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High-Performance Liquid Chromatographic Determination of Urinary Metabolites of 2,4-Dinitrotoluene in Wistar Rats

MIKI SHOJI,*,[a] MASA-AKI MORI,[b] KIYOMI MOTO-O,[b] HIROSHI KOZUKA,[b] and TAKASHI HONDA[c]

Radioisotope Laboratory,[a] Faculty of Pharmaceutical Sciences,[b] and School of Medicine,[c] Toyama Medical and Pharmaceutical University, 2630, Sugitani, Toyama 930–01, Japan

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Urinary metabolites of 2,4-dinitrotoluene (2,4-DNT) were quantitated by high-performance liquid chromatography (HPLC) after administration to male Wistar rats. The urine was extracted with ether after adjusting the pH to 11 and 2. The ether extracts were evaporated to dryness, and each residue was dissolved in aqueous methanol and subjected to HPLC analysis. A 54 μl portion of aqueous methanol solution was analyzed by HPLC on a reversed-phase column. The mobile phases were 40% methanol, 40% methanol in 0.0018 M tetra-*n*-butylammonium chloride (TBACl), 20% methanol in 0.0018 M TBACl, pH 7.4 phosphate buffer and 20% methanol in 0.0027 M sodium 1-hexanesulfonate (HSANa), pH 3.7 acetate buffer, at a flow rate of 0.6 ml/min. 2,4-Dinitrobenzoic acid (2,4-DNBA) was excreted most abundantly, followed by 2,4-dinitrobenzyl alcohol glucuronide (2,4-DNBG), 2-amino-4-acetylaminobenzoic acid (2A4AABA), 2,4-dinitrobenzyl alcohol (2,4-DNB), 4-amino-2-nitro(2-amino-4-nitro)benzyl alcohol glucuronides (4A2N(2A4N)BG), 4-amino-2-nitro(2-amino-4-nitro)benzyl alcohols (4A2N(2A4N)B) and 4-amino-2-nitrotoluene (4A2NT). Though 2,4-dinitrobenzaldehyde (2,4-DNA1), a potent mutagen, was not detected in this study, the oxidative conversion of 2,4-DNB to 2,4-DNBA *in vivo* might be correlated to the carcinogenicity of 2,4-DNT, since 2,4-DNBA and 2,4-DNB are the major metabolites of 2,4-DNT.

Keywords——2,4-dinitrotoluene; metabolism; high-performance liquid chromatographic determination; 2,4-dinitrobenzoic acid; 2,4-dinitrobenzyl alcohol; 2,4-dinitrobenzyl alcohol glucuronide; 2-amino-4-acetylaminobenzoic acid

2,4-Dinitrotoluene (2,4-DNT), an important intermediate in the production of toluenediisocyanate for polyurethan foams, coatings and elastomers, has been shown to be a hepatocarcinogen in Fischer-344[1] and CD-1 rats.[2] We have reported that the urinary metabolites of 2,4-DNT extracted with ether after repeated oral administration to male Wistar rats are 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), 2,4-diaminotoluene (2,4-DAT), 2,4-dinitrobenzyl alcohol (2,4-DNB), 2-amino-4-nitrobenzyl alcohol (2A4NB), 4-amino-2-nitrobenzyl alcohol (4A2NB), 2-nitro-4-acetylaminotoluene (2N4AAT), 2-amino-4-acetylaminotoluene (2A4AAT) and 2-amino-4-acetylaminobenzoic acid (2A4AABA), though they have not been quantitatively determined.[3] Rickert *et al.*[4] showed that one of the abundant urinary metabolites of 2,4-DNT in Fischer-344 rats is 2,4-dinitrobenzoic acid (2,4-DNBA). We have assayed the mutagenicity of the metabolites of 2,4-DNT in Wistar and Fischer-344 rats, as well as that of 2,4-dinitrobenzaldehyde (2,4-DNA1), which was supposed to be an intermediary metabolite in the oxidation of 2,4-DNB to 2,4-DNBA, using the Ames assay. It was found that 2,4-DNA1 shows high mutagenic activity corresponding to about 150 times that of 2,4-DNT (approximately 50 His^+ revertants/μg), whereas 2,4-DNT itself and the metabolites of 2,4-DNT containing an amino, acetylamino, or carboxyl group are weak mutagens.[5] Consequently, it seems to be extremely important to identify and quantify the oxidative metabolites of 2,4-DNT for understanding the carcino-

