[Chem. Pharm. Bull. 32(10)4070—4075(1984)]

Metabolism of Dinitrotoluene Isomers by *Escherichia coli* Isolated from Human Intestine

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(Received January 12, 1984)

Technical grade dinitrotoluene (DNT), an important industrial nitroaromatic compound, not only induces methemoglobinemia but is a potent hepatocarcinogen in man and rats. The purpose of the present study was to identify the metabolites produced from five DNT isomers (2,3-, 2,4-, 2,5-, 2,6- and 3,4-DNT) by *Escherichia coli* (*E. coli*) strain W3110, isolated from human intestine. Data obtained from thin-layer and gas chromatographies or liquid scintillation counting indicated that the metabolites produced by the *E. coli* were two monoaminonitrotoluenes and hydroxylaminonitrotoluenes in all cases. This finding indicates that DNT is reduced *via* hydroxylaminonitrotoluenes to monoaminonitrotoluenes in *E. coli*. In addition, it was found that the reduction reactivities of DNT isomers were 3->2- for 2,3-DNT, 4->2- for 2,4-DNT, 5->2- for 2,5-DNT and 3->4- for 3,4-DNT. The role of bacterial reduction in the toxic actions of DNT is discussed.

Keywords——metabolism and toxicity; dinitrotoluene; *Escherichia coli*; hydroxylaminonitrotoluene; aminonitrotoluene; reduction

Technical grade dinitrotoluene (DNT), which is a mixture consisting of 75% 2,4-DNT, 20% 2,6-DNT and 5% other DNT isomers, has been widely used in the production of polyurethane foams, coatings, elastomers and explosives.

Methemoglobinemia, cyanosis, anemia and jaundice were reported in man as a result of DNT exposure in the workplace.[1] Acute exposure also led to methemoglobinemia and cyanosis, and in some animal species, atrophy of the testes with aspermatogenesis.[2] Rats chronically exposed to DNT in the diet for 1 to 2 years developed hepatocellular carcinomas.[2] In another feeding study, 2,4-DNT was found to be a hepatocarcinogen in rats.[3]

Nitroaromatic compounds are metabolized to nitroso- and hydroxylamino intermediates in liver[4,5] and by intestinal microflora.[6,7] We have shown that the DNT isomers, 2,3-, 2,4-, 2,5- and 3,4-DNT, are principally converted *via* hydroxylamino compounds to monoaminonitrotoluenes in rat liver preparations[8,9] and by *Rodotorula glutinis*[8] (*R. glutinis*), a yeast. More recently, it has been shown by Reddy *et al.*[10] that the *in vivo* induction of methemoglobinemia by nitrobenzene was dependent on the reduction of the compound to active intermediates and reduced metabolites by the gastro-intestinal microflora of rats. Thus, it seems relevant to investigate whether DNT isomers are reduced by intestinal microbial organisms, in order to improve our understanding of the toxic actions of DNT.

In this paper we report the identification and determination of reduction products of five DNT isomers (2,3-, 2,4-, 2,5-, 2,6- and 3,4-DNT) formed by *E. coli*. *E. coli* was chosen for several reasons: first, it is usually present in mammalian intestines; second, it is easy to handle; third, it has the capacity to reduce nitroaromatic compounds such as chloramphenicol[11] and nitrofuran.[12]

Experimental[13]

Synthetic Standards of Substrates and Metabolites——The following compounds were prepared according to the

published procedures: 2,5-DNT,[14] 3-amino-2-nitrotoluene (3A2NT),[15] 3-amino-4-nitrotoluene (3A4NT),[16] 5-amino-2-nitrotoluene (5A2NT),[15] 3-hydroxylamino-2-nitrotoluene (3HA2NT).[8] The purity of each compound was checked chromatographically. 2,3-DNT, 2,4-DNT, 3,4-DNT, 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT), 4-amino-3-nitrotoluene (4A3NT), 2-amino-5-nitrotoluene (2A5NT), 2-amino-3-nitrotoluene (2A3NT) and 2-amino-6-nitrotoluene (2A6NT) were purchased from Wako Chemical Industry Co. Other chemicals were of the highest grade commercially available.

