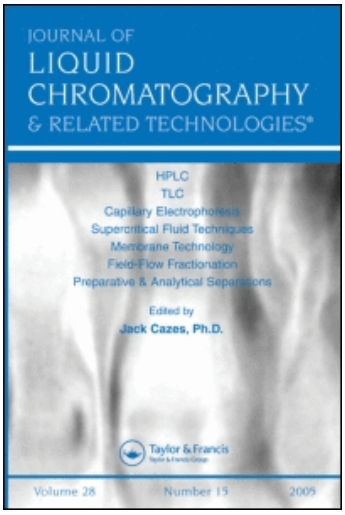

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IDENTIFICATION AND MUTAGENICITY OF THE URINARY METABOLITES OF THE MUTAGENIC NONCARCINOGEN 2,6-DIAMINOTOLUENE

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ABSTRACT

2,6-Diaminotoluene (2,6-DAT) is a high production volume chemical; approximately 100 million pounds are produced annually in the synthesis of several dyes for furs and textiles and in the manufacture of 2,6-toluenediisocyanate for the manufacture of flexible polyurethane foams and elastomers. 2,6-DAT is mutagenic in the **Ames/Salmonella** test requiring metabolic activation, but was not carcinogenic to male or female rats or mice in 2-year bioassays. Following oral administration of ^{14}C-labeled 2,6-DAT, 85% of the radioactivity was excreted in the urine within 24 hours. Resolution of the urine by reversed phase HPLC identified 4 metabolites, and no parent compound was excreted. Analysis by mass spectroscopy and nuclear magnetic resonance spectroscopy identified the metabolites as a) 3-hydroxy-2,6-DAT, b) 5-hydroxy-2-acetylamino-6-aminotoluene, c) 2-acetylamino-6-aminotoluene, and d) 2,6-di(acetylamino)-toluene. Metabolites b and d were found to be mutagenic in the presence of an activation system. This study is an example of the use of liquid chromatography in genetic toxicity studies.

INTRODUCTION

Many carcinogenic chemicals initiate a series of cellular events by interaction with the DNA, either directly such as the alkylating agents or indirectly after they are bioactivated to electrophilic intermediates by enzymes

such as cytochrome(s) P450(1). Mutations resulting from such interactions with DNA may produce heritable alterations in the regulation of the growth and differentiation of daughter cells such that they do not respond to normal cellular signals to stop dividing. Thus begins a series of changes resulting in a tumor.

Identification of chemicals which have the potential to produce tumors is, therefore, of utmost importance in order to prevent or limit their exposure to humans. A major research effort has been the testing of chemicals through lifetime bioassays in laboratory animals in order to identify compounds that are able to produce tumors in rodents, and by extension, in humans. To reduce the time and cost of these bioassays, a variety of short-term genotoxicity tests have been developed in order to evaluate chemicals on the basis of their ability to produce mutations in bacteria or mammalian cells in continuous culture. In order for data from any short-term genotoxicity test to be useful, the results must have an acceptable rate of agreement with the results of the rodent bioassays they are designed to predict. A high rate of false positives would result in the classification of many, potentially useful chemicals as genotoxic and possibly halt their use or further development. A high rate of false negatives in a mutation assay would expose people to mutagenic and potentially carcinogenic chemicals unknowingly, an unacceptable situation.

The perfect short-term mutation assay, therefore, has a high concordance with the bioassay data, for both carcinogenic and noncarcinogenic chemicals. Recently, Tennant, et al (2) evaluated four of the most widely used in vitro assay of genetic toxicity, and determined that the Ames/**Salmonella** assay alone was as accurate at predicting whether a chemical will or will not produce cancer in rodents as any individual or combination of mouse lymphoma, chromosome aberration or sister chromatid exchange assays.

However, the rate of false positives in the Ames/**Salmonella** assay is still quite high; approximately 23% of the chemicals recently reviewed by Zeiger (3) were positive for mutagenicity yet were demonstrated to be noncarcinogenic to animals in lifetime bioassays. One pair of closely related chemicals which are both positive in the Ames/**Salmonella** assay, but only one of which is positive in

the bioassay are 2,4- and 2,6- diaminotoluene (DAT). In order to describe possible mechanisms for the discordance between the positive mutagenicity to **Salmonella** of 2,6-DAT and its negative carcinogenicity in male and female rats and mice, we have been studying the different dispositional factors which exist between the **in vitro** mutation test and the **in vivo** bioassay. This report presents data demonstrating the metabolism of 2,6-DAT in the whole animal which results in several urinary metabolites which cannot be produced in the Ames/**Salmonella** assay and may help explain the discordance between the **in vivo** and the **in vitro** data. Furthermore, this report demonstrates the application of HPLC in the identification and purification of mutagenic metabolites.

EXPERIMENTAL

Chemicals. 2,6-DAT was purchased from Aldrich Chemical Co. (Milwaukee,WI). Lot 9308AV was found to be >99% pure by HPLC analysis and was solely used in this study. [^{14}C]2,6-DAT (generally labeled) was purchased from Chemsyn Laboratories (Lenexa, KS; 22.6 mCi/mmole). The radioactive compound was purified to >99% radiochemical purity by HPLC by the method described below prior to use.