genic action of 2,4-DNT. In this study, we have attempted to establish a method for the determination of 2,4-DNT and its metabolites by high-performance liquid chromatography (HPLC). The urinary metabolites in Wistar rats of orally administered 2,4-DNT (with the ^{3}H-labeled compound as a tracer) were determined by HPLC and liquid scintillation counting based on the established procedure.

Experimental

Reagents——2,4-DNT, 2A4NT, 4A2NT, 2,4-DAT and 2,4-DNBA (99% chromatographically pure after recrystallization from ethanol), methanol, mono- and disodium phosphates, acetic acid, sodium acetate and ether (reagent grade) were obtained from Wako Pure Chemical Ind., Ltd. 2,4-DNB,[6] 2A4NB,[3] 4A2NB,[6] 2N4AAT,[7] 4-nitro-2-acetylaminotoluene (4N2AAT),[7] 2A4AAT,[7] 2,4-diacetylaminotoluene (2,4-DAAT),[7] 2A4AABA[3] and [G-^{3}H] 2,4-DNT (specific activity: 2.14 μCi/mg)[8] were prepared according to the published procedures. 2,4-DNA1 (97% pure) and sodium 1-hexanesulfonate (HSANa) (98% pure) were obtained from Aldrich Chemical Co. Tetra-*n*-butylammonium chloride (TBAC1) was obtained from Nakarai Chemicals Ltd. β-Glucuronidase (bovine liver, 270000 Fishman units/g) was obtained from P-L Biochemicals Inc.

Administration of 2,4-DNT——Five male Wistar rats (weighing 170 to 250 g) were orally given a salad oil solution of [^{3}H]2,4-DNT (75 mg/kg, 0.2 μCi per rat). Rats were kept in metabolic cages and allowed commercial rat diet and water *ad lib.* Urine was collected over a period of 24 h and extracted immediately.

Urine Sample Extraction Procedure——The pH of urine was adjusted to 11 with 10% NaOH solution and the urine was extracted three times with 20 ml of ether. The ether layer was dried over anhydrous Na_2SO_4, and the solvent was evaporated off *in vacuo* at 25 °C to prevent decomposition of 2,4-DNA1. The residue was dissolved in 10 ml of 30% methanol and subjected to HPLC analysis. The pH of the aqueous layer was adjusted to 2 with 10% HCl solution and the aqueous layer was extracted three times with 20 ml of ether. The ether layer was subjected to HPLC analysis in the same way as above.

Enzymatic Hydrolysis——The extracted urine (10 to 15 ml) was mixed with the same volume of sodium acetate buffer (0.2 M, pH 5.0) and 80 mg of β-glucuronidase. Incubation was carried out at 37 °C for 24 h. The hydrolyzed metabolites were extracted and subjected to HPLC analysis according to the urine sample extraction procedure. The aqueous layer was re-incubated with β-glucuronidase as mentioned above.

Determination of Radioactivity in Urine——Radioactivities of portions (300 to 500 μl) of urine samples were counted in TRI-CARB 300C liquid scintillation counter (Packard Instrument Company, Inc.), and quench corrections were made by the external standard method.

