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Further studies on the urinary metabolites of 2,4-dinitrotoluene and 2,6-dinitrotoluene in the male Wistar rat

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1. Conjugates of 2,4-dinitrobenzyl alcohol (2,4-DNB) and 2,6-dinitrobenzyl alcohol (2,6-DNB), which were major urinary metabolites of the male Wistar rat dosed orally with 2,4-dinitrotoluene (2,4-DNT) or 2,6-dinitrotoluene (2,6-DNT), were examined by hplc using potassium 2,4-dinitrobenzyl glucuronide (2,4-DNB-G), potassium 2,6-dinitrobenzyl glucuronide (2,6-DNB-G), pyridinium 2,4-dinitrobenzyl sulphate (2,4-DNB-S), and pyridinium 2,6-dinitrobenzyl sulphate (2,6-DNB-S) as authentic compounds. Other metabolites were also examined by hplc.

2. Conjugates detected from urine following administration of 2,4-DNT and 2,6-DNT were 2,4-DNB-G and 2,6-DNB-G, which accounted for about 10·7 and 17·4% of the administered dose respectively. No peaks corresponding to pyridinium 2,4-DNB-S and pyridinium 2,6-DNB-S were detected in urine samples.

3. 2-Amino-4-nitrobenzoic acid (0·71%), 4-amino-2-nitrobenzoic acid (0·52%) and 4-acetylamino-2-nitrobenzoic acid (3·9%), in addition to known metabolites 4-amino-2-nitrotoluene (0·04%), 2,4-DNB (0·25%), 2,4-dinitrobenzoic acid (6·9%) and 4-acetylamino-2-aminobenzoic acid (3·4%), were detected in ether extracts of urine of rat given 2,4-DNT. 2,6-Dinitrobenzoic acid (0·17%) and two known metabolites, 2-amino-6-nitrotoluene (0·44%) and 2,6-DNB (0·53%), were detected in ether extracts of urine of rat given 2,6-DNT.

Introduction

It has previously been reported that the major urinary metabolites of 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) in the male Wistar (Shoji *et al.* 1985, Mori *et al.* 1989) and Fischer-344 rat (Rickert and Long 1981, Long and Rickert 1982) are conjugates of 2,4-dinitrobenzyl alcohol (2,4-DNB) and 2,6-dinitrobenzyl alcohol (2,6-DNB) respectively. These conjugates have been considered to be glucuronides, since 2,4-DNB and 2,6-DNB are liberated by the hydrolysis of urine samples with β-glucuronidase. However, no direct detection of these conjugates without hydrolysis has been made in the metabolism of 2,4-DNT and 2,6-DNT. The previous study in the male Wistar rat (Shoji *et al.* 1985) has also shown that a few unknown metabolites, in addition to the identified metabolites (2,4-DNB, 4-amino-2-nitrotoluene, 2,4-dinitrobenzoic acid and 4-acetylamino-2-aminobenzoic acid), are present in the ether extract of urine after administration of 2,4-DNT. However, the characterization of these unknown compounds has not been attempted.

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In the present study we attempted the direct determination of urinary conjugates of 2,4-DNB and 2,6-DNB in the male Wistar rat dosed with 2,4-DNT or 2,6-DNT by hplc using potassium 2,4-dinitrobenzyl glucuronide (potassium 2,4-DNB-G), potassium 2,6-dinitrobenzyl glucuronide (potassium 2,6-DNB-G), pyridinium 2,4-dinitrobenzyl sulphate (pyridinium 2,4-DNB-S), and pyridinium 2,6-dinitrobenzyl sulphate (pyridinium 2,6-DNB-S) as authentic compounds. Other metabolites including the unidentified metabolites were also examined by hplc using authentic compounds.

Materials and methods

Materials

2,4-DNT, 2,6-DNT, 2-amino-4-nitrotoluene, 4-amino-2-nitrotoluene, 2-amino-6-nitrotoluene, 2,4-diaminotoluene, 2,6-diaminotoluene, 2,4-dinitrobenzoic acid, and 4-anthranilic acid (2-amino-4-nitrobenzoic acid) were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan) and purified by recrystallization. 2,4-Dinitrobenzaldehyde and 2,6-dinitrobenzaldehyde were obtained from Aldrich Chemical Co. (Milwaukee, WN, USA), and purified by recrystallization. Salad oil (mixed oil of rape and soybean) was obtained from Nitsushin Oil Inc. (Tokyo, Japan). All other chemicals or solvents used were of analytical grade.

