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Intestinal Metabolism of 2,4-Dinitrotoluene in Rats

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2,4-Dinitrotoluene (2,4-DNT), which is an industrial chemical of importance in the production of urethane foams and elastomers, is a hepatocarcinogen in rats. 2,4-Diaminotoluene (2,4-DAT), one of the urinary and hepatic metabolites of 2,4-DNT, is also carcinogenic in rats. We have studied the pathways of metabolism of 2,4-DNT in the cecal microflora of rats. 2,4-DNT was not metabolized by this preparation in the presence of oxygen. Under anaerobic conditions, an ordered sequence of reductive metabolism was observed. 2,4-DNT was reduced to 2-amino-4-nitrotoluene (2A4NT) and 4-amino-2-nitrotoluene (4A2NT) *via* 2-hydroxylamino-4-nitrotoluene (2HA4NT) and 4-hydroxylamino-2-nitrotoluene (4HA2NT), which were identified by mass spectral (MS) comparison with authentic materials. The two aminonitrotoluenes were then reduced to 2,4-DAT. No intermediates in this sequence could be isolated. These findings indicate that rat intestinal microflora catalyze the reductive metabolism of 2,4-DNT and suggest that the reduction of 2,4-DNT to 2,4-DAT may play a role in the carcinogenicity of 2,4-DNT.

Keywords——2,4-dinitrotoluene intestinal metabolism; rat cecal content; rat cecal microflora; hydroxylaminonitrotoluene; aminonitrotoluene; diaminotoluene; dinitrotoluene carcinogenicity

2,4-Dinitrotoluene (2,4-DNT) is an industrial intermediate used in the production of polyurethane foams, elastomers, coatings and explosives. It was found to be a hepatocarcinogen in rats.[1,2] 2,4-Diaminotoluene (2,4-DAT), a possible metabolite of 2,4-DNT, was also found to produce hepatocellular carcinomas in rats.[3-5] 2,4-DAT was first found in the urine of rats surviving for 6 d of daily *p.o.* administration of 2,4-DNT.[6] The reduction of 2,4-DNT to 2,4-DAT in rat liver was mediated mainly by cytosolic xanthine oxidase under anaerobic conditions.[7] Previous studies have demonstrated that intestinal microflora represent an important site for the reductive metabolism of a number of nitroaromatics.[8-10] Thus, it seemed relevant to investigate whether 2,4-DAT is produced in intestinal metabolism of 2,4-DNT, in order to improve our understanding of the carcinogenicity of 2,4-DNT.

It was found that 2,4-DNT was metabolized *via* hydroxylamino intermediates to aminonitrotoluenes, which were subsequently reduced to diaminotoluene. The mechanism of carcinogenic action of 2,4-DNT is discussed.

Experimental[11]

Chemicals——Generally ^{3}H-labeled 2,4-DNT ([^{3}H]-2,4-DNT) with a specific activity of 0.31 μCi/μ mol was prepared by the method previously described.[12] 2,4-Dinitrotoluene (2,4-DNT), 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), 2,4-diaminotoluene (2,4-DAT), dimethyl sulfoxide (DMSO), glucose, peptone (Difco) and yeast extract (Difco) were purchased from Wako Chemical Ind. Ltd. Other chemicals were of the highest grade commercially available.

Preparation of Hydroxylaminonitrotoluenes——4-Hydroxylamino-2-nitrotoluene (4HA2NT) was prepared by applying a method similar to that used for the synthesis of phenylhydroxylamine.[13] A solution of 5.3 g (0.1 mol) of NH_4Cl in 20 ml of H_2O was added to 14.1 g (0.05 mol) of 2,4-DNT dissolved in 400 ml of ethanol. The mixture was stirred vigorously, and 7.8 g (0.12 mol) of zinc dust was added in portions. Stirring was continued for 30 min at 40 °C after all the zinc dust had been added. The solution was filtered and the solvent was evaporated off *in vacuo*. The resulting residue was dissolved in a small amount of ether. This solution was applied to a column of silica gel