Analysis of Urinary Metabolites——The liquid chromatograph used was a Hitachi model 635 with a multi-wavelength ultraviolet (UV) monitor. The reversed-phase column used was Develosil ODS-7 (150 mm × 4.0 mm i.d., particle size 7 μm, Nomura Chemical Co., Seto, Japan). HPLC operating conditions were as follows: flow rate, 0.6 ml/min; UV monitor, 250 nm, 0.04 AUFS; injection volume, 54 μl, column temperature, ambient. The identification of metabolites of 2,4-DNT was carried out by comparing the chromatograms of urine samples from treated rats with those of blank urine samples, and by the co-chromatography of urine samples with authentic samples. The blank urine samples for HPLC analysis were prepared by extracting urine collected for 24 h before administration in the same way as the urine from treated rats. 2,4-DNBA was isolated from the urine by preparative thin layer chromatography (TLC) (1.0 mm thick silica gel plates, Merck Kieselgel 60 F_{254}). The developing solvent used was butanol–acetic acid–water (5 : 1 : 1). Metabolites of 2,4-DNT were quantitated by the external calibration technique, and samples were diluted as necessary.

Results and Discussion

Typical elution profiles of authentic samples of possible metabolites of 2,4-DNT are shown in Fig. 1(A) and (B). 4N2AAT, 2,4-DNB, 4A2NT, 2A4NT, 2,4-DNA1, 2N4AAT and 2,4-DNT were separated in the mobile phase of 40% methanol, and 2,4-DNBA was eluted after 4N2AAT in 40% methanol in 0.0018 M TBAC1 [Fig. 1(A)]. 2,4-DAT, 4A2N(2A4N)B and 2A4AABA were separated in 20% methanol in 0.0018 M TBACl, pH 7.4 (0.001 M NaH_2PO_4, 0.003 M Na_2HPO_4),[9] and 2,4-DAAT and 2A4AAT were separated in 20% methanol in 0.0027 M HSANa, pH 3.7 (0.0095 M acetic acid, 0.0005 M sodium acetate) [Fig. 1(B)]. 4A2NB and 2A4NB were not separated in any of the mobile phase examined. A linear relationship between the amount of each compound and peak height was found over the range of 10 to 150 ng. The detection limit of each compound in this experiment was 10 ng.

