH THEES HOME

Home ID	Types of Sources
1	Gas Cooking Range and Oven Toaster Oil Central Heat
2	Oil Central Heat Electric Range & Oven Microwave Oven 1 Smoker in residence
3	Oil Central Heat Kerosene heater(in living room or kitchen) Electric Range & Oven 2 Smokers in residence
4	Oil Central Heat Kerosene heater (in dining room or hall) Electric heater Gas Range & Oven Microwave Oven
5	Electric Range & Oven Microwave Oven Oil Central Heat
6	Gas Central Heat Kerosene Space Heater Electric Range & Oven Toaster
7	Gas Central Heat Electric Range & Oven Microwave
8	Oil Central Heat Gas Range & Oven Toaster Oven Microwave Oven
9	Oil Central Heat Gas Range & Oven 1 Smoker in residence
10	Oil Central Heat (rarely used) Coal Central Heat (primarily & in living room) Kerostene Heater (in kitchen) Microwave Ovn, Gas Range & Oven

Source: ref. 2

3.3 Sampling methodology

Ideally 24-hr urine would be collected to correlate with the 24-hour exposure samples. This strategy would minimize any effects due to diurnal variations in urinary elimination[3] and provide sample volume for analysis 24-hour collections were not practical for the 14-day study due to participant compliance and storage limitations. The urine sampling strategy employed collected as many voids in a day as possible so that a daily composite sample would be as representative as possible of that day's total elimination.

Participants were requested to collect and record the time of all voids while at home. In addition, participants were asked to record the time of their last void before returning home in the afternoon. This strategy resulted in the collection of samples covering from 8 to 22 hour/day in duration, with the weekend samples of longest duration. Each void was collected separately in a wide-mouthed 220 ml clear high-density polyethylene cup with a snap on cap. The 220 ml cup was placed in a opaque cylindrical container which was used to protect the samples from light. It also had an aesthetic advantage, affording the participants some privacy when the samples were picked up each day. Samples were stored frozen and later aggregated in the lab according to the 24-hour sample period of the diet and personal air exposure samples. The urine samples were aggregated to lag the exposure samples by 2 hours to

compensate for gastric emptying, absorption, etc. The composite samples were stored protected from light in 550 ml amber glass comtainers at -20 C. Study by Jongeneelen et al(1987)[4] indicated that this storing method is reliable.

The urinary sampling is summarized in Table I. The duration of the sample was measured from the time of the void prior to the first collected sample through the collection of the last void.

Table 8. SUMMARY OF THEES PHASE II PRELIMINARY URINE SAMPLING AVERAGE DAILY VALUES

PID	Sex	Sample Duration(hr)	Number of Samples	Total Volume(ml)
001	M	21.8	6	1355
011	F	22.5	4	769
031	F	17.2	5	785
041	F	10.8	2	242
042	F	14.6	4	561
051	F	14.1	4	610
052	M	11.1	4	607
061	F	15.6	4	545
062	M	13.8	3	439
081	F	14.8	3	825

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CHAPTER 4 DETERMINATION OF BENZO(a)PYRENE METABOLITES IN HUMAN URINE

TLC has been widely used for the separation of PAH due to its simplicity of operation, its rapidness, and the possibility of facile sample recovery for further identification of the separated PAH. The principal disadvantage lies in the possibility of degradation of sensitive PAH adsorbed onto the active surface of the stationary phase.[1,2] However, Inscoe[3] reported that recoveries from cellulose acetate plates average around 95% and are much higher than from alumina plates where photochemical oxidation reactions take place during chromatography.

The analytical procedure is based on the reduction of excreted, oxidized metabolites of BaP back to the parent hydrocarbons. After hydrolysis by hydrochloric acid, BaP and its metabolites in the urine sample were extracted by conmercial preparetive cartridges containing C_{18}-modified silica and reduction of metabolites to parent BaP by refluxing hydriodic acid. Subsequente analysis was by thin layer chromatography (TLC) using selective and sensitive fluorescence detection. This method was modified from the Becher and Bjorseth report.[4] In turn, the reduction reaction of hydriodic acid is based on research by Konieczny and Harvey.[5] During the analysis we realized that the recovery

of Becher/Bjorseth procedure was extremely low and we had a detail study of this procedure which is present at Chapter 5. Results presented in this chapter are not corrected for recovery and except indicated are without red phosphorus.

4.1 Chemicals and Apparatus

Benzo(a)pyrene(99%), anthraquinone(97%), anthracene(97%), and pyrene(99%) were purchased from Aldrich Chemical Co.. 3-hydroxy-BaP, 9-benzo(a)pyrenyl-b-d-glucopyranosiduronic acid were purchased from Midwest Research Institute (MRI). BaP-3,6-dione, BaP-4,5-dione, BaP-7,8-dione, BaP-6,12-dione were obtained from IIT Reseach Institute. 1-hydroxypyrene, 2-hydroxypyrene, 4-hydroxypyrene, pyrene-1,6-quinone and pyrene-1,8-quinone were kindly provided by Dr. Joseph Rice Dept. of Pharmaceutical Chemistry School of Pharmacy Rutgers University, Piscataway, N.J.

Hydriodic acid (47-51%), methylene chloride, methanol (HPLC grade) and anhydrous alcohol were purchased from J.T. Baker Chemical Co..Hydriodic acid (65%), acetic acid(99.5%) and potassium disulfate were purchased from Fluka Co.. Phosphorus red (amorphous) was obtained from Fisher Scientific Co.. Hydrochloric acid and sodium sulfate anhydrous were purchased from Mallinckrodt Co..

The precoated thin layer chromatography plates(20x20 250 microns and 20% acetylated cellulose) were purchased from

Analtch Co. an AIS TLC multispotter was used for TLC plate spotting and fluorescence detection was performed with a Perkin-Elmer MFP-44B Fluorescence Spectrophotometer, IBM personal computer with non-linear correction program (which fit a least square parabola having the form $Y = A^{o} + A^{1}X + A^{2}X^{2}$) was used to calculate benzo(a)Pyrene concentrations.

4.2 Procedure of Urine Analysis

100 ml human urine samples were adjusted to pH=2 with concentrated hydrochloric acid and allowed to stand for 30 min. at room temperature.

A sep-pak C_{18} cartridge (Water Associates) was used for separation of the metabolites of BaP. After priming the cartridge with 5 ml methanol followed by 10 ml water, the hydrolyzed sample was passed through the cartridge at a rate of approximately 10 ml/min. Retained solutes were eluted using 5 ml methanol and 5 ml cyclohexane and then elutes were combined. The solvent was evaporated under an air flow and residue refluxed with 1 ml 49% hydriodic acid and 15 ml acetic acid for 24 hrs in an oil bath.

The hot solution was poured into 80 ml of 1% aqueous potassium disulfate solution and extracted twice, first with 40 ml then with 20 ml cyclohexane. The organic phase was washed with water and dried with anhydrous sodium sulfate. The extract was concentrated to 1 ml with constant air flow,

and 100 ul spotted on an acetylated cellulose thin layer chromatography (TLC) plate. The TLC plate was developed using 2 : 1 ethanol/dichloromethane and analyzed spectro-fluorimetrically via plate scanner at 387.0 nm for excitation, 428.6 nm for emission.

4.3 Determination Of Detection Limit

With 1 ng/ml BaP standard, different volumes of the standard were injected on the TLC plate, then developed under the same conditions as the urine samples and the BaP was detected by a fluorescence spectrophotometer.

4.4 Results

Eleven adult urine samples collected daily over fourteen days have been analysed so far. The limit of detection is 5 ng/l (0.005 ug/l) with this procedure (Table 9). The results of measurement in human urine are listed in Table 10 to Table 20. Data presented are not corrected for recovery.

During the preresearch period, urine specimens were collected from two participants (one smoker and one nonsmoker from different families). Indoor air sampling was conducted simultaneously. The indoor air quality and BaP urine excretion appear to be related (Figure 5). The mean concentration of BaP in the smoker's urine was 170 ng/l (For total of six samples, max. 691 ng/l and min. 8 ng/l) compared with 58 ng/l (For total sevene samples, max. 262

ng/l and min. 6 ng/l) for the nonsmoker; concordantlly, the mean indoor air concentrations of BaP in House no. 1 (smoker) was 0.608 ng/m^3 (n=4) compared with 0.334 ng/m^3 (n=5) for House no. 2 (nonsmoker).

BaP exposure and urinary elimination were determined simultaneously. The methods of analysis for personal air and food samples were described elsewhere.[6,7] The data for two participants in Phase II (#42 and #81, see Table 14, 19) are used to model the relationship between exposure to benzo(a)pyrene and BaP metabolite level in human urine.[8] A scatter-plot of elimination verses exposure, Figure 6 indicates all values are detectable and that there is variability in the data. Moreover, in about 80% of the data there appears to be emerging a pattern suggesting a positive association between the variables i.e., elimination increased with exposure.[8]

A second analysis of the BaP exposure/elimination data is presented by comparing the fractional change in exposure and elimination from week 1 to 2, see Figure 7. The fractional change for both exposure and elimination was calculated as the difference between week 1 and 2 divided by the two week mean. Such an analysis compares relative changes from week 1 to week 2 in exposure and elimination. A positive association (data points in quadrantsII and III) is indicated in 7 of 10 cases.

Table 9. Detection Limit of BaP in TLC Separation and Fluorescence Detection

Injection Vol. (ul) of BaP standard (1 ng/l)	Rt (sec.)	Area
10	49	756
20	50	1312
30	50	1940
40	51	2856
50	50	3802
60	51	4587
70	51	5629
80	52	6970
90	53	7333
100	53	7549

*. The minimum integration area of the computer was set to 1000 (as measure of random noise), the limit of detection will be three times this value (Anal. Chem. 1980, 52, 2242-2249).

