Secondary Metabolism of Dinitrobenzyl Glucuronide Related to Production of Genotoxic Compounds of Dinitrotoluene in Male Wistar Rat

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Urinary and biliary metabolites of male Wistar rats dosed orally with 2,4-dinitrobenzyl glucuronide (2,4-DNB-G) and 2,6-dinitrobenzyl glucuronide (2,6-DNB-G) which are major compounds excreted in bile after administration of carcinogenic 2,4-dinitrotoluene (2,4-DNT) and 2,6-dinitrotoluene (2,6-DNT) were examined by HPLC. The object of this study is to determine whether mutagenic 2,4-dinitrobenzaldehyde (2,4-DNBAl) and genotoxic 2-amino-6-nitrobenzyl alcohol (2A6NB) are produced in the secondary metabolism of 2,4-DNB-G and 2,6-DNB-G. Data from HPLC indicated that 2,4-DNBAl (about 1%), in addition to 2,4-DNB-G (about 8.6%), 2,4-dinitrobenzyl alcohol (2,4-DNB, about 0.1%), two aminonitrotoluenes (about 0.2%), two aminonitrobenzyl alcohols (about 0.1%), 4-acetylamino-2-nitrobenzoic acid (4AA2NBA, about 7.4%) and 4-acetylamino-2-aminobenzoic acid (4AA2ABA, about 1.8%) was excreted in the urine or bile after dosing 2,4-DNB-G. This result, together with previous findings, indicates that 2,4-DNBAl is produced not only by oxidation of 2,4-DNB formed from 2,4-DNT, but by oxidation of 2,4-DNB formed from 2,4-DNB-G excreted in bile. In addition, the formation of carcinogenic 2,4-diaminotoluene (2,4-DAT) was ascertained from the metabolic pathway of 2,4-DNB-G based on the metabolites detected. No 2A6NB was found in the urine and bile after dosing 2,6-DNB-G. However, 2-amino-6-nitrobenzoic acid (2A6NBA, about 0.2%), in addition to 2,6-dinitrobenzyl alcohol (2,6-DNB, < 0.1%) and 2,6-DNB-G (about 18%), was detected in the urine or bile after dosing 2,6-DNB-G. This result, together with previous findings, indicates that 2A6NB is an intermediate in the production of 2A6NBA from 2,6-DNB, and further suggests that the production of 2A6NB in the metabolism of 2,6-DNT is coupled to the enterohepatic circulation of 2,6-DNB. The results of this investigation suggest that the production of 2,4-DNBAl and 2,4-DAT, and 2A6NB from 2,4-DNB-G and 2,6-DNB-G may play a role in the hepatocarcinogenicities of 2,4-DNT and 2,6-DNT.

Key words —— 2,4-dinitrobenzyl glucuronide, 2,6-dinitrobenzyl glucuronide, secondary metabolism, 2,4-dinitrobenzaldehyde, mutagenic metabolite

INTRODUCTION

Carcinogenic 2,4-dinitrotoluene (2,4-DNT)[1] and 2,6-dinitrotoluene (2,6-DNT),[2] which are major constituents of technical grade DNT used in the manufacture of explosives and toluene diisocyanate, an intermediate in the production of polyurethane foams,[3] have been shown to be excreted mainly in the bile as 2,4-dinitrobenzyl glucuronide (2,4-DNB-G) and 2,6-dinitrobenzyl glucuronide (2,6-DNB-G) in bile duct-cannulated male Wistar rat.[4] Studies with isolated perfused Fischer 344 rat livers have also shown that the major compounds excreted in bile after dosing of 2,4-DNT and 2,6-DNT are the glucuronides of 2,4-dinitrobenzyl alcohol (2,4-DNB) and 2,6-dinitrobenzyl alcohol (2,6-DNB) respectively from the observation that 2,4-DNB and 2,6-DNB are liberated by incubation of bile with β-glucuronidase.[3,5]

The active metabolite responsible for the carcinogenicity of 2,4-DNT has not yet been reported. 2,4-Dinitrobenzaldehyde (2,4-DNBAl), which is mutagenic in the Ames assay using Salmonella typhimurium strains TA 98 and TA100,[6] and also induces the malignant morphological transformation of C3H/10T 1/2 clone 8 cells[7] has been shown to be a minor biliary metabolite of 2,4-DNT in the bile duct-cannulated Wistar rat.[4,8] This observation in-

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dicates that 2,4-DNBAl is produced by oxidation of 2,4-DNB formed from 2,4-DNT in liver. While, it is reported that 2-amino-6-nitrobenzyl alcohol (2A6NB) is a presumable precursor related to the hepatocarcinogenicity of 2,6-DNT in the male Fischer 344 rat.[9] No 2A6NB has been found in the bile[3] or urine[5] of the rat after dosing 2,6-DNT.

