CONF-870940--4

DE88 007684

PERSISTENCE OF URINARY EXCRETION PRODUCTS OF BENZO(A)PYRENE*

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*Research sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc. and also by Health Effects Research Laboratory, U.S. Environmental Protection Agency, under IAG DOE No. 1739-1739-A1; EPA No. 89931767-01-1

INTRODUCTION

Persistence of DNA-adducts has been observed in a variety of experimental circumstances and has been suggested as one potential mechanism for explaining the long-term delay before expression of proliferative disease (Wogan and Gorelick 1985, Rojas and Alexandrov 1986, Kadlubar et al., 1984, Beland et al., 1980, Alexandrov et al., 1982, and Kotin et al., 1959). In this concept, a stable DNA-adduct, which is a remnant of a prior exposure in a nondividing cell, would not express the genotoxic effect until the cells were stimulated to divide, and thus explain the long-term delay in expression of cancer. An alternative view of the observation of persistent DNA-adducts, described in this communication, is the continuing replenishment of DNA adducts by formation and turnover of these adducts from exposure to a constant supply of the ultimate carcinogenic species derived from a prior exposure. It is of interest to note that virtually all experiments where "persistent" adducts have been observed have been high dose exposures (see Table 1 for examples).

During the course of experiments designed to develop improved methods for detection of DNA adducts and related derivatives derived from polynuclear aromatic hydrocarbons (PAH), we observed that there was a continuous excretion of urinary derivatives of the injected benzo(a)pyrene (BaP) beyond the initial burst of detoxification. This report describes the time dependent distribution of those derivatives in blood, urine, feces, and at the site of injection.

METHODS AND MATERIALS

Tritium labelled, 1,3,6-tri-^{3}H-BaP (^{3}HBaP), (sp act 220 mCi/mmole) was purchased through the National Cancer Institute (NCI) chemical repository program. Stock ^{3}H-BaP was diluted with nonradioactive BaP before use. Nonradioactive BaP was also purchased from the NCI chemical repository. Fischer 344 male and female rats were purchased from Charles River Raleigh in Raleigh, North Carolina. The plastic metabolism cages were designed to minimize contamination by food particles and to keep the urine and feces separated during collection.

Burdick and Jackson (Muskegon, MI) spectrophotometric grade H_2O and methanol were used as solvents for all solutions. Dimethylsulfoxide (DMSO) was spectrophotometric grade purchased from Schwarz/Mann of Spring Valley, NY. Sodium dodecyl sulfate (SDS), molecular biology grade, was purchased from Sigma Chemical Co. of St. Louis, MO. Aquasol (New England Nuclear of Boston, MA) was used as scintillation fluid for radioactive counting. Hydrogen peroxide (30%) was purchased from Fischer Scientific of Fair Lawn, NJ. All counting was done in a Packard scintillation counter. Fluorescence measurements were made with the Perkin-Elmer 650-40 spectrofluorimeter. UV-VIS measurements were made with the IBM 9240 spectrophotometer.

Exposure and collection frequency

Urine was collected from animals placed in the metabolism cages prior to injection. The animals used for the BaP study were put in the cages 2 days prior to injection. Urine was collected at 24 h intervals at about 9:00 AM each day. The animals were given subscapular injections of 0.1 mL of DMSO containing 4 mg (1587 nmole) of BaP with a specific activity of 31 μCi/μmole. All were injected within a 30 min period after mild ether anesthesia.

Collection of products from urine, feces and blood

The urine was collected in 20-mL scintillation vials which initially contained 0.1 mL of toluene. Collected samples were sealed and stored at -15°C. Feces were transferred from the collection screen to 40-mL plastic vials. The total weight of feces was recorded and 1 gm samples were analyzed after H_2O_2 digestion.

Because of the complications of interpreting the effects of periodic blood removal on the levels of BaP metabolites in blood and urine, we have collected blood only at the time of sacrifice. After BaP injection, animals were sacrificed 1,2,3,7,9,11, and 15 days following exposure.

After decapitation, the blood was collected in a beaker previously rinsed with 0.05 M ethylenediamine tetracetic acid pH=7.2. The blood volume was measured and an equal volume of 2% SDS was added. To facilitate subsequent analyses, the blood was digested overnight with added proteinase K at a concentration of 0.1 mg/mL, pH 7 (0.1 M Tris). The blood solution maintained the color of the ferrous-heme complex.