Detection Limit: 50 ul BaP standard injected = 0.05 ng BaP. This means that if the BaP from metabolites in 100 ml of urine is concentrated in 1 ml and a 0.1 ml (100ul) aliquot is analyzed, then the limit of detection for a typical 100 ml urine sample is 0.5 ng/100 ml or 5 ng/l (0.005 ug/l).

Table 10. ANALYTICAL RESULTS OF BaP METABOLITES IN PERSON ID #1 URINE SAMPLES (THEES PHASE II)

Sample Number (Day 1-15)	Results(ug/l)	Sample Volume (ml)
1.	0.011	510
2.	N.D.	500
3.	0.017	420
4.	0.016	470
5.	0.019	490
6.	0.038	510
7.	0.016	500
8.	0.030	480
9.	0.025	490
10.	0.016	480
11.	0.028	470
12.	N.D.	460
13.	0.033	300
14.	0.013	450
15.	0.007	390

N.D.: Not detected.

Table 11. Analytical Results of BaP Metabolites in Person ID # 11 Urine Samples (THEES Phase II)

Sample Number (Day 1 - 15)	Results (ug/l)	Sample Volume (ml)
1.	N.D.	490
2.	0.007	530
3.	N.D.	500
4.	N.D.	540
5.	0.015	450
6.	N.D.	480
7.	N.D.	420
8.	0.026	490
9.	0.006	470
10.	0.023	420
11.	0.01	450
12.	0.015	500
13.	0.007	450
14.	0.005	450
15.	N. D.	

N.D.: Not detected.

Table 12. Analytical Results of BaP Metabolites in Person ID #31 Urine Samples (THEES Phase II)

Sample Number (Day 1-15)	Results(ug/l)	Sample Volume (ml)
1.	0.053	250
2.	0.027	350
3.	0.043	330
4.	0.012	350
5.	0.007	370
6.	0.011	420
7.	0.020	400
8.	0.005	320
9.	0.013	350
10.	N.D.	350
11.	0.010	340
12.	0.010	420
13.	0.023	380
14.	0.007	450
15.	0.015	430

N.D.: Not detected.

Table 13. Analytical Results of BaP metabolites in Person ID #41 Urine Samples (THEES Phase II)

Sample Number (Day 1 - 13)	Results (ug/l)	Sample Volume (ml)
1.	0.006	390
2.	0.032	210
3.	0.010	130
4.	0.007	300
5.	0.032	150
6.	0.010	200
7.	0.017	160
8.	0.021	230
9.	0.015	290
10.	0.012	120
11.	0.006	350
12.	0.016	150
13.	0.016	240

TABLE 14. THE RESULTS OF BaP METABOLITES IN # 42 HUMAN URINE SAMPLES

SAMPLE I.D.	WITH PHOSPHORUS (ug/l)	WITHOUT PHOSPHORUS (ug/l)
1	0.28	
2	0.01	N.D.
3	0.11	
5	0.11	0.05
7	0.38	
9	0.06	
10	0.05	
11	0.21	
14	0.03	
15	0.06	

N.D: Not detected.

Table 15.Analytical Resultsof BaP Metabolites in Person ID #51 Urine Samples (THEES Phase II)

Sample Number (Day 1 - 15)	Results (ug/l)	Sample Volume (ml)
1.	N.D.	480
2.	0.024	390
3.	0.028	260
4.	0.019	440
5.	0.023	450
6.	N.D.	450
7.	0.053	440
8.	0.008	
9.	0.018	
10.		
11.	0.007	
12.	N.D.	420
13.	N.D.	390
14.	0.005	390
15.	N.D.	220

N.N.: Not detected.

Table 16. Analytical Results of BaP Metabolites in Person ID #52 Urine Samples (THEES Phase II)

Sample Number (Day 1 - 14)	Results (ug/l)	Sample Volume (ml)
1.	0.015	450
2		380
a.	0.005	
b.	0.007	
3.	0.023	150
4.	0.010	160
6.	0.026	400
7.	0.012	250
8.	0.013	350
9.	0.016	370
10.	0.015	340
11.	0.017	400
12	0.047	340
13	0.011	300
14	0.018	400
15	0.123	330

a., b.: Duplicated analysis.

Table 17. Analytical Results of BaP Metabolites In Person ID # 61 Urine Samples (THEES Phase II)

Sample Number (Day 1 - 15)	Results (ug/l)	Sample Volume (ml)
1.	0.022	280
2.	0.038	430
3.	0.010	500
4.	N.D.	490
5.	0.014	380
6.	N.D.	380
7.	N.D.	190
8.	0.024	430
9.	0.012	440
10.	0.007	330
11.	N.D.	440
12.	N.D.	380
13.	N.D.	360
14.	0.010	480
15.	N.D.	180

N.D.: Not detected.

Table 18. Analytical Results of Bap Metabolites in Person ID #62 Urine Samples (THEES Phase II)

Sample Number (Day 2 - 14)	Results (ug/l)	Sample Volume (ml)
2.	0.088	330
3.	0.026	320
4.	0.018	450
5.	0.012	200
6.	0.019	340
7.	0.084	350
8.	0.005	240
9.	0.015	450
10.	N. D.	420
11.	N. D.	200
12.	N. D.	400
13.	0.013	350
14.	0.010	330

N.D.: Not detected.

Table 19. Analytical Results of BaP Metabolites in # 81 Urine Samples

Sample I.D.	With Phosphorus (ug/l)	Without Phosphorus (ug/l)
3	0.05	N.D.
4	0.02	0.01
5	0.03	N.D.
6	0.06	0.03
7	0.04	N.D.
8	0.01	0.01
9	0.03	0.02
11	0.21	
12	0.02	N.D.
13	0.10	
14	0.14	

N.D.: Not detected.

Table 20. Analytical Results of BaP Metabolites in # 92 Human Urine

Sample I.D.	With Phosphorus (ug/l)	Without Phosphorus (Ug/l)
6	0.32	
7	0.02	0.006
8	0.06	0.02
9	0.04	0.01
10	0.03	N.D.
14	0.03	
15	0.20	

N.D.--- Not detected.

Figure 5. Indoor Air Exposure

And BaP Urine Excretion

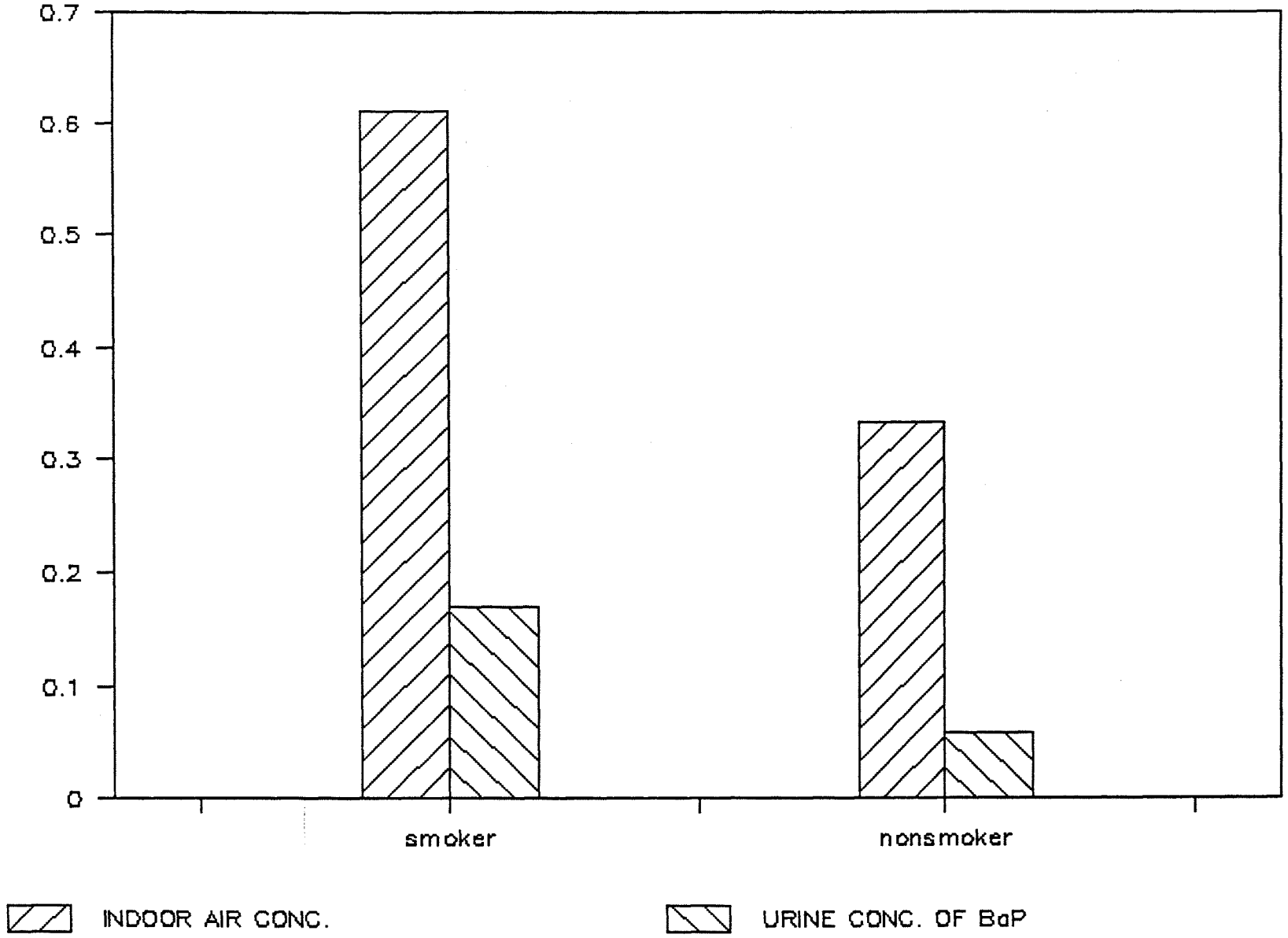
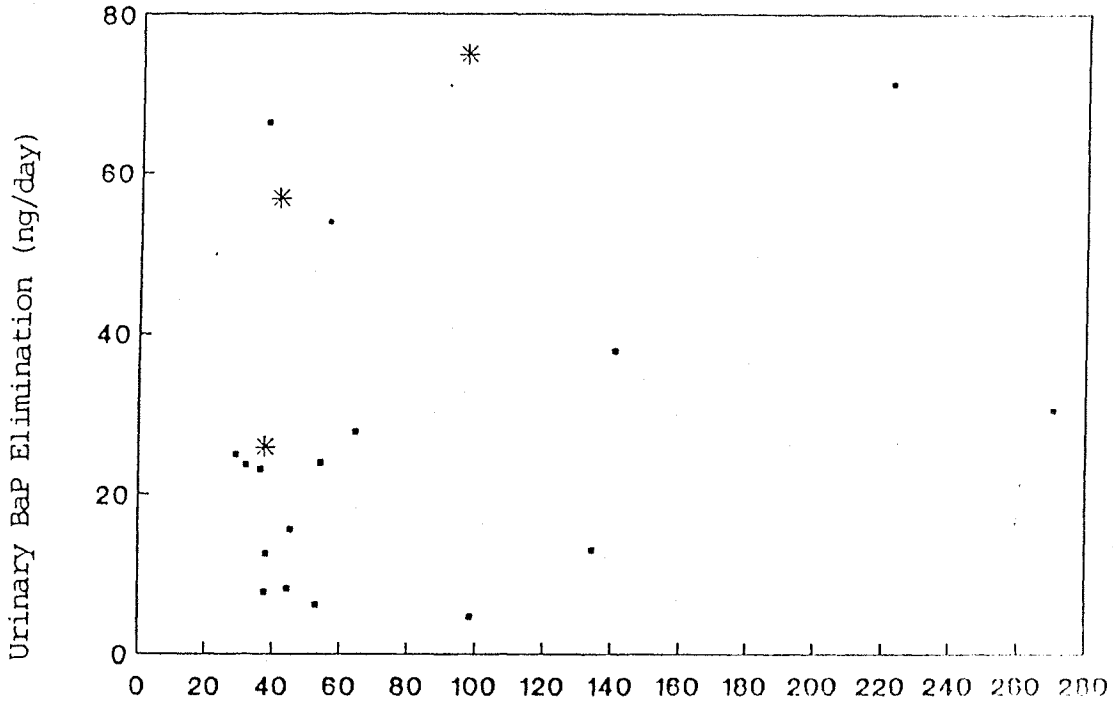