In vitro study of intestinal content of male Wistar rats has shown that 2,4-DNB-G and 2,6-DNB-G are transformed by anaerobic incubation into 2,4-DNB, 2-amino-4-nitrobenzyl alcohol (2A4NB), 4-amino-2-nitrobenzyl alcohol (4A2NB) and 2,6-DNB, 2A6NB, respectively.[4] This observation implies that 2,4-DNB and 2,6-DNB formed in intestine are transported to liver and metabolized to mutagenic 2,4-DNBAl and genotoxic 2A6NB.

Thus, for a better understanding of the carcinogenicities of 2,4-DNT and 2,6-DNT, it is important to determine whether 2,4-DNBAl and 2A6NB are produced in the subsequent metabolism of 2,4-DNB-G and 2,6-DNB-G excreted in bile. For this purpose, we have examined the urinary and biliary metabolites of male bile duct-cannulated Wistar rats dosed orally with 2,4-DNB-G and 2,6-DNB-G by HPLC.

MATERIALS AND METHODS

Chemicals —— 2,4-DNT, 2,6-DNT, 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), 2-amino-6-nitrotoluene, 2,4-diaminotoluene (2,4-DAT), 2,6-diaminotoluene, 2,4-dinitrobenzoic acid and 4-nitroanthranilic acid (2-amino-4-nitrobenzoic acid) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and purified by recrystallization. 2,4-DNBAl and 2,6-dinitrobenzaldehyde (2,6-DNBAl) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, U.S.A.) and purified by recrystallization. All other chemicals or solvents used were of analytical grade.

2,4-DNB, 2,6-DNB, potassium 2,4-DNB-G, potassium 2,6-DNB-G, 2A4NB, 4A2NB, 2A6NB, 2,6-dinitrobenzoic acid, 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 (2A6NBA), 2,4-diaminobenzoic acid, 2,6-diaminobenzoic acid, 2-acetylamino-4-nitrobenzoic acid, 4-acetylamino-2-nitrobenzoic acid (4AA2NBA), 2-acetylamino-6-nitrobenzoic acid, 2-acetylamino-4-aminobenzoic acid, 4-acetylamino-2-aminobenzoic acid (4AA2ABA), 2-acetylamino-6-aminobenzoic acid, 2,4-diacetylaminobenzoic acid, 2,6-diacetylaminobenzoic acid and 2,4-DNBAl-phenylhydrazone were prepared as described previously.[10–12]

Administration of 2,4-DNB-G and 2,6-DNB-G —— Male eight-week old Wistar rats (weighing 200–220 g, Sankyo Laboratories Co.) were dosed orally with a solution of potassium 2,4-DNB-G and potassium 2,6-DNB-G (90 mg/kg each) in 0.9% NaCl (1 ml), and housed individually in metabolic cages with free access to water and commercial rat diet (F-2, Sankyo Laboratories Co.). Five rats per xenobiotic were dosed for each administration. Urine samples were collected over 24 h and stored at – 30°C. Bile duct-cannulated rats were prepared by the method of Abou-el Makarem *et al*.[13] using polyethylene tubing (SP 10, 0.21 mm int. diameter, 0.6 mm overall diameter). The cannulated rats were dosed orally with a solution of 2,4-DNB-G or 2,6-DNB-G (90 mg/kg each) in 0.9% NaCl (1 ml), kept individually in Bollman cages and allowed a commercial rat diet (F-2) and water *ad libitum*. Five rats per xenobiotic were dosed for each administration. Bile samples were collected over 24 h, and stored at – 30°C.