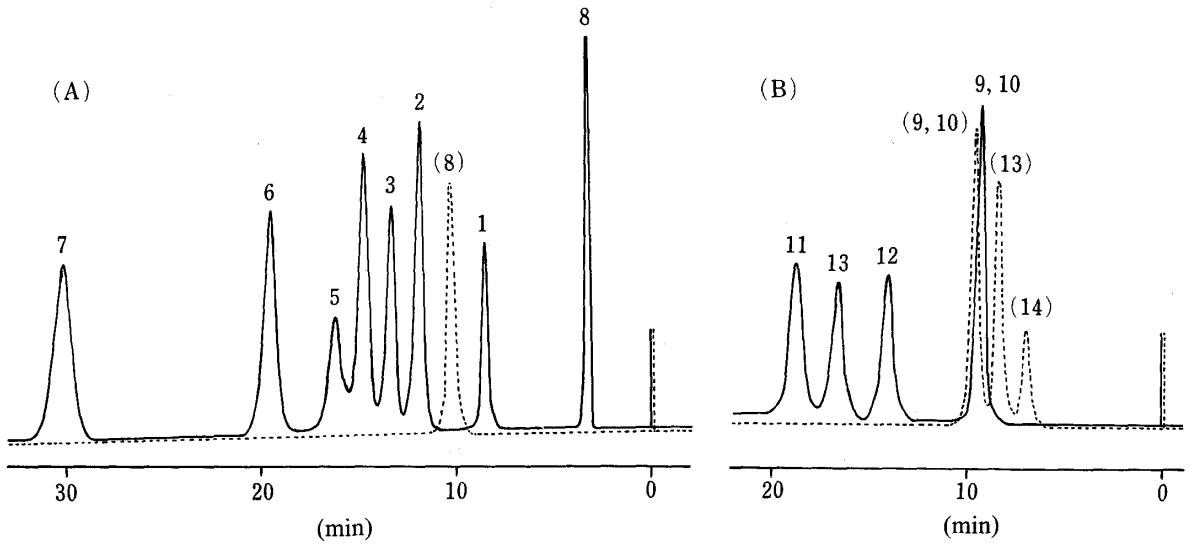

Fig. 1. Separation of 2,4-DNT and Its Derivatives

(A) ——, mobile phase, 40% methanol; ------, mobile phase, 40% methanol in 0.0018 M TBAC1; sample size, 50 ng; other conditions as in text. Compounds: 1=4N2AAT, 2=2,4-DNB, 3=4A2NT, 4=2A4NT, 5=2,4-DNA1, 6=2N4AAT, 7=2,4-DNT, 8=2,4-DNBA, 9=4A2NB, 10=2A4NB, 11=2A4AAT, 12=2,4-DAAT, 13=2A4AABA, 14=2,4-DAT.

(B) ——, mobile phase, 20% methanol in 0.0027 M HSANa, pH 3.7; ------, mobile phase, 20% methanol in 0.0018 M TBAC1, pH 7.4; other conditions as in Fig. 1(A).

TABLE I. Recovery of 2,4-DNT and Its Derivatives in the Ether Extraction Procedure

pH of urine	Compound	Recovery (%)[a]
11	2,4-DNT	96.1±2.1
	4A2NT	96.2±2.4
	2A4NT	95.2±2.1
	2,4-DAT	90.6±3.3
	2N4AAT	96.3±2.0
	4N2AAT	98.1±2.8
	2A4AAT	71.8±2.6
	2,4-DAAT	19.0±0.9
	2,4-DNB	97.2±1.8
	4A2NB	97.8±1.7
	2,4-DNA1	90.8±3.2
2	2,4-DNBA	91.4±1.9
	2A4AABA	61.4±3.2

a) Values are means±S.D. for six samples.

Recovery in the ether extraction procedure was determined (Table I). Six blank urine samples were supplemented with 13 compounds (2,4-DNT and its derivatives) at a concentration of 150 μg/ml and extracted in the same way as test urine samples. The recovery was calculated as follows: the peak height of each compound added to the extracted sample was divided by the peak height of aqueous methanol solution containing 2,4-DNT and its derivatives in the same quantity as the supplemented blank urine. It was confirmed that no interfering substances were present in blank urine extracts. The recoveries for most compounds were more than 90%. Compounds containing $-NHCOCH_3$ and $-NH_2$ moieties were recovered in yields of only 60 to 70%. The diacetyl derivative was hardly extracted be-

cause of its poor solubility in organic solvents.

2,4-DNB, 4A2NT, 2A4NT, 2N4AAT, unchanged 2,4-DNT [Fig. 2(A)], 4A2N(2A4N)B, 2,4-DAT and 2A4AAT [Fig. 2(B) and 2(C)] were detected from ether extracts at pH 11 of urine from treated rats. 2A4AABA [Fig. 3(B)] and peak I [Fig. 3(A)], the retention volume of which was the same as that of 2,4-DNBA, and which was not seen in the blank urine, were detected in ether extracts at pH 2 of urine from treated rats. The compound corresponding to peak I showed the same *Rf* value (0.33) as authentic 2,4-DNBA and mass spectral analysis of the material obtained by preparative TLC gave the molecular ion peak at *m/e* 212 (spectrum not shown). Consequently, peak I was identified as 2,4-DNBA. The aqueous layer after ether extraction was subjected to enzymatic hydrolysis with β-glucuronidase, and 2,4-DNB and 4A2N(2A4N)B were observed (chromatograms not shown). It is considered that 2,4-dinitrobenzyl alcohol glucuronide (2,4-DNBG) and 4-amino-2-nitro(2-amino-4-nitro)benzyl alcohol glucuronides (4A2N(2A4N)BG) were excreted in the urine as metabolites of 2,4-DNT. The amounts of the two compounds released on re-incubation with the enzyme were negligible. The quantitative data are given in Table II for the excretion of urinary metabolites