Figure 6 Scatter-plot of BaP Exposure and Elimination[1].



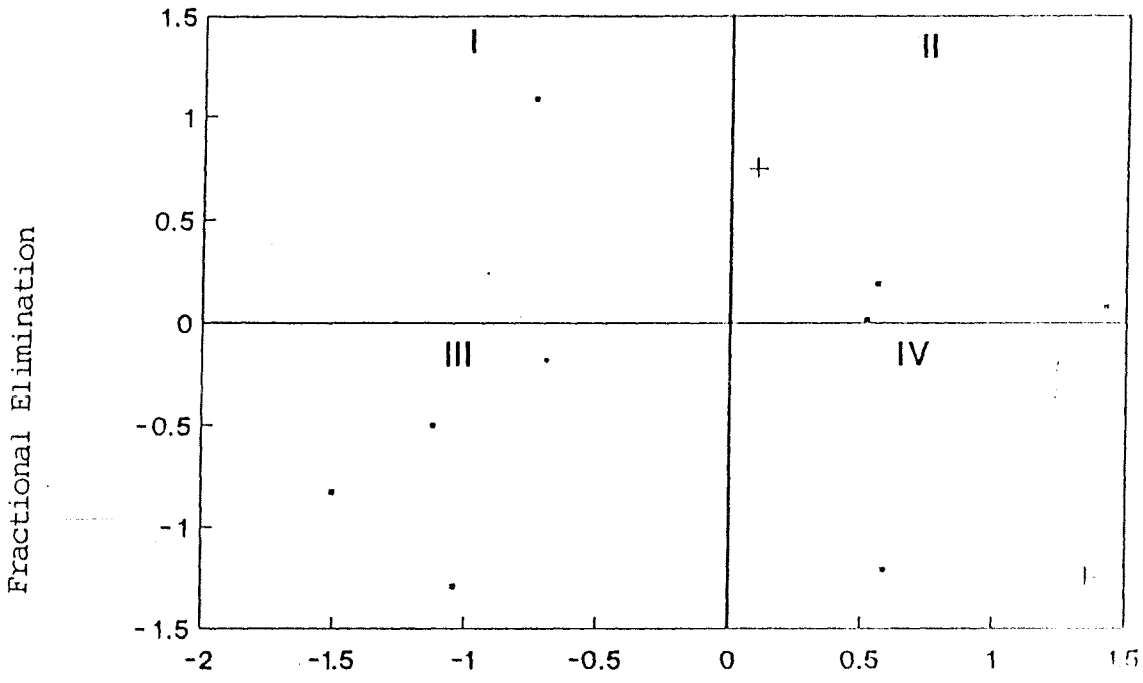
* Urine Analysis without Red Phosphorous

• Urine Analysis with Red Phosphorous

Urinary elimination is not corrected for analytical recoveries.

Source: ref. 6

Figure 7 Week 1 to Week 2 Fractional Elimination and Exposure.



* Urine Analysis with Red Phosphorous

• Urine Analysis without Red Phosphorous

Source: ref. 6

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CHAPTER 5 STUDY OF THE ANALYTICAL PROCEDURE

The principal benefit of the Bjorseth and Becher procedure is that of "reversed metabolism" - recovering of metabolites of PAH in urine in the form of their parent compounds. The difficulties in analyzing PAH in body fluids arise from the fact that the PAH mixture in a work environment frequently contains as many as 100 different PAH compounds[1] and each PAH compound is transformed to a large number of metabolites.[1] However, Becher and Bjorseth did not indicate the total recovery of the procedure. Uncertainties in the urinary BaP determination are likely to be high due to the low recoveries of the method.

In this substudy, research investigation focused on the following issues:

a. Checking the recovery of every step of the procedure.

b. Figuring out the influences of the chemicals utilized (hydriodic acid, acetic acid.) on the reduction of BaP metabolites.

c. Measuring of reduction yields of different PAH metabolites by this procedure.

d. Examining the hydrolysis rate by hydrochloric acid.

e. Developing improved methods.

5.1 The Recovery Yield of BaP And Its Metabolites In The Procedure

The Becher and Bjorseth procedure was tested as shown in Figures 8-12 and the results are achieved as follows:

a. The recovery yield of the steps at hydrochloric acid hydrolysis and Sep-Pak cartridge chromatography is about 30-40% for BaP.

b. The recovery yield of the step involving refluxing with acetic acid and hydriodic acid is only about 30-36% for BaP.

c. The recovery of extraction with cyclohexane (40 ml and 20 ml) is about 80% for BaP.

d. The recovery of blow down of the extracts to 1 ml with air is greater than 90% for BaP.

e. The total recovery of the procedure for BaP is about 8%.

The hydrolysis yield of hydrochloric acid was examined with 9-Benzo(a)pyrenyl-b-d-glucopyranosiduronic acid. The result indicated that the hydrolysis yield is about 50%.

With the [^{3}H]BP treated mouse urine, which was kindly provided from Dr. Regina Santella from the Columbia University School of Public Health, experiments have been

applied on the procedure to check recovery of BaP metabolites in urine. The results are compared with that of radioactivity counts.

As Figure 13 shows, BaP was transformed to several principale metabolites by the mouse. With Becher and Bjorseth procedure, the recovery yield of these metabolites to BaP is about 5 % (n= 6 see Figure 12)*, which is lower than the result of former study[2](Recovery = 8%).

5.2 The Influence of Hydriodic Acid and Acetic Acid on the Reductive Action of BaP Standard

The affect of hydriodic acid on the recovery of BaP standard (70 g/ml, 100 ul) was tested (Fig 9). The recovery yield of refluxing only with glacial acetic acid is almost 99% while the recovery of refluxing with acetic acid and hydriodic acid is 30%. The recovery of refluxing with iodine (equal mole. to 1 ml hydriodic acid) and acetic acid is only 6%. It is clear that HI reacts with BaP probably via release of I_2 the probable attaching reagent.

*. Most recently analysis recovery of comparison of phosphorus effects in reduction were extremely low, which were 4-6% with phosphorus and 1% without phosphorus, probably due to the loss from long period storing.

5.3 Reductive Yield of Selected PAH Metabolites

With different PAH standard metabolites, the precedure in Figure 12 was employed to observe the reduction yield of these compounds. It is clear that recoveries of BaP metabolites are extremely low while those of smaller PAH such as pyrene and anthracene are much higher.

