Cytosolic Acetyl Transfer and *N*-Deacetylation Associated with the Metabolism of Carcinogenic 2,4-Diaminotoluene in Rat Liver

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Acetyl transfer and *N*-deacetylation associated with the metabolism of carcinogenic 2,4-diaminotoluene (2,4-DAT) were examined by detecting the products formed during the incubation of 2,4-DAT and its known urinary metabolites with the liver cytosol fraction from male Wistar rats. Data obtained from the incubation of 4-acetylamino-2-aminotoluene (4AA2AT) with the liver cytosol in the presence of 2,4-diaminobenzoic acid (2,4-DABA) indicated that the cytosol catalyzes the acetyl transfer between 4AA2AT and 2,4-DABA to produce 2,4-DAT and 2-acetylamino-4-aminobenzoic acid (2AA4ABA). The cytosol also catalyzed the acetyl transfer between 2,4-diacetylaminotoluene (2,4-DAAT) and 2,4-DABA to produce 2-acetylamino-4-aminotoluene (2AA4AT) and 2AA4ABA. Without 2,4-DABA, the cytosol catalyzed the *N*-deacetylations of 4AA2AT and 2,4-DAAT to produce 2,4-DAT and 2AA4AT. 2AA4AT was inactive for both the *N*-deacetylation and the acetyl transfer. 2,4-DAT itself was *N*-acetylated to 4AA2AT by incubation with acetyl-CoA, but 2AA4AT and 2,4-DAAT were not detectable in the incubation product. However, both 2AA4AT and 4AA2AT were *N*-acetylated to 2,4-DAAT by incubation with acetyl-CoA. These findings suggest that 2,4-DAT is reproduced either by the *N*-deacetylation of 4AA2AT or by the acetyl transfer between 4AA2AT and 2,4-DABA in the metabolism of 2,4-DAT and further suggest that 2,4-DAAT is produced by the acetyl-CoA-dependent *N*-acetylation of 4AA2AT; 2AA4AT is produced either by the *N*-deacetylation of 2,4-DAAT or by the acetyl transfer between 2,4-DAAT and 2,4-DABA. Based on these results, the metabolic pathway of 2,4-DAT in the rat is proposed.

Key words —— carcinogenic 2,4-diaminotoluene, rat liver cytosol, acetyl transfer, *N*-deacetylation, metabolic pathway

INTRODUCTION

It has been reported that 2,4-diaminotoluene (2,4-DAT), which is an intermediate in the production of polyurethane foam and elastomer, is hepatocarcinogenic in male Wistar rats[1–3] and that it is also a potent mutagen in the Ames assay using *Salmonella typhimurium* strains.[4–6] 2,4-DAT administered to Fischer rats has been shown to be excreted in urine as 2-acetylamino-4-aminotoluene (2AA4AT), 4-acetylamino-2-aminotoluene (4AA2AT), 2,4-diacetylaminotoluene (2,4-DAAT), 4-acetylamino-2-aminobenzoic acid (4AA2ABA), 2,4-diacetylaminobenzoic acid (2,4-DAABA), α-hydroxy-2,4-DAAT, and ring hydroxylation products of 2,4-DAT.[7,8] The major urinary metabolite is 4AA2AT, which accounts for about 6% of the dose.[8] 2,4-Diaminobenzoic acid (2,4-DABA) has been thought to be the precursor of 4AA2ABA and 2,4-DAABA.[9] Since 2AA4AT, 4AA2AT, and 2,4-DAAT have been shown to be nearly nonmutagenic in the Ames assay,[5] the acetylations forming these acetylated products were thought to be the detoxification of the carcinogenic 2,4-DAT. The metabolic reaction associated with the carcinogenic action of 2,4-DAT has not been clarified.

2,4-DAT is converted to 4AA2AT by the liver cytosol fraction from Fischer rats in the presence of acetyl-CoA, but 2AA4AT and 2,4-DAAT are not detectable in the incubation product.[10] The routes of formation of 2AA4AT and 2,4-DAAT in rats have

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not yet been determined. Booth[11] has shown that the liver cytosol from Chester Beatty rats catalyzes the intermolecular acetyl transfer between *N*-hydroxyacetamido derivatives of arylamines and 4-aminoazobenzene. In addition, Bartsch *et al.*[12] have shown that the liver cytosol from CD rats catalyzes the deacetylations of *N*-hydroxyacetylaminoarenes to yield *N*-hydroxyaminoarenes. It was assumed from these observations that *N*-deacetylation and acetyl transfer producing 2AA4AT and 2,4-DAAT, in addition to the acetyl-CoA-dependent *N*-acetylation of 2,4-DAT to produce 4AA2AT, occur during the metabolism of 2,4-DAT. Moreover, if the acetylated derivatives of 2,4-DAT are deacetylated or if the acetyl groups of their acetylated derivatives are transferred to other amino compounds, carcinogenic 2,4-DAT is produced in the liver. Thus, for a better understanding the toxic action of 2,4-DAT, it appears important to determine whether the liver cytosol catalyzes the *N*-deacetylation and acetyl transfer to form 2,4-DAT.