Benzo(a)pyrene remaining at the site of injection was recovered by dissecting the entire cyst from the skin, mincing the tissue in 2 mL of DMSO. The DMSO suspension was subjected to 2 to 3 min of maceration in the Tekmar Tissuemizer (Cincinnati, OH). The extract was allowed to stand in the dark overnight, and then centrifuged to prepare a clear extract.

ANALYSIS OF COLLECTED SAMPLES

Radioactive measurements

The hydrolyzed blood samples totally quenched the radioactive measurements. In order to remove the quenching substances, samples were digested in approximately 3% to 15% hydrogen peroxide (depending on the quenching properties of the sample) before radioactive counting. The sample color was used as an index of digestion. A pale straw yellow color resulted in good counting efficiencies.

The conversion of radioactive data to concentration was done as follows:

$$\text{CONCENTRATION} = \frac{(\text{CPM/mL})*(\text{DILUTION FACTORS})}{(\text{COUNTING EFFICIENCY})*(\text{sp.act.})}$$

The average of triplicate radioactive counts was used for CPM. The routine procedure was to add 5 mL of counting fluid to the sample in a

7 mL plastic counting vial and count for 10 min or count to 0.2% precision. The counting efficiency ranged from 25 to 40%.

Fractionation of samples

The blood and urine were separated into 3 fractions using 1 mL LC-18 cartridges (ODS sorbent) from Supelco, Inc. (Bellefonte, PA) (Fig 1). The cartridges were equilibrated with 1 mL of methanol, followed by 1 mL of H_2O. Cartridges were placed in a 15-mL polypropylene centrifuge tube (Corning, cat #25319 of Corning, NY). The lip on the cartridge prevents the cartridge from sliding to the bottom. Each tube and cartridge was then centrifuged in a clinical centrifuge at top speed for about 30 s to 1 min for each solvent. The urine or blood sample (1 mL) was placed in the cartridge and centrifuged into a clean test-tube. One mL of water was added and the centrifugation was repeated. The combined extracts were the labelled water fraction. One mL of 50% methanol was added to the cartridge and the centrifugation was repeated to obtain the 50% methanol fraction. The process was repeated with 100% methanol to obtain the final fraction. These three fractions represent the water soluble metabolites, the polar organic soluble metabolites and the least polar metabolites and unchanged BaP, if present.

F-1

Assay for ^3H_2O

Volatile ^3H counts were measured by pipetting 0.2 mL aliquots of each fraction into 7 mL plastic counting vials and evaporating the H_2O under vacuum and in the presence of phosphorous pentoxide. Each dried sample was reconstituted to the original volume, mixed thoroughly and counted. The difference in counts between 0.2 mL aliquots of the original solution and the reconstituted dried aliquot was the amount of volatile ^3H_2O.

RESULTS

Persistence at site of injection

The high doses used in these experiments result in retention of much of the BaP at the site of injection. About 20% of the radioactive BaP remains at the site of injection over a period of 10 days post-exposure (Fig. 2). The yellow, insoluble BaP is clearly seen within the cyst at the injection site. Total radioactivity (Fig. 2), UV absorbance and fluorescence measurements (not shown) verified that more than 90% of the radioactivity was BaP. The urine and feces contained about 1% of the injected dose during the first two days. The remaining dose (about 12 μmole) was presumed to be distributed in the rest of the animal.

F-2

Excretion of metabolites

The ratio of total radioactivity in feces to total radioactivity in urine varied from about 6 to 2 during the course of the experiment. The

higher values occurred during the peak excretion period for feces during the second day. Beyond the tenth day following exposure, both sources maintained an essentially constant and low rate of excretion with a ratio of approximately two (Fig. 3).

F-3

The highest levels of urinary excretion occur within the first day following exposure (Fig. 3). These levels decrease from values as high as 80 nmoles per rat per day to plateau levels of about 10 to 20 nmoles per rat per day. The concentration of mixed metabolites in urine was as high as 6.5 μM on the first day following exposure, and decreased to about 2 μM (300 gm rat) after the fourth day post-exposure. This latter level of excretion still represents less than 0.3%/day of the initial dose.

