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IN VIVO AND *IN VITRO* METABOLISM OF 2,4-DINITROTOLUENE IN STRAIN A MICE*

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Abstract—The elimination and metabolism of a single dose (100 mg/kg) of 2,4-dinitrotoluene (2,4-DNT) in A/J mice were examined. After intraperitoneal administration, elimination was rapid, with 70% of the dose appearing in the urine within 4 hr. Four hours after oral administration, only 28.5% of the dose was excreted in the urine, which increased to 66% after 8 hr. Elimination via the feces was minimal (<2.1% of the dose) in both cases. From 0.5 to 4 hr after intraperitoneal administration, 3.6 to 8.8% of the urinary metabolites was unconjugated while 2.4 to 8.8% was present in the glucuronide fraction. After oral administration these amounts were 5.5 to 6.8% and 20.5 to 28.2% respectively. After both intraperitoneal and oral administration, no unchanged 2,4-DNT could be detected in the urine, and 2,4-dinitrobenzyl alcohol (2,4-DNBAlc) represented the most abundant identifiable neutral metabolite. Small amounts of 2,4-diaminotoluene, 2-amino-4-nitrobenzyl alcohol, 2-(*N*-acetyl)amino-4-nitrotoluene, 4-amino-2-nitrotoluene (4A2NT), and 2-amino-4-nitrotoluene (2A4NT) were also present. In almost all cases the largest proportion of metabolites represented unknowns, some of which exhibited the chromatographic properties of carboxylic acid metabolites. Metabolism of 2,4-DNT by liver and lung microsomes yielded mainly 2,4-DNBAlc with lower amounts of 4A2NT and 2A4NT, and their formation was dependent on the presence of oxygen and NADPH. Pretreatment of the animals with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin resulted in increased yields of all three metabolites. Aerobic metabolism of 2,4-DNT by explants of the small intestine, large intestine, or by cecal contents yielded 2,4-DNBAlc, 2A4NT, 4A2NT and 4-(*N*-acetyl)amino-2-nitrotoluene (4Ac2NT). The proportion of reduced metabolites (2A4NT, 4A2NT, and 4Ac2NT) was much higher in these systems than with liver or lung microsomes and their formation by small intestine and cecal contents was enhanced several-fold under anaerobic conditions, while that of 2,4-DNBAlc was abolished. It is concluded that 2,4-DNT metabolism in the A/J mouse is rapid and complete and that the major neutral urinary metabolite is 2,4-DNBAlc. Minor amounts of reduced or partially reduced products appear to be formed mainly in the intestine, with a major role by its microflora.

Dinitrotoluene (DNT‡) has different isomeric forms and is commercially used in the manufacturing of toluenediisocyanate for production of polyurethane foams, coatings and elastomers. DNT isomers may also be present in condensate discharges of 2,4,6-trinitrotoluene production facilities. Minor amounts of DNT are used in the manufacture of explosives, gelatinizing agents, waterproofing compounds and dyes. Hence, because these compounds may pose an occupational and environmental hazard to humans, their carcinogenicity has been studied. Feeding of Fischer-344 rats with technical grade DNT (75.8% 2,4-DNT, 19.5% 2,6-DNT and 4.7% other isomers) results in a dose-dependent incidence of hepatocellular carcinoma [1]. 2,4-DNT induced hepatocellular carcinomas in CD-1 rats at 34 and 45 mg/kg/day [2]. In contrast, a study conducted by the National Cancer Institute revealed that 2,4-DNT is not carcinogenic to either rats or mice [3]. In a study on the carcinogenicity of 2,4-DNT in strain A mice in our laboratory, we showed that 2,4-DNT does not induce lung adenomas after either intraperitoneal or oral administration [4, 5].