2,4-DNB, 2,6-DNB, 2,6-dinitrobenzoic acid, 4-amino-2-nitrobenzyl alcohol, 2-amino-4-nitrobenzyl alcohol, 2-amino-6-nitrobenzyl alcohol, 2-acetylamino-4-nitrotoluene, 4-acetylamino-2-nitrotoluene, 2-acetylamino-6-nitrotoluene, 2-acetylamino-4-aminotoluene, 4-acetylamino-2-aminotoluene, 2-acetylamino-6-aminotoluene, 2,4-diacetylaminotoluene, 2,6-diacetylaminotoluene, 4-amino-2-nitrobenzoic acid, 2-amino-6-nitrobenzoic acid, 2,4-diaminobenzoic acid, 2,6-diaminobenzoic acid, 2-acetylamino-4-nitrobenzoic acid, 4-acetylamino-2-nitrobenzoic acid, 2-acetylamino-6-nitrobenzoic acid, 2-acetylamino-4-aminobenzoic acid, 4-acetylamino-2-aminobenzoic acid, 2-acetylamino-6-aminobenzoic acid, 2,4-diacetylaminobenzoic acid and 2,6-diacetylaminobenzoic acid were prepared in this laboratory (Mori *et al.* 1981, 1986, 1989). Their chemical purities were confirmed by melting point measurement, elemental analysis, mass and ^{1}H-nmr spectrometry.

Pyridinium 2,4-DNB-S was synthesized from 2,4-DNB by reaction with chlorosulphonic acid in pyridine. A solution of 2,4-DNB (3 g) in dry pyridine (3 ml) was added to a mixture of chlorosulphonic acid (5·3 g) and dry pyridine (18 ml). The mixture was kept at 55°C for 30 min, and the reaction mixture was evaporated *in vacuo*. The residue after recrystallization from methanol gave pyridinium 2,4-DNB-S as pale brownish plates; yield 2·4 g; m.p. 134–135°C. Pyridinium 2,6-DNB-S (m.p. 136–137°C) was synthesized from 2,6-DNB (2 g) by treatment with chlorosulphonic acid (3·5 g) and pyridine (12 ml) in the same manner as the synthesis of pyridinium 2,4-DNB-S. The yield was 0·7 g. The purities and structures of the synthetic compounds were confirmed by elemental analysis, ^{1}H-nmr and mass spectrometries (table 1).

Potassium 2,4-DNB-G was synthesized from 2,4-DNB by condensation with a acetylated, brominated and methylated derivative of glucuronic acid, followed by deprotection of the condensate. 2,4-DNB (5 g) was dissolved in dry benzene (150 ml). To this boiling solution, a solution of methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate (15 g), prepared from 1,4-glucuronolactone (Bollenback *et al.* 1955), in dry benzene (300 ml) and Ag_2CO_3 (1 g) were added in small aliquots during 10 h. Benzene was distilled off gradually, and stirring was continued. The reaction mixture was filtered, and the filtrate concentrated to a syrup, which was purified by silica gel column chromatography using chloroform–hexane (1:4 v/v) to give methyl 2,3,4-tri-*O*-acetyl-1-*O*-2,4-dinitrobenzyl-β-D-glucopyranuronate as colourless needles; m.p. 183–185°C; yield 0·3 g. To a solution of this condensate (1 g) in dry methanol (200 ml) was added 0·5 M sodium methoxide (4 ml) and the solution stirred for 3 h at 0°C. The reaction mixture was neutralized with Dowex 50WX8 (H+ form), and filtered. The filtrate was concentrated *in vacuo*. Recrystallization of the residue from ethyl acetate gave methyl 1-*O*-2,4-dinitrobenzyl-β-D-glucopyranuronate as colourless needles; m.p. 183–185°C; yield 0·3 g. To a solution of this deacetylated product (1 g) in methanol (200 ml) was added in portions to 0·4% K_2CO_3 (100 ml) at 0°C. The mixture was then stirred for 1 h at 25°C. The solution was neutralized with Dowex 50WX8 (H+ form), filtered, and evaporated *in vacuo*. Recrystallization from water–methanol–acetone (0·1:2:2 v/v) gave potassium 1-*O*-2,4-dinitrobenzyl-β-D-glucopyranuronate (potassium 2,4-DNB-G) as colourless needles; m.p. (decomp.) 185–191°C; $[\alpha]_D^{31} = -56{\cdot}6°$ ($c = 0{\cdot}01$ in water); yield 0·36 g. Potassium 2,6-DNB-G was synthesized from 2,6-DNB (5 g) by condensation with acetylated, brominated and methylated derivative of glucuronic acid (15 g), followed by deacetylation and demethylation, in the same manner as the synthesis of potassium 2,4-DNB-G. Recrystallization of the residue obtained from the demethylation of deacetylated product, with water–methanol–acetone (0·1:2:2 v/v), gave potassium 1-*O*-2,6-dinitrobenzyl-β-D-glucopyranuronate (potassium 2,6-DNB-G) as colourless needles; m.p. (decomp.) 184–190°C; $[\alpha]_D^{31} = -106°$ ($c = 0{\cdot}01$ in water); yield 0·41 g. The purities and structures of the

Table 1. Elemental analysis, and mass and ^{1}H-nmr spectra measurements of synthetic pyridinium 2,4-dinitrobenzyl sulphate (pyridinium 2,4-DNB-S), pyridinium 2,6-dinitrobenzyl sulphate (pyridinium 2,6-DNB-S), potassium 2,4-dinitrobenzyl glucuronide (potassium 2,4-DNB-G), and potassium 2,6-dinitrobenzyl glucuronide (potassium 2,6-DNB-G).