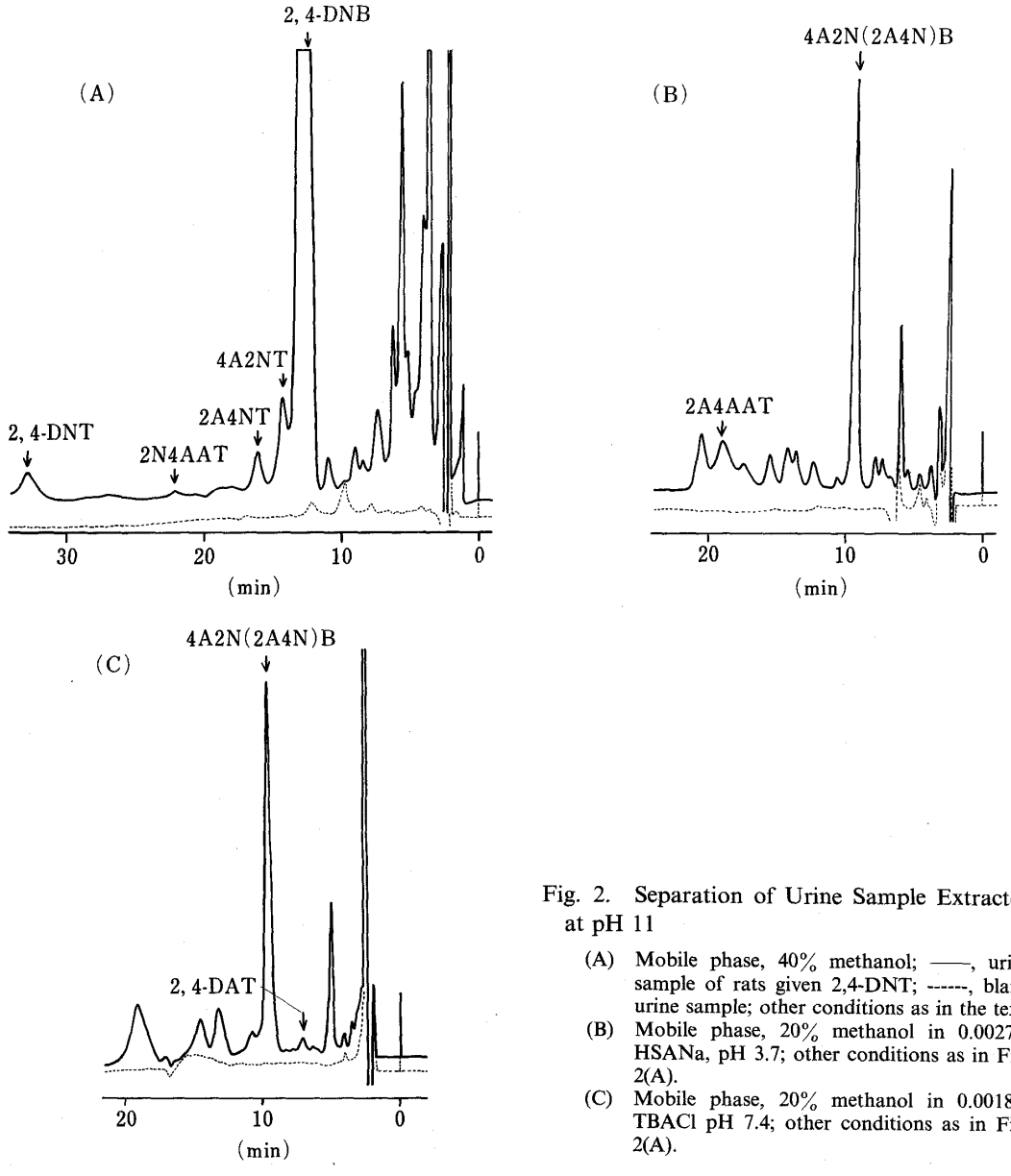

Fig. 2. Separation of Urine Sample Extracted at pH 11

(A) Mobile phase, 40% methanol; ——, urine sample of rats given 2,4-DNT; ------, blank urine sample; other conditions as in the text.
(B) Mobile phase, 20% methanol in 0.0027 M HSANa, pH 3.7; other conditions as in Fig. 2(A).
(C) Mobile phase, 20% methanol in 0.0018 M TBACl pH 7.4; other conditions as in Fig. 2(A).

