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Mutagenicity of Urinary Metabolites of 2,4-Dinitrotoluene to *Salmonella typhimurium*

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2,4-Dinitrotoluene (2,4-DNT) and its urinary metabolites [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), 2-amino-4-acetylaminobenzoic acid (2A4AABA) and 2,4-dinitrobenzoic acid (2,4-DNBA)] and 2,4-dinitrobenzaldehyde (2,4-DNAl), an intermediate in the oxidation of 2,4-DNB to 2,4-DNBA, were tested for mutagenicity in *Salmonella typhimurium* strains TA 98 and TA 100 in the absence or presence of S9 mix. 2,4-DNT itself was only a weak mutagen. 2A4NT, 4A2NT, 2N4AAT, 2A4AABA and 2,4-DNBA were inactive toward strains TA 98 and TA 100 in the absence or presence of S9 mix. In contrast with these metabolites, 2A4AAT, 2A4NB, 4A2NB, 2,4-DAT, 2,4-DNB and 2,4-DNAl were more mutagenic than 2,4-DNT, being increasingly mutagenic in that order. 2A4AAT, 2A4NB and 4A2NB were weak mutagens at mM concentrations, while 2,4-DAT, 2,4-DNB and 2,4-DNAl were mutagenic at μM concentrations. These results suggest that the metabolic conversion of 2,4-DNT to 2,4-DNB, 2,4-DNAl, and 2,4-DAT, a known carcinogen, may contribute to the carcinogenicity of 2,4-DNT.

Keywords——mutagenicity; *Salmonella typhimurium*; urinary metabolite; 2,4-dinitrotoluene; 2,4-dinitrobenzyl alcohol; 2,4-dinitrobenzaldehyde; 2,4-diaminotoluene

Although 2,4-dinitrotoluene (2,4-DNT), which is used in the production of polyurethane foams, coatings and elastomers, has been shown to produce hepatocellular carcinomas when administered in the diet of rats,[1] unequivocal mutagenic activity of this compound has not been detected in Ames' *Salmonella*/microsome system. For example, Couch *et al.*[2] reported that 2,4-DNT produced no significant increase in reversion to histidine protrophy in *S. typhimurium* strains TA 1535, TA 1537, TA 1538. TA 98 or TA 100, with or without the addition of S9 fraction prepared from rats treated with Aroclor 1254. Similarly, 2,4-DNT did not exhibit mutagenic activity in the Ames test in strains TA 98 and TA 100, using S9 fraction of Aroclor 1254-treated rat liver as an activation system.[3] Chiu *et al.*[4] also found that 2,4-DNT produced only small increases in the number of revertants of *S. typhimurium* TA 98 and TA 100. Taken together, these studies suggest that clarification of the metabolic routes of 2,4-DNT is important for the detection of the mutagenic species of 2,4-DNT.

We have reported that the urinary metabolites of 2,4-DNT are 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), 2,4-diaminotoluene (2,4-DAT), 2,4-dinitrobenzyl alcohol (2,4-DNB), 2,4-dinitrobenzoic acid (2,4-DNBA), 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).[5,6] These findings indicate that the primary metabolic reactions of 2,4-DNT in rats are the reduction of the nitro group to an amino group and the oxidation of the methyl group to CH_2OH, and that N-acetylation of the amino group and oxidation of CH_2OH to COOH occur as secondary reactions. Among these metabolic reactions, the secondary reactions appear not to proceed in a metabolic activating system consisting of S9 and a reduced nicotineamide adenine dinucleotide phosphate (NADPH)-generating system, which is usually employed for the Ames assay. Thus, it seemed relevant to examine the mutagenicity of the urinary metabolites, in order to improve our understanding of the carcinogenicity of 2,4-DNT.

In the present study, we have examined the mutagenic activity of 2,4-DNT and ten urinary metabolites described above, using the Ames assay. 2,4-Dinitrobenzaldehyde (2,4-DNA1), a possible intermediate in the oxidation of 2,4-DNB to 2,4-DNBA, was also assayed.