(Wakogel C-200). The material was eluted with chloroform, and the solvent was evaporated off *in vacuo*. The residue was recrystallized from chloroform to give 2.0 g of yellow plates, mp 112—113 °C. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600 (NH), 3340 (OH), 1540 (NO_2). MS *m/e*: 168 (M^+), 151 ($M^+ - OH$). ^{1}H-NMR (200 MHz, in $CDCl_3$) δ: 2.53 (3H, CH_3), 5.42 (1H, OH), 6.88 (1H, NH), 7.11—7.66 (3H, aromatic). This product was converted to 4A2NT by incubation with *E. coli* according to the method previously described.[14] In the preparation of 4HA2NT, a small spot which was visualized with *p*-dimethylaminobenzaldehyde (*p*-DMAB) reagent[15] was detected chromatographically in addition to the large spot corresponding to 4HA2NT. This product was isolated as a pure, crystalline compound, mp 115—116 °C. The yield was 0.35 g. IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3600 (NH), 3340 (OH), 1530 (NO_2). MS *m/e*: 168 (M^+), 151 (M^+–OH). ^{1}H-NMR (200 MHz, in $CDCl_3$) δ: 2.22 (3H, CH_3), 5.26 (1H, OH), 6.68 (1H, NH), 7.11—7.80 (3H, aromatic). This product was converted to 2A4NT by incubation with *E. coli* and was also used as an authentic standard for thin-layer chromatographic (TLC) analysis of biological samples.

Procedure for Anaerobic Incubations——Male Wistar rats (Sankyo Labo. Co.), weighing 200—220 g, were used as experimental animals. Preparations of cecal microflora from intestinal contents were performed according to the method of Ingebrigtsen *et al.*[16] Rats were anesthetized with ethyl ether. The peritoneal cavity was opened, and a portion of the cecal contents (approximately 2 g) was transferred to a sterile centrifuge tube containing 5 volumes of sterile incubation medium (0.5% glucose, 0.5% peptone, 0.5% yeast extract in 0.1 M sodium phosphate buffer, pH 7.4). The suspension was centrifuged at 1500 rpm for 1 min and portions of the supernatant fraction were used to inoculate 5 ml of incubation medium in a sterile Thunberg tube. The side arm of the tube contained [^{3}H]-2,4-DNT, 2,4-DNT, 2A4NT, 4A2NT or 2,4-DAT. After three cycles of evacuation and refilling with N_2, the tubes were sealed and preincubated at 37 °C for 5 min. The reaction was initiated by mixing the substrate in the side arm with the tube contents. Incubations were performed at 37 °C and terminated by admission of air followed by the addition of 2 volumes of ether.

Preliminary experiments utilizing cecal contents demonstrated that 20 mg of cecal contents per ml of incubation mixture was an optimal concentration. Experiments were then performed at three final concentrations of 2,4-DNT or [^{3}H]-2,4-DNT, 25 μM (0.04 μCi), 50 μM (0.08 μCi) and 100 μM (0.16 μCi) in DMSO; the incubation time ranged from 1 to 300 min. Control incubations were performed under aerobic conditions or in the absence of microflora.

Detection of Metabolites of 2,4-DNT by TLC——TLC was carried out by using the *p*-DMAB/zinc system[15]: the spray reagent consisted of 0.25% *p*-DMAB and 0.25 N HCl in ethanol; the plate contained 10% zinc dust in silica gel (Wakogel B-10) as a reducing agent for the spray reagent. The detection of metabolites and unchanged substrate was carried out, utilizing hydroxylaminonitrotoluenes, monoaminonitrotoluenes, 2,4-DAT and 2,4-DNT as reference standards.

Identification of Metabolites of 2,4-DNT by MS Measurements——Sample preparation for the MS measurements was carried out by the following procedure. The combined ether extract from cecal microflora incubated with 2,4-DNT was concentrated to about 0.3 ml, spotted in a line on plates and developed with 5% methanol in dichloromethane. After being visualized with *p*-DMAB reagent, bands corresponding to authentic 2A4NT, 4A2NT, 2HA4NT, 4HA2NT and 2,4-DAT were scraped into tubes, and each material was extracted with ether. The extract was evaporated to dryness under nitrogen, and the residue was introduced on a solid probe. Electron-impact spectra were obtained in a model JMS-D200 instrument. The source pressure was 1×10^{-6} Torr, and the source temperature was 200 °C. The filament was operated at 300 μA at 70 eV.

Determination of Metabolites and Unchanged Substrates——Portions of incubation mixture (4.5 ml) were extracted three times with 2 volumes of ether. The pooled extracts were evaporated to dryness under nitrogen, and the residue was redissolved in about 0.2 ml of methanol. This solution was spotted in a line on TLC plates. After TLC, bands corresponding to 2,4-DNT, 2HA4NT, 4HA2NT, 2A4NT, 4A2NT and 2,4-DAT were scraped off directly into scintillation vials containing 10 ml of scintillation mixture (4 g 2,5-diphenyloxazole (PPO), 0.2 g 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP)/1 toluene). The samples were counted in an Aloka model 903 liquid-scintillation counter. Quench correction was performed by the automatic external standard method. Counting efficiencies were consistently >45%. The amounts of metabolites and unchanged substrate are expressed as a percentage of ^{3}H added.