Compound	Formula	Elemental analysis[a] calculated (found)			Mass[b] (m/z)	^{1}H-nmr[c] (δ)
		C	H	N		
Pyridinium 2,4-DNB-S	$C_7H_6N_2O_8S \cdot C_5H_5N$	40·34 (40·39)	3·10 (3·15)	11·76 (11·85)	437 $[M + C_5H_5N]^+$	5·27 (2H, s, CH_2), 8·00 (2H, t, pyridine), 8·07 (1H, d, aromatic), 8·51 (1H, t, pyridine), 8·61 (1H, dd, aromatic), 8·78 (1H, d, aromatic), 8·88 (2H, d, pyridine)
Pyridinium 2,6-DNB-S	$C_7H_6N_2O_8S \cdot C_5H_5N$	40·34 (40·21)	3·10 (3·12)	11·76 (11·55)	437 $[M + C_5H_5N]^+$	5·14 (2H, s, CH_2), 7·78 (1H, t, aromatic), 8·01 (2H, t, pyridine), 8·20 (2H, d, aromatic), 8·53 (1H, t, pyridine), 8·90 (2H, d, pyridine
Potassium 2,4-DNB-G	$C_{13}H_{13}N_2O_{11}K \cdot 1/2H_2O$	37·06 (37·34)	3·35 (3·30)	6·65 (6·63)	413 $[M + H]^+$	3·14–3·26 (4H, m, glucuronic), 4·38 [1H, d, glucuronic), 5·18 (2H, ABq, CH_2), 8·34 (1H, d, aromatic), 8·59 (1H, dd, aromatic), 8·80 (1H, d, aromatic)
Potassium 2,6-DNB-G	$C_{13}H_{13}N_2O_{11}K \cdot 1/2H_2O$	37·06 (36·92)	3·35 (3·39)	6·65 (6·54)	413 $[M + H]^+$	2·84–3·24 (4H, m, glucuronic), 4·14 [1H, d, glucuronic), 4·98 (2H, ABq, CH_2), 7·83 (1H, t, aromatic), 8·23 (2H, dd, aromatic)

[a] Samples were dried over P_2O_5 for 3 h at 40°C.

[b] Mass spectra (positive-ion fast atom bombardment) were recorded on a JEOL JMS-DX 300 at 10 kV ion source accelerating potential using *m*-nitrobenzyl alcohol as the matrix.

[c] ^{1}H-nmr [in $(CD_3)_2SO$] were recorded on a Varian XL-200 with Me_4Si ($\delta = 0$) as internal standard. s, singlet; d, doublet; t, triplet; m, multiplet; ABq, quarter.

synthetic compounds were confirmed by elemental analysis, ^{1}H-nmr and mass spectrometries (table 1). β-Configuration of the glucuronide linkages of potassium 2,4-DNB-G and potassium 2,6-DNB-G was supported by the fact that they were hydrolyzable with β-glucuronidase (data not shown).

Administration of 2,4-DNT and 2,6-DNT

Male Wistar rats (body weight 200–230 g) obtained from Sankyo Laboratories (Tokyo, Japan) were used. Rats were dosed orally with a solution of 2,4-DNT and 2,6-DNT (75 mg/kg each) in 1 ml salad oil, and housed individually in metabolic cages with free access to water and commercial diet (F-2, Sankyo Laboratories). Six rats per xenobiotic dosed were used in each administration. Urine samples after administration of xenobiotics were collected over 24 h, on ice. Control urine was collected for 24 h before administration of the chemicals. Urine samples were stored at − 30°C.

Analysis of conjugated metabolites by hplc

The stored 24-h urine samples (5 ml) were filtered using a 0·45-μm syringe filter. Aliquots (10 μl) of the filtrates were injected into a high-performance liquid chromatograph equipped with a multi-wavelength UV monitor (Hitachi model 655). A reversed phase column packed with TSK gel ODS-80TM (4·6 mm i.d. × 150 mm, particle size 5 μm; Toso Co., Tokyo, Japan) was used with mobile phases A or E (tables 2 and 3). Hplc operating conditions were as follows: flow rate, 1 ml/min; UV monitor, 250 nm, column temperature, ambient. Under these conditions pyridinium 2,4-DNB-S and potassium 2,4-DNB-G, pyridinium 2,6-DNB-S and potassium 2,6-DNB-G were separated (tables 2 and 3).