Experimental

Chemicals——2,4-DNT, 2A4NT, 4A2NT and 2,4-DNBA (>99% chromatographically pure after recrystallization from methanol), 4-nitroquinoline (4NQO, reagent grade), benzo[*a*]pyrene (BP, ultra grade) and dimethylsulfoxide (DMSO, ultra grade) were obtained from Wako Pure Chemical Industries Co. 2,4-DNA1 (98% pure) and 2-nitrofluorene (2NF, 98% pure) were obtained from Aldrich Chemical Co. 2A4NB, 4A2NB, 2A4AAT and 2A4AABA (>99% chromatographically pure) were synthesized as previously described.[5] Other chemicals were obtained from the following sources: phenobarbital-Na (PB) and 5,6-benzoflavone (BF) from Wako Pure Chemical Industries Co.; NADPH, reduced nicotineamide adenine dinucleotide (NADH) and glucose-6-phosphate (G-6-P) from Sigma Chemical Co., Bacto-agar and Bacto nutrient broth from Difco Laboratories.

Preparations of S9 Fraction and S9 Mix——Male Sprague-Dawley rats (180—200 g) obtained from Sankyo Laboratories Co., were pretreated with PB (80 mg/kg in 0.9% NaCl *i.p.* 96, 72, 48 and 24 h before death) and BF (80 mg/kg in corn oil *i.p.* 48 h before death). The S9 fractions and S9 mix were prepared according to the method of Ames *et al.*[7] Animals were killed by decapitation. The livers were removed, minced and homogenized in 3 volumes of ice-cold 1.15% KCl. The homogenates were centrifuged for 10 min at 9000 *g* and the supernatant (S9 fraction) was used for the preparation of S9 mix. The S9 mix contains, per ml: S9 (0.3 ml, 2 mg protein), $MgCl_2$ (8 μmol), KCl (33 μmol), G-6-P (5 μmol), NADPH (4 μmol), NADH (4 μmol) and sodium phosphate buffer, pH 7.4 (100 μmol). All manipulations were performed at 4 °C under sterile conditions.

Assay of Mutagenicity——*S. typhimurium* strains TA 98 and TA 100 were kindly provided by Prof. I. Tomita, Shizuoka College of Pharmacy, Shizuoka (Japan). Mutagenicity assay was carried out according to the method of Ames *et al.*[7] The strains were grown in nutrient broth and shaken for 15 h at 37°C. A 0.1 ml aliquot of the bacterial suspension (approximately 1×10^7 cells), 0.5 ml of phosphate buffer (pH 7.4) or 0.5 ml of S9 mix and 0.1 ml of the test compound in DMSO were mixed with 2 ml of molten top agar containing 50 μM histidine and 50 μM biotin by agitation and poured onto a minimum agar plate. Metabolic activations were carried out for 20 min at 37 °C before the addition of molten top agar. A control plate contained 0.1 ml of DMSO. Colonies of his^+ revertant were enumerated after incubation in the dark at 37 °C for 2 d, and compounds inducing more than twice the number of revertants of the control were considered as mutagenic. Each compound was tested at doses ranging between 0 and 2000 μg per plate. The mutagenicities of 2NF, 4NQO and BP were determined in each experiment to monitor the sensitivity of TA 98 and TA 100. The mutagenic activity is given as the number of revertants per plate ± standard deviation (S.D.) or per μmole of test compound determined from the linear part of the dose-response curve.

Results

It was found that 1.0 μg of 2NF and 0.1 μg of 4NQO induced 452 ± 16 (S.D.) and 748 ± 36 (S.D.) revertants/plate in TA 98 and TA 100 in the absence of S9 mix, while 3 μg of BP induced 326 ± 12 (S.D.) and 478 ± 21 (S.D.) revertants/plate in TA 98 and TA 100 in the presence of S9 mix.