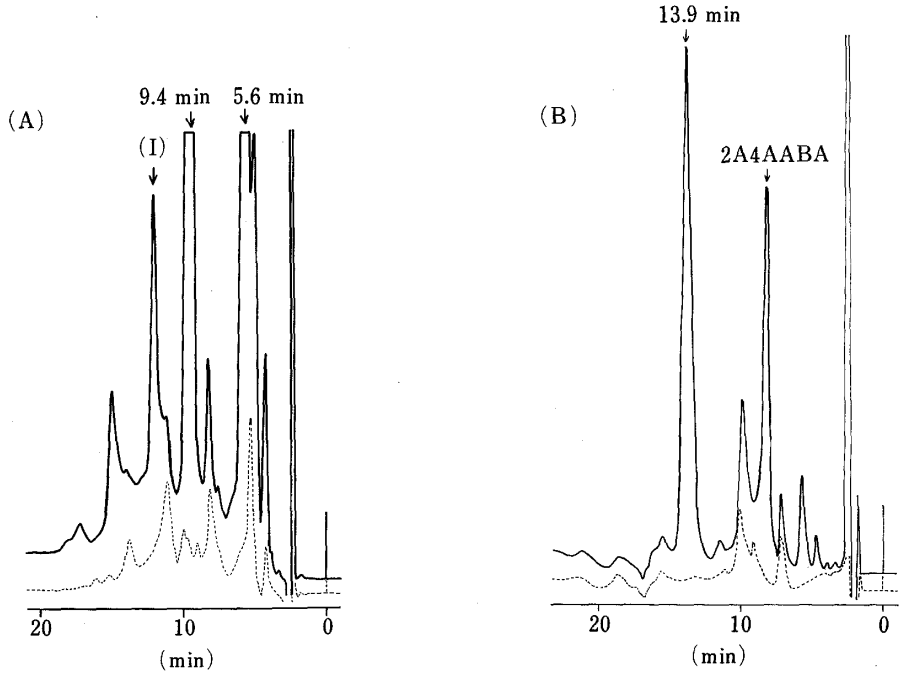

Fig. 3. Separation of Urine Sample Extracted at pH 2

(A) Mobile phase, 40% methanol in 0.0018 M TBAC1; ——, urine sample of rats given 2,4-DNT; ------, blank urine sample; other conditions as in the text.
(B) Mobile phase, 20% methanol in 0.0018 M TBAC1, pH 7.4; other conditions as in Fig. 3(A).

TABLE II. Quantitation of 2,4-DNT Metabolites in Rat Urine

Metabolite	Percent of dose excreted[a)]
2,4-DNB	0.83±0.52
4A2NT	0.03±0.01
4A2N(2A4N)B	0.16±0.10
2,4-DNBA	5.91±3.06
2A4AABA	1.85±1.12
2,4-DNBG	3.15±0.95
4A2N(2A4N)BG	0.45±0.11

a) Values are means±S.D. for five rats.

TABLE III. Percent of Radioactivity Found in Each Fraction

	Urine fraction	Percent of radioactivity[a)]
Before hydrolysis	Ether extract at pH 11	1.5±0.4
	Ether extract at pH 2	1.5±0.2
After hydrolysis	Ether extract at pH 11	4.7±1.2
	Ether extract at pH 2	0.4±0.2
	Aqueous layer	5.6±1.6
	Total	13.7±2.4

a) Values are means±S.D. for five rats.