Detection of metabolites was carried out by the co-chromatography of samples and blanks with authentic compounds. The limit of detection for each conjugate was 0·2 μg/ml. Quantities of metabolites were determined from standard curves plotted as peak areas calculated automatically by a Hitachi 655-61 processor A. A linear relationship between the amount of each compound and peak area was found over the range 0·4–200 μg/ml (data not shown).

Analysis of unconjugated metabolites by hplc

The stored 24-h urine samples (5–10 ml) were adjusted to pH 11 with 2 M Na_2CO_3 and extracted three times with ether (30 ml) (neutral-basic fractions). The extracted urine samples were adjusted to pH 2 with 2 M HCl and extracted three times with ether (30 ml) (acidic fractions). The ethereal neutral-basic and acidic fractions were dried over anhydrous Na_2SO_4, and the solvents evaporated off under a stream of N_2 and dissolved in 10 ml methanol–water (30 : 70 v/v). Aliquots (10–50 μl) of these solutions were injected into a high-performance liquid chromatograph. The hplc conditions were the same as those described above except that solvents of B, C, D or F used as mobile phases (tables 2 and 3). Under the conditions, 2,4-DNT and its derivatives, except 2-amino-4-nitrobenzyl alcohol and 4-amino-2-nitrobenzyl alcohol, 2,6-DNT and its derivatives were separated (tables 2 and 3). The detection limit of each compound was 0·1 μg/ml. The range of linearity of each compound was 0·2–100 μg/ml (data not shown).

Results

Figure 1 shows a representative high-performance liquid chromatogram of the urine of rats dosed orally with 2,4-DNT. A peak with retention time of 16·1 min, which co-eluted with potassium 2,4-DNB-G, was detected in the urine. As shown in figure 2, a peak with retention time of 20·9 min, corresponding to potassium 2,6-DNB-G, was detected in the urine after administration of 2,6-DNT. Peaks corresponding to pyridinium 2,4-DNB-S and pyridinium 2,6-DNB-S were not detected (figures 1 and 2). This result indicates that the conjugates of 2,4-DNB and 2,6-DNB are 2,4-DNB-G and 2,6-DNB-G respectively.

2-Amino-4-nitrobenzoic acid, 4-amino-2-nitrobenzoic acid and 4-acetylamino-2-nitrobenzoic acid, in addition to known metabolites (2,4-dinitrobenzoic acid and 4-acetylamino-2-aminobenzoic acid) (Shoji *et al.* 1985), were detected in the ethereal extract from the acidic fraction of urine after administration of 2,4-DNT (figure 3). Other known metabolites, 4-amino-2-nitrotoluene and 2,4-DNB were detected from the neutral-basic fraction (chromatogram not shown). The metabolites detected from the neutral-basic fraction of urine after administration of 2,6-DNT were 2-amino-6-nitrotoluene and 2,6-DNB (chromatogram not shown), which were known metabolites (Mori *et al.* 1989). A small peak corresponding to 2,6-dinitrobenzoic acid, which was not seen in our previous study (Mori *et al.* 1989), was detected from the acidic fraction (figure 4).

Table 2. Retention times of 2,4-dinitrotoluene (2,4-DNT) and its derivatives on hplc.

Compound	Retention time (min) mobile phase[a]			
	A	B	C	D
2,4-Diaminotoluene	2·0	7·8		
2-Acetylamino-4-aminotoluene	2·2	9·0		
2-Amino-4-nitrobenzyl alcohol	6·3	9·8		
4-Amino-2-nitrobenzyl alcohol	6·3	9·8		
4-Acetylamino-2-aminotoluene	3·3	16·3		
2,4-Diacetylaminotoluene	6·3	18·0		
2-Acetylamino-4-nitrotoluene	24·6		5·5	
2,4-DNB	35·4		7·4	
4-Acetylamino-2-nitrotoluene	61·2		8·1	
2,4-Dinitrobenzaldehyde			9·1	
4-Amino-2-nitrotoluene			10·4	
2-Amino-4-nitrotoluene			11·3	
2,4-DNT			21·9	
2,4-Diaminobenzoic acid	3·5			4·2
4-Amino-2-nitrobenzoic acid	8·0			6·3
4-Acetylamino-2-aminobenzoic acid	6·3			7·7
2-Acetylamino-4-aminobenzoic acid	8·9			9·8
4-Acetylamino-2-nitrobenzoic acid	5·9			10·9
2,4-Diacetylaminobenzoic acid	11·4			21·6
2,4-Dinitrobenzoic acid	5·5			25·0
2-Amino-4-nitrobenzoic acid	42·6			32·4
2-Acetylamino-4-nitrobenzoic acid	27·9			118·6
Potassium 2,4-DNB-G	16·1			
Pyridinium 2,4-DNB-S	21·4			

[a]A, 10 mM potassium phosphate buffer (pH 3)–acetonitrile (85:15 v/v); B, 4 mM sodium phosphate buffer (pH7·4)–methanol (85:15 v/v); C, water–acetonitrile (65:35 v/v); D, 0·1% tetrabutylammonium bromide in water–methanol (70:30 v/v).