Table. 21 Reduction Yields of PAH metabolites

PAH Metabolites	Recovery[a] With HI & HAc
3-hydroxy-BaP	0.3%
BaP-3,6-dione	0.2% or N.D[b]
BaP-4,5-dione	0.1%
BaP-7,8-dione	0.1%
BaP-6,12-dione	N.D.
9-Benzo(a)pyrenyl-b-d-glucopyranosiduronic acid	N.D.
Pyrene[c]	120%
1-hydroxypyrene	85%
2-hydroxypyrene	65%
4-hydroxypyrene	92%
Pyrene-1,6-quinone	50%
Pyrene-1,8-quinone	25%
Anthraquinone	110%

a. Recovery: BaP metabolites are recovered as BaP. Pyrene metabolites are recovered as pyrene and anthraquinone is recovered as anthracene.

b. N.D. : Not Detected.

c. The limit of detection for pyrene is 0.1 ng/ml=10 ng/l[3] Excitation: 335.5 nm. Emission: 390.0 nm.

5.4 Improving the Reduction Yield with Red Phosphorus

Disappointed by the very poor recoveries of BaP (Fig. 12) and its metabolites (Table 21), attempts were made to increase the yield.

Following Gardon and Wolloam's procedure[4], BaP metabolite standard and [^{3}H]BP treated mouse urine were reacted with sodium borohydride in acidic media. However, the recovery was still extremely low. The results of reduction with zinc were unsuccessful also (Table 22).

Table 22. Recovery of 3-OH-BaP and BaP Metabolites In Radio-labelled Mouse Urine By Reduction of Sodium Borohydride or Zinc In Acidic Media

	Reduction With $NaBH_4$	Reduction With Zinc
	Recovery	Recovery
3-OH-BaP:	Not detected	2%
Mouse Urine:	1.6%	--

The key to the Bjorseth procedure[1] is the reduction by hydriodic acid which was based on the report of Konieczny and Harvey[5]. In their experiments, Konieczny and Harvey originally improved upon a procedure which involved

phosphorus when they were successful omitting it and thus allowing a cleaner procedure. However, they tested their reaction on smaller ring systems and never reached the pentacyclic PAH. The larger rings are more easily oxidized and this is especially true for BaP. Thus, the procedure was not an improvement for BaP and in fact lowered the yield.

Improvement was made by simply returning to the original procedure (refluxing hydriodic acid with red phosphorus at acid media, see Figure 14). The results show that this change has a significantly increased the recovery of BaP and BaP metabolites by 3-4 fold.

For the BaP standard, the results of refluxing with acetic acid, hydriodic acid and red phosphorus (0.2g) is 3 time that of without phosphorus(Table 23).

With [^{3}H]BP treated mouse urine, the modified procedure was tested. The recovery of BaP metabolites in mouse urine are listed in Table 23 and comparison of recovery with and without phosphorous is shown in Figure 10.

Table. 23 Recovery of BaP Metabolites in Radio-labelled Mouse Urine

	WITHOUT PHOSPHOROUS		WITH PHOSPHOROUS	
	RESULTS	RECOVERY	RESULTS	RECOVERY
Mouse Urine:				
	42 ng/ml	2.1%	160 ng/ml	8%
	43 ng/ml	2.2%	240 ng/ml	13%
BaP Standard: (70 ng/ml)				
	7 ng/ml	10%	22 ng/ml	32%
	12 ng/ml	17%	23 ng/ml	33%
3-OH BaP: (10 ug/l)	28 ng/ml	0.3%	150 ng/l	1.5%

Duplicate comparisons were made on collected human urine samples to observe the function of phosphorus in the reductive action(Fig. 16-18 and Table 24). The average improvement of the overall recovery was a factor of 3-4 (Mean 3.8). It is clear that red phosphorus allows for more quantitative reduction of BaP metabolites.

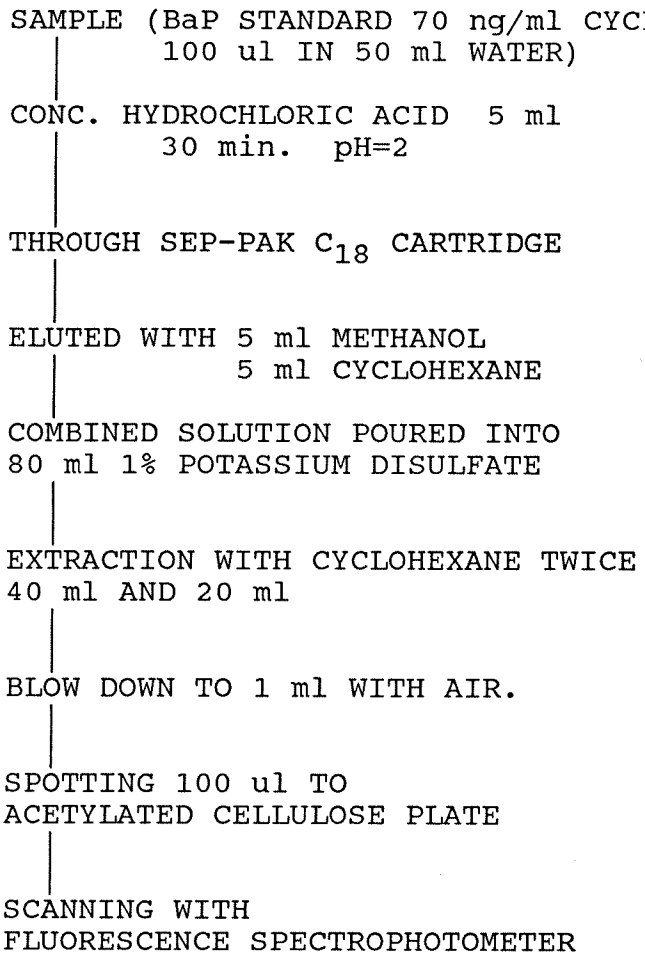
Table 24. Summary of Comparison of The Effects of Red Phosphorus in The Recovery of BaP Metabolites

Sample I.D.	With Phosphorus (ug/l)	Without Phosphorus (ug/l)	P/NP[a] Ratio
ID 42 D02	0.01	N.D.[b]	4.0
ID 42 D05	0.11	0.05	2.2
ID 81 D03	0.05	N.D.	20.0
ID 81 D04	0.02	0.01	2.0
ID 81 D05	0.03	N.D.	12.0
ID 81 D06	0.06	0.03	2.0
ID 81 D07	0.04	N.D.	16.0
ID 81 D08	0.01	0.01	1.0
ID 81 D09	0.03	0.02	1.5
ID 81 D12	0.02	N.D.	8.0
ID 92 D07	0.02	0.006	3.3
ID 92 D08	0.06	0.02	3.0
ID 92 D09	0.04	0.01	4.0
ID 92 D10	0.03	N.D.	12.0
		Mean:	6.50
		S.D.:	5.88

a. P/NP - The ratio of analytical results with red phosphorus and the results without red phosphorus.

b. N.D. - Not detected.

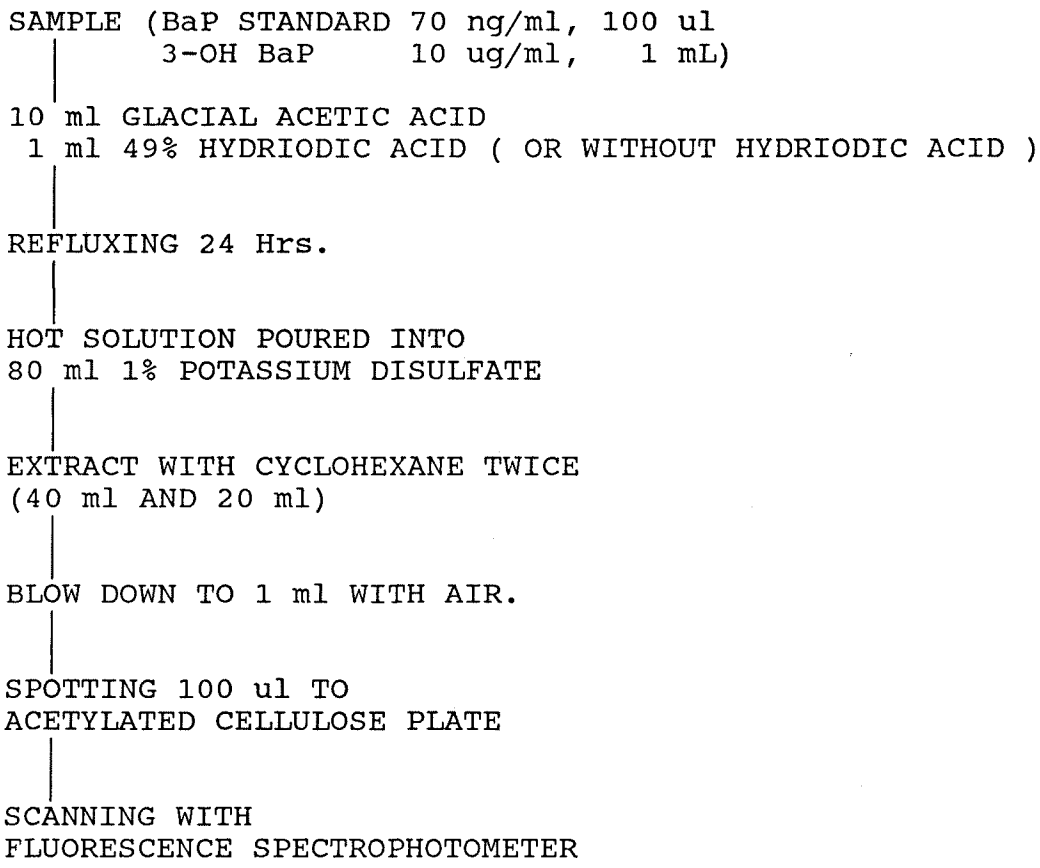
FIGURE 8. THE RECOVERY OF BaP THROUGH CHROMATOGRAPHY STEP



RECOVERY:

BaP STANDARD 30-40 % (n=2)