Analysis of Urinary and Biliary Conjugates by HPLC —— The stored 24-h urine (5 ml) and bile samples (5 ml) were filtered using a 0.45 μm membrane filter. Aliquots (10 μl) of the filtrates were injected into a high-performance liquid chromatograph equipped with a multi-wavelength UV monitor (Hewlett Packard HP 1100). A reversed-phase column packed with TSK gel ODS-80 TM (4.6 mm × 150 mm, particle size 5 μm; Toso Co., Tokyo, Japan) was used with mobile phases A or E (Tables 1 and 2). HPLC operating conditions were as follows: flow rate, 1 ml/min; UV monitor, 250 nm; column temperature, 25°C. Detection of conjugates was carried out by the co-chromatography of samples and blanks with authentic compounds. The limit of detection for 2,4-DNB-G and 2,6-DNB-G was 0.2 μg/ml. Quantities of conjugates were determined from standard curves plotted as peak areas calculated automatically by HP 1100 Chemstation software. A linear relationship between the amount of each compound and peak area was found over the range 0.4–400 μg/ml.

Table 1. 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	8.4		
2-Acetylamino-4-aminotoluene	2.2	9.5		
2-Amino-4-nitrobenzyl alcohol (2A4NB)	12.4	21.0		
4-Amino-2-nitrobenzyl alcohol (4A2NB)	6.3	11.0		
4-Acetylamino-2-aminotoluene	3.3	18.3		
2,4-Diacetylaminotoluene	6.3	20.0		
2-Acetylamino-4-nitrotoluene	24.6		5.2	
2,4-Dinitrobenzyl alcohol (2,4-DNB)	30.0		6.8	
4-Acetylamino-2-nitrotoluene	61.2		7.5	
2,4-Dinitrobenzaldehyde (2,4-DNBAl)			8.6	
4-Amino-2-nitrotoluene (4A2NT)			9.4	
2-Amino-4-nitrotoluene (2A4NT)			10.2	
2,4-Dinitrotoluene (2,4-DNT)			19.3	
2,4-Diaminobenzoic acid	3.5			6.0
4-Amino-2-nitrobenzoic acid	8.0			8.1
4-Acetylamino-2-aminobenzoic acid (4AA2ABA)	6.3			11.8
2-Acetylamino-4-aminobenzoic acid	8.9			16.1
4-Acetylamino-2-nitrobenzoic acid (4AA2NBA)	5.9			17.2
2,4-Diacetylaminobenzoic acid	11.4			45.8
2,4-Dinitrobenzoic acid	5.5			36.7
2-Amino-4-nitrobenzoic acid	42.6			53.3
2-Acetylamino-4-nitrobenzoic acid	27.9			
Potassium 2,4-dinitrobenzyl glucuronide (Potassium 2,4-DNB-G)	13.2			

a) A, 10 mM potassium phosphate buffer (pH 3)–acetonitrile (85 : 15); B, 4 mM sodium phosphate buffer (pH 7.4)–methanol (85 : 15); C, water–acetonitrile (65 : 35); D, 0.1% tetrabutylammonium bromide in water–methanol (80 : 20).

Table 2. Retention Times of 2,6-Dinitrotoluene (2,6-DNT) and Its Derivatives on HPLC

Compound	Retention time (min), mobile phase[a)]			
	E	B	F	G
2,6-Diaminotoluene	2.0	4.7		
2-Acetylamino-6-aminotoluene	2.5	5.6		
2,6-Diacetylaminotoluene	5.0	7.1		
2-Amino-6-nitrobenzyl alcohol (2A6NB)	16.6	16.9	4.4	
2-Acetylamino-6-nitrotoluene	31.0		5.7	
2,6-Dinitrobenzyl alcohol (2,6-DNB)	26.0		7.0	
2-Amino-6-nitrotoluene			12.3	
2,6-Dinitrobenzaldehyde (2,6-DNBAl)			12.9	
2,6-Dinitrotoluene (2,6-DNT)			30.0	
2,6-Diaminobenzoic acid	2.0			3.2
2,6-Dinitrobenzoic acid	3.0			3.9
2-Acetylamino-6-nitrobenzoic acid	4.8			8.0
2-Acetylamino-6-aminobenzoic acid	5.2			9.9
2-Amino-6-nitrobenzoic acid (2A6NBA)	11.5			13.9
2,6-Diacetylaminobenzoic acid	8.0			22.0
Potassium 2,6-dinitrobenzyl glucuronide (Potassium 2,6-DNB-G)	14.0			

a) E, 10 mM potassium phosphate buffer (pH 3)–acetonitrile (90 : 10); F, water–acetonitrile (70 : 30); G, 10 mM potassium phosphate buffer (pH 3)–acetonitrile (95 : 5). B is the same as in Table 1.