Table 3. Retention times of 2,6-dinitrotoluene (2,6-DNT) and its derivatives on hplc.

Compound	Retention time (min) mobile phase[a]			
	E	B	C	F
2,6-Diaminotoluene	1·8	5·3		
2-Acetylamino-6-aminotoluene	2·4	6·4		
2,6-Diacetylaminotoluene	5·2	8·3		
2-Amino-6-nitrobenzyl alcohol	22·0	10·3	4·0	
2-Acetylamino-6-nitrotoluene	36·2		4·8	
2,6-DNB	31·0		6·5	
2-Amino-6-nitrotoluene			10·5	
2,6-Dinitrobenzaldehyde			11·6	
2,6-DNT			24·4	
2,6-Diaminobenzoic acid	2·7			3·5
2,6-Dinitrobenzoic acid	3·2			4·7
2-Acetylamino-6-nitrobenzoic acid	5·4			10·6
2-Acetylamino-6-aminobenzoic acid	5·5			12·5
2-Amino-6-nitrobenzoic acid	13·7			20·7
2,6-Diacetylaminobenzoic acid	9·1			29·7
Potassium 2,6-DNB-G	20·9			
Pyridinium 2,6-DNB-S	26·8			

[a]E, 10 mM potassium phosphate buffer (pH 3)–acetonitrile (90:10 v/v); F, 10 mM potassium phosphate buffer (pH 3)–acetonitrile (95:5 v/v). B and C are the same as in table 2.

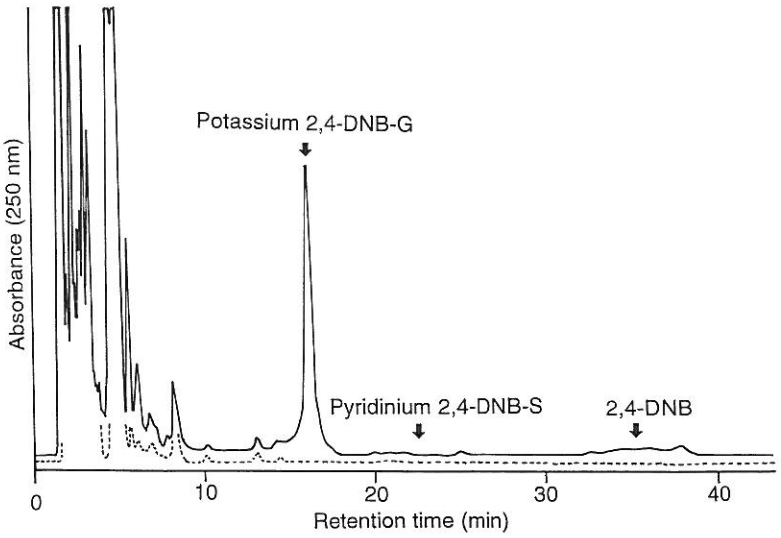

Figure 1. High-performance liquid chromatogram of urine from rat dosed with 2,4-dinitrotoluene (2,4-DNT). Arrows show the retention times of the authentic compounds. Potassium 2,4-DNB-G, potassium 2,4-dinitrobenzyl glucuronide; pyridinium 2,4-DNB-S, pyridinium 2,4-dinitrobenzyl sulphate; 2,4-DNB, 2,4-dinitrobenzyl alcohol. Hplc operating conditions were as follows: reversed-phase column, TSK gel ODS-80TM; mobile phase A, 10 mM potassium phosphate buffer (pH 3)-acetonitrile (85:15 v/v); flow rate, 1 ml/min; column temperature, ambient. ——, Sample; - - - - -, blank.