Experiment with Cultured *E. coli*——*E. coli* strain W3110, which was kindly provided by Dr. S. Okamura of this University, was maintained on agar slants consisting of yeast extract, peptone and glucose, and was stored at 2 °C prior to use. Incubation culture was prepared from the following mineral base: 0.1% NH_4Cl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.4% glucose, 0.7% $Na_2HPO_4 \cdot 12H_2O$, 0.3% KH_2PO_4 and 0.05% NaCl.[17] Media were sterilized at 120 °C for 20 min. One loopful of surface growth from slants of the *E. coli* was inoculated into 50 ml of the incubation culture and then incubated aerobically at 37 °C for 24 h. After incubation for one day, cells were washed with 20 ml of saline solution and then suspended in 50 ml of saline solution. Next, 1.0 ml of 1.0% ethanol solution of the substrate was added to 50 ml of the cell suspension (corresponding to 1.1 mM), and the mixture was then centrifuged at 2500 rpm for 15 min to remove cells. The supernatant obtained was extracted 3 times with ether. The combined ether layers were dried over anhydrous Na_2SO_4 and concentrated to about 1 ml. This sample was subjected to thin-layer chromatography (TLC) or subjected to sample preparation procedures for mass spectral (MS) and ultraviolet (UV) spectral measurements and chromatographic analysis as described below.

Detection of Metabolites of DNT Isomers by TLC——TLC was carried out on plates of silica gel (0.25 mm thick, Wakogel B-10) containing 10% zinc dust and developed with dichloromethane; the chromatogram was visualized with *p*-dimethylaminobenzaldehyde (*p*-DMAB) reagent.[18] The detection of metabolites was carried out by utilizing monoaminonitrotoluene and hydroxylaminonitrotoluene as reference standards.

Identification of Metabolites of DNT Isomers by MS and UV Spectral Measurements——Sample preparation for the MS and UV spectral measurements was carried out by the following procedure. Portions of ether extract were subjected to preparative TLC. The material obtained by this process was extracted 3 times with ether by shaking for 10 min. The combined ether layer was concentrated to about 0.1 ml and was subjected to MS and UV spectral measurements and gas chromatographic analysis.

Determination of Metabolites of DNT Isomers by Gas Chromatography——A Shimadzu GC-6A gas chromatograph equipped with a hydrogen flame ionization detector was used. The column was a glass tube, 2.0 m × 4 mm, packed with 5% OV-17 on Shimalite W (80—100 mesh). Chromatographic conditions were as follows: temperatures were 200 or 220 °C for the column or sample chamber and detector cell; nitrogen was used as the carrier gas at a flow rate of 40 ml/min.

Incubation of *E. coli* with 2,4-DNT Containing [^{3}H]2,4-DNT——Generally ^{3}H-labeled 2,4-DNT with a specific activity of 0.31 μCi/μmol, which was prepared by the method previously described,[19] was used as a tracer. Except for the addition of [^{3}H]2,4-DNT (0.05 μmol, 15.5 mμCi) to 0.055 mmol of 2,4-DNT as a substrate, the incubation system was similar to that described above. The incubation was carried out aerobically at 37 °C for 0—28 h. The ether extracts from incubation mixtures were spotted in a line on plates and developed with dichloromethane. After being visualized with *p*-DMAB reagent, bands corresponding to 2A4NT (*Rf* 0.71), 4A2NT (*Rf* 0.61) and two hydroxylaminonitrotoluenes (*Rf* 0.21 and 0.30) were scraped off directly into scintillation vials containing 12 ml of scintillation mixture (4 g PPO, 0.2 g 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 amount of metabolites produced is expressed as a percentage of ^{3}H added after subtraction of background radioactivity (35 dpm).

Results

A representative thin-layer chromatogram of an ether extract from *E. coli* incubated with 2,3-DNT is shown in Fig. 1. Metabolites which gave *Rf* values corresponding to those of authentic 2A3NT, 3A2NT and 3HA2NT were detected in the sample (Fig. 1). As shown in Figs. 2 and 3, the MS and UV spectra of corresponding authentic and biological samples indicated that they were identical. Similarly, metabolites corresponding to monoaminonitrotoluenes were also detected in the biological samples from 2,4-, 2,5-, 2,6- and 3,4-DNT and were characterized as 2A4NT and 4A2NT for 2,4-DNT, 2A5NT and 5A2NT for 2,5-DNT, 2A6NT, for 2,6-DNT, and 3A4NT and 4A3NT for 3,4-DNT by comparison of the MS of authentic and biological samples. In addition, one or two spots giving *Rf* values of around 0.2 to 0.3 were detected chromatographically in the biological samples from 2,6- and 3,4-DNT or 2,4- and 2,5-DNT. The MS of each material obtained by preparative TLC showed a peak at