The excretion of radioactive metabolites into feces follows a pattern similar to urine with a peak on the second day following exposure followed by exponential decrease of the radioactivity to essentially a plateau (Fig. 3). The highest observed values ranged from 165 to 256 nmoles per day per rat, which decreased to plateau values of 30 to 45 nmoles per day per rat.

The number of nmoles of radioactivity present in blood follows the pattern for feces and peaked during the second day. In contrast to the feces and urine, the level of radioactivity in blood samples remained constant for the remainder of the experiment (Fig. 4). Most of the radioactivity was found to be volatile when the blood sample was dried (Table 2).

F-4,T-2

Distribution of metabolites

Urine was separated into three fractions to determine the distribution of radioactivity into water soluble, polar organic, and nonpolar fractions (Fig. 5) (see Methods). The water fraction from the reverse-phase fractionation scheme contained about one-half of the total radioactivity in urine. The 50% methanol extract contained about one-third to one-half of the radioactivity, and the 100% methanol extracts contained only a few percent (but constant level) of the total urine radioactivity. Measurable quantities of volatile ^{3}H-H_2O was observed in the water extracts from urine (Table 3). About 60% of the urinary tritium counts are volatile from day 1 post-exposure rising to about 90% of the counts by day 16 post-exposure (Table 3). Since there is no evidence for significant exchange of these ^{3}H atoms on BaP without prior oxidation, the release of tritium is still a measure of *in situ* metabolism (e.g. phenol formation 3,4).

F-5, T-3

DISCUSSION

The daily levels of metabolites found in urine, blood and feces indicated that the process of mobilization and conversion of the stored portion of the injected BaP into excretable forms is slow. Thus approximately 85% of the dose is still retained beyond day 6 post-exposure, and is made available as excretable metabolites at a rate of about 70 nmole per day (total of urine and fecal excretion). This rate of turnover would require about 60 days to completely eliminate 1 mg of absorbed BaP. The pathways of BaP metabolism are well understood (5,9,1), and the ultimate carcinogenic form is a major intermediate both for detoxification and for DNA-adduct formation. This phenomenon coupled with the long time required to completely excrete large doses suggests that a mechanism of replenishment of DNA adducts from the continuous mobilization of stored BaP may also explain adduct persistence. Thus the single measurement of an adduct at sacrifice may not be sufficient to characterize when the adduct was initially formed.

The essentially constant level of radioactivity in blood, combined with an essentially constant excretion rate, suggests a steady-state set of processes controlling the mobilization and excretion of BaP and its metabolites. This saturation of the pharmacokinetics of elimination may provide a useful basis for modelling the pharmacokinetics of PAH metabolism.

The high percentage of $^{3}H\text{-}H_2O$ in the total urine provides an interesting caveat in the interpretation of distribution of ^{3}H-BaP into adducts during <u>in vivo</u> experimentation. At this time, the source of $^{3}H_2O$ would appear to be either nonspecific oxidation of the labelled BaP to quinones, or through the NIH shift (5) accompanying spontaneous formation of phenol from arene oxides.

ACKNOWLEDGEMENTS

We thank Dr. F. F. Knapp for the use of his animal facility, and we thank Dr. J. Ma for use of her scintillation counter.

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FRACTIONATION OF TISSUE EXTRACTS