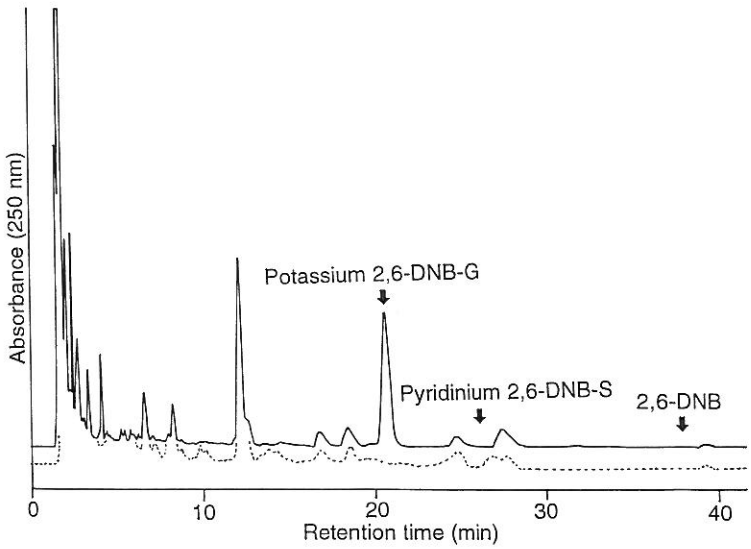

Figure 2. High-performance liquid chromatogram of urine from rat dosed with 2,6-dinitrotoluene (2,6-DNT). Arrows show the retention times of the authentic compounds. Potassium 2,6-DNB-G, potassium 2,6-dinitrobenzyl glucuronide; pyridinium 2,4-DNB-S, pyridinium 2,4-dinitrobenzyl sulphate; 2,6-DNB, 2,6-dinitrobenzyl alcohol. Hplc operating conditions were as follows: reversed-phase column, TSK gel ODS-80TM; mobile phase, E, 10 mM potassium phosphate buffer (pH 3)-acetonitrile (90:10 v/v); flow rate, 1 ml/min; column temperature, ambient. ——, Sample; - - - - -, blank.

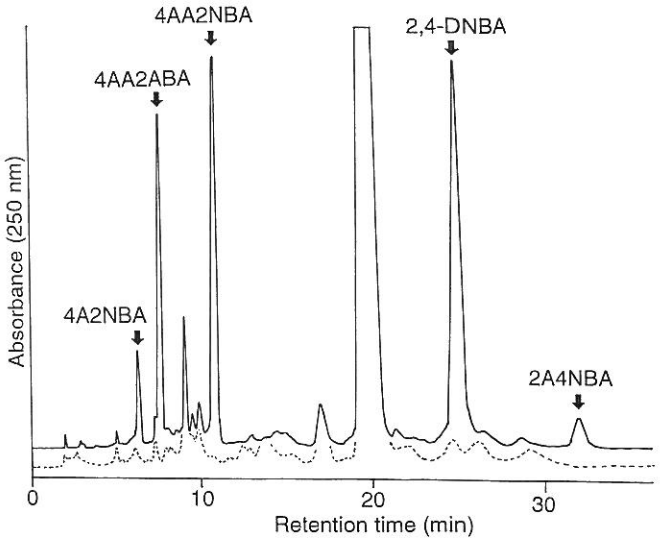

Figure 3. High-performance liquid chromatogram of ether extract from acidic fraction of urine after administration of 2,4-dinitrotoluene (2,4-DNT) to rat. Arrows show the retention times of the authentic compounds. 2,4-DNBA, 2,4-dinitrobenzoic acid; 2A4NBA, 2-amino-4-nitrobenzoic acid; 4A2NBA, 4-amino-2-nitrobenzoic acid; 4AA2NBA, 4-acetylamino-2-nitrobenzoic acid; 4AA2ABA, 4-acetylamino-2-aminobenzoic acid;. Hplc operating conditions were as follows: reversed-phase column, TSK gel ODS-80TM; mobile phase D, 0·1% tetrabutylammonium bromide in water–methanol (70:30 v/v); flow rate, 1 ml/min; column temperature, ambient. ——, Sample; - - - - -, blank.

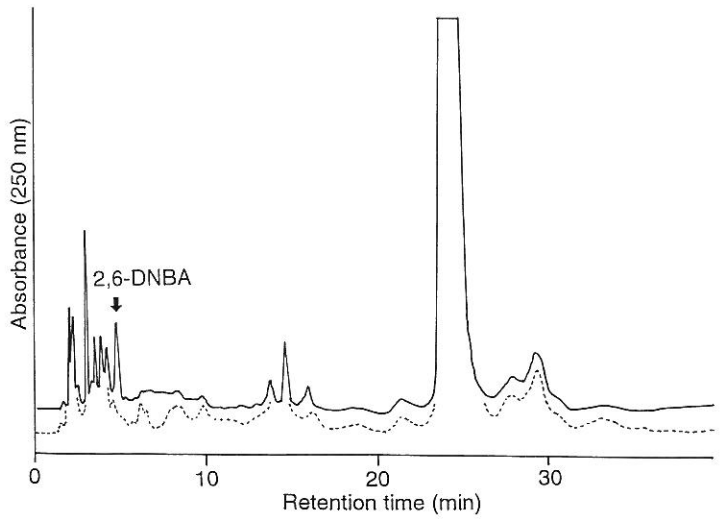

Figure 4. High-performance liquid chromatogram of ether extract from acidic fraction of urine after administration of 2,6-dinitrotoluene (2,6-DNT) to rat. Arrow shows the retention time of the authentic compound. 2,6-DNBA, (2,6-dinitrobenzoic acid). Hplc operating conditions were as follows: reversed-phase column, TSK gel ODS-80TM; mobile phase F, 10 mM potassium phosphate buffer (pH 3)-acetonitrile (95:5 v/v); flow rate, 1 ml/min; column temperature, ambient. ——, Sample; - - - - -, blank.

