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A METHOD FOR THE SEPARATION, IDENTIFICATION AND QUANTITATION OF 3,4-BENZPYRENE FROM RUBBER TIRE DUST

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A METHOD FOR THE SEPARATION, IDENTIFICATION AND QUANTITATION

OF 3,4-BENZPYRENE FROM RUBBER TIRE DUST

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Carbon Blacks and Rubber

In 1755, a London physician named Percival Pott (1) published his observations concerning the appearance of scrotal and facial cancers among those employed as chimney sweeps. Kennaway (2) in a review paper covering the work of his group from the 1920's to the 1950's, describes the isolation of the polycyclic aromatic hydrocarbon, 3,4-benzpyrene or benzo[a]pyrene, (BAP) from coal tar and the demonstration that it is a potent carcinogen. In 1951, Falk et al. (3) demonstrated the presence of BAP and other carcinogenic hydrocarbons in benzene extracts of laboratory rubber stoppers. In the following year, Falk and Steiner (4) showed that the source of BAP in rubber is carbon black.

With our present degree of medical sophistication, emphasis is being placed more and more upon prevention as a means of controlling diseases. Materials and conditions suspected of being capable of causing diseases are now properly within the realm of medical investigation. Nau et al. (5) reported that carbon black (commercial soot) may reach us by various routes: directly as coloring matter in jelly beans and licorice

and indirectly from rubber goods used in food preparation, such as conveyor belts, tubing, seals, gaskets, etc.

Carbon blacks of commerce are produced by a variety of methods from several different substances. The various carbon blacks differ somewhat in physical and chemical properties. Channel blacks are produced by the impingement of natural gas flames upon slowly moving channel irons. The size of channel black particles ranges from about 5 to about 30 millimicrons in diameter. Historically, channel blacks were one of the first commercially produced carbon blacks. Due to the development of pipelines and an increased demand for natural gas, coupled with a low yield, channel blacks are today among the most expensive. There is evidence that the American manufacturers will discontinue making channel blacks in the near future (6). Furnace blacks are produced by the partial combustion of gas or oils, including refinery sludge, and range in size from about 30 to about 200 millimicrons in diameter. These blacks are used to a large extent in rubber. The smaller the size, the more strength and hardness the carbon gives the rubber. The larger sizes also give some degree of strengthening and are termed semi-reinforcing blacks. The larger the carbon particles, the more black can be "loaded" into the rubber stock, thus lowering the price by diluting out the valuable rubber latex. The largest particle size carbon blacks are produced by passing a mixture of petroleum vapor (natural gas, oil, or a mixture) and air through a preheated brick lattice work. The process is efficient enough to be economically feasible using natural gas. Blacks produced by this method are termed thermal blacks and range in size from 120 to 500 millimicrons in diameter. They are used mainly as diluents and have very

little effect on any of the physical characteristics of the rubber stock.

It has been found that the composition of carbon blacks varies with manufacturing methods. For example, according to Smith (7) furnace blacks and thermal blacks, which are water quenched, may contain up to one per cent ash due to adsorption of minerals from the hard water used in the quenching process. In addition to minerals, carbon blacks contain hydrogen as a residual from the hydrocarbons, and chemisorbed oxygen. Thomas et al. (8) report that X-ray crystallography indicates that carbon black particles consist of a series of graphite-like plates having the symmetry and overall structure of large polynuclear aromatic molecules in random orientation. In other words, a carbon black particle looks like a ball made up of randomly oriented flat pieces of chicken wire. They indicate that channel blacks have a porous structure while furnace and thermal blacks do not. Their studies further indicate that polycyclic aromatic hydrocarbons are attached to the surface of the particles by adsorption alone. Falk and Steiner (4,9) showed that there was no detectable BAP in channel blacks of any size. In the case of furnace blacks, it was found that more BAP could be extracted from the larger sizes. It was also noted that the smaller blacks, up to about 17 millimicrons in diameter, would actually irreversibly adsorb added BAP.

According to Smith (7) various carbon blacks are used in tire rubber at loadings from about 30 to about 50 per cent. Depending upon the use of the rubber, either a single black or a blend of different blacks with different properties may be used. The smaller blacks give stiffening, mechanical strength and abrasion resistance. Large sized blacks have little reinforcing ability but may be used in large quantities

without decreasing the resiliency of the stock.

Biological Activity of BAP

In order to discuss the possible health implications of BAP, it would be of use first to consider some of the biological factors which may affect the actions of compounds. Some of the factors are: absorption, distribution, metabolism and elimination. In order for a substance to have a biological effect, it must first be capable of being taken into the body. This requires that the substance not only be present in the environment but also that it must be in a state such that it can cross biological membranes without being inactivated. Next, the material must be in a position to reach biological membranes which it is capable of crossing. Finally, the substance must be present in concentrations sufficient to produce an effect.

Having crossed the biological membranes and gained access to the body, the substance must then be transported to the proper tissue in order to have an effect. In the simplest case, the substance may have a purely local action at the site of absorption. In other cases, the substance must be transported, most commonly by the blood, to other organs and tissues. The rate of absorption can play a major role in determining the possibility of biological action. In the case of slow absorption, it is possible that blood levels sufficient to produce effective concentrations in target tissues will not be attained. In the case of locally acting substances, it is possible that rapid transport will lower the concentrations so that only a brief effect will be observed.

Once a substance gains access to the blood, there are a number of factors which may influence subsequent interactions. Many substances

are bound rather firmly to plasma proteins and are thus held in a relatively inactive state. Enzymes are present in the blood, such as the nonspecific esterases, which can either activate or inactivate substances. There is a difference in the quantity of blood going to different tissues so that uneven distribution will result. The liver, kidneys, lungs, spleen and brain have a high perfusion rate while fat, skeletal muscle, bone and other tissues have a low perfusion rate. The significance is: if a material is capable of being stored in one or more of the tissues having a low perfusion rate, it will take a reasonably long exposure to achieve appreciable levels of the substance in these tissues; but once it becomes present in these tissues, it may be expected to be eluted from them in low levels for a prolonged period. The highly perfused organs will be expected to reflect the blood levels of absorbed materials when the possibilities of metabolism and storage by these tissues are purposely overlooked.

The question of metabolism involves a number of factors. The possibility of local metabolism at the original site of absorption has already been mentioned as has the possibility of some metabolism in the blood. Nearly all venous blood is returned to the heart and lungs by way of the liver, which is the major organ for the metabolism of exogenous compounds. In the liver, there is the possibility that the metabolism of one compound may affect the rate of metabolism of another compound. In certain cases, previous exposure to a compound will result in a significant, rapid rise in the rate of metabolism of that compound.

There are various methods by which the body rids itself of materials. In the case of volatile substances, the lung may provide an exit. Minor excretion routes include desquamated skin, hair and nails, saliva

and perspiration. The major routes of excretion are the urine and feces. The rate of urinary excretion depends upon several factors; the most important of these are plasma concentration, binding of the substance to plasma proteins and whether the material is secreted actively by the renal tubules or is passively filtered out. Reabsorption of the substance by the kidney is also a possibility. There also exists the possibility for fecal excretion of materials absorbed by routes other than oral. In this case, the substance is usually metabolized in the liver and the metabolite is concentrated in the bile and thus passes to the intestine. In a few instances, metabolites concentrated in the bile and moved to the lumen of the intestine are reabsorbed by the intestine and transported to the kidney for excretion in the urine.

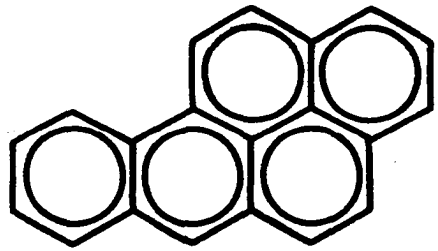
In 1943, Doniach et al. (10) painted BAP onto the skins of mice and observed changes in the color of skin fluorescence with time, thus indicating that BAP may be absorbed through the skin and that at least some local metabolism may be possible. They also injected BAP intravenously into various animals and noticed transient fluorescence in the kidneys, liver and lungs. Fluorescence was found in the feces after 24 hours. Heidelberger and Weiss (11) injected BAP labelled with carbon-14 into mice both intravenously and subcutaneously. All together, they were able to account for 97.2 per cent of the administered dose at the end of 24 hours. Of the total dose, 92 per cent was found in the feces, digestive tract and bile. An additional 2.5 per cent was found in the fat and the rest was found in various other tissues in extremely low concentrations. Virtually none of the BAP was metabolized to CO_2. In the case of subcutaneous administration, the biological half-life of BAP was found

to be 1.75 weeks with approximately 80 per cent of the injected radioactivity recoverable in the pooled feces after 12 weeks. When the C^{14}-labelled BAP was administered to the intact skin, the half-life was found to be 1.66 days for the initial rapid disappearance phase and 4.33 days for the slow disappearance phase. At the end of 20 weeks more than 80 per cent of the administered radioactivity had been accumulated in the pooled feces.