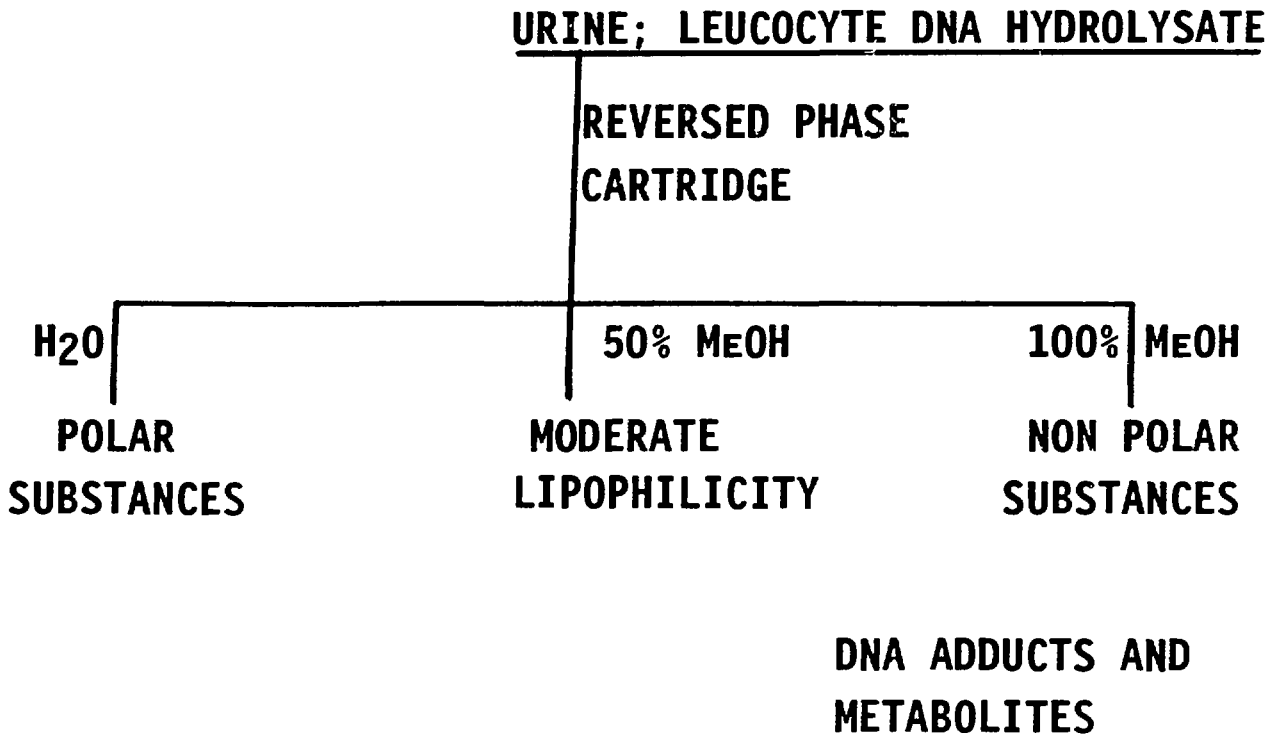

FIGURE 1. THE SEPARATION OF BaP METABOLITES INTO WATER-SOLUBLE, POLAR ORGANIC, AND NONPOLAR FRACTIONS ON REVERSED PHASE CARTRIDGES.