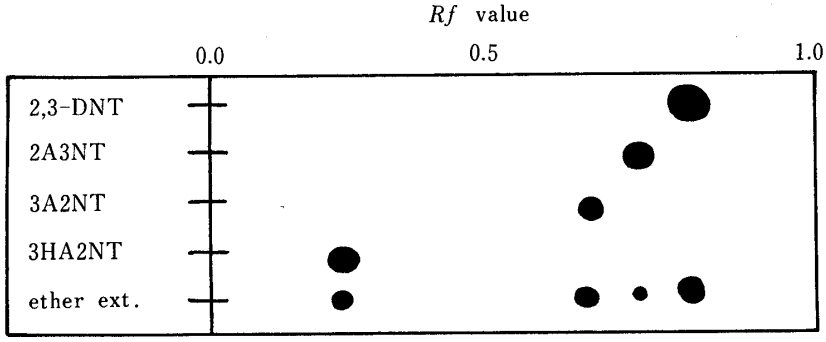

Fig. 1. Schematic Diagram of a Thin-Layer Chromatogram of the Ether Extract from *E. coli* Incubated with 2,3-DNT

Plate, silica gel+Zn; solvent, CH_2Cl_2; detection, *p*-DMAB reagent. Abbreviations: 2,3-DNT, 2,3-dinitrotoluene; 2A3NT, 2-amino-3-nitrotoluene; 3A2NT, 3-amino-2-nitrotoluene; 3HA2NT, 3-hydroxylamino-2-nitrotoluene.

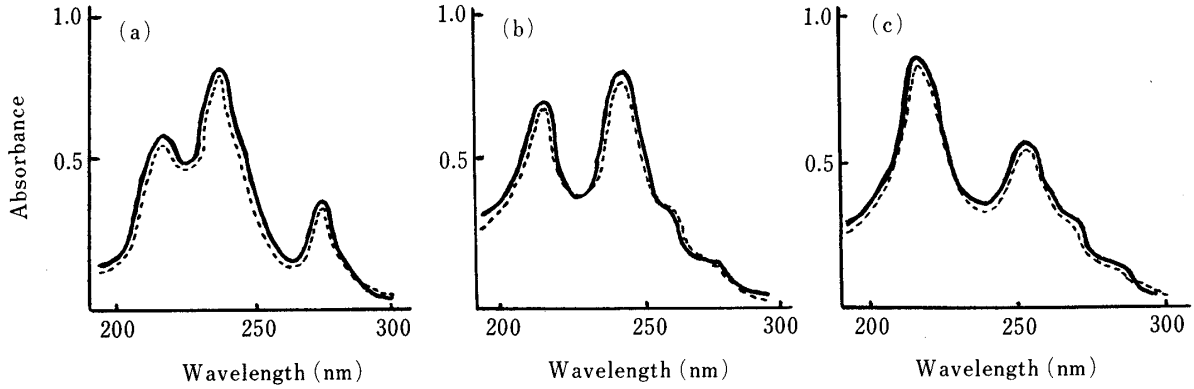

Fig. 2. UV Absorption Spectra of Samples from *E. coli* Incubated with 2,3-DNT

Biological sample, ——; authentic sample, ------. (a) 2A3NT (2-amino-3-nitrotoluene). (b) 3A2NT (3-amino-2-nitrotoluene). (c) 3HA2NT (3-hydroxylamino-2-nitrotoluene).