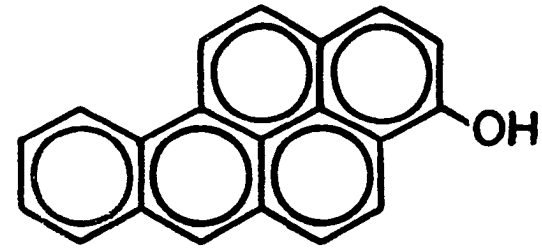
Kotin et al. (12) showed that C^{14}-labelled BAP injected intravenously into the rat is essentially completely cleared from the blood within 10 minutes. Absorption of an intratracheal instillation of an ethylene glycol solution of BAP was shown to be rapid and complete. Metabolism by the liver, secretion into the bile and excretion in the feces was the major pathway of elimination. Rigdon and Neal (13) fed BAP to chickens and found that the chickens showed fluorescence in their feet, feathers and eggs. Rigdon and Neal (14) also administered BAP by intratracheal injection to ducks and found the feathers and other tissues to fluoresce. Rees et al. (15) incubated everted rat intestine with BAP and various metabolic inhibitors. Their results indicate that intestine absorbs BAP by a purely passive diffusion mechanism and that there is no active uptake mechanism.

In an early paper, Peacock (16) found that when BAP was injected intravenously, a blue fluorescence soon appeared in the bile. Sims (17) using a rat liver preparation showed that BAP has three metabolites as shown in Figure 1.

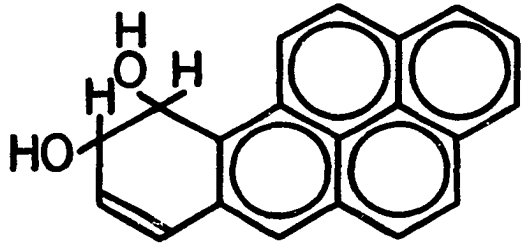
Conney et al. (18) determined that the system responsible for metabolizing BAP, named BAP hydroxylase, is a component of the hepatic



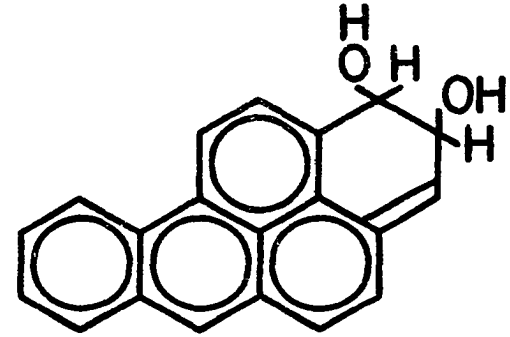
3,4-BENZPYRENE
PARENT COMPOUND



3-HYDROXYBENZPYRENE
MAJOR METABOLITE



9,10-DIHYDRO-9',10'-DIHYDROXY-
BENZPYRENE
MAJOR METABOLITE



1,2-DIHYDRO-1',2'-DIHYDROXY-
BENZPYRENE
MINOR METABOLITE

Figure 1. Structure of 3,4-Benzpyrene and its Metabolites.

microsomal system. It was found that the system requires reduced triphosphopyridine nucleotide and oxygen. Silverman and Talalay (19) developed a highly refined in vitro BAP hydroxylase system and demonstrated that it contains cytochrome P-450. Wattenberg and Leong (20) using a histochemical technique, demonstrated BAP hydroxylase activity in renal cortex, liver, adrenal, thyroid, testis and lung. Liver was found to be the most active tissue, with the others showing only trace activity.

A number of factors were found to influence the activity of BAP hydroxylase. Sims (17) indicates that many polycyclic hydrocarbons are metabolized by the system more readily than BAP. This would indicate that there is a possibility of competitive inhibition when more than one polycyclic hydrocarbon is present.

Conney et al. (18) found that the injection of 0.1 to 1 milligram of BAP into weanling rats could cause up to a 5-fold increase in BAP hydroxylase activity by the end of 24 hours. As administration of the protein synthesis inhibitor ethionine inhibits this response, it is likely that enzymatic induction in this case is due to new enzyme synthesis. Alvares et al. (21) showed that pretreatment with BAP causes an increase in cytochrome P-450, while pretreatment with phenobarbital seems to have the dual effect of stimulating cytochrome P-450 production as well as increasing the affinity of the enzyme system for its substrate.

Arcos et al. (22) found that many different polycyclic hydrocarbons can cause an increase in BAP hydroxylase activity. Creaven and Parke (23) and Buu-Hoi and Hien (24) showed that carcinogenicity and the ability of a polycyclic hydrocarbon to induce BAP hydroxylase are not related. Levine (25) demonstrated that induction of BAP hydroxylase

stimulates both the rate of metabolism of BAP and its rate of biliary elimination.

In addition to induction and competitive inhibition of BAP hydroxylase, there appear to be some species and sex factors involved in BAP metabolism. Wattenberg and Leong (20) found that not all species could metabolize BAP in tissues other than liver. Kodama (26) found a 2 to 3-fold difference in the ability of mice of various strains to metabolize BAP. Levine (25) found that male rats metabolize BAP more rapidly than female rats.

Carcinogenicity

The carcinogenic potential of soot has been appreciated for some time. One of the first references to soot as a potential carcinogen is that of Percival Pott (1). He described the appearance of "soot wart" among chimney sweeps. Falk et al. (27) report that scrotal cancer did not appear among German chimney sweeps until coal replaced wood as the chief fuel. They propose that one explanation is that wood combustion produces little BAP while coal produces much. In a review paper covering the work of his group from the 1920's to the 1950's Kennaway (2) became interested in finding the carcinogenic component in coal tar. Over a several year period he was successful in separating, identifying and demonstrating the carcinogenicity of BAP. This work was the first evidence for the carcinogenicity of BAP. Cottini and Mazzone (28) in 1939 demonstrated that BAP is a carcinogen for man when applied to the skin. Rhoads et al. (29) confirmed that BAP is a human carcinogen. In addition to skin tumors, Rigdon et al. (30) and Uematsu (31) demonstrated the production of leukemia in mice fed BAP. Recent literature provides hundreds

of accounts of the carcinogenicity of BAP.

There are several known mechanisms by which the carcinogenesis of BAP may be altered. Van Duuren (32) reports that a series of compounds isolated from croton oil, named phorbol esters, have a potentiating effect. Even though the half-life of these compounds was found to be only 23 hours, there was found to be a significant increase in tumor production when BAP was applied to the skin after a delay of as much as 380 days following treatment with phorbol esters. Wattenberg and Leong (33) demonstrated that compounds which potentiate BAP hydroxylase activity greatly reduce the carcinogenicity of BAP. Homburger and Treiger (34) found that quinones inhibit the carcinogenicity of BAP. Falk et al. (35) found that some polycyclic aromatic hydrocarbons such as phenanthrene and anthracene are able to inhibit the carcinogenicity of BAP. It appears possible that this type of inhibition may be due to the ability of the other hydrocarbons to occupy physically the cellular sites where BAP is active in producing cancer.

Nau et al. (36, 37) exposed mice to carbon black for prolonged periods. They found no incidence of carcinogenic effect when exposure was by ingestion or direct skin contact. It was found that a hot benzene extract of the carbon blacks used was carcinogenic. In a later paper, Nau et al. (38) attempted to extract materials from carbon black with gastric juice, blood plasma and a mixture of gastric juice and ten per cent cooking oil. In no case was anything shown to be extracted. The discrepancy between these findings and the evidence of Falk et al. (35) who did find that plasma elutes BAP from carbon blacks larger than 100 millimicrons in diameter could be due either to differences in analytical

methods or to the use of different carbon blacks having quite different properties. Nau et al. (39) exposed mice, rabbits, guinea pigs, hamsters and monkeys in dust chambers to carbon black for prolonged periods. There was evidence of carbon accumulation in the lungs but no evidence of malignancies. In the past several years, Nau (40) has exposed monkeys to rubber dust in dust exposure chambers, with no evidence of malignancies. The failure to produce tumors under these circumstances may be due to any of the factors mentioned above, but are apparently due to binding of the carcinogenic materials to the carbon and rubber particles in a manner making them inaccessible to the body.

Analytic Methods

The goal of any quantitative chemical analytical procedure is to determine in a reproducible, accurate fashion, the quantity of material in question. In order to attain the goal, several practical considerations must be examined. The desired substance is rarely found alone. It is thus nearly always necessary to separate the substance from similar substances capable of interfering with the measurement of the desired compound. The degree of isolation required depends, to a large extent, upon the detection and measurement system to be employed. If a detector is easily misled by other substances, extensive sample preparation may be required. If a detector is available which is quite insensitive to substances other than the one in question, relatively little separation is required. Another characteristic to consider in a detector besides specificity is sensitivity. The detection method employed must be sufficiently sensitive so that it may respond to the levels of material available. Due to low detector sensitivity, especially in trace analysis,

it is frequently necessary to concentrate a sample in order to be able to measure it. Detector selection also depends to a large degree upon the required degree of sensitivity and accuracy. Other factors entering into any practical consideration of analysis include expense and time. These factors are dependent to a large extent upon the anticipated sample load as well as the required degree of sensitivity and accuracy. It may be perfectly reasonable to employ a method yielding but a single determination per week, requiring considerable expense in time and materials when sample variation is expected to be low and small numbers of determinations may be expected to yield statistically valid results. In the case of biological data where individual variation may cause wide discrepancy between values, it is ordinarily necessary to use large numbers of determinations, requiring fast, efficient, inexpensive procedures. Of course, in each case, the accuracy of the method must be such that valid data may be obtained. It is desirable to determine in advance the required degree of accuracy; thus it may be possible to avoid the waste inherent in an overdeveloped system. One other important consideration is that not only does each additional step in a determination represent an expenditure in time and money, it also represents a potential for sample loss or contamination. Any quantitative method intended for routine use should be simple enough that it may be employed by laboratory technicians in reasonable time with reasonable accuracy, where "reasonable" is determined by the requirements of the experimental design.