FIGURE 9. THE RECOVERY OF BaP AND 3-OH BaP IN REFLUXING PROCEDURE



RECOVERY

WITH HYDRIODIC ACID & ACETIC ACID

BaP	21 % - 31 % (n=2)

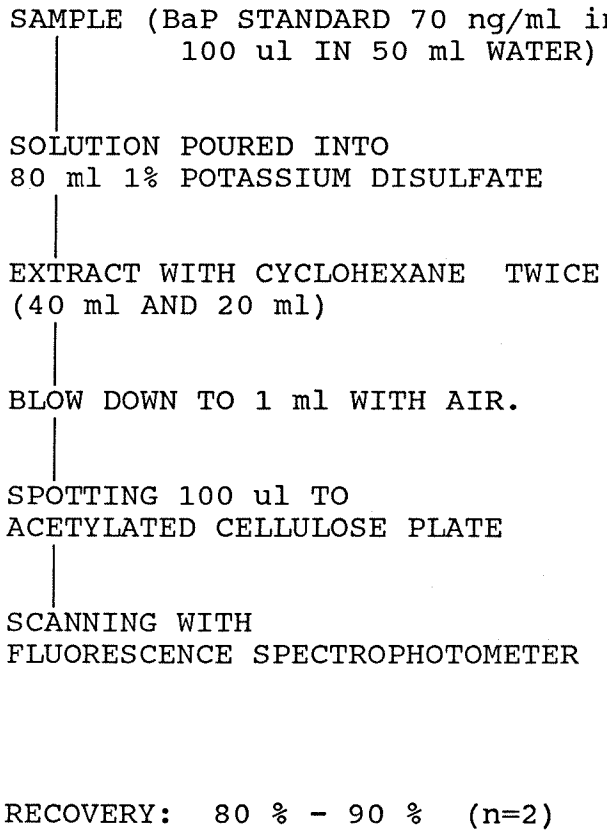
WITHOUT HYRIODIC ACID

BaP	80 % - 99 % (n=2)

NORMAL REFLUXING PROCEDURE

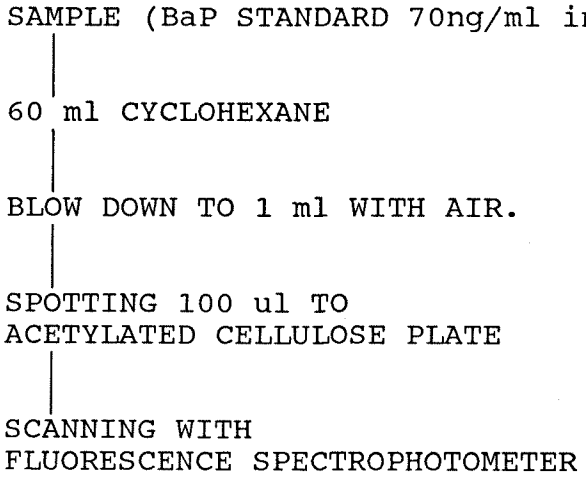
3-OH BaP	0.3 %

FIGURE 10. THE RECOVERY OF BaP IN THE EXTRACTION PROCEDURE



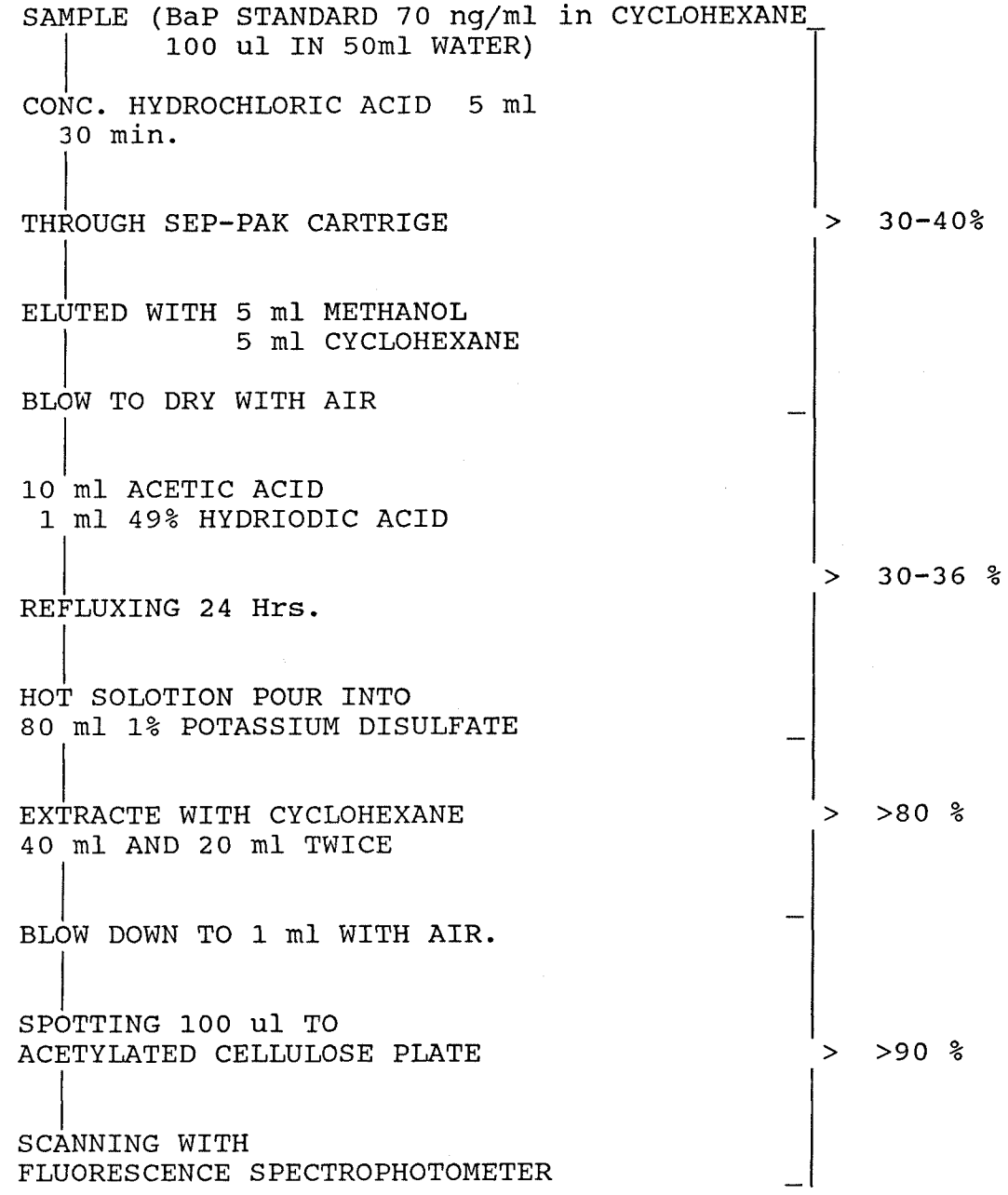
RECOVERY: 80 % - 90 % (n=2)

FIGURE 11. THE RECOVERY OF BaP IN THE STEP OF BLOW DOWN BY AIR



RECOVERY: 86 % - 107 % (n=2)

FIGURE 12. SUMMARY OF THE RECOVERY OF BaP IN THE NORMAL BECHER/BJORSETH PROCEDURE



Total Estimated Recovery = 35% x 33% x 80% x 90% = 8%

Figure 13. HPLC OF RADIO-LABELLED MOUSE URINE
(Courtesy of Dr. Regina Santella)