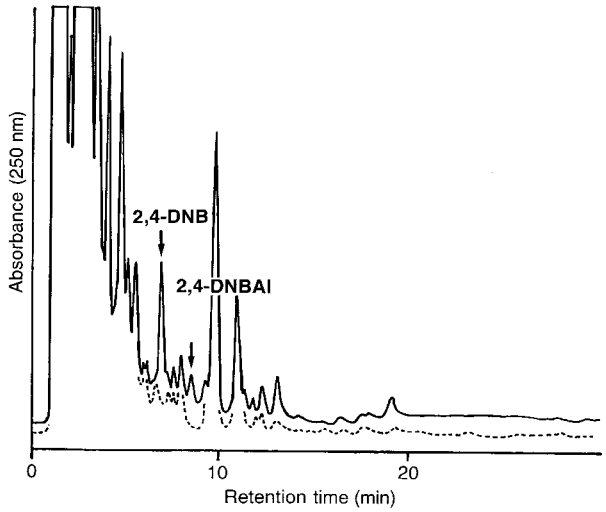

Fig. 1. High-Performance Liquid Chromatogram of Neutral-Basic Fraction from Urine of Rats Dosed with Potassium 2,4-Dinitrobenzyl Glucuronide (Potassium 2,4-DNB-G)
——, sample; - - - - - -, blank. Arrows show the retention times of authentic compounds.

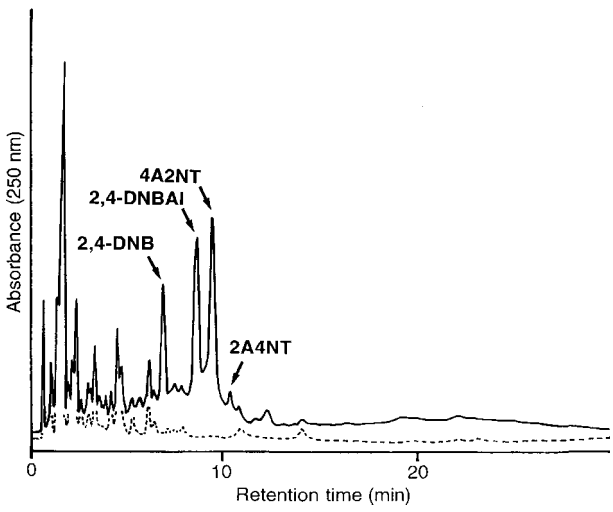

Fig. 2. High-Performance Liquid Chromatogram of Neutral-Basic Fraction from Bile of Rats Dosed with Potassium 2,4-Dinitrobenzyl Glucuronide (Potassium 2,4-DNB-G)
——, sample; - - - - - -, blank. Arrows show the retention times of authentic compounds.

Analysis of Urinary and Biliary Unconjugated Metabolites by HPLC —— The stored 24-h urine and bile samples (5 ml) were adjusted to pH 11 with 2 M Na_2CO_3 and extracted three times with diethylether (30 ml) (neutral-basic fractions). The extracted urine and bile samples were adjusted to pH 2 with 2 M HCl and extracted three times with diethylether (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 nitrogen, and the residue was dissolved in 10 ml of methanol–water (30 : 70). Aliquots (5–50 μl) of these solutions were injected into an HPLC. The HPLC conditions were the same as those described above except for mobile phases of B, C, D, F and G (Tables 1 and 2). The detection of metabolites was carried out by comparing the chromatograms of samples from treated rats with those of blank samples, and by the co-chromatography of samples with authentic compounds. The detection limit of each authentic compound was 0.04 μg/ml. The range of linearity of each authentic compound was 0.04–200 μg/ml.

RESULTS

Tables 1 and 2 show the retention times of 2,4-DNB-G and 2,6-DNB-G, and their possible metabolites in HPLC using various mobile phases. Neutral-basic compounds, acidic compounds and conjugated compounds were separated in the mobile phases of B, C or F; D or G; A or E, respectively.