Presently there are three systems which are used for the detection of BAP. These are 1) gas-liquid chromatography, 2) ultraviolet absorption and 3) fluorescence spectral analysis. According to Sawicki et al. (41) the minimum quantity of BAP detectable using gas-liquid

chromatography is 5 x 10^{-6} grams. According to Jager (42) the minimum sensitivity for ultraviolet absorption is 10^{-6} gram per milliliter, and the minimum level for fluorescence is 10^{-10} gram per milliliter. Even though gas-liquid chromatography offers the lowest sensitivity of any of the detection methods, it offers one overwhelming advantage. Todd (43) was able to demonstrate the presence of a number of polycyclic aromatic hydrocarbons on carbon black directly by using a pyrolysis unit in a gas chromatograph to cause thermal desorption of the adsorbed materials. Although it was not possible to quantitate the various hydrocarbons in this study, the advantage of being able to detect the presence of a variety of compounds without lengthy sample preparation makes the method extremely valuable, especially for screening purposes. This method, however, is not sufficiently sensitive for general trace analysis.

There are many methods in the literature utilizing ultraviolet absorption for detection. It is unfortunate that ultraviolet spectra generally occur as a series of rounded humps. The rather nonspecific nature of ultraviolet absorption spectra usually requires extensive sample preparation, generally involving a combination of chromatographic steps and solvent partition (44, 45, 46, 47, 48, 49). These methods require from two to five days per analysis.

Given proper instrumentation, fluorescence analysis is specific as well as sensitive. The most useful instrument for fluorescence analysis is the spectrophotofluorimeter. This instrument together with an X-Y recorder allows one to determine the wavelengths which cause the material in question to fluoresce, to determine the wavelengths given off by the fluorescing material and to record these spectra. As two

spectra, an activation spectrum and a fluorescence spectrum are involved, it is usually possible to pick an activation line and a fluorescence line such that other fluorescing materials which may be present will not interfere. Zdrojewski et al. (50) have shown that BAP may be easily detected by fluorescence analysis in the presence of materials which make its detection by ultraviolet absorption impossible. Analytical methods range from the technique of Monkman et al. (51) who were able to simply extract high volume air filters with solvent and determine the BAP from the crude extract to rather elaborate separation methods such as those used by Dubois et al. (52) and Davis et al. (53).

CHAPTER II

PURPOSE AND SCOPE

Recently the United States Food and Drug Administration has ruled that up to ten per cent carbon black produced specifically by the furnace method may be used in rubber products for food processing (54). It is a known fact that desorbable BAP is an adsorbed component of carbon blacks with the exception of channel blacks. It has also been shown that BAP on carbon black becomes a component of rubber when the carbon black is incorporated into the rubber formulation. Some of the rubber items used in food processing include conveyor belts, gaskets, tubing, seals, carrying containers and electrical insulators. It is expected that these items will be exposed to a certain amount of abrasion and that rubber particles may be found in the foods which are exposed to rubber. It is also to be expected that the rubber particles may contain BAP. The possibility of a health hazard due to the ingestion of rubber dust contaminated food has not been determined.

There are a number of factors which may affect the possible action of BAP present in the rubber particles. It is quite possible that the BAP is so firmly adsorbed onto the carbon particles that it may not be removed by normal physiological processes. There is also a possibility that the noncarbon components of rubber effectively prevent physiological contact with all but the carbon particles exposed on the surface of the

rubber particles. If it is possible for BAP to be absorbed from rubber particles, it is possible that this process may be so slow that a biologically effective concentration of free BAP may not be reached. The attainment of a biologically effective concentration of BAP depends upon total dose, dose rate and individual sensitivity. It has been shown (12) that a single small dose of BAP may be cleared from the blood of the rat in about ten minutes. It has also been shown (18, 25) that a low level exposure to BAP has a rapid and large stimulating effect upon the biochemical machinery responsible for the metabolism of BAP. It is possible that a large dose of BAP absorbed over a sufficiently long time might be completely metabolized to inactive substances. It is also possible that if this same dose were absorbed rapidly enough, it could reach sensitive tissues in a high enough concentration to cause a carcinogenic response.

The Department of Environmental Health at the University of Oklahoma has been concerned with the possibility of a health hazard due to contact exposure to carbon black and rubber dust. It has been found that these materials apparently have no untoward effect on the species tested when administered by the cutaneous and respiratory routes (37,38,39). There is no information concerning the possible adverse effects of rubber dust administered by the oral route. As has been pointed out above, because of wear and tear of rubber products used in food processing, humans may be exposed to rubber particles in their food. It is felt that it would be desirable to study some of the factors involved in attempting to determine the possibility of a health hazard due to ingested rubber particles.

A survey of the literature has shown that there is no method available at this time which will allow one to determine rapidly the

quantity of BAP present in a complex mixture such as feces. It is felt that it would be worthwhile to find a method allowing the rapid determination of BAP from feces. This would be accomplished by housing rats in metabolic cages and feeding them chow spiked with rubber dust. As rats are coprophagic, housing them in metabolic cages would insure that the rubber collected in the feces would have passed through the gastrointestinal tract only once. The material collected from the metabolic cages would supply material to be used in finding a method for analysis. In addition, the use of metabolic cages would allow one to quantitate the BAP intake of the rats and also to determine the BAP output in the feces. Any difference in these numbers would indicate that BAP was being removed from the rubber dust.

CHAPTER III

MATERIALS, INSTRUMENT AND PROCEDURE[1]

Reagents

The rubber dust used in this study was obtained from a local tire recapping shop. It was dried at room temperature for a short time and then screened, using a set of sieves and a shaking machine. A fraction consisting of pass 80 mesh was used.

Iso-octane (i-C8) was obtained from Phillips Petroleum Company. It was 99 mol per cent and was repurified by distillation, discarding the first and last ten per cent. Benzene (Bz) was Baker reagent grade, purified by triple distillation, discarding the first and last ten per cent at each step. The methanol used was Baker absolute methanol purified by refluxing four liters with 20 grams of potassium hydroxide and 50 grams of zinc dust for three hours and then slowly distilling, discarding the first and last ten per cent. Every effort was made to keep the methanol as anhydrous as possible. Baker anhydrous reagent grade ether was used as received. Matheson, Coleman and Bell dimethylformamide, spectroquality, was used as received. Nitrogen, prepurified grade, was used.

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[1]The entire procedure is presented in flow chart form in Appendix B, page .

The chromatography paper employed was Whatman number 1, 19.5 by 56.5 centimeters, prewashed with i-C8. Florsil, 60 to 100 mesh, was obtained from Fisher and was prepared by washing 500 gram portions with 4 liters of redistilled benzene, 3 liters of redistilled methanol and then drying in a vacuum oven at 25 to 26 inches of mercury and 50 degrees centigrade for 16 hours. The sodium sulfate was Fisher reagent grade, anhydrous. The various BAP standard solutions were prepared using Baker pure grade BAP, melting point 176 to 178°C.

Animals

The rats used were Cheek-Jones line of Sprague-Dawley albino rats. Both male and female young adults were used. The rats were housed in the animal facilities for six weeks before use. They were kept in stainless steel pans and were provided with Rockland rat chow and tap water ad libitum. They were handled frequently so that being handled during the experiment would not induce additional stress, possibly affecting their eating habits.

Animal Diet

The standard chow employed was compounded as follows: 200 grams of all purpose white flour, 750 grams of finely ground Rockland rat chow, 250 milliliters of Crisco cooking oil and 750 milliliters of 5 per cent carboxymethyl cellulose were combined with 50 grams of rubber dust for the experimental group and without the rubber dust for the control group. The mass was rolled into sheets about 2 millimeters thick and scored so that it could be broken into pieces roughly 5 millimeters square after being dried at 65°C for 24 hours. It was found that using small pieces

of food was a reasonable compromise with the rats. It was not practical to restrain the rats in the feeding area of the metabolic cages. Powdered food was not accepted by the rats and was easily scattered by them, thus contaminating the fecal samples and making a quantitative determination impossible. Large pieces of food were usually carried into the body of the metabolic cage, nibbled on for a short time and dropped. While these large pieces were easily separated from the feces, it was found that the rats were not actually getting much to eat. While the smaller pieces were also dropped into the bottom of the metabolic cages, there was more tendency to consume them and the fragments were separated from the feces.

Animal Feeding Experiment

Sixty rats were divided into four groups in the following manner: fifteen male controls, fifteen male experimentals, fifteen female controls and fifteen female experimentals. Each group was housed two rats per metabolic cage except for the fifteenth animal of each group which was housed individually. Chow either with or without rubber dust was fed at the rate of ten grams per rat per 24 hours. Tap water from the same source was available freely. Fecal collections and exact chow intake determinations were made for 24 hour periods on the following days: minus 4, plus 1, plus 2, plus 4 and plus 8. Collections were pooled for each group for each collection period. All fecal samples were stored frozen in the dark until processed.

Feces samples were thawed and dried at room temperature in the dark overnight and subsequently extracted for 48 hours in Soxhlet extractors with redistilled benzene. The extract was reduced in volume sufficiently to be transferred quantitatively to a volumetric flask and diluted

to exactly 50 milliliters. The samples were stored in the dark in glass stoppered bottles until analyzed.