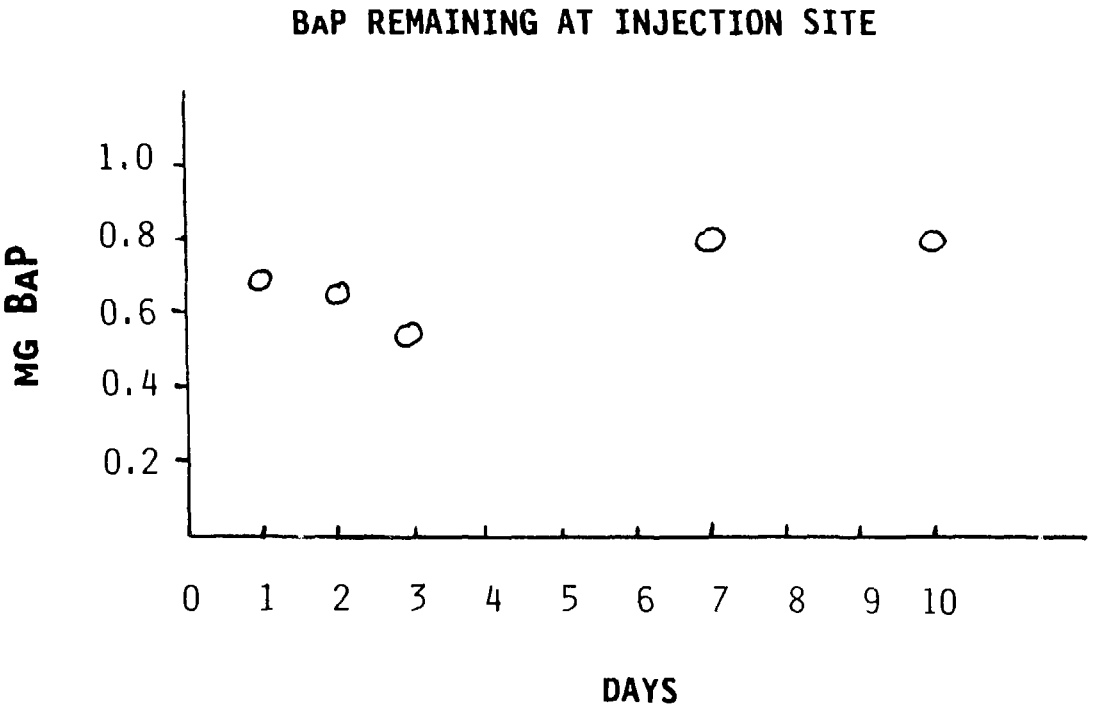

FIGURE 2. THE AMOUNT OF BaP FOUND AT THE SITE OF INJECTION OVER A PERIOD OF 10 DAYS POST-EXPOSURE.