Metabolism studies have shown that the metabolism of 2,4-DNT occurs principally in two organs. In the rat, the liver and the gastrointestinal tract were shown to be the major sites for oxidative and reductive metabolism of 2,4-DNT respectively [6–11]. Metabolism studies in CD-1 rats [6] have indicated that 2,4-DNT is excreted mainly as 2,4-DNBAlc and 2A4NBAlc in the urine. Fischer-344 rats excrete 2,4-DNT as 2,4-DNBAlc glucuronide, 4Ac2NBAcid, 2A4NBAcid and 2,4-DNBAcid [12]. The major product of *in vitro* incubation of 2,4-DNT with rat hepatic microsomes [9] or isolated rat hepatocytes [7] is 2,4-DNBAlc, whereas rat and mouse cecal contents form predominately amino-nitrotoluenes [10]. Our studies on the distribution

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‡ Abbreviations: DNT, dinitrotoluene; 4Ac2NBAcid, 4-(*N*-acetyl)amino-2-nitrobenzoic acid; 2,4-DNBAcid, 2,4-dinitrobenzoic acid; 2A4NBAcid, 2-amino-4-nitrobenzoic acid; DAT, diaminotoluene; 2A4NBAlc, 2-amino-4-nitrobenzyl alcohol; 2Ac4NT, 2-(*N*-acetyl)amino-4-nitrotoluene; 2,4-DNBAlc, 2,4-dinitrobenzyl alcohol; 4A2NT, 4-amino-2-nitrotoluene; 2A4NT, 2-amino-4-nitrotoluene; 4Ac2NT, 4-(*N*-acetyl)amino-2-nitrotoluene; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and HPLC, high pressure liquid chromatography.

and elimination of 2,4-DNT in the strain A mouse have shown its rapid elimination from tissues and excretion in the urine [4]. It was suggested, by analogy to the situation in the rat [6–11], that the liver and the gastrointestinal tract may be major sites of 2,4-DNT metabolism in this mouse strain [4]. In conjunction with our carcinogenicity studies [4, 5] and in order to determine whether species differences in 2,4-DNT carcinogenicity [1–5] are related to differences in metabolism, we have examined the *in vivo* metabolism of 2,4-DNT following intraperitoneal and oral administration as well as its *in vitro* metabolism by liver and lung microsomes, intestinal explants and cecal contents of the strain A mouse.

MATERIALS AND METHODS

Animals. Mice were kept on corn cob bedding in temperature and humidity controlled rooms and were given food and water *ad lib.* (NIH-07 diet, Ziegler Brothers, Gardner, PA). Young, adult (6–8 weeks) mice were used in this study and were derived from our breeding colony established with breeding sets of A/Jax mice obtained from Jackson Laboratories (Bar Harbor, ME).

Chemicals. [3-^{3}H]2,4-DNT (sp. act. 1.4 Ci/mmole) was obtained from Moravek Biochemicals (Brea, CA) and was >99.4% radiochemically pure by HPLC [C_{18}-μBondapak column (Waters, Milford, MA) developed isocratically with methanol–water, 1:1]. 2,4-DNT, 2,4-DNBAcid, and 2,4-DAT were obtained from the Aldrich Chemical Co. (Milwaukee, WI). 2A4NBAcid, 2A4NT and 4A2NT were purchased from ICN Pharmaceuticals (Plainview, NY). 4Ac2NBAcid, 2A4NBAlc, 2Ac4NT, and 4Ac2NT were gifts from Drs. Douglas E. Rickert and John G. Dent of the Chemical Industry Institute of Toxicology (Research Triangle Park, NC). TCDD was obtained from KOR Isotopes (Cambridge, MA).

In vivo *elimination and metabolism studies.* Mice were injected intraperitoneally with a tricaprylin solution (0.25 ml/mouse) containing 2,4-DNT and a tracer amount (2–3 μCi) of [^{3}H]2,4-DNT to give a dose of 100 mg/kg. In separate experiments mice were given 100 mg/kg of 2,4-DNT by gavage in tricaprylin (0.25 ml/mouse) along with a tracer amount (2–3 μCi) of [^{3}H]2,4-DNT. The mice were killed after 30 min–8 hr (four mice/time point) and their bladder including contents, and large intestine including contents, were removed. For time periods up to 1 hr after dosing, mice were placed individually in glass containers and any voided urine was collected by several rinses with small volumes of distilled water and the rinses were then combined with the bladder contents [4]. For longer periods (>1 hr) mice were placed individually in metabolic cages as before [5], and urine and feces were collected separately. An aliquot of each urine was removed for quantitation of radioactivity. Feces and contents of the large intestine of each mouse were combined and, after homogenization in 3 ml of distilled water, an aliquot was removed for quantitation of radioactivity. For analysis of metabolites the combined urines of each time point were extracted twice with 3 vol. of ethyl acetate–acetone (3:1). The combined organic phases were dried at 40° under a stream of nitrogen, and the resulting residue was designated the unconjugated fraction. The aqueous phase was adjusted to pH 6.8 by the addition of 0.1 ml of 0.75 M phosphate buffer, pH 6.8. After addition of β-glucuronidase (400 units/ml, Type VII, Sigma), the samples were incubated at 37° for 16 hr and then extracted as described above. This fraction was termed the glucuronide fraction. Both the unconjugated and glucuronide fractions were reconstituted in 0.4 to 0.6 ml of water–methanol (1:1) containing a mixture of authentic standards (10 μg/ml each) and were then filtered through a 0.22 μm Swinnex filter (Millipore). An aliquot was removed for the determination of total radioactivity, and another aliquot was used for analysis by HPLC as described below.