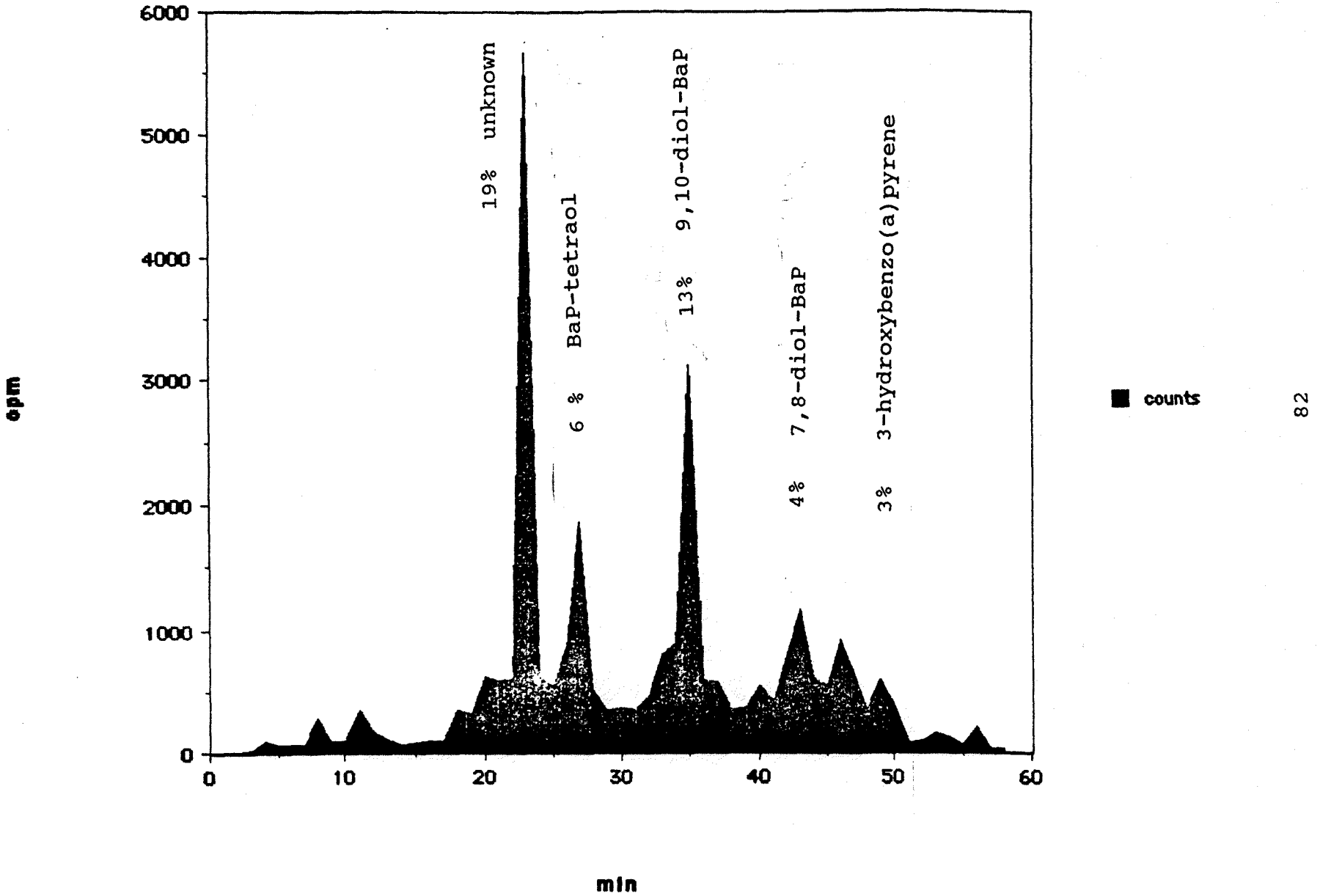


FIGURE 14. RECOVERIES OF 3-OH BaP AND MOUSE URINE IN NORMAL PROCEDURE WITH RED PHOSPHORUS AND WITHOUT RED PHOSPHORUS

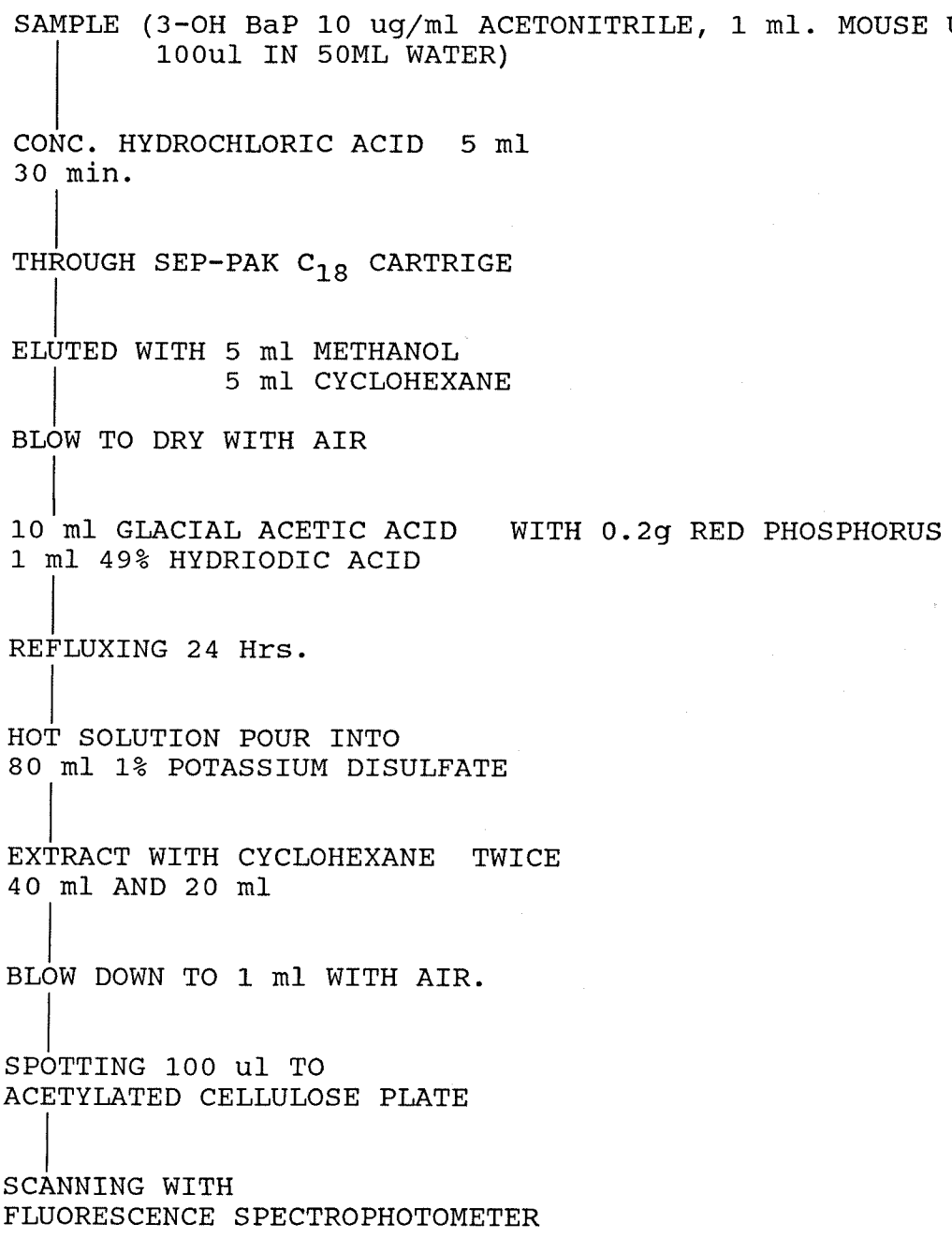


Figure 15. Compare BaP In Mouse Urine

With Phosphorus Modified Reduction