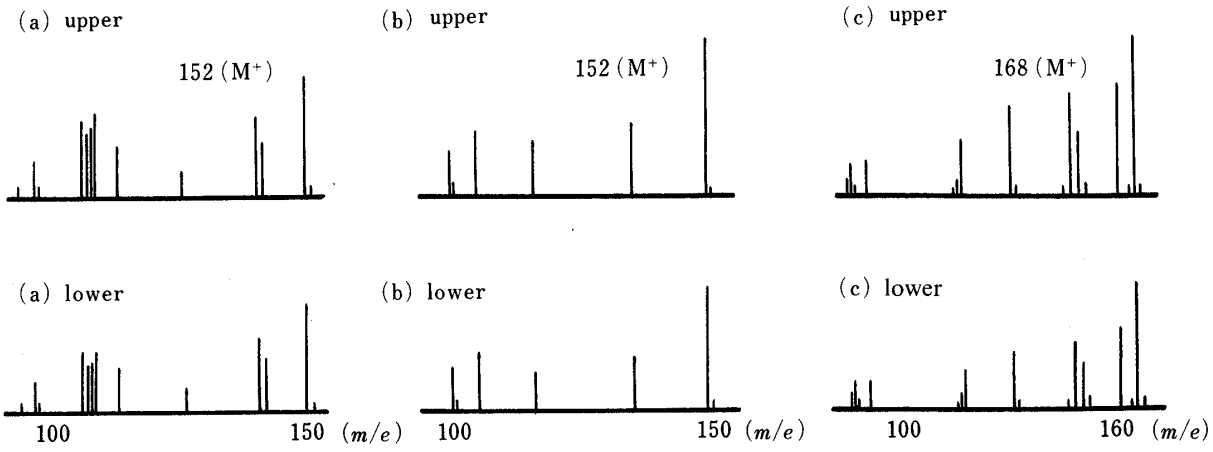

Fig. 3. MS of Samples from *E. coli* Incubated with 2,3-DNT

(a) 3A2NT (3-amino-2-nitrotoluene). (b) 2A3NT (2-amino-3-nitrotoluene). (c) 3HA2NT (3-hydroxylamino-2-nitrotoluene). Upper plots show biological samples and lower plots show authentic samples.

m/e 168 assignable to a parent ion. This finding together with the mobilities of hydroxylaminonitrotoluenes and monoaminonitrotoluenes indicated that the one or two spots giving *Rf* values of 0.2 to 0.3 described above are 2HA6NT for 2,6-DNT, 2HA4NT and

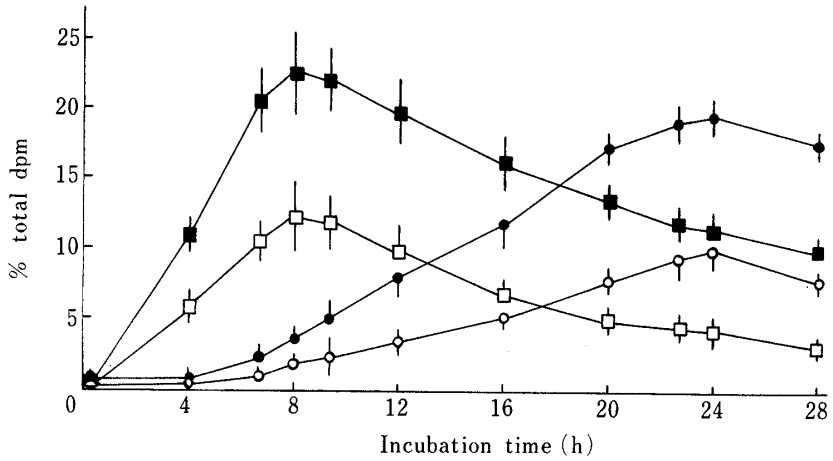

Fig. 4. Time Course of Production of Metabolites from 2,4-DNT by *E. coli*

E. coli was incubated with 2,4-DNT (1.1 mM) containing [^{3}H]2,4-DNT (1 μM) for various times. Metabolites were separated by preparative TLC and quantified by scintillation counting. Each value is the mean of three samples with the standard deviation.
●, 4A2NT; ○, 2A4NT; ■, 4HA2NT; □, 2HA4NT.

TABLE I. Determination of Unchanged Substrates and Monoaminonitrotoluenes Produced by *E. coli*, Expressed as Percentage of DNT Isomer Added

Substrate	Unchanged DNT (%)	Monoaminonitrotoluene (%)	
		3A2NT	2A3NT
2,3-DNT	40.0±3.8	25.1±3.2	13.2±1.2[a)]
		4A2NT	2A4NT
2,4-DNT	59.5±4.1	18.8±2.0	10.2±1.8[a)]
		5A2NT	2A5NT
2,5-DNT	50.7±4.3	21.8±3.0	12.2±1.6[a)]
		2A6NT	
2,6-DNT	30.2±2.8	45.7±5.0	
		3A4NT	4A3NT
3,4-DNT	46.7±3.7	15.8±1.9	10.9±1.3[a)]

Each value is the mean of four experiments with the standard deviation.
a) Significance difference between the values for the two monoamine forms for each substrate at $p<0.01$.

4HA2NT for 2,4-DNT, and 2HA5NT and 5HA2NT for 2,5-DNT. One hydroxylaminonitrotoluene detected in the sample from 3,4-DNT could not be identified; presumably it is either 3HA4NT or 4HA3NT.