Among the compounds tested at doses ranging between 0 and 2000 μg/plate, 2A4NT, 4A2NT, 2N4AAT, 2A4AABA and 2,4-DNBA showed almost no mutagenic activity toward strains TA 98 and TA 100 in the absence or presence of S9 mix (data not shown). The dose-response curves of seven compounds (2,4-DNT, 2,4-DAT, 2,4-DNB, 2A4NB, 4A2NB, 2A4AAT and 2,4-DNA1) are shown in Figs. 1 to 7. As shown in Fig. 1, the linear regions for

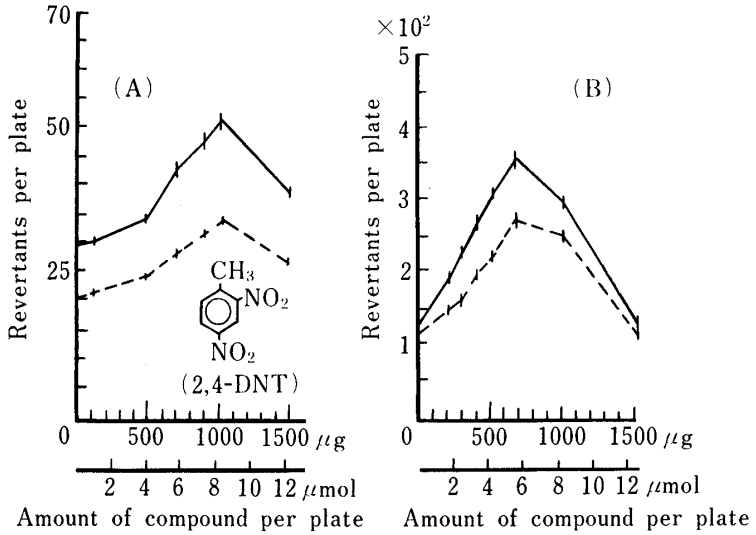

Fig. 1. Dose–Response Curves of 2,4-Dinitrotoluene (2,4-DNT) Tested with TA 98 (A) and TA 100 (B)

Each bar represents the average number of induced revertant colonies with the standard deviation on 3 to 5 plates from three separate experiments. ——, with S9 mix; ----, without S9 mix.

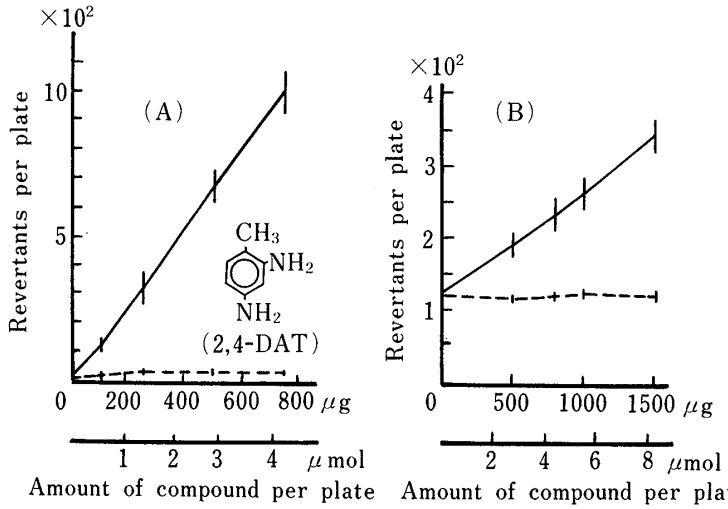

Fig. 2. Dose–Response Curves of 2,4-Diaminotoluene (2,4-DAT) Tested with TA 98 (A) and TA 100 (B)

Experimental conditions were as indicated in the legend to Fig. 1.

2,4-DNT occurred at doses of 500 to 1000 μg (without S9 mix) and 400 to 1000 μg (with S9 mix) for TA 98 (Fig. 1A). For TA 100 the linear regions were from 200 to 700 μg in the absence and presence of S9 mix (Fig. 1B). With 2,4-DNT, the addition of S9 mix tended to increase the revertant number/plate in both strains TA 98 and TA 100. 2,4-DNT itself, however, appeared to be only a weak mutagen, because the number of revertants/plate induced by this compound was less than 3 times that induced spontaneously in strains TA 98 and TA 100. This finding is in accord with the results of Chiu *et al.*[4] 2,4-DAT showed almost no mutagenic activity toward both tester strains in the absence of S9 mix, but with S9 mix this compound showed higher mutagenic activity toward strains TA 98 (Fig. 2A) and TA 100 (Fig. 2B). This finding indicates that the mutagenesis of 2,4-DAT is indirect, requiring metabolic activation. Similarly, the mutagenesis of 2A4AAT is indirect in TA 98, requiring metabolic activation, as shown in Fig. 3; however, this compound showed no mutagenic activity toward