Figure 1 shows a representative high-performance liquid chromatogram of the neutral-basic fraction from urine of rats dosed orally with 2,4-DNB-G. Peaks with retention times of 6.8 and 8.6 min, which co-eluted with authentic 2,4-DNB and 2,4-DNBAl, were detected in the neutral-basic fraction by HPLC using mobile phase of C. The excretion of 2,4-DNBAl was also confirmed chromatographically by detecting the peak which co-elutes with authentic 2,4-DNBAl-phenylhydrazone from the neutral-basic fraction treated with phenylhydrazine. Two aminonitrobenzyl alcohols (2A4NB and 4A2NB) were detected in the neutral-basic fraction by using mobile phase of B. In addition, 4AA2NBA and 4AA2ABA were detected in the acidic fraction and 2,4-DNB-G was detected in the filtered urine, respectively. While, 2,6-DNB-G (filtered urine), 2,6-DNB (neutral-basic fraction) and 2A6NBA (acidic fraction) were detected in the urine after dosing 2,6-DNB-G.

As shown in Fig. 2, 2,4-DNB, 2,4-DNBAl, 2A4NT and 4A2NT were detected in the neutral-basic fraction of bile following administration of 2,4-DNB-G. The excretion of 2,4-DNBAl was also confirmed by HPLC using authentic 2,4-DNBAl-phenylhydrazone and neutral-basic fraction treated with phenylhydrazine, as described above. Both 2A4NB and 4A2NB were also detected in the neutral-basic fraction by using mobile phase B. In addi-

Table 3. Amounts of Compounds Detected from Urine and Bile of Male Wistar Rats Dosed with 2,4-Dinitrobenzyl Glucuronide (2,4-DNB-G) and 2,6-Dinitrobenzyl Glucuronide (2,6-DNB-G)

Compound	Percentage of the dose excreted in 24 h	
	Urine	Bile
2,4-DNB-G		
2,4-Dinitrobenzyl alcohol (2,4-DNB)	0.02 ± 0.01[a)]	0.10 ± 0.05
2,4-Dinitrobenzaldehyde (2,4-DNBAl)	0.05 ± 0.02	1.02 ± 0.42
4-Amino-2-nitrotoluene (4A2NT)		0.19 ± 0.05
2-Amino-4-nitrotoluene (2A4NT)		0.03 ± 0.01
4-Acetylamino-2-nitrobenzoic acid (4AA2NBA)	7.05 ± 3.60	0.39 ± 0.15
4-Acetylamino-2-aminobenzoic acid (4AA2ABA)	1.78 ± 0.62	
4-Amino-2-nitrobenzyl alcohol (4A2NB)	0.04 ± 0.02	0.01 ± 0.01
2-Amino-4-nitrobenzyl alcohol (2A4NB)	0.03 ± 0.02	0.01 ± 0.01
2,4-DNB-G	3.44 ± 1.35	5.16 ± 1.76
2,6-DNB-G		
2,6-Dinitrobenzyl alcohol (2,6-DNB)	0.01 ± 0.01	0.02 ± 0.02
2-Amino-6-nitrobenzoic acid (2A6NBA)	0.21 ± 0.19	
2,6-DNB-G	2.21 ± 0.54	15.78 ± 5.74

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

tion, 2,4-DNB-G and 4AA2NBA were detected in the filtered bile and in the acidic fraction, respectively. Compounds detected in bile after dosing 2,6-DNB-G were 2,6-DNB (neutral-basic fraction) and 2,6-DNB-G (filtered bile). The amounts of urinary and biliary compounds detected are shown in Table 3. The proposed metabolic pathways of 2,4-DNB-G and 2,6-DNB-G which are based on the results of Table 3 and the previous findings from in vitro study with rat intestinal content[4)] are shown in Figs. 3 and 4, respectively.

It has been demonstrated that various xenobiotics, including aminocephalosporin antibiotics[14,15)] and aminobenzoic acids,[16)] are absorbed from the rat intestinal lumen by a carrier-mediated transport system. Thus, although it remains to be determined whether 2,4-DNB-G and 2,6-DNB-G administered are absorbed from the intestine, it is possible to presume that a part of 2,4-DNB-G and 2,6-DNB-G excreted in bile and urine is derived from parent glucuronides. As shown in Table 3, nine compounds, including 2,4-DNBAl and 2,4-DNB-G, were detected in the administration of 2,4-DNB-G, whereas compounds detected in the administration of 2,6-DNB-G were 2,6-DNB, 2A6NBA and 2,6-DNB-G. The finding that 2,4-DNBAl is one of the metabolites of 2,4-DNB-G indicates that 2,4-DNBAl is produced by oxidation of 2,4-DNB formed in the intestinal hydrolysis of 2,4-DNB-G (Fig. 3). The metabolic pathway shown in Fig. 3 also suggests that 2,4-DAT which is a known hepatocarcinogen[17,18)] and is also a mutagen[6,19)] is produced from 2A4NT and its isomer (4A2NT) in the metabolism of 2,4-DNB-G.