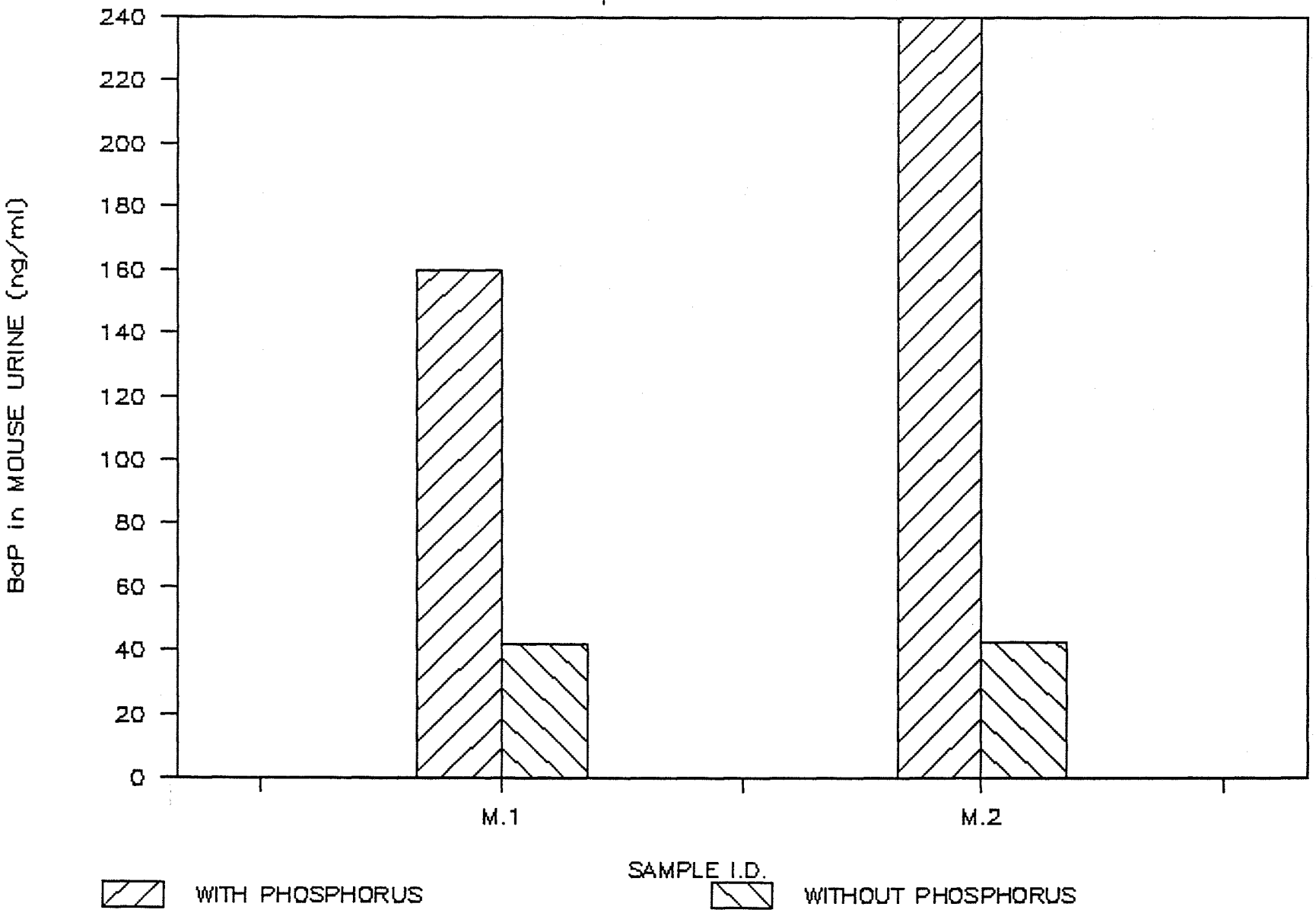


Figure 16. Compare Effect Of Phosphorus

In The Reduction Of BaP Metabolites

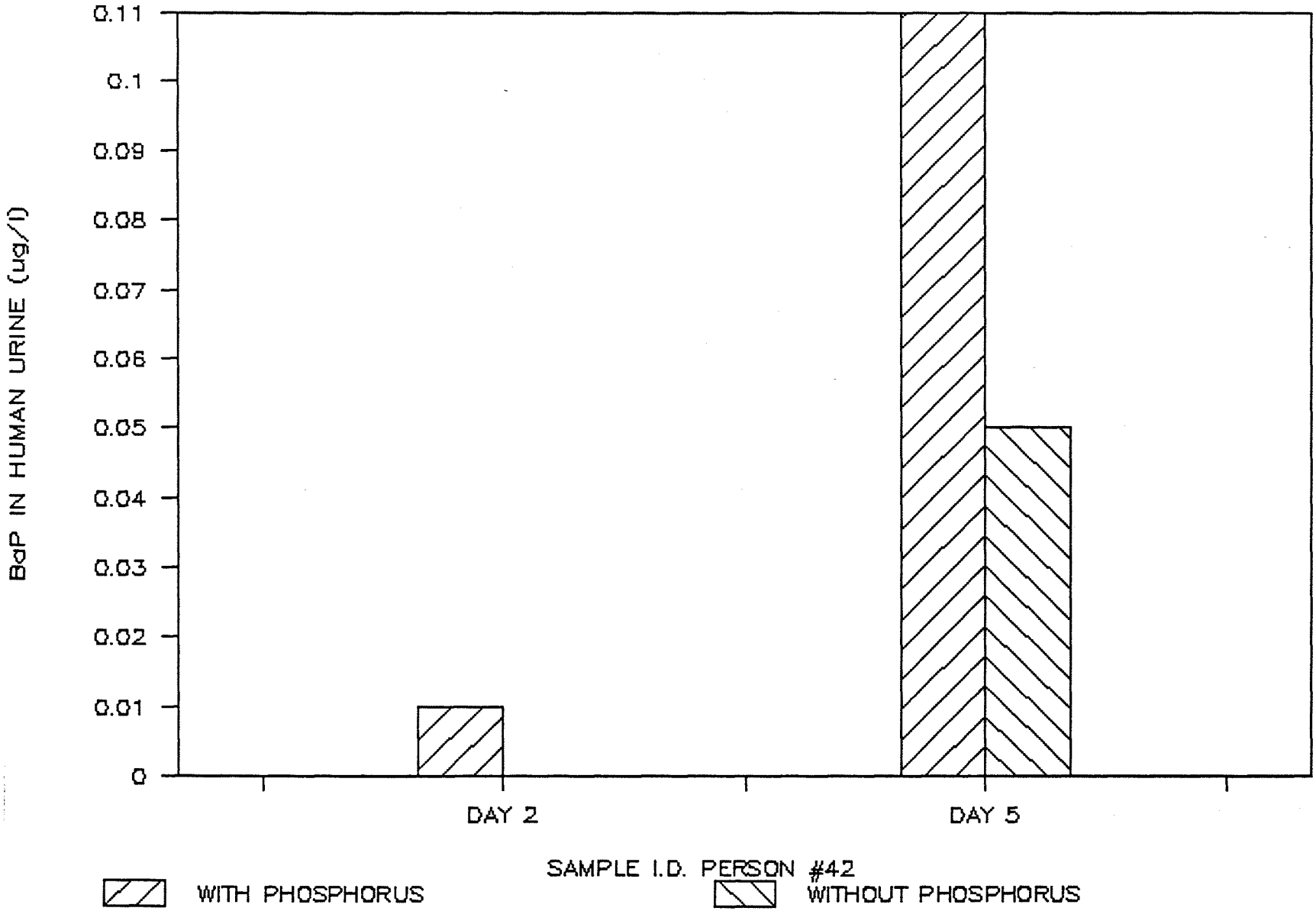


Figure 17 Compare Effect Of Phosphorus

In Recovery Of BaP Metabolites

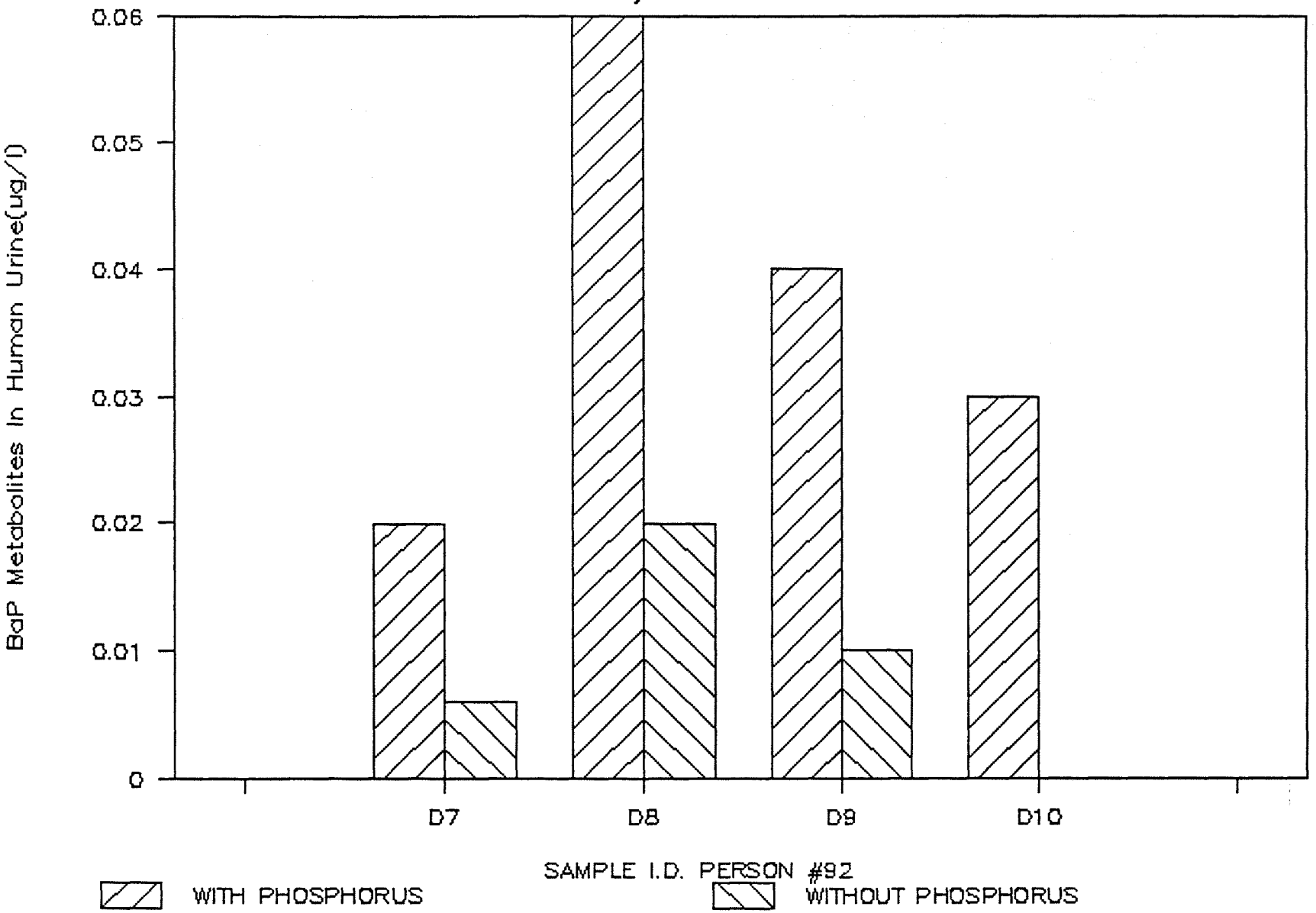
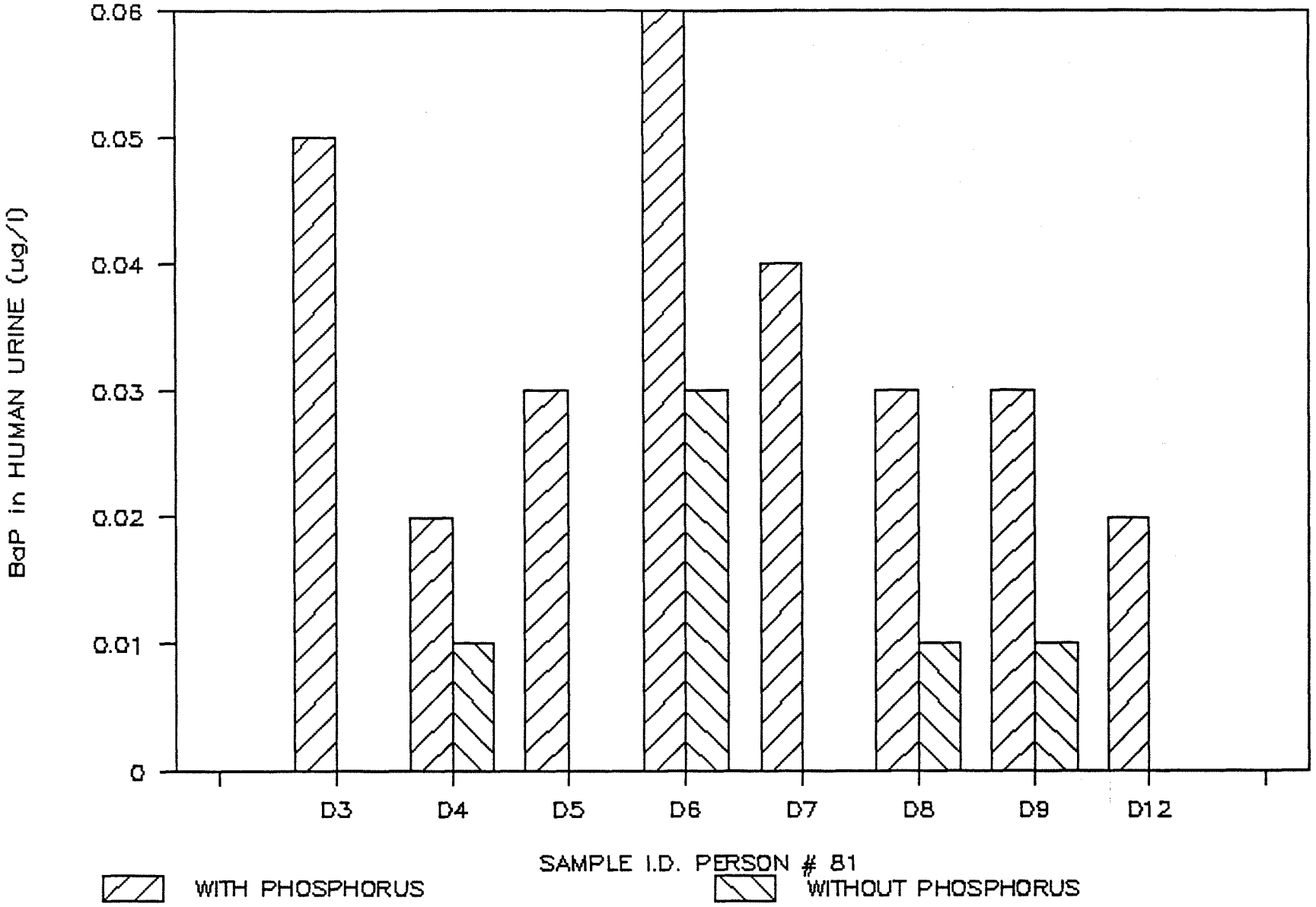