Sample Preparation

It was found that it was possible to determine BAP by using a two step chromatography procedure followed by fluorescence analysis. Because BAP is light sensitive, all procedures were carried out in a darkened environment. Into a glass chromatography column with an inside diameter of approximately 27 millimeters were placed 15 grams of prepared Florsil with a 15 gram layer of anhydrous sodium sulfate on top. The column was prewashed with 25 ml of i-C8 followed by another 25 ml of i-C8. Immediately after pipetting this second 25 ml of i-C8 onto the column, a 0.500 ml aliquot of the 50 ml sample was added as rapidly as possible. The column was allowed to stop dripping, the walls were washed down with a small quantity of i-C8 and the column was extracted with 150 ml of i-C8. The i-C8 fractions were discarded. The column was then eluted with five 25 ml portions of benzene which were pooled. The benzene fraction was evaporated to a small volume on a steam bath under a stream of nitrogen and transferred quantitatively to a 25 ml glass stoppered flask with 5 ml of benzene. The benzene extract was then evaporated to approximately 0.2 ml. This benzene extract was then applied to paper in a band approximately 0.5 by 11 cm. A reference spot of BAP was also applied at one edge of the paper. The paper was coated with a stationary phase of 25 per cent dimethyl formamide in diethyl ether and developed for approximately four hours with i-C8 by descending chromatography.

The chromatogram was then dried briefly and examined in the dark with ultraviolet light. A band corresponding to the reference material

was outlined in pencil and cut out with scissors. The paper strip was then cut into small squares and placed in a sintered glass pressure filtering funnel. The material was then eluted from the paper with small portions of hot methanol totalling approximately 40 ml. Dry nitrogen gas was used to supply pressure for filtering. The methanol was then evaporated to a small volume using the steam bath and a stream of nitrogen. The flask containing the residue was then held in the hand and taken to dryness in a stream of nitrogen. The walls of the flask were washed with 1 ml of benzene, the contents transferred quantitatively to a 5 ml volumetric flask and diluted to the mark.

Sample Analysis

Principles of Operation of the Spectrophotofluorimeter

The spectrophotofluorimeter, abbreviated SPF, consists of a high-intensity xenon light source, two monochromators, a sample compartment and a light detector. Figure 2 is a diagrammatic representation of the instrument. The light source is located at the input of the first monochromator so that its light may be dispersed into whatever wavelength is desired. The output of the first monochromator is directed into the sample compartment. The input of the second monochromator comes from the sample compartment at right angles from the output of the first monochromator; thus, it is impossible for light to travel directly from the output of the first monochromator directly into the input of the second monochromator. The sample acts as a light source for the input of the second monochromator. The light input to the second monochromator may be either exactly the same wavelength as the wavelength of the output

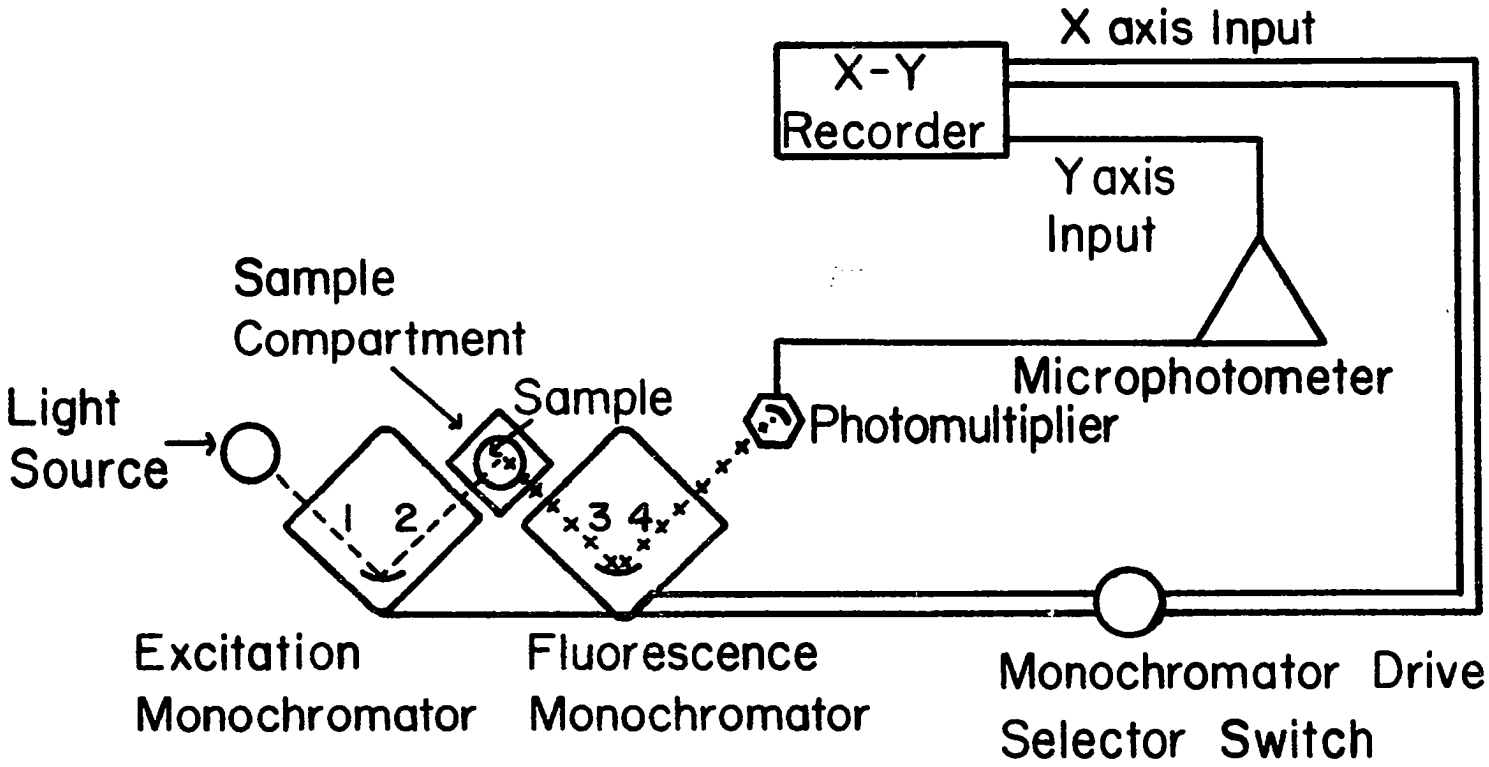

Figure 2. Diagrammatic Representation of the Spectrophotofluorimeter.

of the first monochromator due to light scattering by "dust" in the sample or may be of a different wavelength due to fluorescence of the sample. As photons of light energy strike a substance, it is possible for photons of exactly the right energy to interact with an orbital electron of the substance and boost that electron to a higher energy level. This creates an unstable situation, and the electron usually immediately drops back to its original energy level, giving off a photon of somewhat lower energy than the original striking photon. The process of absorbing a photon and having an electron boosted to a higher energy level is called excitation; giving off a lower energy photon as the electron drops back to its original energy level is called fluorescence. The higher the energy of a photon, the shorter its wavelength. As photons of lower energy than the original photon are given off in the fluorescence process, fluorescence always occurs at longer wavelengths than the wavelengths required for excitation. The degree of spectral shift is some index of the amount of energy required for activation of the substance. In the case of simple scattering, the photons simply "bounce off" particles in the solvent without interacting with them and appear radiated in all directions without undergoing a spectral shift.

A light detector is mounted at the output of the second monochromator. This arrangement allows one to find the wavelengths which will excite a substance and then to find the wavelengths the substance emits when it fluoresces. In addition, by using stable electronics, it is possible to quantitate the light reaching the detector and to relate this to the concentration of material in the sample.

The instrument used in this case was an Aminco-Bowman model

4-8203 SPF equipped with an off-axis ellipsoidal mirror condensing system. This was used in conjunction with an American Instrument Company solid state blank subtract photomultiplier microphotometer and a 1P28 photomultiplier. The data were recorded using an X-Y recorder, model 814A made by Bolt, Beranek and Newman. All slits were set at one millimeter. All determinations were made with a sensitivity setting of 100 per cent and the meter multiplier value was set such that the recordings did not go off scale. The output of the microphotometer activates the Y axis to the recorder and the monochromators the X axis.

Calibration and Standardization

The operating parameters of the SPF were first studied. It was soon found that the sensitivity control on the microphotometer does not respond in a linear fashion; in order to be able to compare results using different meter multiplier values, it is necessary to use the same sensitivity position. The value of 100 per cent was chosen as the standard value for the sensitivity control for all determinations. A value of 1 millimeter for all slits was found to give good sensitivity as well as excellent spectral resolution. Figure 3 shows the excitation and fluorescence spectra for BAP in benzene. It was found experimentally that using a wavelength of 386 nanometers for excitation and 405 nanometers for fluorescence gave the greatest response. These values were chosen as standards for quantitating BAP in benzene. Solvents other than benzene may cause spectral shifts. If they are used, it is necessary to determine the excitation and fluorescence spectra before using that solvent, and to use these spectra to find the optimum excitation and fluorescence wavelengths.