In vitro *metabolism studies.* Male mice were treated intraperitoneally with TCDD (0.4 mg/kg) dissolved in tricaprylin (0.25 ml/mouse) and controls received tricaprylin only. After 24 hr the animals were killed by cervical dislocation. Liver and lungs were removed and washed in 0.15 M KCl. A 20% homogenate (w/v) of liver and lungs was made in 0.1 M potassium phosphate buffer, pH 7.4, containing 0.15 M KCl. The homogenate was first centrifuged at 800 *g* for 10 min followed by centrifugation at 9000 *g* for 20 min at 4°. The resulting supernatant fraction was further centrifuged at 100,000 *g* for 60 min. The 100,000 *g* pellet was washed once with the suspending buffer and recentrifuged at 100,000 *g* for 60 min at 4°, after which it was suspended (5–10 mg protein/ml) in 0.1 M potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol. This suspension was termed the microsomal fraction, and it was stored at −80° until use [^{3}H]2,4-DNT (1.0 μCi) and 2,4-DNT (200 μM final concentration) were incubated in 60 mm tissue culture dishes with liver and lung microsomes (0.8 to 1.0 mg microsomal protein/dish) from untreated or TCDD-pretreated animals. The incubation medium consisted of 1.0 ml of 0.1 M potassium phosphate buffer, pH 7.4, containing 70 mM $MgCl_2$ and 0.56 mM NADPH. Dishes were placed in air-tight chambers which were gassed (to 3 psi) with either air or nitrogen and rocked at 5 cycles/min at 37°. After 60 min, the mixture was extracted as described above and analyzed by HPLC as described below.

Small and large intestine were removed, opened along their longitudinal surface, and washed free of contents. Explants (0.5 cm^2 each) were placed in 60 mm tissue culture dishes (four explants/dish, two explants on opposite sides) with the epithelium oriented towards the gaseous phase. Medium was added (3 ml/dish), and the dishes were then incubated in air-tight chambers and gassed and rocked as described above, so that the explants were alternately exposed to medium and atmosphere. For incubations with cecal contents, the combined contents of the ceca from four mice were suspended in the incubation medium and equally distributed among four dishes. At the end of the incubation period (60 min), the medium was extracted as described above and analyzed by HPLC as described below. The DNA concentration in homogenates of the explants was determined by the diphenylamine method [13].

HPLC analysis. HPLC analysis was performed using the method of Rickert and Long [12] with a slight modification. An aliquot of the sample, containing a mixture of authentic standards, was injected into the high pressure liquid chromatograph (Waters, model 334). Recovery of sample radioactivity was always >83% of the amount injected. The mixture of standards contained the following metabolites (10 μg/ml each): 4Ac2NBAcid, 2,4-DNBAcid, 2A4NBAcid, 2,4-DAT, 2A4NBAlc, 2Ac4NT, 2,4-DNBAlc, 4A2NT, 2A4NT, 4Ac2NT, and 2,4-DNT. The metabolites were separated at ambient temperature on a 4.6 × 25 cm column (Ultrasphere ODS, Altex). A constant flow rate of 1.0 ml/min and a 30-min linear gradient from 15% methanol in 0.005 M potassium phosphate buffer, pH 7.4, to 100% methanol was used. Fractions (0.5 ml) of eluate were collected into counting vials, and the radioactivity in each fraction was quantitated by standard liquid scintillation methods. Under these conditions the retention times of the various metabolites, which were monitored at 254 nm, were as follows: 4Ac2NBAcid, 2.6 min; 2,4-DNBAcid, 4.9 min; 2A4NBAcid, 6.2 min; 2,4-DAT, 9.7 min; 2A4NBAlc, 14.7 min; 2Ac4NT, 17.6 min; 2,4-DNBAlc, 18.9 min; 4A2NT, 19.6 min; 2A4NT, 20.2 min; 4Ac2NT, 21.1 min; and 2,4-DNT, 23.3 min. These retention times were essentially the same as the published values [12].