The time course of metabolites produced by incubating 2,4-DNT containing [^{3}H]2,4-DNT with *E. coli* is shown in Fig. 4. The first metabolites to appear in the reaction mixture were 4HA2NT and 2HA4NT, which reached maximal concentrations at about 8 h. As the concentrations of hydroxylaminonitrotoluenes declined, there was a corresponding increase in the concentrations of 2A4NT and 4A2NT, reaching maxima by about 24 h. The materials corresponding to hydroxylamino intermediates were isolated after an 8 h incubation, and the production of 2A4NT or 4A2NT was detected chromatographically in the reaction mixture by reincubating each material obtained with *E. coli*. In addition, the rate of production of monoaminonitrotoluenes from other DNT isomers examined was also maximal at about 24 h. These findings indicate that DNT is converted *via* hydroxylaminonitrotoluene to two monoaminonitrotoluenes by *E. coli*. The possibility of production of other metabolic products, including nitroso and diamino forms, was carefully examined, but none of them

were detected.

Subsequently, determination of unchanged substrates or monoaminonitrotoluenes produced by incubating DNT isomers with *E. coli* for 24 h was carried out by gas chromatography, which showed a very good separation of DNT (t_R, about 2.3) and two monoaminonitrotoluenes (t_R, about 4.3 and 5.8) under the conditions described in Experimental. As shown in Table I, all the DNT isomers (except for 2,6-DNT) show characteristic patterns of monoaminonitrotoluenes production. Thus, 3A2NT, 4A2NT, 5A2NT and 4A3NT are formed predominantly in metabolic reduction of 2,3-, 2,4-, 2,5- and 3,4-DNT, respectively. This tendency is in accordance with the results obtained with rat liver preparations[8,9] or *R. glutinis*.[8] In addition, it was found that the major metabolites determined by gas-liquid chromatography (GLC) were the two monoaminonitrotoluenes for each DNT isomer, and these accounted for about 25 to 45% of the substrate added. The determination of hydroxylaminonitrotoluenes was not carried out in the present study, because two peaks were found on GLC.

Discussion

The purpose of this investigation was to characterize the metabolites formed from five DNT isomers by *E. coli* isolated from human intestine, in order to understand the role of bacterial metabolism in toxic actions of DNT. We characterized the metabolic products of the DNT isomers as two kinds of monoaminonitrotoluenes by comparison with authentic samples. In addition, the production of hydroxylaminonitrotoluenes as reduction intermediates was demonstrated by thin-layer chromatographic analysis, MS measurements or reincubation studies.

Reddy *et al.*[10] demonstrated that the *in vivo* induction of methemoglobinemia by nitrobenzene was dependent on the reduction of the compound to active intermediates and reduced metabolites by the gastro-intestinal microflora. Our previous results showed that the formation of methemoglobin in rats ingesting 2,4-DNT was significantly increased compared with that in untreated rats.[20] These observations together with the findings that aminonitrotoluenes and hydroxylaminonitrotoluenes are the major reduced metabolites of DNT isomers in rat liver preparations[8,9] and *E. coli* (Fig. 4 and Table I) indicate that the hepatic and bacterial reductions of DNT isomers are correlated to the formation of methemoglobin, and suggest that the reduced metabolites may contribute to DNT-related methemoglobinemia, cyanosis and anemia observed in man[1] and rats.[2]

The reduction reactivities of DNT isomers, which are 3- > 2- for 2,3-DNT, 4- > 2- for 2,4-DNT, 5- > 2- for 2,5-DNT and 3- > 4- for 3,4-DNT (Table I), correspond well with the results obtained in rat liver preparations[8,9] and *R. glutinis*;[8] this suggests that the position of the nitro group, relative to the methyl group, may be correlated to the rate of reduction. Since Īda[21] has demonstrated that the reduction of nitro groups occurs by the movement of electrons to the N atom from the enzyme (in other words, a nucleophilic displacement reaction for the N atom), it appears that the N atom which is more accessible to electrons should be much more readily reduced than the other N atom in each DNT. Thus, the slower formations of 2HA4NT and 2A4NT compared with those of 4HA2NT and 4A2NT (Fig. 4) may be due to interaction between the methyl hydrogens and the nitro groups or to steric hindrance by the methyl group.

A number of hydroxylamines are known to be proximate carcinogens and it is thought that the formation of the hydroxylamines *in vivo* is important process in carcinogenic action.[22,23] It may be concluded, therefore, that the reduction of DNT to hydroxylaminonitrotoluenes by *E. coli*, one of the intestinal microbial organisms, is probably involved in the induction of methemoglobinemia, and may also be involved in the carcinogenicity of

DNT.

Acknowledgement We are grateful to Dr. S. Okamura of this University for supplying *E. coli* strain W3110 and to Mr. M. Morikoshi of the Analysis Room of this University for measurement of MS.

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