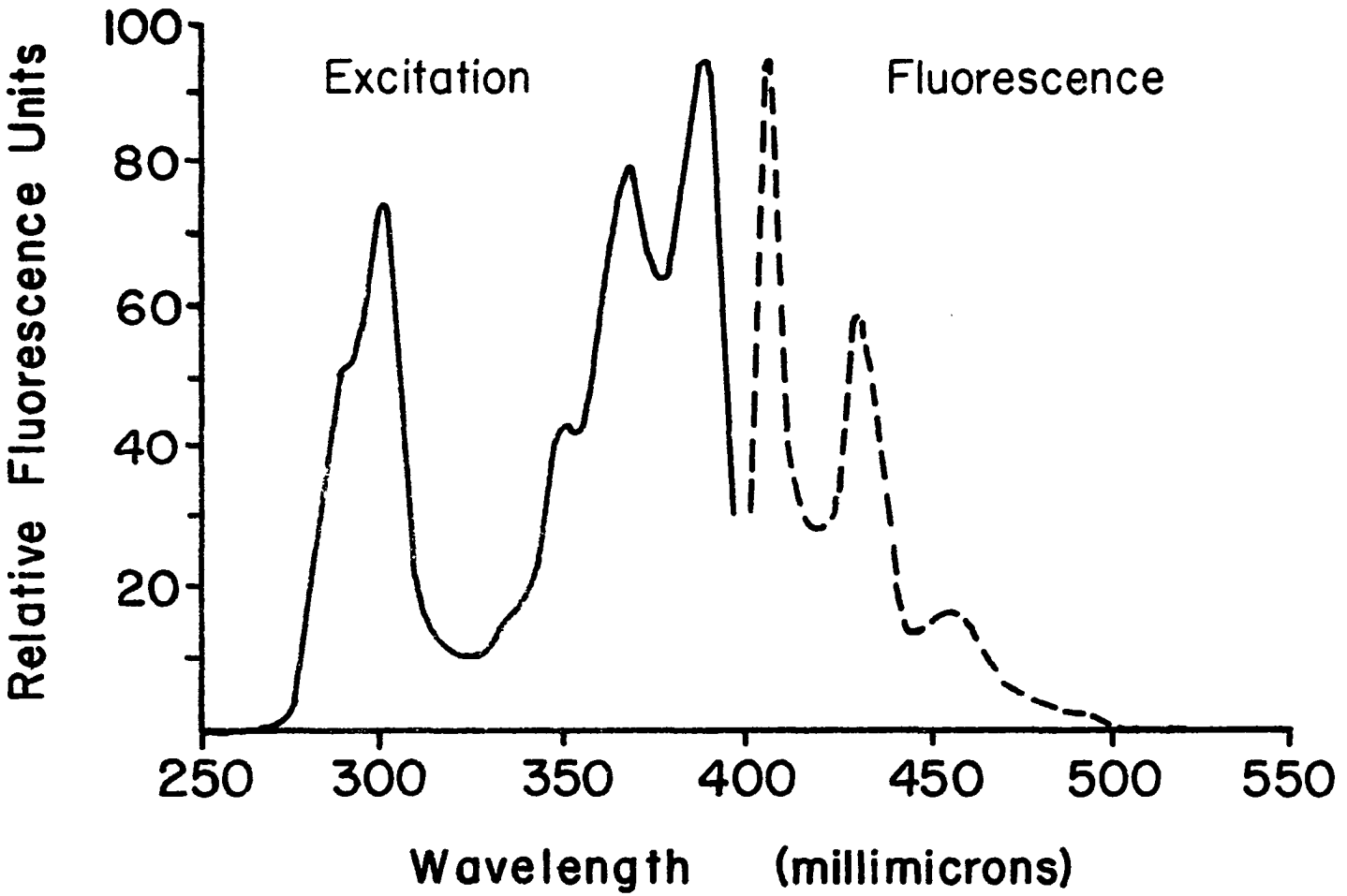

Figure 3. Excitation and Fluorescence Spectra of 3,4-Benzpyrene in Benzene.

Figure 4 shows the instrument response to different concentrations of BAP. It is interesting to notice that the response is linear between 10^{-9} grams per milliliter and 10^{-6} grams per milliliter. It was found that 10^{-6} grams per milliliter is approaching the highest concentration which can be measured with the instrumental conditions used without diluting the sample. Concentrations less than 10^{-9} grams per milliliter can be measured with the instrument used in this study, but much below 10^{-9} grams per milliliter an unknown material in the benzene begins to interfere with the measurements. Whatever the material is, it appears to be present in extremely minute amounts and is only slightly reduced by additional distillations. It is present in such small quantities that a good spectrum of it is not readily obtainable and it serves only to make uncertain the spectrum of BAP.

In analyzing the samples, each unknown was scanned and its excitation and fluorescence spectra recorded. A value in arbitrary fluorescence units was determined by multiplying the height of the excitation peak times the meter multiplier value. A series of standard dilutions of pure BAP was prepared in benzene, and a suitable dilution of standard BAP was also recorded on the same sheet as the unknown. The value of the unknown was then determined by comparing the ratio of the value in fluorescence units and concentration of the standard to the value in fluorescence units of the unknown. Evidence justifying the direct linear interpolation ov values comes from the determination of the linear response to concentrations of BAP ranging from 10^{-6} grams per milliliter to 10^{-9} grams per milliliter.

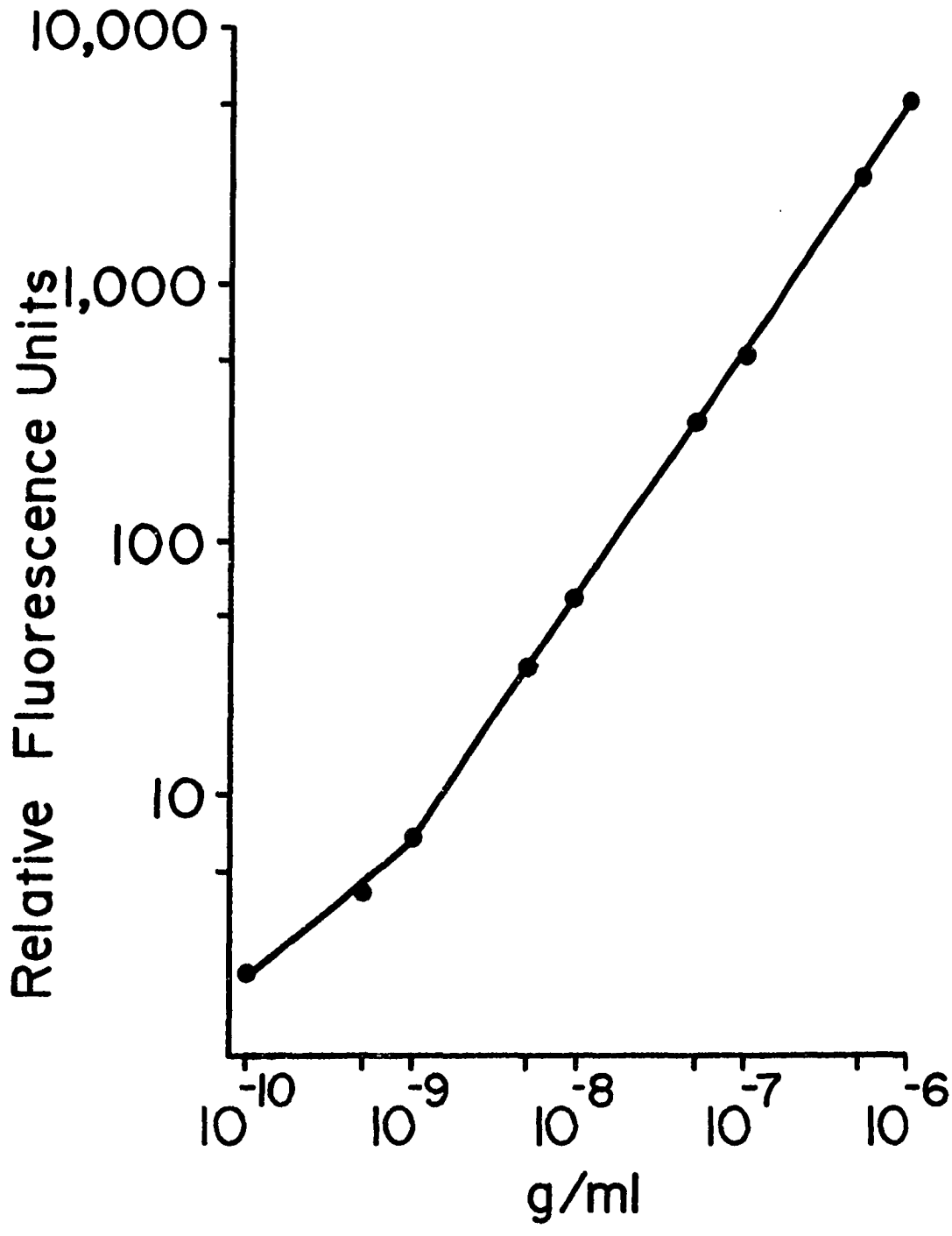

Figure 4. Calibration curve for 3,4-Benzpyrene.

CHAPTER IV

RESULTS AND DISCUSSION

A series of determinations was made to find the reproducibility and sensitivity of the method. The determinations were run exactly as if a biological sample were being used except that instead of using a biological sample, an appropriate quantity of standard BAP solution was used. The results are summarized below in Table 1 and in Figure 5.