RESULTS

After intraperitoneal administration, excretion of radioactive material in the urine was rapid, with approximately 70% of the dose excreted in 4 hr.

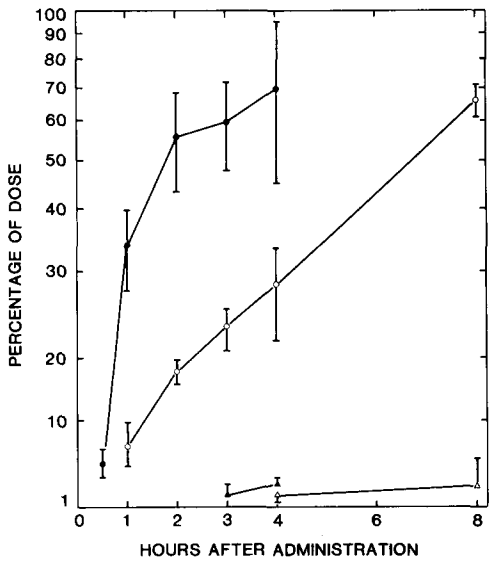

Fig. 1. Elimination of 2,4-DNT after i.p. and p.o. administration to A/J mice. Young adult (6–8 weeks of age) male A/J mice were given 100 mg 2,4-DNT/kg, either i.p. (●,▲), or p.o. (○,△), along with a tracer amount of [^{3}H]2,4-DNT (2–3 μCi), both dissolved in tricaprylin (0.25 mg/mouse). Elimination of radioactive material in the urine (●,○) and feces (▲,△) was followed for up to 8 hr. Each point represents the mean (±S.D.) of four mice.

Excretion after oral administration was much slower with only 28.5% of the dose (100 mg/kg) appearing in the urine after 4 hr and 66% after 8 hr (Fig. 1). During these time periods, elimination in the feces was minimal (1.1 to 2.1% of the dose; Fig. 1), after either intraperitoneal or oral administration.

After intraperitoneal administration the amount of radioactive material in the unconjugated fraction of the urine represented 3.6 to 8.8% of the dose, while that in the glucuronide fraction constituted 2.4 to 7.5% of the dose. The corresponding amounts after oral administration were 5.5 to 6.8 and 20.5 to 28.2% respectively. Radioactive material remaining in the aqueous phase was not further characterized, except that, upon HPLC analysis of the spent urine, almost all of the radioactivity (>86% in all cases) eluted immediately after the column void volume, i.e. together with the three carboxylic acid standards (4Ac2NBAcid, 2,4-DNBAcid, and 2A4NBAcid). Several additional, incompletely separated compounds appeared in this area of the chromatogram and no attempts were made to resolve and characterize these metabolites.

Only the unconjugated fraction of the urine obtained after intraperitoneal administration was analyzed for metabolites, and the most abundant neutral metabolite present in this fraction was 2,4-DNBAlc (1.58 to 5.59% of the dose). Minor amounts of 2,4-DAT, 4A2NT, and 2A4NT were also detected (Table 1). The fraction termed "Acids" in Table 1 most likely represents a small portion of the water-soluble material that partitioned into the organic phase upon extraction of the urine with ethyl acetate–acetone (3:1). This material was termed acidic, similar to the remaining radioactive material in the spent urine (see above), because it eluted with retention times covering the range of those of the three carboxylic acid standards (4Ac2NBAcid, 2,4-DNBAcid and 2A4NBAcid). Water-soluble conjugates of neutral metabolites, such as the glucuronide of 2,4-DNBAlc, elute with higher retention times than those of the carboxylic acids [12]. Substantial amounts of unknown metabolites were also detected (Table 1). After oral administration, most of the neutral metabolites in the unconjugated fraction represented unknowns (1.73 to 2.60%, Table 1), but the most abundant identifiable metabolite of this fraction was 2A4NBAlc (0.47 to 1.53% of the dose). In contrast, in the glucuronide fraction, 2,4-DNBAlc was the major neutral metabolite (5.45 to 16.13%, Table 1). Also, in both the unconjugated and the glucuronide fractions, a small proportion of the water-soluble metabolites partitioned into the organic phase and eluted with chromatographic properties similar to those of the carboxylic acid standards (see above). In all cases after oral administration, minor amounts of 2,4-DAT, 2Ac4NT, 4A2NT and 2A4NT could be detected (Table 1). Except for an apparent increase in the amounts of the alcohols (2A4NBAlc and 2,4-DNBAlc), no other time-related changes in the patterns of metabolites were observed during the 0.5 to 8 hr of urine collection (Table 1). Feces and contents of the large intestine were not analyzed for metabolites.