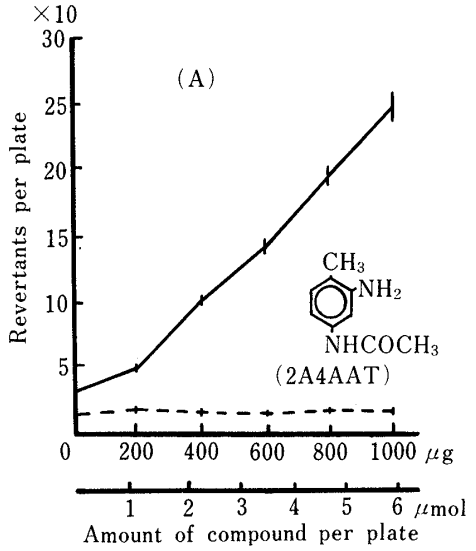

Fig. 3. Dose–Response Curve of 2-Amino-4-acetylaminotoluene (2A4AAT) Tested with TA 98

Experimental conditions were as indicated in the legend to Fig. 1.

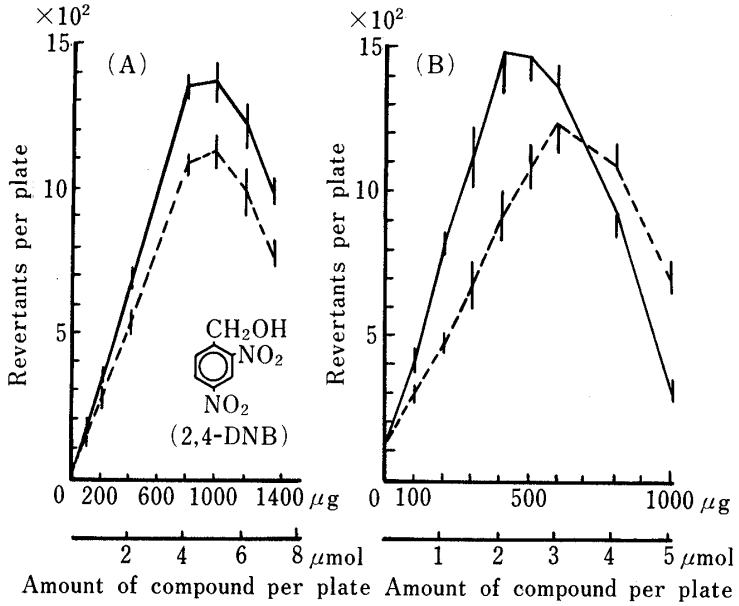

Fig. 4. Dose–Response Curves of 2,4-Dinitrobenzyl Alcohol (2,4-DNB) Tested with TA 98 (A) and TA 100 (B)

Experimental conditions were as indicated in the legend to Fig. 1.

TA 100 in the absence or presence of S9 mix (data not shown). 2,4-DNB showed a higher mutagenic activity toward strains TA 98 (Fig. 4A) and TA 100 (Fig. 4B), and the activity in the presence of S9 mix was slightly higher than that in the absence of S9 mix. 2A4NB showed a dose-dependent increase in the number of revertants with TA 98 (Fig. 5A) and TA 100 (Fig. 5B). 4A2NB also showed a dose-dependent increase in the number of revertants with TA 98 (Fig. 6A) and TA 100 (Fig. 6B). Among the compounds tested, 2,4-DNA1 showed the highest activity toward both strains TA 98 (Fig. 7A) and TA 100 (Fig. 7B). The number of revertants/plate reached about 2400/plate with TA 98 and about 2800/plate with TA 100 at a dose of 50 μg in the absence of S9 mix. With 2,4-DNA1 the addition of S9 mix tended to decrease the revertant number/plate in both strains TA 98 and TA 100.