The excretion of 2A6NB itself was not seen in the administration of 2,6-DNB-G, but 2A6NBA was detected in the urine. This finding indicates that 2A6NB is a precursor of 2A6NBA and is produced from 2,6-DNB in intestine (Fig. 4). In addition, the finding that the biliary excretion of 2,6-DNB-G is about 15.8% of the dose suggests that enterohepatic circulation of 2,6-DNB occurs.

DISCUSSION

It has been shown that the biliary excretion of 2,4-DNBAl and 2,4-DNB-G in the administration of 2,4-DNT (40 mg/kg) are about 0.1 and 35% of the dose, respectively.[4)] When 2,4-DNB-G (90 mg/kg) which corresponds to about 2.8 fold of the biliary excretion (35%) was administered, the biliary excretion of 2,4-DNBAl is about 1.0% of the dose (Table 3). These findings indicate that the rate of 2,4-DNBAl excreted in bile after dosing 2,4-DNB-G is comparable to about four times of that of 2,4-DNBAl excreted in bile after dosing 2,4-DNT. In addition, the rate (about 1.0%) of biliary excretion of 2,4-DNBAl (Table 3) is approximately equal to that (about 1.1%) of biliary excretion of 2,4-DNBAl in the administration of 2,4-DNB (40 mg/kg).[20)] Therefore, it may be concluded that the production of 2,4-

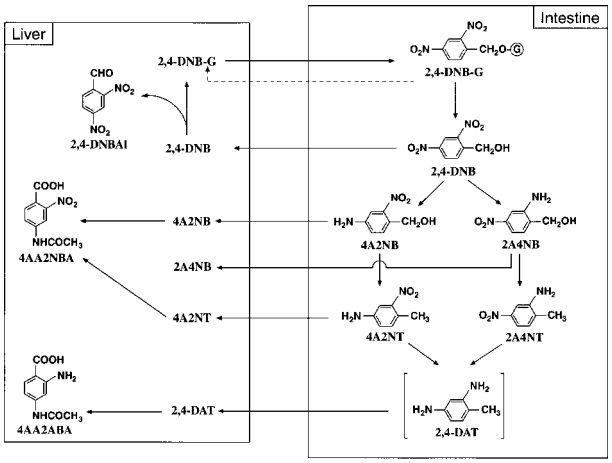

Fig. 3. Proposed Metabolic Pathway of 2,4-Dinitrobenzyl Glucuronide (2,4-DNB-G)

Arrow with broken line shows a possible absorption route of 2,4-DNB-G.

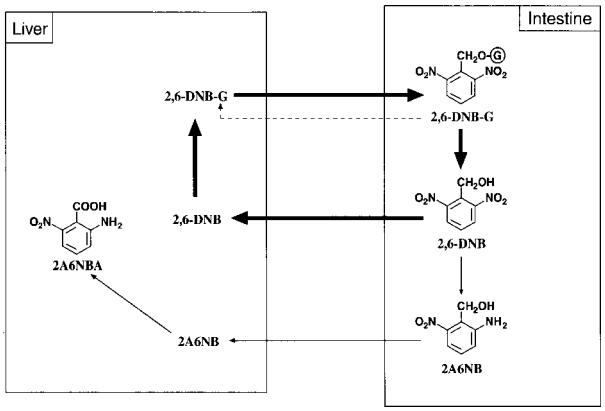

Fig. 4. Proposed Metabolic Pathway of 2,6-Dinitrobenzyl Glucuronide (2,6-DNB-G)

Arrow with broken line shows a possible absorption route of 2,6-DNB-G. Bolder arrows show enterohepatic circulation of 2,6-dinitrobenzyl alcohol (2,6-DNB).