In vitro metabolism of 2,4-DNT by liver and lung microsomes yielded three identifiable products, 2,4-

Table 1. Distribution of urinary metabolites of 2,4-DNT after i.p. or p.o. administration (100 mg/kg) to A/J mice*

Time after administration (hr)	Fraction†	Percentage of dose‡								
		Acids	2,4-DAT	2A4NBAlc	2Ac4NT	2,4-DNBAlc	4A2NT	2A4NT	2,4-DNT	Unknown
					i.p. Administration					
0.5	U	0.01	—§	—	—	5.59	0.03	0.01	0.03	3.04
1	U	0.58	0.06	—	—	2.46	0.07	0.06	0.08	0.19
2	U	0.50	0.16	—	—	1.58	0.03	0.01	—	1.32
					p.o. Administration					
1	U	3.09	0.07	0.47	0.04	0.17	0.05	0.01	—	2.60
	G	10.01	0.10	0.21	0.18	5.45	0.25	0.10	—	4.12
2	U	2.55	0.08	1.12	0.06	0.13	0.07	0.02	0.01	2.15
	G	6.35	0.11	0.11	0.17	16.13	0.25	0.11	—	4.96
4	U	1.48	0.10	1.26	0.02	0.23	0.04	0.03	0.01	2.43
	G	3.98	0.13	0.17	0.79	11.50	0.19	0.13	—	4.41
8	U	2.68	0.05	1.53	0.20	0.52	0.03	0.04	—	1.73
	G	5.63	0.10	0.13	1.13	14.08	0.20	0.13	—	3.63

* Mice (four animals/time point) received 2,4-DNT (100 mg/kg, i.p. or p.o.), and their urine was collected, extracted and analyzed by HPLC as described in Materials and Methods.

† U, unconjugated fraction; and G, glucuronide fraction.

‡ Values represent the average of duplicate HPLC runs (C.V. < 15%).

§ Non-detectable.

Table 2. Metabolism of 2,4-DNT by liver and lung microsomes from A/J mice*

Animal pretreatment	Souce of microsomes	Incubation atmosphere	Metabolite†				
			2,4-DNBAlc	4A2NT	2A4NT	Unknown	2,4-DNT
			pmoles/mg protein/hr			% of total chromatographed	
Vehicle	Liver	Air	7019	1117	432	2.5	83.0
Vehicle	Lung	Air	4442	892	496	4.3	92.3
Vehicle	Liver	Nitrogen	70	14	<10	3.4	95.0
Vehicle	Liver (boiled)	Air	26	—‡	—	5.9	92.4
Vehicle	Liver (no NADPH)	Air	<10	—	—	—	97.3
TCDD	Liver	Air	13,758	2460	721	3.5	68.5
TCDD	Lung	Air	5496	1766	1388	4.8	92.4

* Microsomes (0.8 to 1.0 mg protein) were incubated for 1 hr in 0.1 M potassium phosphate buffer, pH 7.4 (1 ml), containing 70 mM $MgCl_2$, 0.56 mM NADPH, 200 μM 2,4-DNT, and 1 μCi [^{3}H]2,4-DNT. Metabolites were extracted and analyzed by HPLC as described in Materials and Methods.

† Values represent the average of duplicate experiments (C.V. < 28%).

‡ Non-detectable.