in Wistar rats up to 24 h after administration of 2,4-DNT. The percentages of major metabolites excreted were 5.91% for 2,4-DNBA, 3.15% for 2,4-DNBG, 1.85% for 2A4AABA and 0.83% for 2,4-DNB, and those of other metabolites were 0.45% for 4A2N(2A4N)BG,

0.16% for 4A2N(2A4N)B and 0.03% for 4A2NT. 2A4NT, 2N4AAT, 2,4-DAT and 2A4AAT, which were detected in urine of rats after repeated doses in our previous study,[3] were also observed in the urine of some of the rats, but in very small amounts. The percentage of radioactivity found in each fraction is given in Table III. The total percentage excreted in urine corresponded quite well with the result in the previous study (13.52%).[8] The percentage of radioactivity found in the ether extract at pH 2 was only 1.5%, though the amount of two carboxylic metabolites determined by HPLC analysis in the same fraction was 7.76% (Table II). One of the reasons for the above difference may be that ^3H was eliminated as a result of oxidation of the methyl group of 2,4-DNT. About one-third of the radioactivity was retained in the aqueous layer after enzymatic hydrolysis. It was reported that the major component of the metabolites of 2,4-DAT in rats is 5-hydroxy-2,4-diaminotoluene.[10] Therefore the remaining radioactivity in the aqueous layer may be partly due to phenolic metabolites of 2,4-DNT, though it remains to be confirmed what kind of metabolites are contained in the aqueous layer.

From the results of this study, it may be concluded that 2,4-DNT is mainly metabolized by oxidation of the methyl group, followed by the formation of glucuronide conjugates in the case of alcoholic metabolites, in male Wistar rats after a single oral administration. The amounts of 2A4NT, 2N4AAT, 2,4-DAT and 2A4AAT, which were detected in our previous repeated administration study,[3] were neglibible, and 2A4AABA was the major form of acetylamino metabolites in urine. These differences are thought to be due to the difference in the method of administration. In our previous study, when the urine was extracted at pH 4.0, 2,4-DNBA was not detected.[3] This may be because of the strong acidity of 2,4-DNBA. 2,4-DNBA and 2,4-DNBG found in this study are the same metabolites as those