It is seen in Figure 5 that a recovery curve must be plotted if it is necessary to determine exact quantities of BAP. If it is desired only to determine relative quantities, then the raw values may be used. The results of the feeding experiments are presented in Table 2. These values are summarized for the male and female experimental groups in Figures 6 and 7. It is seen that there is a delay in the appearance of BAP in the feces and that there is an overshoot. It is believed that the delay in appearance of BAP is most probably due to the approximately 24 hours required for material to pass through the gastrointestinal tract of the rat. The reason for the overshoot is not apparent and no explanation is given. Integrating the areas under the curves shows a 112.2 per cent recovery for the male experimental group and a 93.2 per cent recovery for the female group. As these values are based on single observations, they are not statistically important. Because the quantity of BAP excreted is the same as the quantity ingested, it is felt that the

TABLE 1

RECOVERY DATA FOR VARIOUS CONCENTRATIONS
OF BAP RUN THROUGH THE SYSTEM

Concentration g/ml	Value RFU	Average Value, RFU	Average Value Corrected to Zero, RFU	Standard Error	Per Cent Recovery
zero	10.5 9.5 9.1	9.7	0	0.42	-
10^{-10}	17.0 15.0 14.0	15.3	5.6	0.94	124[a]
10^{-9}	20.5 22.3 22.8	21.9	12.2	0.70	84
10^{-8}	90.0 96.0 97.2	94.4	84.7	2.25	81
10^{-7}	855 880 909	881.3	871.6	15.6	88

[a]Recovery in a range greater than 100 per cent has been found repeatedly for this concentration. It is believed that this may be due to a contribution from the unknown substance present in the benzene.

TABLE 2

3,4- BENZPYRENE RECOVERY DATA FROM FEEDING EXPERIMENTS*

Day	Male Control Excreted	Female Control Excreted	Male Experiment Ingested	Male Experiment Excreted	Female Experiment Ingested	Female Experiment Excreted
minus 4	0.60	0.50	0.20	0.48	0.20	0.36
zero	0.85	0.44	0.20	0.78	0.18	0.42
plus 1	0.85	0.43	35.5	9.90	30.5	11.98
plus 2	0.60	0.58	32.6	35.70	26.9	22.46
plus 4	0.70	1.88	31.4	47.15	27.4	33.90
plus 8	0.90	0.54	37.8	43.65	25.3	23.90

*Values are given as micrograms of BAP. Ingestion values were determined by quantitating the BAP present in the diet presented in the 24 hour period immediately preceeding the start of the feces collection period.

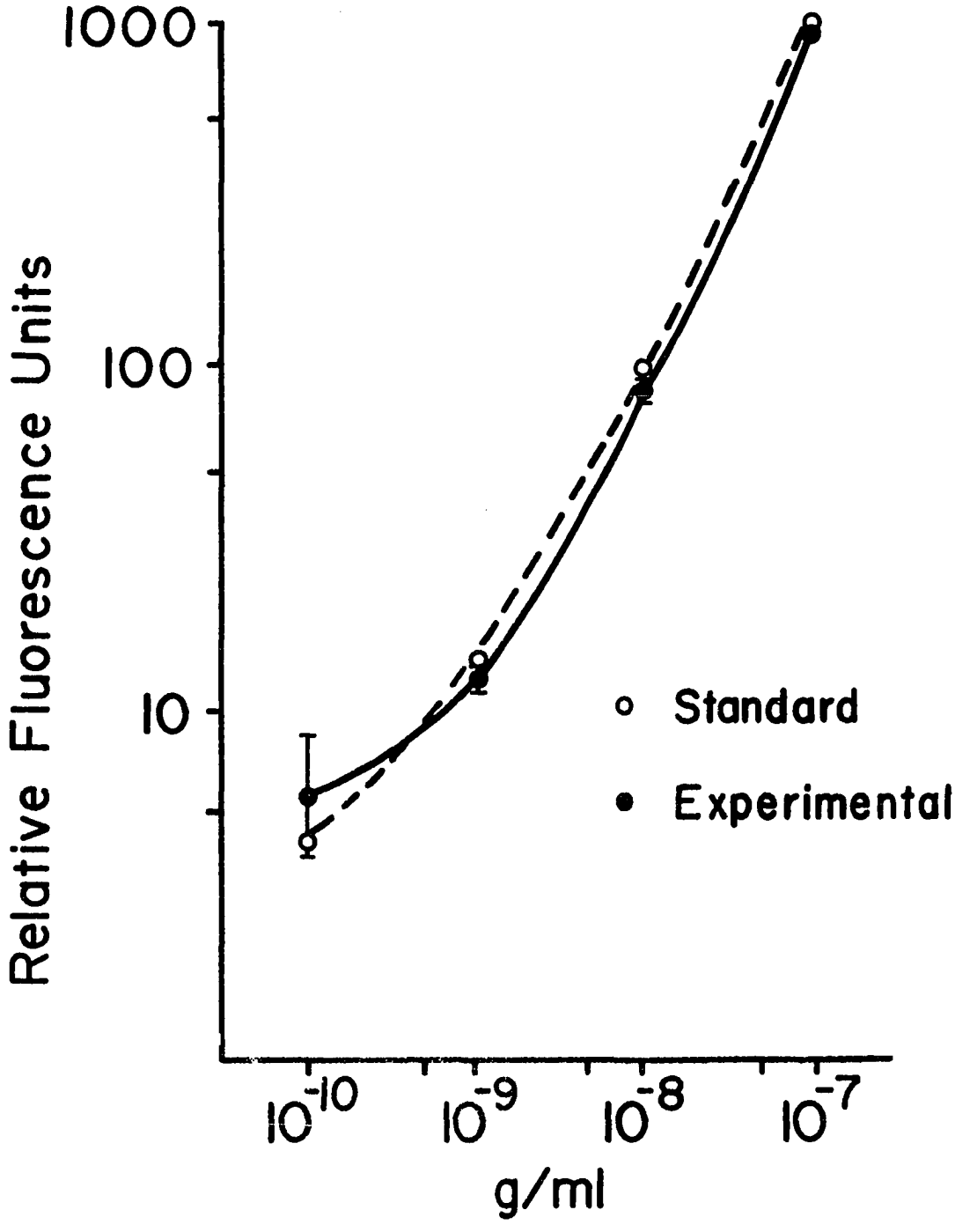

Figure 5. Recovery Using Various Concentrations of 3,4-Benzpyrene.

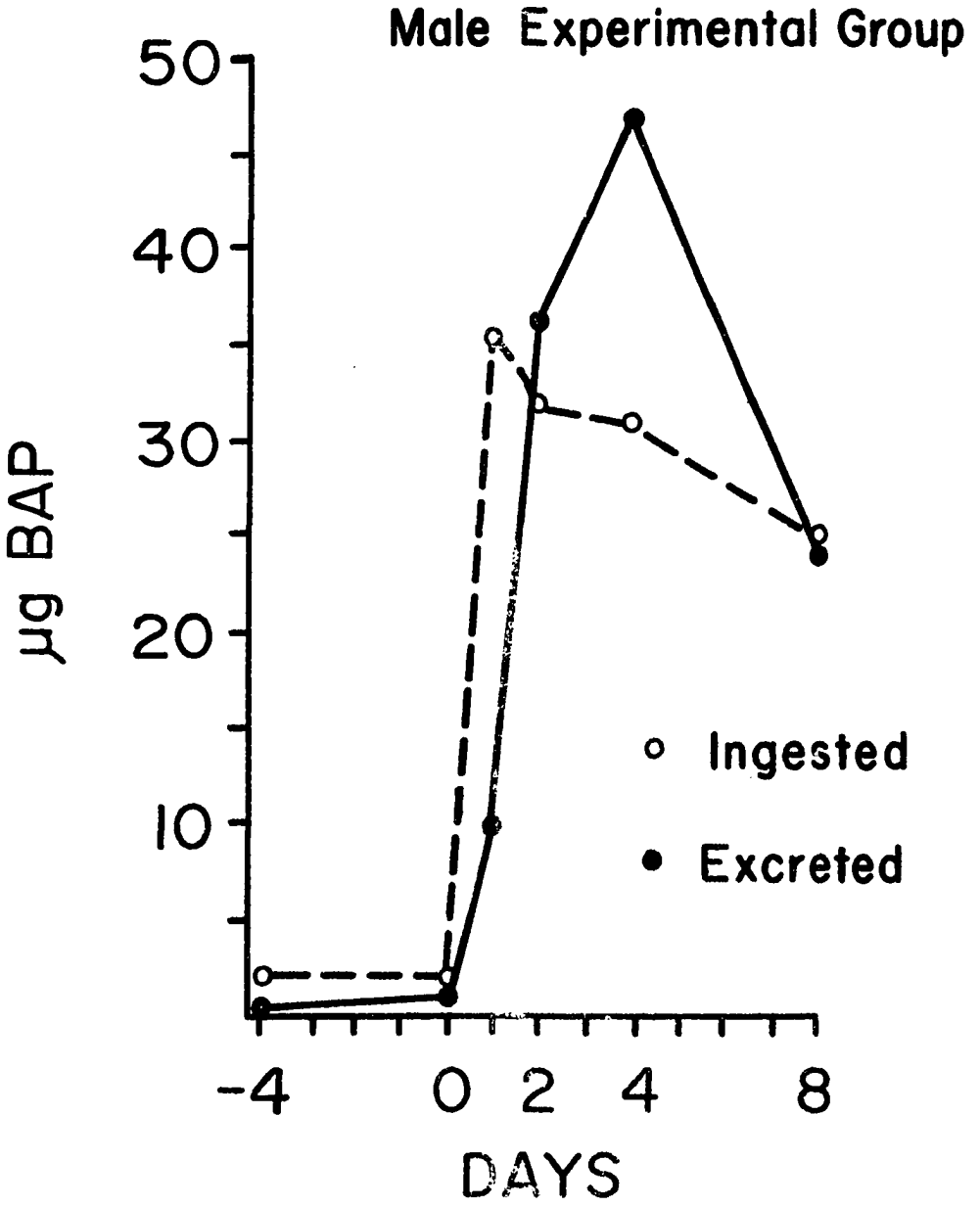

Figure 6. 3,4-Benzpyrene Recovery Curve for the Male Experimental Group.