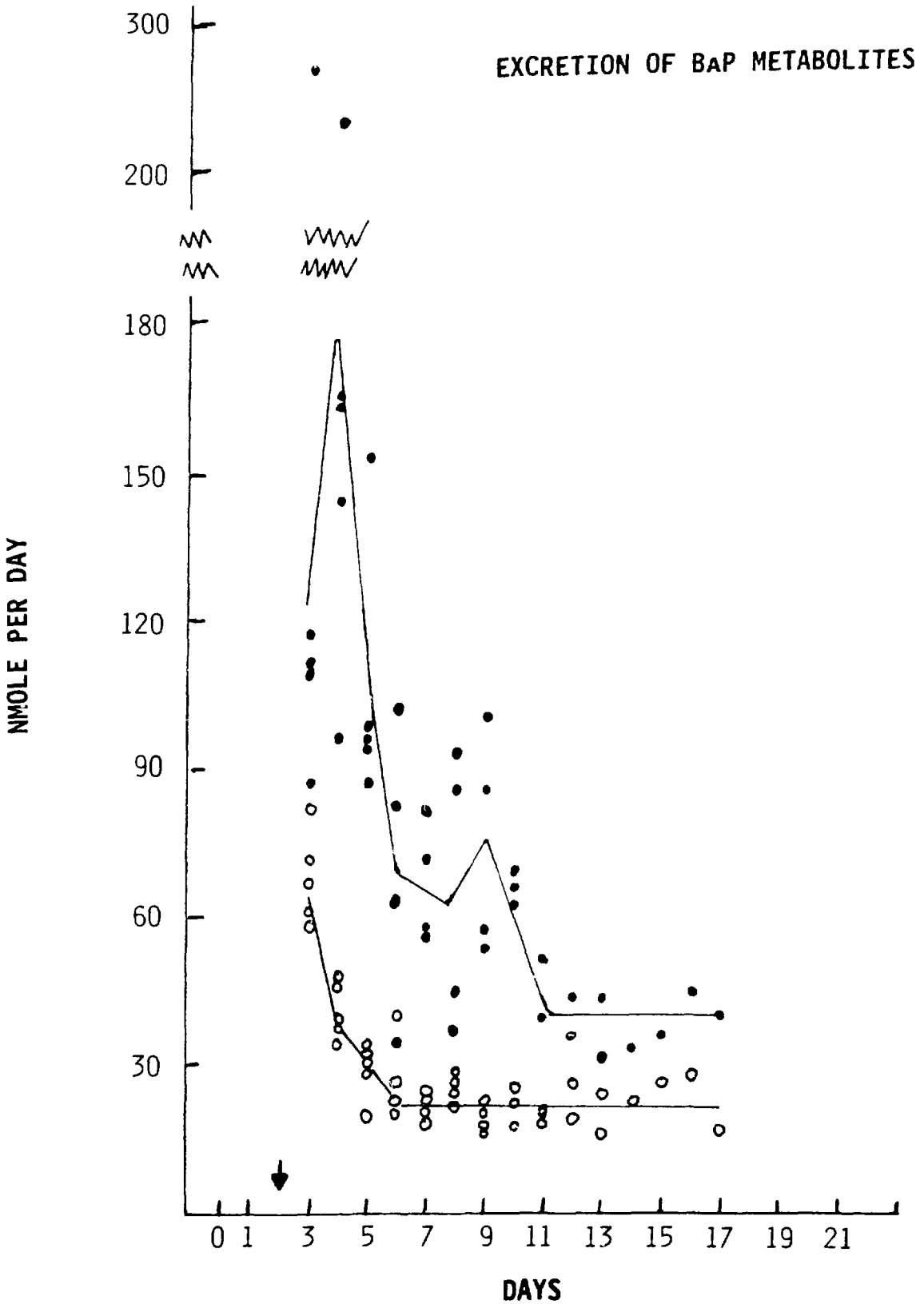

FIGURE 3. THE AMOUNTS OF BaP METABOLITES FOUND IN URINE AND FECES.
● FECES; ○ URINE

TOTAL BaP AND METABOLITES IN BLOOD

ORNL DWG 87-9810

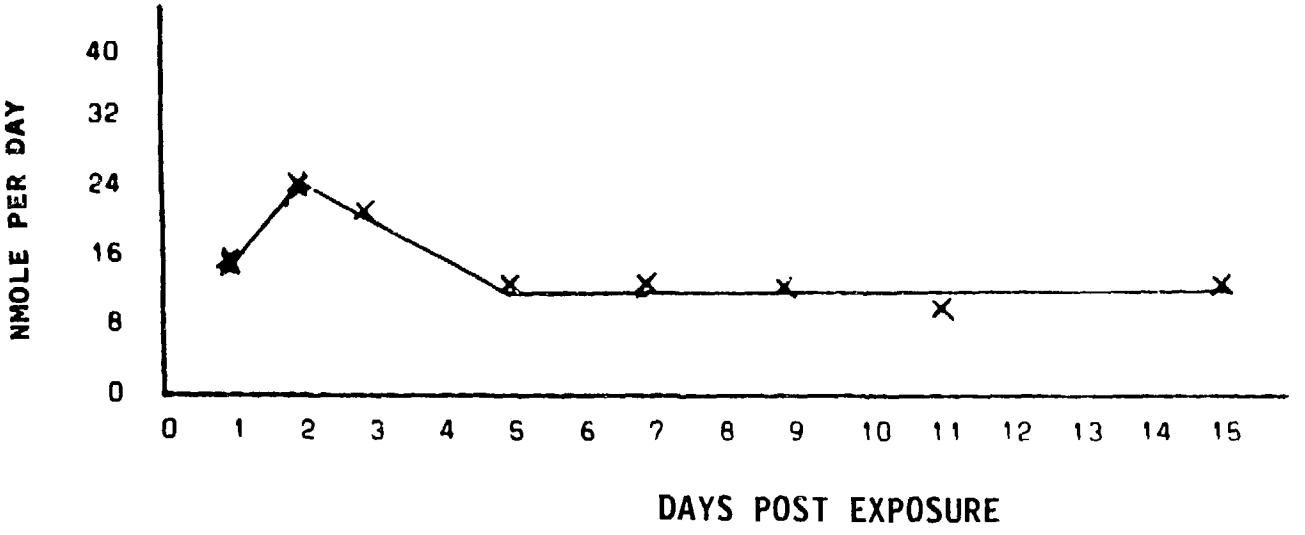

FIGURE 4. THE NUMBER OF NMOLES OF RADIOACTIVITY PRESENT IN BLOOD. THIS VALUE WAS ESTIMATED USING A BLOOD VOLUME VALUE OF 8% OF BODY WEIGHT.