When 2,4-DNT, 2A4NT, 4A2NT and 2,4-DAT were used as substrates, the determination of metabolites and unchanged substrates was carried out by use of a densitometer equipped with ultraviolet UV detector (Shimadzu, model CS 910). Portions of pooled extracts were spotted on plates (not containing zinc dust) and developed with 5% methanol in dichloromethane. After development, the plates were put on the stage and scanned linearly. The conditions were as follows: wavelength, 230 nm for samples and 600 nm references; slit width, 10 × 0.25 mm; scanning rate, 40 mm/min. The amounts of unchanged substrates and metabolites produced were calculated from the standard curves by measuring the peak areas. Approximately 0.1 μg of 2,4-DNT, 2A4NT, 4A2NT, 2HA4NT, 4HA2NT and 2,4-DAT could be detected by this detection system.

Results

A representative thin-layer chromatogram of extracts from cecal microflora incubated

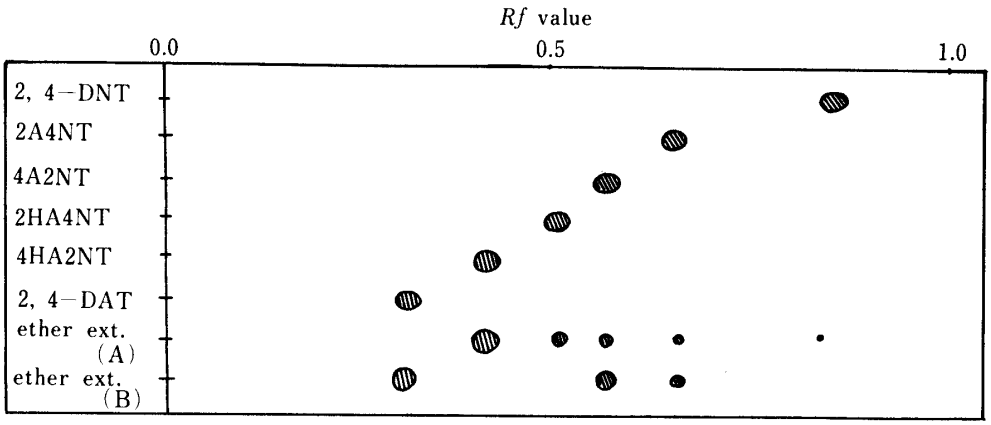

Fig. 1. Schematic Thin-Layer Chromatogram of Ether Extracts from Cecal Microflora Incubated with 2,4-DNT for 20 min (A) and 80 min (B) Under Anaerobic Conditions

Plate, silica gel+Zn; solvent, 5% MeOH in CH_2Cl_2; detection, *p*-DMAB reagent. Abbreviations: 2,4-DNT, 2,4-dinitrotoluene; 2A4NT, 2-amino-4-nitrotoluene; 4A2NT, 4-amino-2-nitrotoluene; 2HA4NT, 2-hydroxylamino-4-nitrotoluene; 4HA2NT, 4-hydroxylamino-2-nitrotoluene; 2,4-DAT, 2,4-diaminotoluene.