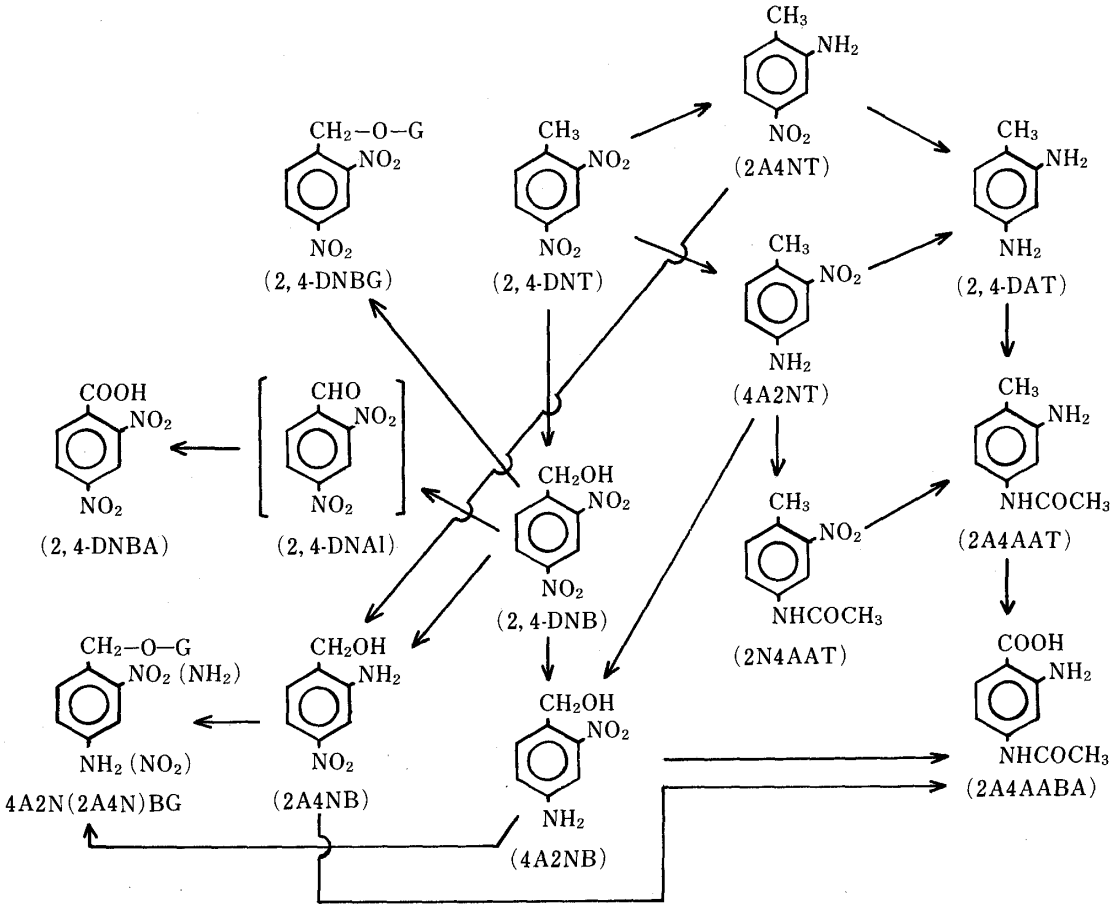

Chart 1. Probable Pathways of 2,4-DNT Metabolism in Rats

identified by Rickert *et al.* in Fischer-344 rats.[4] In addition to those metabolites, 2-nitro-4-acetylaminobenzoic acid (2N4AABA) and 2-amino-4-nitrobenzoic acid (2A4NBA) were detected in their study. 2,4-DNB, 4A2N(2A4N)BG and 2A4AABA, which were not detected in their work,[4] were detected in this study. These differences may be due to the difference in the strains of rats used. In the chromatograms of urine from treated rats, unknown peaks were found at retention times of 5.6, 9.4, [Fig. 3(A)] and 13.9 min [Fig. 3(B)] which were not seen in those of blank urine, and work is in progress to clarify the nature of these peaks. Based on the results of this study and the previous repeated administration study, the probable metabolism of 2,4-DNT in rats can be summarized as in Chart 1.

2,4-DNBA was found to be a non-mutagen in our mutagenicity assay using *S. typhimurium* strains TA98 and TA100, and 2,4-DNB, 4A2NB, 2A4NB and 4A2NT were weak mutagens (3 His^+ revertants/μg).[5] These compounds appear to be some of the mutagenic metabolites of 2,4-DNT in rats. Furthermore, 2,4-DNA1, a putative metabolite of 2,4-DNT, showed high mutagenicity (about 50 His^+ revertants/μg),[5] and 2,4-DNA1 may be an intermediary metabolite in the oxidation of 2,4-DNB to 2,4-DNBA. 2,4-DAT was shown to be a hepatocarcinogen in Wistar[11,12] and Fischer-344[13] rats. However, 2,4-DAT is probably not related to the carcinogenicity of 2,4-DNT, because 2,4-DAT was not detected in the urine, feces,[14,15] blood, kidney, liver[16] or bile[17] of Fischer-344 rats administered 2,4-DNT. In this investigation, 2,4-DNBA and 2,4-DNB were excreted in urine as oxidative metabolites of 2,4-DNT in Wistar rats. It has also been reported that two of the major urinary metabolites of 2,4-DNT in Fischer-344 rats are 2,4-DNBA and 2,4-DNBG,[4] and that 2,4-DNA1 is a potent mutagen.[5] Thus it seems likely that the hepatocarcinogenicity of 2,4-DNT[1,2] may involve the formation of 2,4-DNA1 from 2,4-DNT in the liver. Further studies on the metabolism of 2,4-DNB or 2,4-DNA1 *in vitro* using rat liver homogenates will be necessary to confirm this.

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