FRACTIONATION OF URINE ON REVERSED PHASE CARTRIDGES

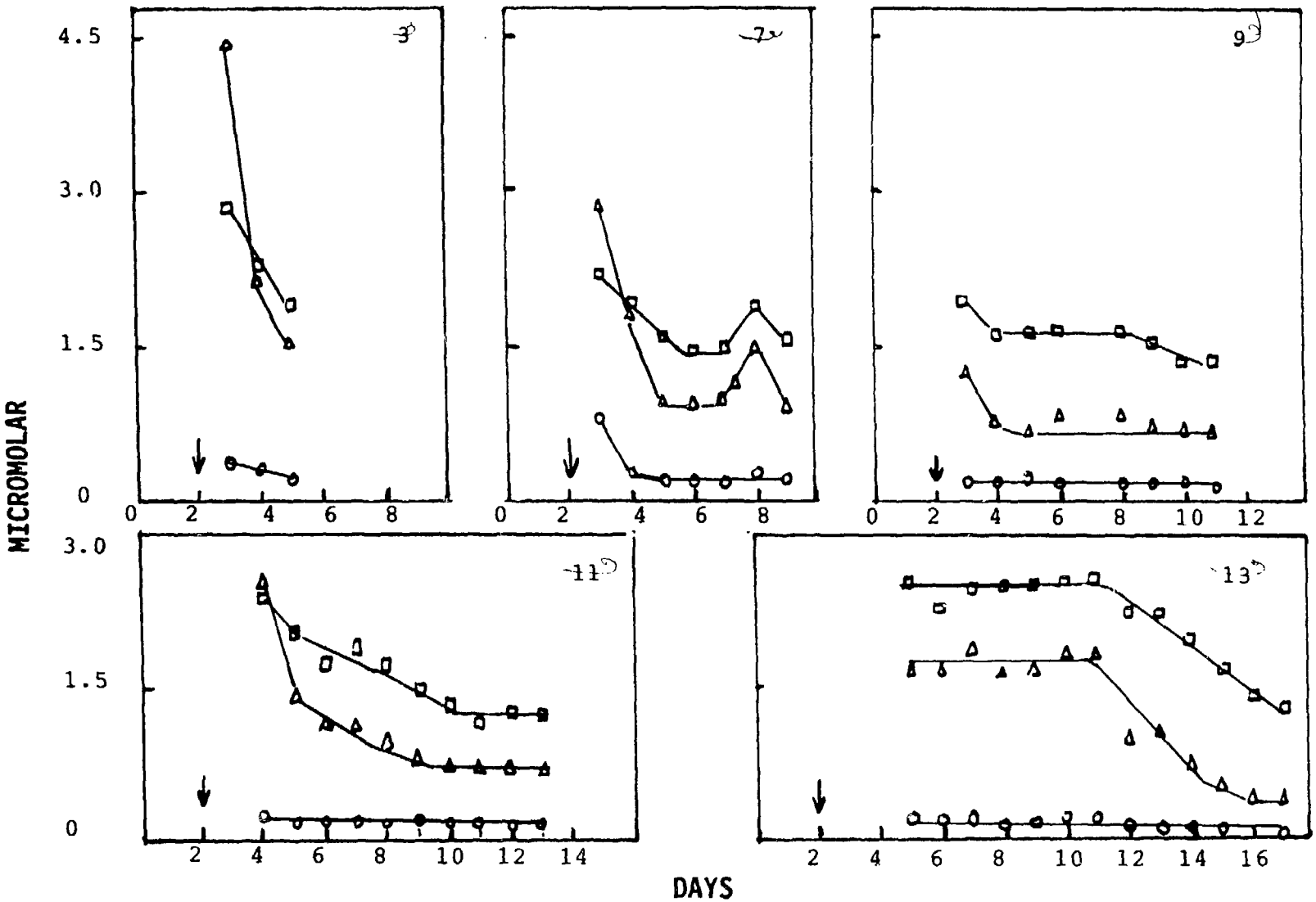

FIGURE 5. THE DISTRIBUTION OF METABOLITES AND DERIVATIVES IN URINE BY RATS EXPOSED TO BAP (12 MG/KG). THE ARROW INDICATES THE TIME OF INJECTION OF BAP. ⊟ H_2O SOLUBLE; Δ 50% MEOH SOLUBLE; ⊖ 100% MEOH SOLUBLE

TABLE 1

PERSISTENCE[A] AND DOSE

CHEMICAL[B]	DOSE/ANIMAL	MEASURE	DAYS[C]	REFERENCE
BP-4,5-EPOXIDE	1 MG/KG RODENTS	DNA ADDUCT	21	ROJAS AND ALEXANDROV 1986
MNU	3 MG/KG RAT	DNA ADDUCT	21	KADLUBAR ET AL. 1984
MAB	42 MG/KG RAT	DNA ADDUCT	14	BELAND ET AL. 1980
BPDE	8.8 MG/KG MOUSE	DNA ADDUCT	21	ALEXANDROV ET AL. 1982
BP	3 MG/KG MOUSE	RETENTION	7	KOTIN ET AL. 1959

[A] PERSISTENCE IS DEFINED AS THE OBSERVATION OF THE CHEMICAL, ITS METABOLITES, OR ITS DNA ADDUCTS AT LEAST 2 DAYS POST-INJECTION.