DNBAlc, 4A2NT and 2A4NT, with 2,4-DNBAlc being the most abundant metabolite in all cases (Table 2). No other metabolites were found under these conditions. The amounts of metabolites formed by lung microsomes were always lower than those formed by liver microsomes, except for 2A4NT after pretreatment of the animals with TCDD (Table 2). Using boiled liver microsomes or incubating liver microsomes in an atmosphere of nitrogen, or in the absence of NADPH resulted in negligible metabolism (Table 2). Pretreatment of the animals with TCDD resulted in an approximate doubling of the amounts of metabolites formed by liver microsomes and of the amounts of 4A2NT and 2A4NT formed by lung microsomes, but had a negligible effect on the amount of 2,4-DNBAlc formed by lung microsomes (Table 2). A small amount of unknown material (<5.9% of the total, Table 2) was found in almost all cases.

When intestinal explants or cecal contents were incubated with 2,4-DNT, 2,4-DNBAlc, 2A4NT, 4A2NT, and 4Ac2NT could be detected as metabolites (Table 3). In an atmosphere of air the most abundant metabolite formed by the small intestine was 2,4-DNBAlc (2327 pmoles/mg DNA/hr), with lower amounts of the others (837–1582 pmoles/mg DNA/hr, Table 3). The large intestine formed approximately equivalent amounts of all four metabolites and the amount of 2,4-DNBAlc (1002 pmoles/mg DNA/hr) was less than half of the amount of this metabolite formed by the small intestine (Table 3). Regarding the relative amounts of the two isomeric

Table 3. Metabolism of 2,4-DNT by intestinal explants and cecal contents from A/J mice*

Tissue or material incubated†	Incubation atmosphere	Metabolite‡					
		2,4-DNBAlc	2A4NT	4A2NT	4Ac2NT	Unknown	2,4-DNT
		pmoles/mg DNA/hr§				% of total chromatographed	
Small intestine	Air	2327	1488	1582	837	4.4	84.0
Small intestine	Nitrogen	—‖	2592	2186	1296	1.1	95.3
Large intestine	Air	1002	1107	1620	1134	4.6	91.0
Large intestine	Nitrogen	—	378	162	162	1.6	92.9
		pmole/hr					
Cecal contents	Air	135	621	486	243	0.4	95.0
Cecal contents	Nitrogen	47	4644	4482	648	0.1	46.1

* Intestinal explants or cecal contents were incubated for 1 hr in 0.1 M potassium phosphate buffer, pH 7.4 (3 ml), containing 70 mM $MgCl_2$, 0.56 mM NADPH, 100 μM 2,4-DNT, and 0.5 μCi [^{3}H]2,4-DNT. Metabolites were extracted and analyzed by HPLC as described in Materials and Methods.

† See Materials and Methods for further details of the incubation conditions.

‡ Values represent the average of duplicate experiments (C.V. < 19%).

§ The amount of DNA in the samples from the small intestine (twelve explants combined) varied from 1.1 to 1.35 mg. and that in the large intestine (twelve explants combined) was 1.2 to 1.3 mg.

‖ Non-detectable.

aminonitrotoluenes (2A4NT and 4A2NT), explants of both the small and large intestine formed higher amounts of 2A4NT (Table 3) than do liver and lung microsomes (Table 2). In an atmosphere of nitrogen, 2,4-DNBAlc could not be detected, but the amounts of 2A4NT, 4A2NT and 4Ac2NT formed by the small intestine were increased, while the amounts of these metabolites formed by the large intestine were decreased under these conditions (Table 3). Incubation of cecal contents in an atmosphere of nitrogen resulted in a 7- to 9-fold increase in the amounts of 2A4NT and 4A2NT, and in a 2.6-fold increase in the amount of 4Ac2NT, when compared to incubation in the presence of air (Table 3). The small amount of 2,4-DNBAlc that could be detected in the medium after incubation in air (135 pmoles/hr) decreased to 47 pmoles/hr when the incubation was performed in an atmosphere of nitrogen (Table 3). In all cases small amounts (<4.6% of the total chromatographed) of unknowns could be detected on the chromatogram.