Table I shows the mutagenic potencies of 2,4-DNT and its metabolites, expressed as revertants per μmol of test compound determined from the linear part of the dose–response curves (Figs. 1 to 7). The mutagenic potency of 2A4AAT in TA 98 was about 5.5 times that of 2,4-DNT in the presence of S9 mix. The mutagenic potencies of 2A4NB and 4A2NB in TA

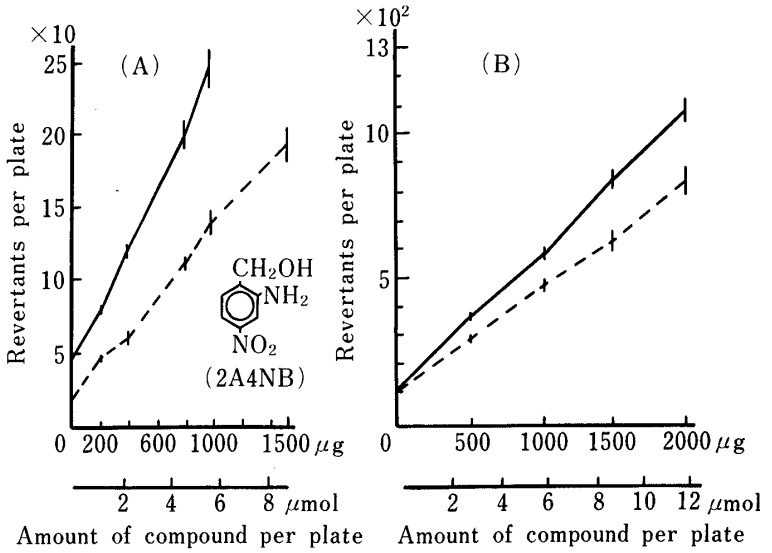

Fig. 5. Dose–Response Curves of 2-Amino-4-nitrobenzyl Alcohol (2A4NB) Tested with TA 98 (A) and TA 100 (B)

Experimental conditions were as indicated in the legend to Fig. 1.

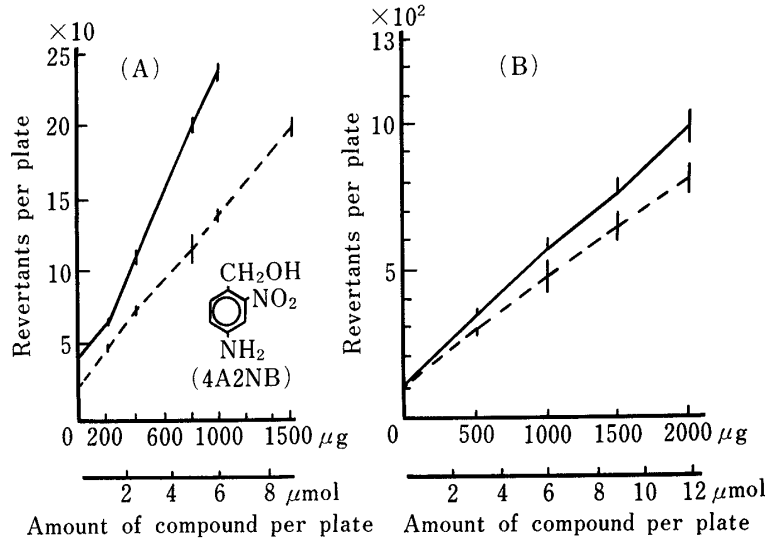

Fig. 6. Dose–Response Curves of 4-Amino-2-nitrobenzyl Alcohol (4A2NB) Tested with TA 98 (A) and TA 100 (B)

Experimental conditions were as indicated in the legend to Fig. 1.

98 were about 5 times that of 2,4-DNT in the absence of S9 mix, but were approximately equal to that of 2,4-DNT in TA 100. 2,4-DAT showed no mutagenicity in the absence of S9 mix, but with S9 mix its mutagenic potency was about 32 times that of 2,4-DNT in TA 98, whereas the sensitivity of TA 100 was much less. The mutagenic potency of 2,4-DNB in TA 98 was higher than those of 2,4-DNT, 2A4AAT, 2A4NB and 4A2NB, and was about 60 times that of 2,4-DNT in the absence of S9 mix. The mutagenic potencies of 2,4-DNA1 in TA 98 and TA 100 were about 2000 and 170 times that of 2,4-DNT in the absence of S9 mix.