DNBAl in the metabolism of 2,4-DNT depends on the secondary metabolism of 2,4-DNB-G rather than the metabolism of 2,4-DNT itself. The preponderance of secondary metabolism of 2,4-DNB-G in the production of 2,4-DNBAl is at least partly because the hepatic concentration of 2,4-DNB produced from 2,4-DNB-G is higher than that of 2,4-DNB produced from 2,4-DNT. Since it is thought that the lipophilicity of 2,4-DNT is greater than that of 2,4-DNB with a benzylalcoholic group, the higher concentration of 2,4-DNB in liver may be because the intestinal hydrolysis of 2,4-DNB-G surpasses the hepatic oxidation of 2,4-DNT with respect to the production of 2,4-DNB and/or the extent of intestinal reduction of 2,4-DNB is lower than that of intestinal reduction of 2,4-DNT. Moreover, the metabolic difference between hepatic oxidation of 2,4-DNB to 2,4-DNBAl in the co-existence of intestinal products from 2,4-DNB-G and hepatic oxidation of 2,4-DNT to 2,4-DNBAl in the co-existence of intestinal products from 2,4-DNT may also be responsible for the preponderance of secondary metabolism of 2,4-DNB-G in the production of 2,4-DNBAl.

The excretion of 2,4-DAT was not seen in the administration of 2,4-DNB-G; however, aminonitrotoluenes (2A4NT and 4A2NT) and 4AA2ABA were detected in the urine or bile (Table 3). This finding suggests that 2,4-DAT is produced in the intestinal metabolism of 2,4-DNB-G and is transported to liver (Fig. 3). The production of 2,4-DAT from 2A4NT and 4A2NT was supported from the observation that 2A4NT and 4A2NT are metabolized to 2,4-DAT by anaerobic incubation with rat intestinal contents[20] and with rat cecal contents.[21] Since *p*-hydroxybenzyl alcohol and vanillyl alcohol are metabolized to *p*-cresol and 4-methylguiacol by rat intestinal microflora, respectively,[22] the reduction of 2A4NB and 4A2NB to 2A4NT and 4A2NT was also thought to be a reasonable metabolic route. The excretion of 2,4-DAT is not seen in the single administration of 2,4-DNT (40 mg/kg),[4] whereas 2,4-DAT is detected in the urine of rats dosed with 2,4-DNT (25 mg/kg/d) for 6 d.[23] Thus, it is suggested that the intestinal metabolic route shown in Fig. 3, in addition to the intestinal reduction of 2,4-DNT, may also play a role in the production of 2,4-DAT after continuous dosing of 2,4-DNT.

Kedderis *et al*.[9] have proposed a hypothesis relating to the hepatocarcinogenicity of 2,6-DNT in male Fischer 344 rat. They have postulated that the bioactivation of 2,6-DNT requires intestinal deconjugation of glucuronide of 2,6-DNB (2,6-DNB-G) to 2,6-DNB, intestinal reduction of 2,6-DNB to aminonitrobenzyl alcohol (2A6NB), transportation of 2A6NB to liver, and further metabolism of 2A6NB to genotoxic compounds, including hydroxylamino derivative of 2A6NB and its sulfate. The finding that 2A6NBA which is a urinary metabolite of 2,6-DNB-G (Table 3) is produced from 2A6NB in the intestinal metabolism of 2,6-DNB-G (Fig. 4) provides evidence in support of their hypothesis that 2,6-DNB-G excreted in bile is converted into 2A6NB in intestine and 2A6NB formed in intestine is transported to liver. In addition, the finding that the rates of biliary excretion of 2,6-DNB-G

in the administration of 2,6-DNT (40 mg/kg) and 2,6-DNB-G (90 mg/kg) was about 52[4] and 15.8% of the dose (Table 3) indicates that enterohepatic circulation of 2,6-DNB occurs in the metabolism of 2,6-DNT, and further suggests that transportation of 2A6NB to liver (Fig. 4) is continual. Since a number of hepatocarcinogenic aromatic amines have been shown to be metabolized to intermediates which bind covalently to hepatic DNA,[24,25] it is possible to assume that a part of 2A6NB which is transported to liver is converted into a hydroxylamino derivative of 2A6NB. The failure to detect the presumed hydroxylamino compound may be due to its lability.

