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Identification of Urinary Metabolites of 2,4-Dinitrotoluene (2,4-DNT) in Rats

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Nine metabolites of 2,4-dinitrotoluene (2,4-DNT) in rat urine were detected by thin-layer chromatography (TLC) after repeated oral administration to male Wistar rats. These metabolites, 2-amino-4-nitrotoluene (2A4NT, M-I), 4-amino-2-nitrotoluene (4A2NT, M-II), 2,4-diaminotoluene (2,4-DAT, M-III), 2,4-dinitrobenzyl alcohol (2,4-DNB, M-IV), 2-amino-4-nitrobenzyl alcohol (2A4NB, M-V), 4-amino-2-nitrobenzyl alcohol (4A2NB, M-VI), 2-nitro-4-acetylaminotoluene (2N4AAT, M-VII), 2-amino-4-acetylaminotoluene (2A4AAT, M-VIII) and 2-amino-4-acetylaminobenzoic acid (2A4AAB, M-IX), were identified by comparison of ultraviolet (UV) and mass spectral data with those of authentic samples. The probable metabolism of 2,4-DNT in rats is discussed on the basis of these results.

Keywords——2,4-dinitrotoluene; metabolism; 2-amino-4-nitrotoluene; 4-amino-2-nitrotoluene; 2,4-diaminotoluene; 2-amino-4-nitrobenzyl alcohol; 4-amino-2-nitrobenzyl alcohol; 2-nitro-4-acetylaminotoluene; 2-amino-4-acetylaminotoluene; 2-amino-4-acetylaminobenzoic acid

2,4-Dinitrotoluene (2,4-DNT) is an industrial intermediate used in the production of explosives and dyes. However, several reports appeared in the 1940's describing toxic effects in man and animals. For example, McGee *et al.* observed symptoms of cyanosis, anemia and jaundice in man.[1] Bedow and Jung reported the formation of methemoglobin in the plasma of cats after administration of 2,4-DNT.[2] However, studies on the metabolism of 2,4-DNT, which are essential for understanding the toxic actions of this compound, have not been reported. In recent years, the carcinogenicity of 2,4-diaminotoluene (2,4-DAT) was revealed through a number of studies with rats[3,4] and mice.[5] Since the active metabolites of 2,4-DNT might be coincident with those of 2,4-DAT, it seems to be of importance to examine the metabolism of 2,4-DNT in detail.

In the present study on the metabolism of 2,4-DNT in rats, nine metabolites, M-I, M-II, M-III, M-IV, M-V, M-VI, M-VII, M-VIII and M-IX were detected by thin-layer chromatography (TLC). This paper deals with the identification of these metabolites and the probable routes of metabolism of 2,4-DNT.

Experimental[6]

Synthetic Standards——The following compounds were prepared according to the published procedures: 2,4-dinitrobenzyl alcohol (2,4-DNB),[7] 4-amino-2-nitrobenzyl alcohol (4A2NB),[7] 2-nitro-4-acetylaminotoluene (2N4AAT),[8] 2-amino-4-acetylaminotoluene (2A4AAT)[7]. Each compound showed the reported melting point and its purity was checked chromatographically. In the synthesis of 4A2NB according to the method of Gal'bershtam *et al.*,[7] a small spot which was visualized with *p*-dimethylaminobenzaldehyde (*p*-DMAB) reagent[9] was detected chromatographically in addition to the spot corresponding to 4A2NB. This product was isolated as a pure, crystalline compound, mp 120—121.[7] The mass and IR spectra of this compound indicated it to be 2-amino-4-nitrobenzyl alcohol (2A4NB), and this compound was also used as an authentic sample for thin-layer chromatographic analysis of urinary metabolites. 2,4-DNT, 2-amino-4-nitrotoluene (2A4NT), 4-amino-2-nitrotoluene (4A2NT) and 2,4-DAT were purchased from Wako Pure Chemical Ind., Ltd. and recrystallized from MeOH.

Administration of 2,4-DNT——One milliliter aliquots of 2,4-DNT solution in salad oil, 6.2 mg/ml, were administered orally to each of 30 male Wistar strain rats (weighing 250 g) to provide daily doses of 25 mg/kg for 6 days. The dose of 2,4-DNT totalled 1.1 g through the experiment. The urines were collected daily

for 7 days after giving the first dose.

Extraction and Fractionation——The pH of urine was adjusted to 4.0 with 10% HCl solution and the urine was extracted with ether for 6 hr in a continuous extractor. The ether layer was dried over anhydrous Na_2SO_4, and the solvent was evaporated off. The resulting residue was dissolved in a small amount of MeOH. This solution was applied to a column of silica gel (Wakogel C-200, 300 g). Successive elution with $CHCl_3$ (1000 ml), $CHCl_3$–MeOH (98: 2, 800 ml) and $CHCl_3$–MeOH (90: 10, 500 ml) gave fractions 1, 2 and 3, respectively.