[B] BP-4,5-EPOXIDE IS 4,5-EPOXY-BENZO(A)PYRENE; MNU IS N-METHYL, N-NITROSOUREA; MAB IS N-METHYL-4-AMINOAZOBENZENE; BPDE IS BENZO(A)PYRENEDIOLEPOXIDE; AND BP IS BENZO(A)PYRENE.

[C] THIS IS THE TIME OF THE LAST MEASUREMENT AND IS THE NUMBER OF DAYS POST-EXPOSURE.

TABLE 2
VOLATILE ^{3}H DPM IN BLOOD FROM
ANIMALS EXPOSED TO BaP[A]

H_2O FRACTION FROM BLOOD

DAYS POST EXPOSURE	DEHYDRATED	INTACT	% CHANGE
1	0.33	0.61	45
2	0.38	1.0	62
3	0.46	0.86	47
9	0.29	0.56	48
11	0.33	0.60	46
15	0.40	0.53	24

[A] THE NUMBER OF NAMOMOLES/ML OF RADIOACTIVE MATERIAL THAT DID NOT ABSORB TO THE ODS SORBENT WAS TESTED FOR THE PRESENCE OF $^{3}H_2O$ BY DRYING AT ROOM TEMPERATURE IN A DESSICATOR IN THE PRESENCE OF PHOSPHOROUS PENTOXIDE. SEE METHODS FOR CONVERSION OF CPM TO NMOLES. THESE VALUES WERE ADJUSTED FOR DILUTIONS OCCURRING DURING SAMPLE PREPARATION.

TABLE 3

PERCENT ^3H_2O IN WATER SOLUBLE FRACTION FROM URINE SAMPLES OF ANIMALS EXPOSED TO BaP[A]

ANIMAL/DAY[B]	1	2	3	4	5	6
1	60	60	70	74	71	67

ANIMAL/DAY[B]	5	6	7	8	9	10	11	12	13	14	15	16
2	67	69	60	72	73	74	71	84	81	82	87	88

[A] THE PERCENT OF ^3H_2O WAS CALCULATED BY MEASURING THE AMOUNT OF NONVOLATILE TRITIUM DPM, WHICH WAS OBTAINED BY SUBTRACTING THE DEHYDRATED SAMPLE VALUE FROM THE NUMBER OF DPM IN THE SAMPLE BEFORE DEHYDRATION, AND CALCULATING THE PERCENTAGE BASED ON THE SAMPLE BEFORE DEHYDRATION. THE OBSERVED DPM VALUES USED TO CALCULATE THESE PERCENTAGES, RANGED FROM A LOW OF 6000 TO A HIGH OF 40,000.

[B] THESE NUMBERS ARE THE DAYS POST-EXPOSURE.

TABLE 4

PERCENT ^3H_2O IN WATER SOLUBLE FRACTION FROM URINE SAMPLES OF ANIMALS EXPOSED TO BaP[A]

ANIMAL/DAY[B]	1	2	3	4	5	6
1	60	60	70	74	71	67

ANIMAL/DAY[B]	5	6	7	8	9	10	11	12	13	14	15	16
2	67	69	60	72	73	74	71	84	81	82	87	88

A THE PERCENT OF ^3H_2O WAS CALCULATED BY MEASURING THE AMOUNT OF NONVOLATILE TRITIUM DPM, WHICH WAS OBTAINED BY SUBTRACTING THE DEHYDRATED SAMPLE VALUE FROM THE NUMBER OF DPM IN THE SAMPLE BEFORE DEHYDRATION, AND CALCULATING THE PERCENTAGE BASED ON THE SAMPLE BEFORE DEHYDRATION. THE OBSERVED DPM VALUES USED TO CALCULATE THESE PERCENTAGES, RANGED FROM A LOW OF 6000 TO A HIGH OF 40,000.

B THESE NUMBERS ARE THE DAYS POST-EXPOSURE.