In conclusion, our results provide substantial evidence that 2,4-DNBAl is produced by the secondary metabolism of 2,4-DNB-G formed in the metabolism of 2,4-DNT and that the production of 2,4-DNBAl depends more on the secondary metabolism of 2,4-DNB-G than the metabolism of 2,4-DNT itself. We also showed that 2,4-DAT and 2A6NB are produced in the secondary metabolism of 2,4-DNB-G and 2,6-DNB-G, and are transported to liver. These results provide important information for understanding the metabolic activation responsible for the hepatocarcinogenicities of 2,4-DNT and 2,6-DNT in rats.

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REFERENCES

1) Ellis H.V., Hagensen J.H., Hodgson J.R., Minor J.L., Hong C.B., Ellis E.R., Girvin J.D., Helton D.O., Herndon B.L., Lee C.C., Final Report no. 7, Project 3900-B, Midwest Research Institute, Kansas City, Mo., 1979.
2) Leonard T.B., Popp J.A., *Proc. Amer. Assoc. Cancer Res.*, **22**, 82 (1981).
3) Bond J.A., Medinsky M.A., Dent J.G., Rickert D.E., *J. Pharmacol. Exp. Ther.*, **219**, 598–603 (1981).
4) Mori M., Sayama M., Shoji M., Inoue M., Kawagoshi T., Maeda M., Honda T., *Xenobiotica*, **27**, 1225–1236 (1997).
5) Long R.M., Rickert D.E., *Drug Metab. Dispos.*, **10**, 455–458 (1982).
6) Mori M., Miyahara T., Moto-o K., Fukukawa M., Kozuka H., Miyagoshi M., Nagayama T., *Chem. Pharm. Bull.*, **33**, 4556–4563 (1985).
7) Mori M., Dohrin M., Kozuka H., Kaji T., Yamamoto T., Sakamoto T., Proceeding of The 114th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, March 1994, Vol. 3, p. 184.
8) Sayama M., Mori M., Ishida M., Okumura H., Kozuka H., *Xenobiotica*, **19**, 83–92 (1989).
9) Kedderis G.L., Dyroff M.C., Rickert D.E., *Carcinogenesis*, **5**, 1199–1204 (1984).
10) Galbershtam M.A., Budarina Z.N., *Zh. Org. Khim.*, **5**, 953–960 (1969).
11) Mori M., Inoue M., Nunozawa T., Miyahara T., Kozuka H., *Chem. Pharm. Bull.*, **34**, 4859–4861 (1986).
12) Mori M., Dohrin M., Sayama M., Shoji M., Inoue M., Kozuka H., *Chem. Pharm. Bull.*, **46**, 145–147 (1998).
13) Abou-el-Makarem M.M., Millburn P., Smith R.L., *Biochem. J.*, **105**, 1269–1273 (1967).
14) Nakashima E., Tsuji A., Mizuno H., Yamana T., *Biochem. Pharmacol.*, **33**, 3345–3352 (1984).
15) Okano T., Inui K., Takano M., Hori R., *Biochem. Pharmacol.*, **35**, 1781–1786 (1986).
16) Yamamoto A., Sakane T., Shibukawa M., Hashida M., Sezaki H., *J. Pharm. Sci.*, **80**, 1067–1071 (1991).
17) Ito N., Hiasa Y., Konishi Y., Marugami M., *Cancer Res.*, **29**, 1137–1145 (1969).
18) Cardy R.H., *J. Natl. Cancer Inst.*, **62**, 1107–1116 (1979).
19) Ames B.N., Kammen H.O., Yamasaki E., *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 2423–2427 (1975).
20) Mori M., Kudo Y., Nunozawa T., Miyahara T., Kozuka H., *Chem. Pharm. Bull.*, **33**, 327–332 (1985).
21) Guest D., Schnell S.R., Rickert D.E., *Toxicol. Appl. Pharmacol.*, **64**, 160–168 (1982).
22) Scheline R.R., *Pharmacol. Rev.*, **25**, 451–523 (1973).
23) Mori M., Naruse Y., Kozuka H., *Chem. Pharm. Bull.*, **29**, 1147–1150 (1981).
24) Kriek E., Westra J.G., "Chemical Carcinogenesis and DNA," Vol. 2, Grover P. (ed.), CRC Press, FL, pp. 1–28, 1979.
25) Hashimoto Y., Shuto K., Okamoto T., *Farumashia*, **17**, 326–330 (1981).