Figure 18. Compare Effect Of Phosphorus

In The Reduction Of BaP Metabolites



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CHAPTER 6 DISCUSSION AND CONCLUSIONS

6.1 Comparison of BaP Exposure to Urinary Excretion

The urinary BaP levels reported in this preliminary study are consistent with values reported by other investigators (See Chapter Two). BaP levels in the exposure and urine samples are above detection and are highly variable (>20-fold) for the 11 non-smoking, non-occupationally exposed participants. A positive association between the variables may be emerging. (Fig. 7) With this preliminary and limited data set, the predictive association bettween exposure and elimination is tenuous. Conclusions about this data will await further analyses of this data and the inclusion of pending analyses.

The relationship of exposure to elimination will be explained further by the inclusion of the remaining Phase II data along with Phase III data. The Phase III data will include a comparison of the Becher and Bjorseth method of urine analyses with that of the method used by Jongeneelen.[1]

Cigarette smoking is considered an important source of PAH exposure. A smoker of filter cigarettes might inhale 0.4 ug benzo(a)pyrene per pack.[2] This is reflected in a significant increase in the excretion of PAH and PAH metabolites in smokers' urines as compared to a non-smoking

control group. Similar results were obtained by Repetto and Martinez[3] for benzo(a)pyrene and by Maly for dibenzo(a,l)pyrene.[4] From our results, it seems that a positive relationship between smoking and excretion of BaP and BaP metabolites may be inferred. However, with these limited data derivation of a conclusion is premature.

6.2 Development of Analytical Protocol

The idea of using the reductive action of HI in the Bjorseth and Becher method was derived from the Konieczny and Harvey paper.[5] These latter omitted phosphorus from the hydriodic reagent. However, this modification was only tested on PAH with less than four aromatic rings. Moreover, in their summary the authors indicate that for resistant cases(e.g. 1-hydroxynaphthalene and 9-hydroxyphenanthrene), HI needed to be employed with red phosphorus. It is also known that relative yields of reduction of different aromatic compounds are anticipated to be a function of energies to surmount the unfavorable keto-phenol equilibria at each stage[5] (Fig. 19). Thus, the yields may vary within a given group of isomers.

As mentioned before, most carcinogenic PAH are larger than tetracyclic(Fig. 1) and are transformed extensively in vivo. With its five rings, BaP is more readly oxidized than its benzenoid isomers as well as its smaller homologues.

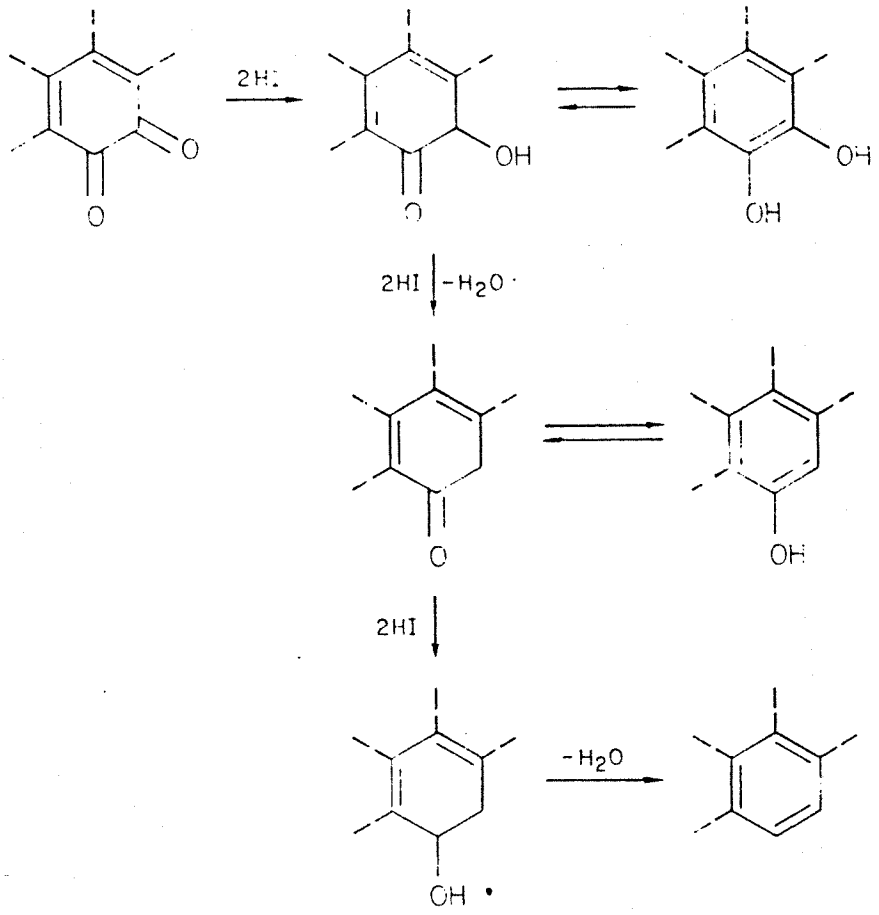

Fig. 19 Protonation and Hydride Transfer from HI with Formation of I and dehydration at Each Stage

Source: ref. 5

Hydrogen iodide dissociates at higher temperatures to iodine and hydrogen, which effects hydrogenations. The reaction is reversible. Its equilibrium is shifted in favor of the decomposition by the reaction of hydrogen with organic compounds to be reduced but it can also be affected by removal of iodine; this can be accomplished by allowing iodine to react with phosphorus to form phosphorus triiodide which decomposes in the presence of water to phosphorous acid and hydrogen iodide. In this way, by adding phosphorus to the reaction mixture hydrogen iodide is recycled and the reducing efficiency of hydriodic acid is enhanced[6]

$$HI + \text{Hydroxy-BaP} \longrightarrow H_2O + BaP + I_2$$

$$P + H_2O + I_2 \longrightarrow H_3PO_4 + HI$$

In the Becher/Bjorseth procedure, iodine formed during reflux may convert BaP to a mixture of quinones and further oxidation destroys the aromatic nucleus[7]. Iodine may also form complexes with BaP.[8] The results of our study support these assumptions.

The recovery of BaP standard refluxed with iodine is 4%; with iodine and acetic acid it is 6%, with hydriodic acid and acetic acid the recovery is 30%, while with acetic acid alone recovery is 99%. For BaP metabolites, the Becher/ Bjorseth standard reduction procedure was very low in

efficiency. In an attempt to improve recoveries, the chemical preparation of the sample was modified to included red phosphorus. Red phosphorus is believed to quench iodine's reaction with the parent BaP. With this modification, recoveries have approximately tripled. It is likely that with appropriate modification of reactant ratios and experimental conditions, the reduction may be controlled to afford the most satisfictory recovery.

Another weakness of the Becher/Bjorseth procedure is the loss from C_{18} cartridge chromatography. Our low recovery (35%) for this step is in line with the Becher/Bjorseth report (40%)[9]. Tests will be made to find a more efficient extraction method.

The low hydrolysis yield will be improved by substituting enzyme hydrolysis for chemical hydrolysis.

6.3 Investigation of Pyrene Metabolites as a Biomarker

The rational for development of pyrene metabolites as a biomarker of human exposure was introduced in Chapter 2. Three reasons to select pyrene as a biomarker of PAH air pollutant are:

a. Pyrene is less reactive than BaP in air. Pyrene is oxidized with greater difficulty.

b. In the atmosphere, the pyrene concentration is higher

than that of BaP.[1]

c. Pyrene metabolizes almost exclusively to 1-hydroxypyrene in contrast with the more than twenty kinds of metabolites obtained from BaP.

Pyrene and its metabolites had acceptable recoveries in the procedure employed. However, we failed to detect pyrene and its metabolites in human urine, probably dueto difficulties in chromatographic separation. The possibility of using pyrene metabolites as a surrogate for PAH metabolites will be investigated in futher studies.

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