DISCUSSION

Urinary excretion of 2,4-DNT and its metabolites by the strain A mouse was rapid, especially after intraperitoneal administration (Fig. 1). Elimination in the feces did not amount to more than 2.1% of the dose (100 mg/kg). These results are at variance with those of Lee *et al.* [6] who reported that 81–84% of a single oral dose (195 mg/kg) of 2,4-DNT administered to CD-1 or B6C3F1 mice appears in the feces after 24 hr while only 7–11% is excreted in the urine. The reasons for this discrepancy are unknown but are possibly related to the different mouse strains and/or to the different doses administered. In studies on the elimination of single oral doses of 2,4-DNT in other species (rat, rabbit, dog, monkey), the urine was found to be the major route of elimination throughout a 24-hr period after administration, and only minor amounts (<9%) appeared in the feces [6]. After 8 hr, the strain A mouse had eliminated 66% of an oral dose of 100 mg/kg (Fig. 1), while in Fischer-344 rats <35% of a similar dose appears in the urine during this time period [12]. This difference in rates of elimination is paralleled by the much shorter half-lives of 2,4-DNT and its metabolites in liver and kidney of the strain A mouse [4] than those in the Fischer-344 rat [14].

Identified neutral urinary 2,4-DNT metabolites (unconjugated and glucuronides) represented only a small percentage (2.4 to 8.8%) of the total amount of metabolites present in the urine, both after intraperitoneal and after oral administration (see Results). The vast majority of the balance of the metabolites appeared to represent carboxylic acid derivatives of 2,4-DNT as judged by their chromatographic behavior (see Results). This is in contrast to the situation in the Fischer-344 rat where 18–22% of the urinary metabolites isolated 16 hr after an oral dose of 100 mg 2,4-DNT/kg represents carboxylic acid derivatives and approximately 16% of the neutral metabolites is conjugated with glucuronic acid [12]. While we have termed as "Acids" the radioactive material eluting with retention times similar to those of the three carboxylic acid standards (4Ac2NBAcid, 2,4-DNBAcid, 2A4NBAcid), the possibility that some of this material represents conjugates other than glucuronides or sulfates (the β-glucuronidase preparation used was not free of sulfatase contamination) cannot be excluded. Further chromatographic and identification studies will be necessary to elucidate this.

After oral administration, a much larger proportion (20.5 to 28.2%) of metabolites was conjugated with glucuronic acid than after intraperitoneal administration (2.4 to 7.5%). However, in all cases, 2,4-DNBAlc was the major neutral identifiable metabolite (Table 1). Significant amounts of unknown neutral metabolites were also formed (Table 1), a result identical to that obtained in the CD-1 rat [6] and in the Fischer-344 rat [12]. 2A4NBAlc formed from 2,4-DNT after oral administration did not appear to be conjugated with glucuronic acid, as almost all of this metabolite was present in the unconjugated fraction (Table 1). This metabolite is detected, also in the unconjugated form, after incubation of 2,4-DNT with rat hepatocytes [7] and after perfusion of the rat liver [15]. Similar to the strain A mouse (Table 1), the CD-1 rat metabolizes 2,4-DNT to a number of reduced or partially reduced products [6], but in the Fischer-344 rat the only identifiable neutral urinary metabolite is 2,4-DNBAlc [12]. 2,4-DAT, which is a hepatocarcinogen in rats [16, 17], and in female mice [18], is not formed in the Fischer-344 rat [14], but minor amounts can be detected as a urinary 2,4-DNT metabolite in CD-1 rats [6] and in strain A mice (Table 1). 2,4-DAT is not carcinogenic in the strain A mouse lung tumor bioassay [19].

Liver and lung microsomes metabolized 2,4-DNT to mainly 2,4-DNBAlc and to small amounts of the reduced products 4A2NT and 2A4NT (Table 2). A similar pattern is observed in the metabolism of 2,4-DNT by 10,000 *g* liver supernatant fractions of various species, including the mouse [6]. Rat liver microsomes also form these three metabolites in similar proportions and, in addition, can form 2Ac4NT under anaerobic conditions [9]. Under the latter conditions, the formation of all three metabolites by mouse liver microsomes was negligible (Table 2). Regarding the lack of formation of 2,4-DNBAlc in the absence of oxygen, this finding confirms similar results obtained with rat liver microsomes [9]. However, anaerobic conditions have been found to result in an increase in the formation of reduced 2,4-DNT metabolites (4A2NT and 2A4NT) by rat liver microsomes [9]. This difference between the rat and the mouse may be related to differences in oxygen sensitivity of the liver microsomal nitroreductases of these two species. Interspecies differences in the catalytic activities of liver nitroreductases have been reported [20]. Except for the apparent dependence of the liver microsomal nitroreductase on NADPH (Table 2), this enzyme was not characterized further. Microsomes isolated from the lung, which is the target organ in the lung tumor bioassay, formed the same metabolites as did liver microsomes but in lower amounts (Table 2). Such a quantitative difference in the metabolic capacity of these two organs was also apparent from our *in vivo* studies on 2,4-DNT elimination in this mouse strain [4].