Detection of Metabolites of 2,4-DNT by TLC——TLC was carried out on plates of silica gel (0.25 mm thick, Wakogel B-10) containing 10% zinc dust: the plates were activated at 110° for 60 min before use. The developing solvents used were A), CH_2Cl_2 for fraction 1; B), EtOH–dioxane–benzene–NH_4OH (5: 40: 50: 5) and C), $CHCl_3$–MeOH (3: 1) for fraction 2; and D), BuOH–MeOH–$CHCl_3$ (2: 2: 1) for fraction 3. The chromatograms were visualized with *p*-DMAB reagent.[9] The detection of metabolites was carried out with 2A4NT and 4A2NT as reference standards for fraction 1 and 2,4-DNB, 2A4NB, 4A2NB, 2,4-DAT, 2N4AAT and 2A4AAT as reference standards for fraction 2.

Identification of Metabolites of 2,4-DNT by Mass and UV Spectral Measurements——Sample preparation for the mass and UV spectral measurements was carried out by the following procedure. Portions of fractions 1 and 2 were subjected to preparative TLC. The materials obtained by this process were each extracted 3 times with 10 ml of $CHCl_3$–MeOH (3: 1) solution by shaking for 10 min. The combined organic layer was concentrated to about 0.3 ml and subjected to mass and UV spectral measurements.

Isolation of Metabolite M-IX——After the TLC of fraction 3, the solvent was evaporated off *in vacuo*. The residue was dissolved in a small amount of MeOH–$CHCl_3$ (1: 1) and the solution was applied to a column of silica gel (Wakogel C-200, 50 g). The metabolite M-IX was eluted with $CHCl_3$–MeOH (98: 2), and fractions of about 20 ml were collected. The fractions positive to *p*-DMAB reagent[9] were combined, and the solvent was evaporated off *in vacuo*. The residue was recrystallized from cold MeOH to give 30 mg of light-brownish needles (M-IX), mp 194—195°. *Anal.* Calcd for $C_9H_{10}N_2O_3$: C, 56.67; H, 5.15; N, 14.43. Found: C, 56.80; H, 5.05; N, 14.39. IRν_{max}^{KBr} cm^{-1}: 3510, 3380 (OH, NH_2); 1665, 1560, 1320 (NHCO). MS *m/e* 194 (M^+), 176 (M^+-OH), 149 (M^+-COOH).

Results and Discussion

Fractions 1 and 2 obtained by silica gel column chromatography of the urinary extract were analyzed by TLC. The structures of metabolites were identified by comparison of UV and mass spectral data with those of authentic samples.

Fraction 1 contained two metabolites, M-I and M-II, which gave *Rf* values corresponding to authentic 2A4NT and 4A2NT with a much smaller spot corresponding to unchanged 2,4-DNT (Fig. 1). The mass and UV spectra of the authentic and biological samples were identical.

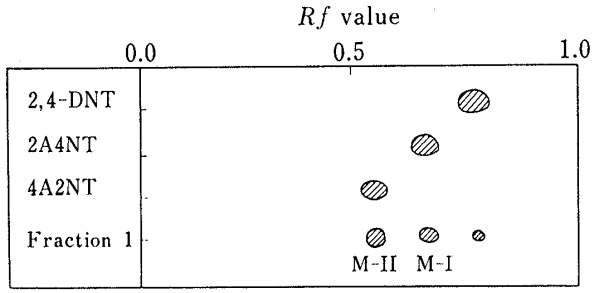

Fig. 1. Schematic Thin-Layer Chromatogram of Fraction 1 Obtained by Column Chromatography on Silica Gel

Plate, silica gel + Zn; solvent system, (A) CH_2Cl_2; detection, *p*-DMAB reagent.
Abbreviations: 2,4-DNT, 2,4-dinitrotoluene; 2A4NT, 2-amino-4-nitrotoluene; 4A2NT, 4-amino-2-nitrotoluene.

Six metabolites, M-III, M-IV, M-V, M-VI, M-VII and M-VIII, were detected chromatographically from fraction 2 (the chromatogram is not shown). By comparison of the mass and UV spectra of authentic and biological samples, the metabolites were identified.

Only one metabolite M-IX was detected chromatographically from fraction 3. It was obtained as a pure, crystalline compound with mp 194—195°, which is in good accord with the melting point of 2-amino-4-acetylaminobenzoic acid (2A4AAB), 193—194°.[10] The metabolite M-IX was characterized as 2A4AAB from the results of elemental analysis, and mass (Fig. 2) and IR (Fig. 3) spectral measurements.