Table 4. Amounts of urinary metabolites of 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) in the male Wistar rat.

Metabolite	Percentage of the dose excreted in 24 h
2,4-DNT	
4-Amino-2-nitrotoluene	0·04 ± 0·01
2,4-DNB	0·25 ± 0·16
2,4-Dinitrobenzoic acid	6·90 ± 0·60
2-Amino-4-nitrobenzoic acid	0·71 ± 0·05
4-Amino-2-nitrobenzoic acid	0·52 ± 0·11
4-Acetylamino-2-nitrobenzoic acid	3·90 ± 1·00
4-Acetylamino-2-aminobenzoic acid	3·40 ± 0·49
2,4-DNB-G	10·70 ± 1·00
2,6-DNT	
2,6-DNT	0·09 ± 0·05
2-Amino-6-nitrotoluene	0·44 ± 0·26
2,6-DNB	0·53 ± 0·51
2,6-Dinitrobenzoic acid	0·17 ± 0·10
2,6-DNB-G	17·40 ± 4·60

Values are means ± SD for six rats.

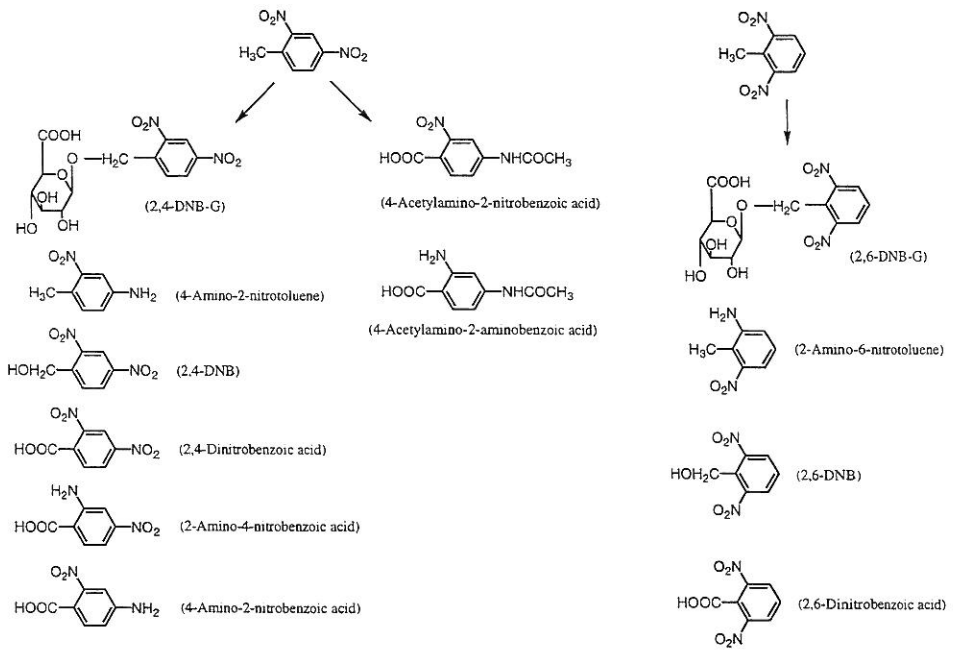

Figure 5. Structure of metabolites of 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) in urine after administration to the male Wistar rat.

Table 4 shows the amounts of urinary metabolites detected in the urine of the male Wistar rat given 2,4-DNT and 2,6-DNT. The structure of metabolites determined is shown in figure 5. From the results in table 4 it was found that the major urinary metabolites of 2,4-DNT and 2,6-DNT were 2,4-DNB-G and 2,6-DNB-G, accounting for about 10·7 and 17·4% of the dose respectively. Three

new metabolites, 4-acetylamino-2-nitrobenzoic acid, 2-amino-4-nitrobenzoic acid and 4-amino-2-nitrobenzoic acid accounted for about 3·9, 0·71 and 0·52% of the dose respectively. The amounts of other metabolites, 2,4-dinitrobenzoic acid (6·9%), 4-acetylamino-2-aminobenzoic acid (3·4%), 2,4-DNB (0·25%) and 4-amino-2-nitrotoluene (0·04%) corresponded well to the amounts (0·03 − 6·0%) of those in our previous study (Shoji *et al.* 1985). The amounts of 2,6-DNB (0·53%), 2-amino-6-nitrotoluene (0·44%) and 2,6-DNT (0·09%) were also approximately equal to the amounts (0·01–0·25%) of those in our previous study (Mori *et al.* 1989).