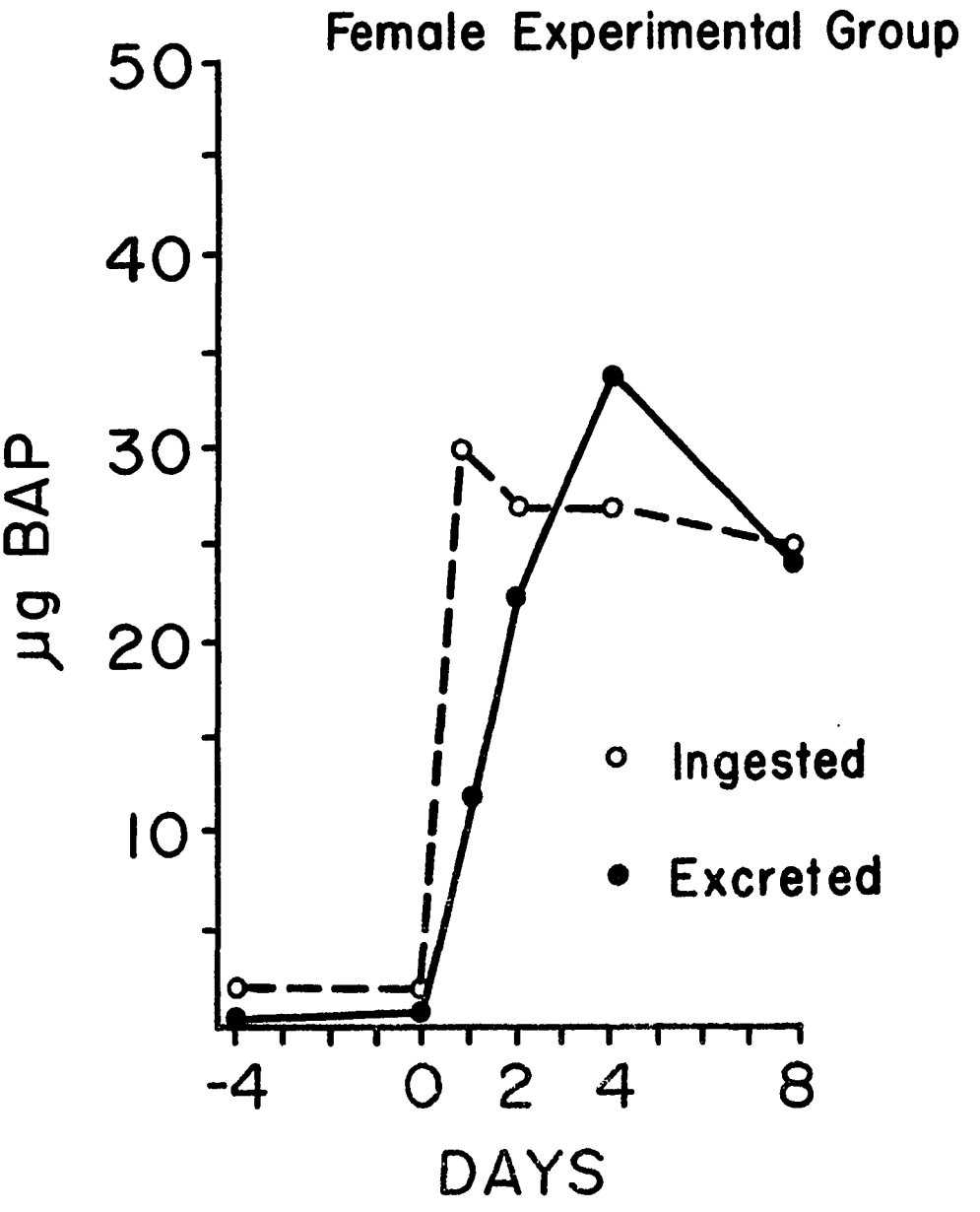

Figure 7. 3,4-Benzpyrene Recovery Curve for the Female Experimental Group.

results indicate that it is likely that none of the BAP present in rubber is absorbed from the gastrointestinal tract of the rat under the conditions of this experiment. Figures 8 and 9 show that the control values remained quite constant throughout the experiment and were insignificant compared to the experimental values.

Rubber dust was fed to rats in order to obtain a sample of rubber containing feces. These feces provided the material for developing a sensitive, reproducible analytic method. Having developed the method, it was decided to attempt to find the quantitative relationship between the quantity of BAP ingested in the rubber and the quantity of BAP excreted in the feces. This data is presented in Table 2 and Figures 6 and 7. Recoveries of 112.2 per cent for the male group and 93.2 per cent for the female group, although not statistically valid, do indicate that there is probably no removal of BAP from the lumen of the gastrointestinal tract of the rat from rubber dust under normal physiological conditions. These values also show that it is possible to apply this analytical method to an actual situation. In addition, the sizes of the per cent recoveries for the male and female experimental groups indicate that the method is reproducible.

It is felt that it would be worthwhile to mention some of the difficulties encountered in this project. Initially, the benzene was stored in an unairconditioned solvent shed before distillation. Apparently the summer sun in Oklahoma has an effect on benzene, for it was found to take on a strong yellow color after several months of storage. The discoloring was avoided by storing the benzene in a cold room. It was soon found that most lots of Florsil will retain a great deal of BAP from

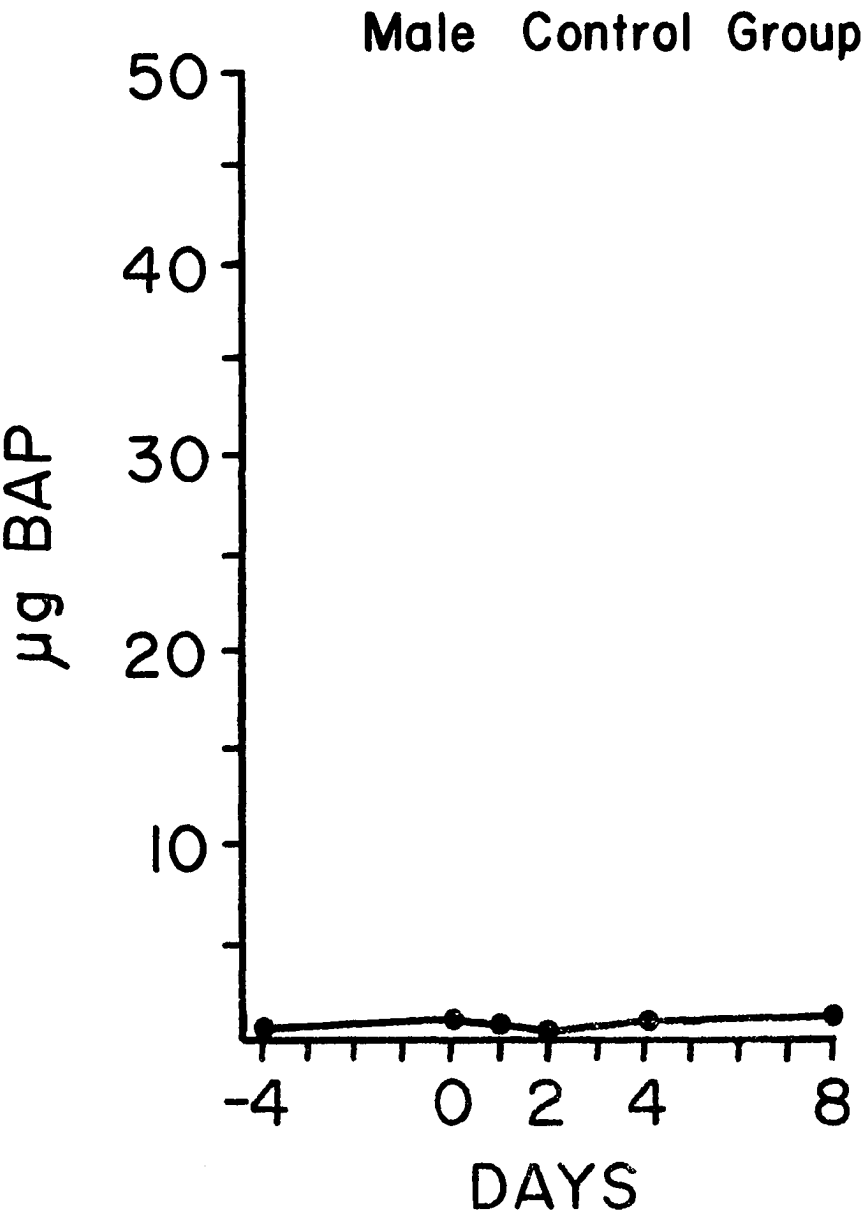

Figure 8. 3,4-Benzpyrene Recovery Curve for the Male Control Group.