During the analysis of the urinary metabolites, the possibility of the occurrence of other metabolites, including hydroxylaminonitrotoluenes as reduction intermediates of metabolites M-I and M-II, and 4-nitro-2-acetylaminotoluene, 4-amino-2-acetylaminotoluene and 4-amino-

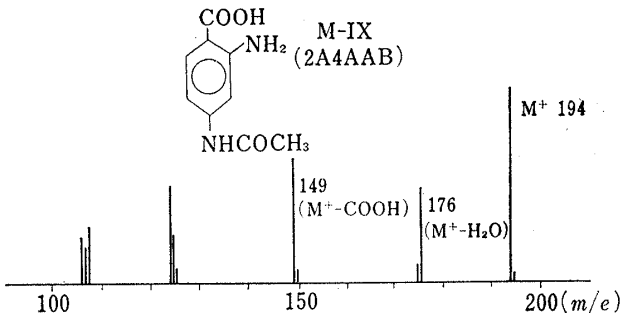

Fig. 2. Mass Spectrum of Metabolite M-IX Obtained from Fraction 3, Identified as 2A-4AAB (2-amino-4-acetylaminobenzoic acid)

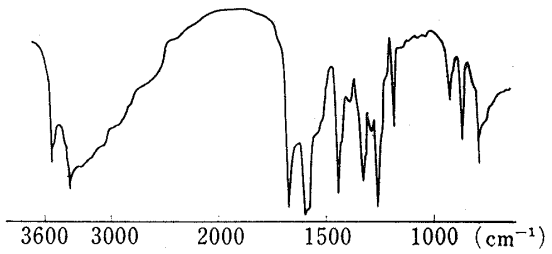

Fig. 3. IR spectrum of Metabolite M-IX Obtained from Fraction 3, Identified as 2A4AAB (2-amino-4-acetylaminobenzoic acid)

2-acetylaminobenzoic acid, (isomers of metabolites M-VII, M-VIII and M-IX), was carefully examined, but none was detected.

Previously, we demonstrated that 2A4NT and 4A2NT were formed by rat liver homogenate.[12] Glinsukon *et al.* reported that 2,4-DAT was mostly N-acetylated by hamster liver cytosol to 2-amino-4-acetylaminotoluene (2A4AAT), and to a much lesser extent to 4-amino-2-acetylaminotoluene (4A2AAT).[13] Based on the results mentioned above, the probable metabolism of 2,4-DNT in rat can be summarized as follows (Chart 1).

It is clear from Chart 1 that the primary metabolic reactions of 2,4-DNT in the rat are the reduction of a single nitro group to an amino group and the oxidation of the methyl group

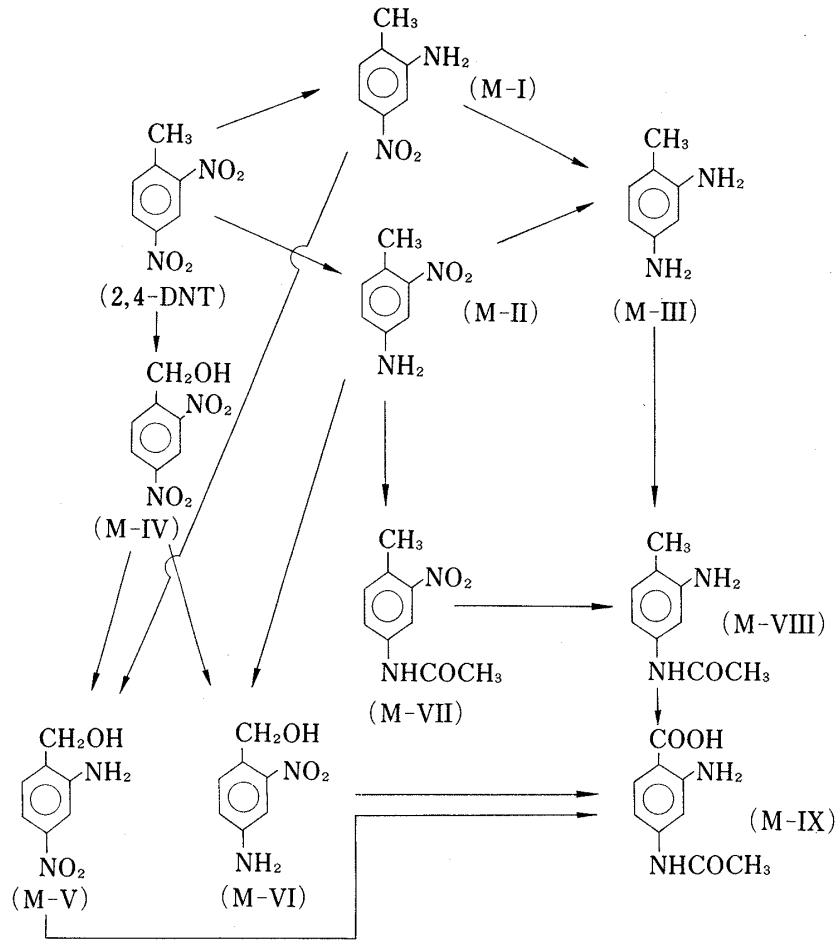

Chart 1. Probable Routes of 2,4-Dinitrotoluene Metabolism in Rats

to CH_2OH, and that N-acetylation of the amino group of the 4-position and subsequent oxidation of CH_2OH to COOH occur as secondary reactions. In addition, it was found that 2A4AAB (M-IX) was a final metabolic product, and could be obtained from the ether extract of urine samples.

Since 2,4-DAT, one of the urinary metabolites, is carcinogenic,[3-5] it is clearly of interest to determine whether 2,4-DNT itself possesses mutagenic and/or carcinogenic activities.

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