Animals. Male F344 rats (150-175 grams) were purchased from Charles River Breeding Laboratories (Raleigh, NC). Animals were fed a standard NIH 31 diet **ad libitum** and were allowed two weeks adaptation time prior to dosing.

Treatment. Rats were randomly assigned to treatment groups, and 24 hours prior to dosing animals were placed in individual glass metabolism cages to adapt to the new environment. A single dose of 2,6-DAT (10 mg/animal;90 μCi/mmole) was administered by gavage in 0.01 N HCl and the rats returned to their cage with free access to food and water.

Separation of Urinary Metabolites. 24 hour urine samples were collected from animals treated with [^{14}C] 2,6-DAT and analyzed for metabolites by HPLC using a μBondapak C18 column (Waters Associates, Milford, MA) with a linear gradient of 50 mM phosphate buffer (pH 4.5) to 100% methanol in 25 min. at 1.5 ml/min. Detection was accomplished using a model 171 radioisotope detector (Beckman

Instruments, Fullerton, CA) with a splitless solid scintillator flow cell for the nondestructive detection of the ^{14}C-labeled metabolites. All peaks containing radioactivity were collected for further analysis.

Identification of Urinary Metabolites. Following separation and collection of urinary metabolites by HPLC as described above, the fractions containing radiolabel were rechromatographed in 100% water to 100% methanol to remove the phosphate buffer and further purify the metabolites. Samples were concentrated under reduced pressure at room temperature and analyzed in deuterated methanol by nuclear magnetic resonance (NMR) spectroscopy (General Electric model QE-300) and electron impact (VG 12-250 at 70 ev from m/z 35-510 at one second/scan) and fast atom bombardment (VG 7070S, mass resolution of 1000; glycerol and thioglycerol matrix; positive and negative ion mode) mass spectroscopy.

Mutagenesis Assays. Cultures of **Salmonella typhimurium** TA 98 (generously provided by Dr. Bruce Ames, University of California, Berkeley) were grown overnight in Oxoid nutrient broth No. 2 with shaking at 37°C(4). A 0.1 ml aliquot of the overnight culture was added to 2.0 ml molten top agar, 0.1 ml test chemical, and an activation mix containing 0.05 mM biotin and histidine, 4 mM NADP, 100 mM sodium phosphate, pH 7.4, and 0.5 mg/ml S9 protein obtained from rats induced with Aroclor-1254 (500 mg/kg body weight) for 5 days. This protein concentration was found to produce optimum mutagenesis of 2,6-DAT in preliminary scaling experiments. These components were vortexed briefly and poured onto petri plates containing 20 ml minimal agar and the mutants allowed to grow into colonies for 48 hours at 37°C. The number of colonies was determined by means of a Biotran III automatic colony counter (New Brunswick Scientific Co., Edison, NJ). Protein concentration was estimated by the method of Lowry, et al(5) .

RESULTS

Analysis of 24 hour urine samples by reverse phase HPLC demonstrated the presence of four peaks of radioactivity (Figure 1). None of the peaks comigrated with the parent compound (Figure 1 arrow) indicating that each peak represents a unique metabolite.

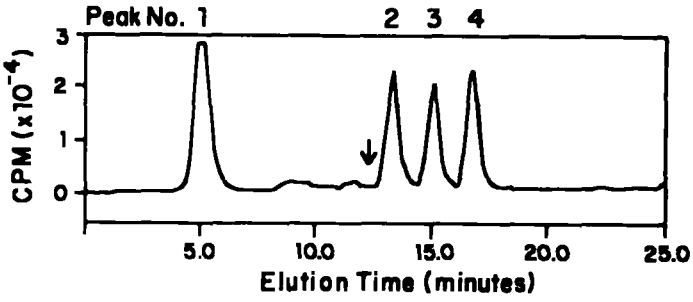


FIGURE 1. Radiochromatogram of the urinary metabolites of 2,6-DAT. The arrow indicates the retention time for 2,6-DAT.

After repeated purification by HPLC as described above, the compounds in each peak were analyzed by NMR and mass spectroscopy and the data are presented in Tables 1 and 2.

Based on the data presented in Tables 1 and 2, the proposed structures of the urinary metabolites of 2,6-DAT are presented in Figure 2. The hydroxyl group on metabolite 2 was assigned the 5 position based on the similarity of the proton shifts for peaks 1 and 2, although the possibility that substitution is at the 3 position cannot be ruled out unequivocally.

Two of the urinary metabolites of 2,6-DAT expressed significant mutagenicity in the Ames/Salmonella assay with S9 activation (Table 3). 5-hydroxy-2-acetylamino-6-aminotoluene (metabolite 2) and 2,6-di(acetylamino)-toluene (metabolite 4) produced dose-dependent increases in the mutation rate in the presence but not the absence of S9 activation.