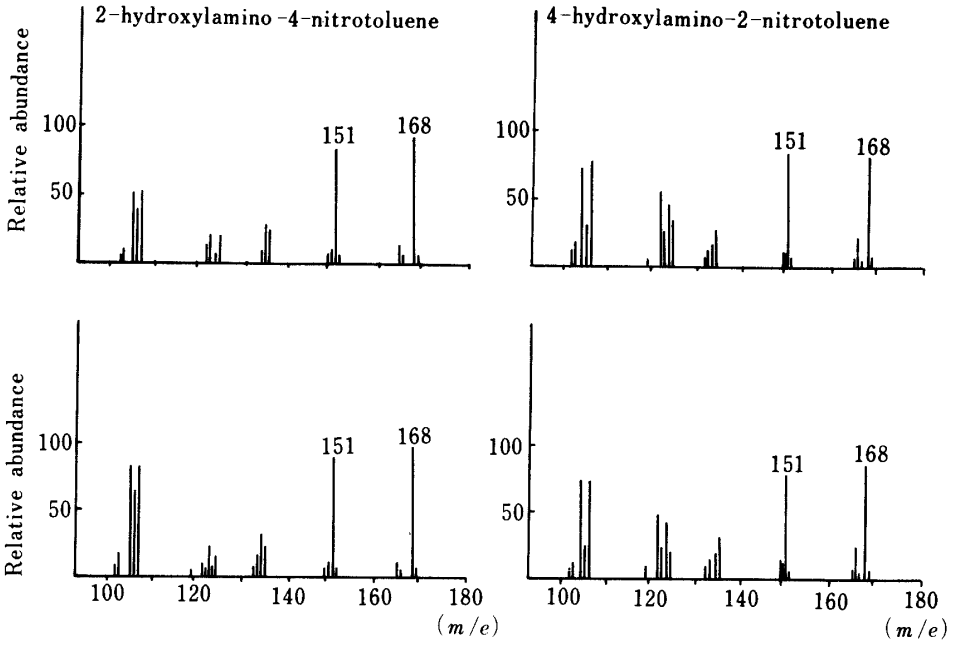

Fig. 2. Mass Spectra of 2-Hydroxylamino-4-nitrotoluene and 4-Hydroxylamino-2-nitrotoluene

Lower panels are spectra of biological samples; upper panels are spectra of authentic samples.

with 2,4-DNT for 20 min (A) and 80 min (B) is shown in Fig. 1. Four metabolites which gave *Rf* values corresponding to those of authentic 2A4NT, 4A2NT, 2HA4NT and 4HA2NT were detected in sample A. The materials corresponding to hydroxylamino intermediates were isolated from sample A, and the metabolites were identified by comparison of the MS of authentic and biological samples (Fig. 2). Similarly, metabolites corresponding to monoamino-nitrotoluenes and diaminotoluene were isolated from sample B, and were characterized as 2A4NT, 4A2NT and 2,4-DAT by comparison of the MS with those of authentic samples (spectra not shown).

When [^{3}H]-2,4-DNT (100 μM) was incubated with cecal microflora, a rapid decrease in

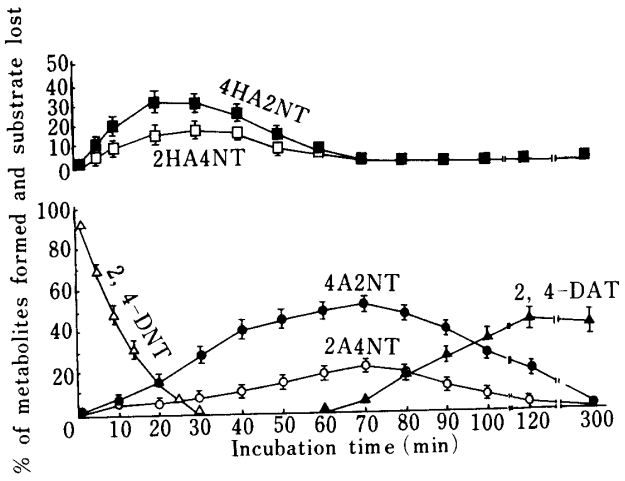

Fig. 3. Metabolism of 2,4-DNT by Rat Cecal Microflora Under Anaerobic Conditions

Cecal microflora were incubated under nitrogen with [^{3}H]-2,4-DNT (100 μM) for various times. Metabolites were separated by TLC and quantified by scintillation counting. Each value is the mean of five samples with the standard deviation. (△), 2,4-DNT; (○), 2A4NT; (●), 4A2NT; (□), 2HA4NT; (■), 4HA2NT; (▲), 2,4-DAT. Chromatographic conditions and abbreviations are as indicated in the legend to Fig. 1.

substrate concentration was observed. [^{3}H]-2,4-DNT was completely consumed within 30 min (Fig. 3). The first metabolites to appear in the reaction mixture were 2HA4NT and 4HA2NT, which reached maximal concentrations between 20 and 80 min. As the concentrations of hydroxylamino compounds declined, there was a corresponding increase in the concentrations of 2A4NT and 4A2NT which reached maxima within 70 min. As the concentrations of these aminonitro compounds decreased, a corresponding increase in the amount of 2,4-DAT was detected. At no time were azoxy compounds detected.