Discussion

The present results provided unequivocal evidence in support of the previous enzymic observations that the urinary conjugates of 2,4-DNB and 2,6-DNB are 2,4-DNB-G and 2,6-DNB-G respectively (Shoji *et al.* 1985, Mori *et al.* 1989). Namely, the conjugate of 2,4-DNB detected directly, without enzymic hydrolysis, from the urine following administration of 2,4-DNT was 2,4-DNB-G itself (figure 1). Similarly, the only conjugate of 2,6-DNB confirmed was 2,6-DNB-G (figure 2). It was found from the direct determination of these glucuronides that the major urinary metabolites of 2,4-DNT and 2,6-DNT are 2,4-DNB-G and 2,6-DNB-G, corresponding to about 10·7 and 17·4% of the dose respectively (table 4).

In our previous study, the amounts of 2,4-DNB-G and 2,6-DNB-G, which were quantified from 2,4-DNB and 2,6-DNB liberated by the hydrolysis with β-glucuronidase (10 000 units, 24 h), were about 3·2 and 1·5% of the dose respectively (Shoji *et al.* 1985, Mori *et al.* 1989). The lower percentages in our previous study may be due to uncomplete enzymic hydrolysis of these glucuronides. The urinary excretion (0·2%) of 2,6-dinitrobenzoic acid, which was not seen in the previous study (Mori *et al.* 1989), was confirmed in this study (figure 4). That no 2,6-dinitrobenzoic acid was detected was considered to be due to the poor separation of peaks of 2,6-dinitrobenzoic acid and of components in blank urine.

4-Amino-2-nitrotoluene (0·04%) and its oxidized and *N*-acetylated derivatives, 4-amino-2-nitrobenzoic acid (0·52%), 4-acetylamino-2-nitrobenzoic acid (3·9%) and 4-acetylamino-2-aminobenzoic acid (3·4%) were detected in the urine after administration of 2,4-DNT, but the metabolite detected as the derivative of 2-amino-4-nitrotoluene was 2-amino-4-nitrobenzoic acid (0·71%) only (table 4). This difference was thought to be based on the intestinal metabolism of 2,4-DNT since the rate of formation of 4-amino-2-nitrotoluene was much greater than that of 2-amino-4-nitrotoluene in incubations of 2,4-DNT with intestinal contents of rat (Guest *et al.* 1982, Mori *et al.* 1985).

The urinary excretion of 2-amino-4-nitrobenzoic acid (0·71%), 4-amino-2-nitrobenzoic acid (0·52%) and 4-acetylamino-2-nitrobenzoic acid (3·9%), in addition to known 4-acetylamino-2-aminobenzoic acid (3·4%), was confirmed in the administration of 2,4-DNT, whereas the urinary excretion of oxidized and *N*-acetylated derivatives of 2-amino-6-nitrotoluene was not observed after the administration of 2,6-DNT (table 4). This difference highlights the metabolic differences between 2,4-DNT and 2,6-DNT in the male Wistar rat.

Rickert and Long (1981) have shown that the urinary metabolites in the male Fischer-344 rat dosed orally with 2,4-DNT are conjugates of 2,4-DNB (11·4%), 2,4-dinitrobenzoic acid (21·3%), 2-amino-4-nitrobenzoic acid (2·2%) and 4-acetyl-

amino-2-nitrobenzoic acid (10·6%) respectively. They have also shown that the urinary metabolites of 2,6-DNT are conjugates of 2,6-DNB (21·7%), 2,6-dinitrobenzoic acid (21·1%) and 2-amino-6-nitrobenzoic acid (14·0%) (Long and Rickert 1982). The difference between the Wistar and Fischer-344 rat in the urinary metabolites of 2,4-DNT and 2,6-DNT is that no 4-acetylamino-2-aminobenzoic acid and 4-amino-2-nitrobenzoic acid were found in the urine of the Fischer-344 rat, no 2-amino-6-nitrobenzoic acid was found in the urine of the Wistar rat, and the excretion of 2,6-dinitrobenzoic acid was much greater in the Fischer-344 (21·1%) than in the Wistar rat (0·17%). These findings indicate that the metabolism of 2,4-DNT and 2,6-DNT differs between the two strains of rat.

In conclusion, our results substantiate previous indications that 2,4-DNB-G and 2,6-DNB-G are the major urinary metabolites of 2,4-DNT and 2,6-DNT, and there are metabolic differences between 2,4-DNT and 2,6-DNT in the male Wistar rat. This conclusion is based on the direct determination of 2,4-DNB-G and 2,6-DNB-G, and on the characterization of unknown metabolites.

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