DISCUSSION

The value of short-term mutation tests for prediction of carcinogenesis is compromised by the occurrence of false positive results. The Ames/Salmonella test has recently been shown to be as good or better than any other short-term

TABLE 1

NMR Data for the Urinary Metabolites of 2,6-DAT

	δ(ppm)	#Hs	J(Hz)	Assignment
METABOLITE 1	1.95	3		CH_3
	6.15	1	8	ArH
	6.86	1	8	ArH
METABOLITE 2	1.95	6		$ArCH_3$ & $COCH_3$
	6.10	1	9	ArH
	6.90	1		ArH ortho
METABOLITE 3	1.90	3		$ArCH_3$
	2.12	3		$COCH_3$
	6.61	1	9	5-ArH
	6.93	1	9	4-ArH
	7.20	1		3-ArH
METABOLITE 4	2.00	3		$ArCH_3$
	2.05	6		$COCH_3$
	7.20	3		ArH

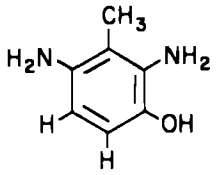
TABLE 2

Mass Spectral Data for the Urinary Metabolites of 2,6-DAT

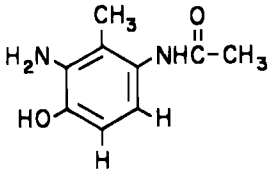
Metabolite	m/z M+	Fragments	Molecular Formula
Metabolite 1	138		$C_7H_{10}N_2O$
Metabolite 2	180		$C_9H_{12}N_2O_2$
	138	M+ - $COCH_3$	
	122	M+ - $NHCOCH_3$	
Metabolite 3	164		$C_9H_{12}N_2O$
	122	M+ - $COCH_3$	
	105	M+ - $NHCOCH_3$	
Metabolite 4	206		$C_{11}H_{14}N_2O_2$
	164	M+ - $COCH_3$	
	122	M+ - $COCH_3$	
		- $COCH_3$	

tests or combination of tests using mammalian cells. However, the high rate of false positives in the Ames/**Salmonella** test is unacceptable, especially for aromatic amines. Research into understanding the mechanisms whereby chemicals are mutagenic yet noncarcinogenic is therefore part of a greater effort to understand and utilize **in vitro** test results to accurately predict the potential of chemicals to induce cancer in test animals and humans.

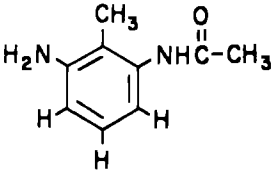
The results of this study demonstrate that the mutagenic noncarcinogen 2,6-DAT is biotransformed **in vivo** to four major metabolites which are excreted in urine. Three of these metabolites are N-acetylated derivatives of 2,6-DAT.



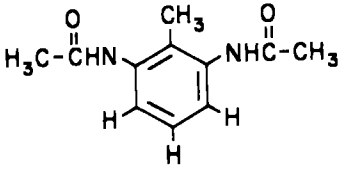
Metabolite 1: 3-Hydroxy-2,6-diaminotoluene



Metabolite 2: 5-Hydroxy-2-acetylamino-6-aminotoluene



Metabolite 3: 2-Acetylamino-6-aminotoluene



Metabolite 4: 2,6-Di(acetylamino)-toluene

FIGURE 2: Proposed structures of the urinary metabolites of 2,6-DAT.

TABLE 3

Mutagenicity of urinary metabolites of 2,6-DAT

Metabolite	Revertants per Plate (μmoles per plate) 0.15	0.30
1--3-hydroxy-2,6-diaminotoluene	39	44
2-- 5-hydroxy-2-acetylamino-6-aminotoluene	240	633
3-- 2-acetylamino-6-aminotoluene	21	60
4-- 2,6-di(acetylamino)-toluene	80	230
2,6-diaminotoluene	122	235

[a] Results are the average of two determinations. The background mutation rate of 30 mutants per plate has been subtracted from each value. All assays were conducted in the presence of S9.

None of these three metabolites can be formed in the standard Ames/**Salmonella** test since it is deficient in the cofactor needed for acetylation reactions, acetyl Coenzyme A. Interestingly, two of these acetylated metabolites are further activated to mutagenic species by S9. Only one metabolite, 5-hydroxy-2,6-DAT, is common to both the Ames/**Salmonella** assay and the bioassay in the rat (6). However, it was found to be not mutagenic in the presence or absence of S9 activation and is not likely to be responsible for the mutagenicity of 2,6-DAT observed in the Ames/**Salmonella** assay.

We have recently identified the putative mutagenic metabolite of the carcinogenic isomer 2,4-DAT formed by S9 in the Ames/**Salmonella** assay and have determined that it is probably not formed in vivo (7). A similiar metabolite was not detected in studies with 2,6-DAT. Further, the present study has identified

two mutagenic metabolites of the noncarcinogenic isomer 2,6-DAT which were formed in the intact animal which cannot be produced in the Ames/Salmonella assay. Therefore, it appears that formation of mutagenic metabolites of a chemical by an intact animal may not necessarily be sufficient to induce cancer even with lifetime exposure. Thus, the source of the nonconcordance observed between this in vitro short-term mutagenicity assay and the in vivo carcinogenicity bioassay may lie in the capacity of the intact animal to clear the putative mutagens prior to further activation or prior to their interaction with DNA.

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