After aerobic incubation of cecal microflora for up to 120 min, no radioactivity other than that of [^{3}H]-2,4-DNT could be detected in the chromatogram (chromatogram not shown). Under anaerobic conditions, with [^{3}H]-2,4-DNT concentrations of 25, 50 and 100 μM, the pattern of metabolites produced was independent of substrate concentration (data not shown). Furthermore, at the lowest substrate concentration (25 μM), all of the [^{3}H]-2,4-DNT was consumed within 15 min; at the two higher [^{3}H]-2,4-DNT concentrations, the consumption of substrate was independent of their concentration at 15 min.

It was not possible to isolate or identify nitrosonitrotoluenes, nitrosoaminotoluenes or hydroxylamino-aminotoluenes. However, since it has been assumed that biological reduction

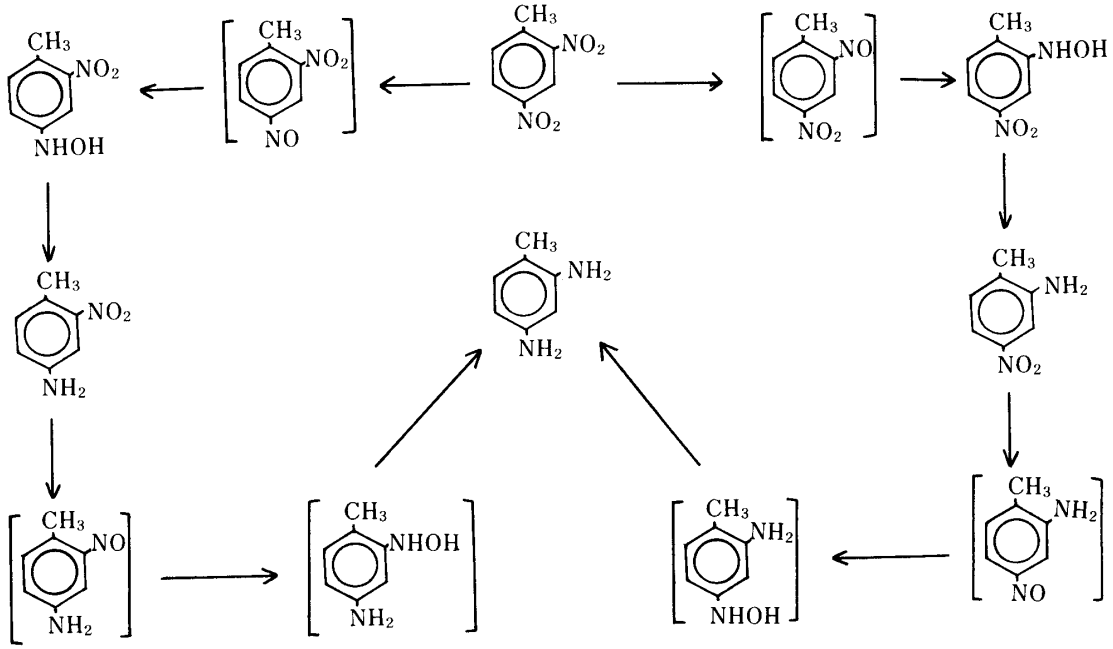

Chart 1. Proposed Pathway for the Anaerobic Metabolism of 2,4-DNT in Rat Intestinal Microflora

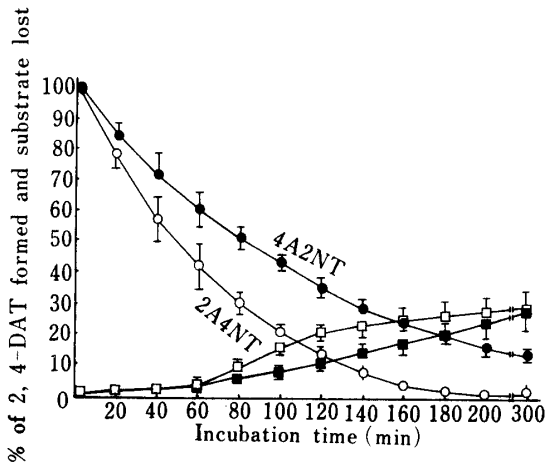

Fig. 4. Metabolism of Aminonitrotoluenes by Rat Cecal Microflora Under Anaerobic Conditions

Cecal microflora were incubated under nitrogen with 100 μM 2A4NT or 4A2NT for various times. Disappearance of substrate and appearance of 2,4-DAT were measured at various times by TLC-densitometric analysis. Each value is the mean of four samples with the standard deviation. (○), 2A4NT; (●), 4A2NT; (□), 2,4-DAT from 2A4NT; (■), 2,4-DAT from 4A2NT. Chromatographic conditions and abbreviations are as indicated in the legend to Fig. 1.

of the nitro group of aromatics proceeds through nitroso and hydroxylamino intermediates,[17] it is possible that 2,4-DNT is metabolized to 2,4-DAT *via* nitroso and hydroxylamino intermediates as proposed in Chart 1.