From these results, it may be concluded that: (a) 2,4-DNT itself is only a weak mutagen; (b) 2A4NT, 4A2NT, 2N4AAT, 2A4AABA and 2,4-DNBA are non-mutagens: (c) 2A4AAT, 2A4NB and 4A2NB are weak mutagens at mM concentrations; (d) 2,4-DAT, 2,4-DNB and 2,4-DNA1 are potent mutagens at μM concentrations: (e) mutagenesis of 2A4AAT and 2,4-

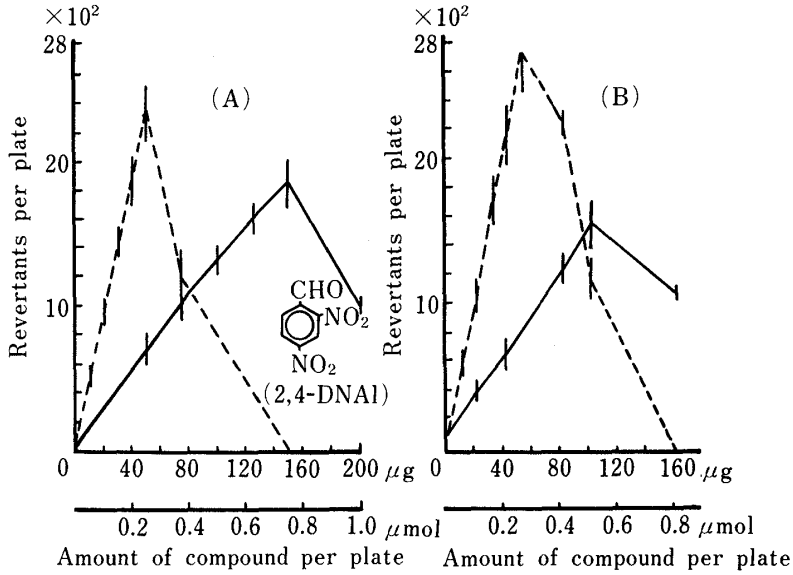

Fig. 7. Dose–Response Curves of 2,4-Dinitrobenzaldehyde (2,4-DNAl) Tested with TA 98 (A) and TA 100 (B)

Experimental conditions were as indicated in the legend to Fig. 1.

TABLE I. Comparison of Mutagenic Activities of 2,4-DNT and Its Metabolites in *S. typhimurium* TA 98 and TA 100

Test compound	His⁺ revertants/μmol[a]			
	TA 98		TA 100	
	Without S9 mix	With S9 mix	Without S9 mix	With S9 mix
2,4-DNT	4.5±0.3	7.2±1.0	62.1±6.6	76.1±13.2
2A4AAT	<1.0	39.3±2.3	<1.0	<1.0
2A4NB	23.2±1.8	44.0±4.5	73.7±4.3	95.0±2.6
4A2NB	24.2±3.3	44.2±4.5	71.6±6.5	87.0±6.5
2,4-DAT	<1.0	233.2±11.6	<1.0	52.5±10.5
2,4-DNB	269.4±9.0	337.5±6.9	467.5±51.7	764.7±42.0
2,4-DNAl	9289.5±938.3	2550.8±111.3	10703.0±1187.0	3230.5±436.9

a) Values are means with standard deviations for four to six doses.

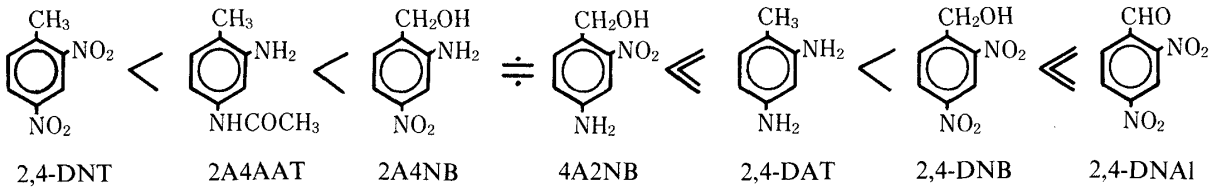

Fig. 8. Relative Mutagenic Activities of 2,4-DNT and Its Urinary Metabolites (2A4AAT, 2A4NB, 4A2NB, 2,4-DAT and 2,4-DNB) and 2,4-DNAl

DAT is indirect, requiring metabolic activation. The relative mutagenic activities are shown in Fig. 8.