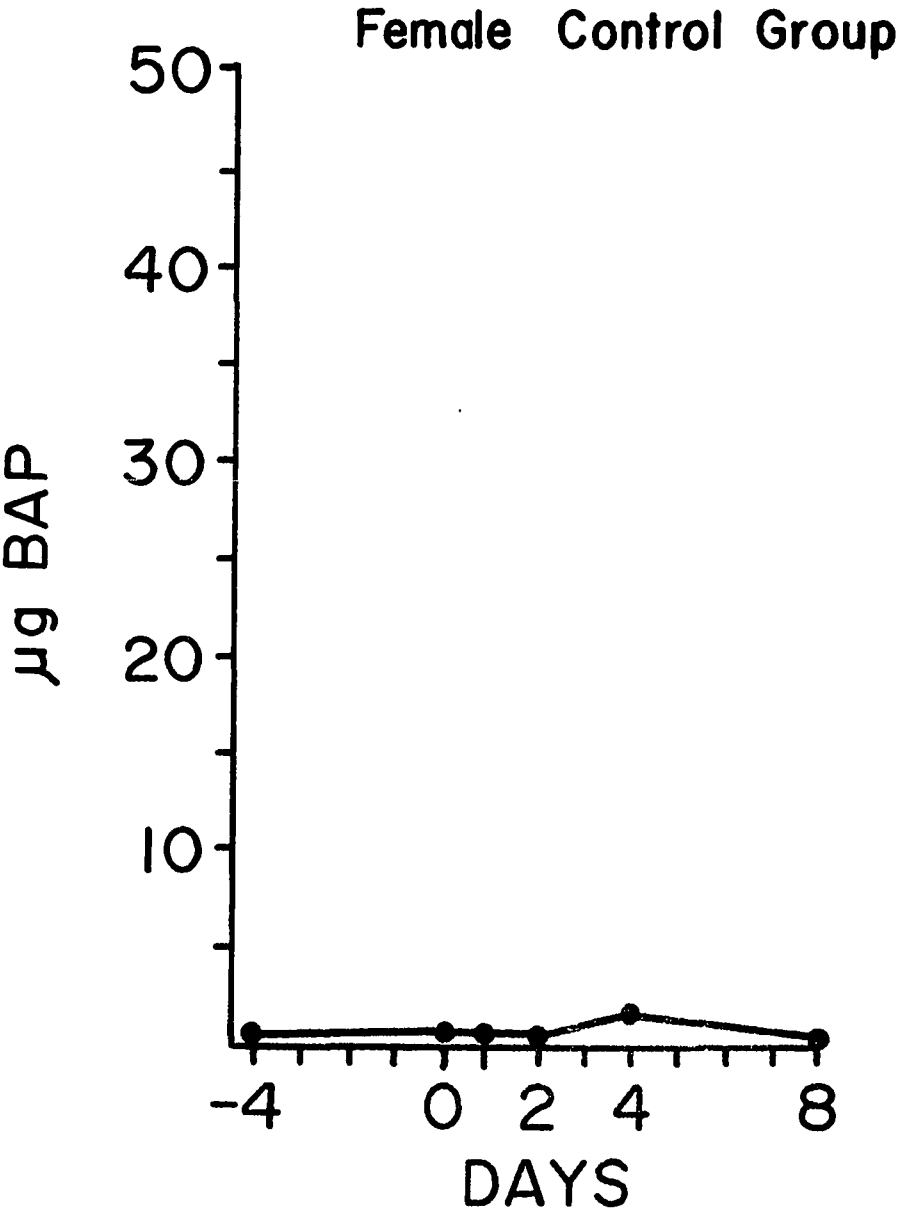

Figure 9. 3,4-Benzpyrene Recovery Curve for the Female Control Group.

the sample if they are used as received. Washing with benzene and methanol and drying in a vacuum oven before use greatly improves the recovery of the BAP.

It was found that rats will carry food off and nibble it and drop pieces. In order to keep food contamination of the feces to a minimum, it was found useful to prepare the food in the form of small wafers about 2 mm thick by 5 mm square. These were consumed quite well with a minimum of dropping and the pieces dropped were easily separated from the feces.

It was found that from time to time the lamp in the SPF will strike its arc to the envelope of the lamp instead of across the electrodes. It is necessary to observe the lamp as it is lit and to turn it off immediately and try again if the arc is not satisfactory in order to avoid ruining the lamp. Also, the arc rarely strikes in exactly the same place twice and the lamp output is somewhat different from use to use. This problem may be compensated for by running a standard with each determination.

The usefulness of the method lies in its comparative speed, reproducibility and sensitivity. The method uses small quantities of reagents and is somewhat less expensive than other methods. Even though the method requires the purchase of an SPF, which costs about $10,000, the savings in time and the increased sensitivity easily justify the expenditure for laboratories doing more than casual analysis for polycyclic aromatic hydrocarbons.

Using this method, it was possible for the author to perform fifteen determinations in eight working days. The older methods would have required approximately one week per determination. Although analyses for polycyclic aromatic hydrocarbons other than BAP were not made,

it is probable that the method would be of use in determining others. This could most likely be achieved by simply spotting small quantities of the additional materials on the paper chromatogram along with the BAP standard and removing, eluting and reading the corresponding bands. It is also felt that it would be worthwhile to repeat the feeding experiments in order to obtain statistically significant data. The author looks forward to the time when this may be possible.

CHAPTER V

CONCLUSIONS

A sensitive, accurate, rapid and reproducible method was developed for the separation, identification and quantitation of 3,4-benzpyrene from rubber dust and rat feces. The method uses hot benzene extraction of the sample, chromatography on a short column of specially prepared Florsil, descending paper chromatography and detection using a spectrophotofluorimeter. Using this method, it has been possible for a single worker to analyze up to twenty samples in two five-day weeks. The older methods reported in the literature require from two to five days per sample. The particular instrument used in this study allowed the direct measurement of 3,4-benzpyrene in concentrations from 5×10^{-10} to 10^{-6} grams per milliliter. Concentrations outside this range may be measured by concentrating or diluting the sample as required.

The rat chow containing rubber dust and the fecal samples used in developing the method were analyzed in a quantitative manner in order to gain information about the fate of BAP in rubber as it passes through the digestive system of the rat. While the results of a single experiment are not statistically valid, evidence was obtained which indicates that BAP is not removed from rubber dust as it passes through the digestive system. Additional studies will be required to confirm this evidence.

Besides BAP, there are other fluorescent polycyclic aromatic

hydrocarbons of biological interest. It is possible that the method developed for the detection of BAP may also be suitable for the detection of these other substances. At this time studies are being made to determine this possibility.

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APPENDIX A

LIST OF ABBREVIATIONS USED IN THIS TEXT

Abbreviation	Name
BAP	benzo a pyrene
Bz	benzene
i-C8	iso-octane
ml	milliliter
RFU	relative fluorescence unit
SPF	spectrophotofluorimeter

APPENDIX B

FLOW SHEET FOR BAP DETERMINATION

A. Reagents

1. Benzene: reagent grade, triple distilled, discarding ten per cent of the tops and bottoms at each step.
2. Iso-octane: 99 mol per cent, redistilled once, discarding ten per cent of the tops and bottoms.
3. Methanol: Reagent grade purified by refluxing 4 liters with 50 grams of zinc dust and 20 grams of potassium hydroxide for three hours and then distilling, discarding ten per cent of the tops and bottoms.
4. Dimethylformamide: spectro grade, used as received.
5. Ether: Diethyl ether, anhydrous reagent grade, used as received.
6. Nitrogen: prepurified grade.
7. Chromatography paper: Whatman number 1, cut to 19.5 by 56.5 centimeters, then washed with i-C8 and dried.
8. Florsil: 60 to 100 mesh, 500 gram portions washed with 4 liters of redistilled benzene followed with 3 liters of redistilled methanol and then dried in a vacuum oven at 25 to 26 inches of mercury and 50 degrees centigrade for 16 hours.
9. Sodium sulfate: Anhydrous reagent grade.
10. Nitrogen: prepurified grade

B. Method

1. Because BAP is light sensitive, carry out all operations in a darkened environment.
2. Place 15 grams of Florsil into a glass chromatography column with an inside diameter of approximately 27 millimeters.

3. Place 15 grams of anhydrous sodium sulfate on top of the Florsil.
4. Prewash with two 25 ml portions of i-C8.
5. Immediately after adding the second 25 ml portion of i-C8 add 0.500 ml of the sample using a calibrated micropipette.
6. Allow the column to stop dripping and wash the walls with a small quantity of i-C8.
7. Extract the column with 125 ml of i-C8 and discard this i-C8 fraction.
8. Extract the column with five 25 ml portions of benzene and pool the benzene fraction.
9. Evaporate the benzene fraction to a small volume on the steam bath using a stream of nitrogen.
10. Transfer the benzene fraction quantitatively to a 25 ml glass stoppered Erlenmeyer flask using 5 ml of benzene.
11. Evaporate the benzene fraction to approximately 0.2 ml using the steam bath and a stream of nitrogen.
12. Apply the benzene extract to a sheet of prepared chromatography paper in a band approximately 0.5 by 11 centimeters.
13. Apply a reference spot of BAP to one edge of the paper at the same position as the sample.
14. Apply a stationary phase of 25 per cent dimethylformamide in diethyl ether being careful to avoid immersing the applied band of sample.
15. Using i-C8 as the developing solvent, develop the chromatogram by descending chromatography for approximately four hours.
16. Dry the chromatogram briefly in the dark.

17. Examine the chromatogram under ultraviolet light and cut out the portion corresponding to the BAP reference spot.
18. Cut the selected strip into small pieces and place the pieces in a small sintered glass pressure filtering funnel.
19. Using dry nitrogen for pressure, elute the paper with small quantities of hot methanol totalling approximately 40 ml.
20. Using a stream of nitrogen and the steam bath, evaporate the methanol extract to a small volume.
21. Holding the flask in the hand to insure a low temperature, take the methanol extract to dryness using a stream of nitrogen.
22. Wash the walls of the flask with 1 ml of redistilled benzene and transfer quantitatively to a 5 ml volumetric flask.
23. Dilute to the mark with redistilled benzene and read on the SPF.