When cecal microflora were incubated with either 2A4NT or 4A2NT, the only product which could be detected was 2,4-DAT (Fig. 4). Of the two aminonitro compounds, 2A4NT was more rapidly metabolized to 2,4-DAT. The amount of 2,4-DAT formed did not totally account for the disappearance of either substrate. When 2,4-DAT (100 μM) was added to cecal microflora, a 50% loss of 2,4-DAT was observed over a 2 h incubation. No spots or peaks other than 2,4-DAT could be detected by TLC and densitometric analysis.

Discussion

The metabolites produced from 2,4-DNT by rat cecal microflora were two hydroxylaminonitrotoluenes, two monoaminonitrotoluenes and 2,4-DAT. The time-course study indicated that 2,4-DNT undergoes an ordered reduction. The nitro groups are first reduced to hydroxylamino and then to amino groups. The failure to isolate the presumed intermediate nitroso compounds suggests that these intermediates are very labile. Similarly, the inability to isolate either the nitroso- or hydroxylamino-amino intermediates in the reduction of the monoaminonitro compounds to 2,4-DAT suggests that these intermediates are highly reactive. The apparent instability or reactivity of these intermediates leaves open their possible role in the mechanism of 2,4-DNT carcinogenicity. It remains to be established whether they are sufficiently stable to travel from the intestine to the liver. It is possible that these or other metabolites bind to macromolecules in microflora. This hypothesis may explain why the quantified metabolites accounted for only 50 to 90% of the 2,4-DNT which disappeared from the incubation mixture (Fig. 3). This hypothesis may also explain the discrepancy between the disappearance of 2A4NT and 4A2NT and the formation of 2,4-DAT (Fig. 4).

The position of the nitro group, relative to the methyl group, appears to be an important determinant of the rate of reduction. 4A2NT is present in approximately two-fold excess over 2A4NT after a 70 min incubation of cecal microflora with [^{3}H]-2,4-DNT (Fig. 3). Similarly, the formation of 4HA2NT occurs predominantly in metabolic reduction of 2,4-DNT to hydroxylaminonitrotoluenes (Fig. 3). This is similar to the chemical reduction of 2,4-DNT to the hydroxylaminonitrotoluenes (see Experimental). Moreover, when 2A4NT and 4A2NT were used as substrates, 2A4NT was more rapidly converted to 2,4-DAT than 4A2NT (Fig. 4). The slower reduction of the nitro group *ortho* to the methyl group may be due to interaction between methyl hydrogens and the nitro oxygens or to steric hindrance by the methyl group.

2,4-DAT is a potent hepatocarcinogen in rats,[3-5] but its proximate or ultimate forms are not known. The observations that 2,4-DAT is non-mutagenic in the absence of S9 mix[18,19] and is mutagenic in the presence of S9 mix[18] strongly suggest that the mutagenic activity is due to the oxidation of 2,4-DAT by the enzymes contained in the S9. Goldman has shown that amine- and amide-containing carcinogens are N-hydroxylated by the cytochrome P-450 system of the liver in the obligatory first step of the metabolic sequence leading to the formation of the electrophilic metabolites which react with cellular macromolecules to initiate carcinogenesis.[10] In addition, Clayson and Garner have demonstrated that aromatic amines are toxic or form toxic metabolites in the body after being adsorbed from the intestine.[20] Our previous finding[7] showed that 2,4-DAT could be produced *via* 2A4NT and 4A2NT from 2,4-DNT by rat liver cytosol preparations under anaerobic conditions. Thus, it seems that the conversion of 2,4-DAT produced in the intestinal and/or hepatic metabolism to the hydroxylamino intermediates in the liver is involved in the 2,4-DNT carcinogenicity.

The data presented here indicate that 2,4-DAT can be produced *via* hydroxylaminonitrotoluenes and monoaminonitrotoluenes in intestinal metabolism of 2,4-DNT. It is possible that the formation of 2,4-DAT is involved in 2,4-DNT carcinogenicity.

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