Treatment of mice with TCDD results in an increase in hepatic microsomal cytochrome P-448 [21]. Pretreatment of strain A mice with TCDD resulted in an increase in the *in vitro* formation of 2,4-DNT metabolites by both liver and lung microsomes (Table 2). This finding, together with the observed dependence of metabolite formation on the presence of NADPH and oxygen (Table 2), suggests, but does not prove, that the formation of these metabolites is catalyzed by microsomal cytochrome P-450-dependent enzymes. The involvement of such enzymes in the *in vitro* formation of 2,4-DNBAlc from 2,4-DNT by rat liver microsomes [9] and in that by rat hepatocytes [7] has been demonstrated.

Incubation of intestinal explants or cecal contents with 2,4-DNT resulted in the formation of oxidative (2,4-DNBAlc) and reductive (2A4NT and 4A2NT) metabolites, that were similar to those formed by liver and lung microsomes. In addition, significant amounts of 4Ac2NT were formed (Table 3). These results represent the first demonstration that the rodent intestinal epithelium is capable of metabolism of 2,4-DNT by conventional and axenic rats such as rat hepatocytes [7] and the perfused rat liver [15], also form 2,4-DNBAlc as the main 2,4-DNT metabolite, together with minor amounts of 2A4NBAlc, 2A4NT, 4A2NT, 2Ac4NT, and 4Ac2NT. Relative to the amount of 2,4-DNBAlc, intestinal explants form much higher amounts of the reduced metabolites 2A4NT and 4A2NT (Table 3) than do liver or lung microsomes (Table 2). This is possibly due to the contribution of soluble 2,4-DNT nitroreductases, which have been shown to be active in rat liver cytosol [9]. The importance of the intestinal microflora in the reductive metabolism of 2,4-DNT was realized from studies on the *in vivo* metabolism of 2,4-DNT by conventional and axenic rats [8]. It was further confirmed by *in vitro* metabolism studies with intestinal microflora from mice, rats and man [10]. Our present results indicate that the microflora of the strain A mouse also plays a major role in the reductive metabolism of 2,4-DNT (Table 3). The effect of incubating explants of the large intestine in an atmosphere of nitrogen (Table 3) was similar to that observed after incubation of liver microsomes under anaerobic conditions (Table 2) in that the formation of both the oxidative metabolic product (2,4-DNBAlc) and reduced metabolites (2A4NT, 4A2NT) was decreased. In contrast, with both the small intestine and with cecal contents, anaerobic conditions inhibited the formation of 2,4-DNBAlc and enhanced the formation of reduced metabolites (Table 3). This difference may be related to the different properties of microsomal and/or soluble nitroreductases of these systems. It is known that liver microsomal and cytosolic nitroreductases have different activities [22].

The formation of N-acetylamino derivatives of 2,4-DNT by rodents ([7–9, 12]; Tables 1 and 3) is of interest since another aromatic N-acetylamine, 2-acetylaminofluorene, is a potent carcinogen in a number of animal species [23]. When administered *in vivo*, a rapid equilibrium is established between this compound and its N-deacetylated derivative, 2-aminofluorene [23]. Similar to the situation in rabbits and humans [24], inbred mouse strains can be classified as either "slow acetylators" or "fast acetylators" of certain arylamine drugs and carcinogens [25]. It has been suggested that the capacity for N-acetylation may regulate both the occurrence of tumors in specific organs and the susceptibility to carcinogenesis from aromatic amines with the "slow acetylator" being more susceptible [26, 27]. Support for this possibility has been derived from experiments in which the genotoxic response of cultured rabbit hepatocytes to certain aromatic amines was shown to be related to the acetylator phenotype [28]. Inasmuch as neither 2,4-DNT nor 2,4-DAT is carcinogenic in the strain A mouse [4, 19], and since A/J mice are slow acetylators [25], it appears unlikely that the acetylator status of this mouse strain is related to susceptibility to the carcinogenic effects of DNTs. However, since the acetylator phenotype has been established based on studies in the liver [25], a more detailed analysis of extrahepatic phenotyping is warranted, especially in A/J mice where the lungs are the target organ in the short-term assay.

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