Discussion

The objective of this investigation was to identify potent mutagenic metabolites of 2,4-DNT in order to understand the role of metabolism in the carcinogenicity of this compound.

The present results showed that the mutagenic potencies of 2A4AAT, 2A4NB, 4A2NB, 2,4-DAT, 2,4-DNB and 2,4-DNA1 were higher than that of 2,4-DNT itself. Among them, 2,4-DAT has been shown to be a hepatocarcinogen in feeding experiments in rats.[8-10] 2,4-DAT has also been shown to be a mutagen requiring metabolic activation in the Ames assay using strain TA 1538 and S9 mix prepared from PB- or BF-treated rat livers.[11] In agreement with Aune *et al.*,[11] we also found that 2,4-DAT was mutagenic toward both strains TA 98 and TA 100 only in the presence of S9 mix.

The observation that 2,4-DNB and 2,4-DNBA are major metabolites of 2,4-DNT[5,6] suggests that 2,4-DNA1, an intermediate in the oxidation of 2,4-DNB to 2,4-DNBA, is an important mutagen. The TA 98 and TA 100 used as tester strains have an aerobic nitroreductase,[12] so that bacterial reduction of 2,4-DNA1 to a nitroso or N-hydroxyl derivative(s) might be responsible for the mutagenicity. However, since compounds containing a chemically active functional group such as $-CHO$, $-CN$ or $-CH_2Cl$ damage bacterial DNA, as do nitro compounds,[4] the finding that 2,4-DNA1 is significantly more mutagenic than the other mutagenic nitro compounds (Table I) suggests that the potent mutagenic activity of 2,4-DNA1 may be more dependent on the presence of $-CHO$ in the molecule than on the bacterial reduction of the nitro groups.

Bond and Rickert[13] have shown that the formation of 2,4-DNB from 2,4-DNT is mediated by cytochrome P-450-dependent monooxidase using the isolated hepatocytes of rat liver. In addition, *p*-nitrobenzyl alcohol is oxidized to *p*-nitrobenzaldehyde by an NAD-dependent enzyme localized in the hepatic soluble fraction.[14] Thus, it appears that the formation of 2,4-DNA1 from 2,4-DNT in rat liver may depend on the microsomal cytochrome P-450 and cytosolic aldehyde dehydrogenase.

Uehleke[15] demonstrated that the N-hydroxylation of aromatic amines was mediated by cytochrome P-450 enzymes. In addition, McCoy *et al.*[16] suggested that arylhydroxylamines are either direct-acting mutagens or become so following nonenzymatic conversion to aryl nitrenium ions. Thus, it seems likely that the mutagenic activities of 2,4-DAT and 2A4AAT in the presence of S9 mix (Figs. 2 and 3) may be due to the hydroxylamino derivatives formed by the metabolic activation system. Sundvall *et al.*[17] showed that 2,4-DNBA was non-mutagenic or only weakly mutagenic towards TA 98 or TA 100 because of the absence of production of hydroxylamino derivatives by the S9 mix. We also found that 2,4-DNBA was non-mutagenic in strains TA 98 and TA 100 (data not shown). Since the S9 fraction or tester strains have an N-hydroxylating ability[15] or nitro-reducing ability,[12] as described above, it is difficult to determine the active forms of compounds containing nitro and/or amino groups. In addition, the fact that arylhydroxylamines are either direct-acting or become so following esterification to electrophiles[16] may further complicate attempts to assess the mutagenicity of compounds containing nitro and/or amino groups. The lack of mutagenic effect of 2A4NT, 4A2NT and 2N4AAT containing amino and/or nitro groups is so far unexplained.

More recently, we have demonstrated that 2,4-DAT, a known carcinogen, is produced by incubating 2,4-DNT with hepatic cytosol fractions[18] and with intestinal microflora of rats.[19] This observation, together with the finding that 2,4-DAT, 2,4-DNB and 2,4-DNA1 are potent mutagens, suggests that the metabolic conversion of 2,4-DNT to 2,4-DNB, 2,4-DNA1 and 2,4-DAT may contribute to the carcinogenicity of 2,4-DNT in rats. Experiments are in progress to determine the formation of 2,4-DNA1 in 2,4-DNT metabolism *in vitro*.

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