In the present study, we examined the transfer of acetyl groups from 2AA4AT, 4AA2AT, and 2,4-DAAT to 2,4-DABA and the *N*-deacetylations of 2AA4AT, 4AA2AT, and 2,4-DAAT using rat liver cytosol. The cytosolic transfers of acetyl groups from acetyl-CoA to 2,4-DAT, 2AA4AT, and 4AA2AT were also investigated.

MATERIALS AND METHODS

Chemicals —— 2,4-DAT and acetyl-CoA were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals or solvents used were of analytical grade. 2,4-DABA, 2AA4AT, 4AA2AT, 2,4-DAAT, 2-acetylamino-4-aminobenzoic acid (2AA4ABA), 4AA2ABA, and 2,4-DAABA were prepared as described previously.[13,14] Their chemical purities and structures were confirmed by elemental analysis and ^{1}H-NMR and MS spectrometry.

Preparation of Liver Cytosol Fraction —— Male Wistar rats weighing 200–220 g (Sankyo Laboratories Co., Tokyo, Japan) were used. Animals were decapitated and the liver was removed. The livers were perfused with ice-cold 1.15% KCl and homogenated in 2 volumes of the same solution. The homogenate was centrifuged at 9000 × *g* for 45 min. The 9000 × *g* supernatant was further centrifuged at 10, 5000 × *g* for 60 min. The resulting supernatant was dialyzed at 4ºC overnight against 2500 volumes of 0.1 M sodium phosphate buffer (pH 7.4). The dialyzed supernatant was used for the enzymic incubation. The protein concentration was determined by the method of Lowry *et al.*[15]

Incubation of Cytosol with Acetylated Derivatives of 2,4-DAT in the Presence and Absence of 2,4-DABA —— The incubation mixtures contained 0.6 ml of 0.1 M sodium phosphate buffer (pH 7.4), 8 μmol of 2,4-DAAT, 4AA2AT, and 2AA4AT dissolved in 0.05 ml of dimethyl sulfoxide (DMSO), 8 μmol of 2,4-DABA dissolved in 0.05 ml of DMSO or 0.05 ml of DMSO alone, and 0.3 ml of cytosol fraction (6.6 mg of cytosolic protein). In some experiments, 1, 2, 4, 6, 8 and 10 mM of 2,4-DABA were used. Incubations were carried out at 37ºC for 0–60 min. The reaction was terminated by the addition of 2 ml of 1 M Na_2CO_3, and the reaction mixture was extracted three times with 5 ml of diethyl ether (neutral-basic fraction). The extracted aqueous layer was adjusted to pH 2 with concentrated HCl and extracted three times with 5 ml of diethyl ether (acidic fraction). These ether neutral-basic and acidic fractions were dried over anhydrous Na_2SO_4, and the solvents were evaporated under a stream of nitrogen. The residues were dissolved in 1.0 ml of methanol. Aliquots (10 μl) of these solutions were subjected to HPLC. Controls were run with buffer substituted for cytosol.

Incubation of Cytosol with 2,4-DAT, 2AA4AT, and 4AA2AT in the Presence of Acetyl-CoA —— The incubation mixtures consisted of 0.8 ml of 0.1 M sodium phosphate buffer (pH 7.4) containing 4 μmol of acetyl-CoA, 0.5 μmol of 2,4-DAT, 2AA4AT, and 4AA2AT dissolved in 0.1 ml of DMSO, and 0.1 ml of cytosol fraction (2.6 mg of cytosolic protein). Incubations were carried out at 37ºC for 0–30 min. The reaction was terminated by the addition of 2 ml of 2 M Na_2CO_3, and the reaction mixtures were extracted three times with 5 ml of diethyl ether (neutral-basic fraction). This ether fraction was dried over anhydrous Na_2SO_4 and the solvent was evaporated under a stream of nitrogen. The resulting residue was dissolved in 0.5 ml of methanol. Aliquots (10 μl) of this solution were subjected to HPLC. Controls were run with buffer substituted for cytosol.

Analysis of Products by HPLC —— HPLC analyses were conducted on a Hitachi model 655 high-performance liquid chromatograph equipped with a multi wavelength UV monitor. A reverse-phase column packed with TSK-gel ODS-80TM (4.6 mm i.d. × 150 mm, particle size 5 μm, Toso Co., Tokyo, Japan) was used with mobile phases A and B (Table 1).

Table 1. Retention Times of 2,4-DAT and its Related Compounds on HPLC

Compound		Retention Time (min) Mobile Phase[a)]	
		A	B
2,4-Diaminotoluene	(2,4-DAT)	8.7	
2-Acetylamino-4-aminotoluene	(2AA4AT)	10.0	
4-Acetylamino-2-aminotoluene	(4AA2AT)	17.5	
2,4-Diacetylaminotoluene	(2,4-DAAT)	20.4	5.5
2,4-Diaminobenzoic acid	(2,4-DABA)		4.4
2-Acetylamino-4-aminobenzoic acid	(2AA4ABA)		14.9
4-Acetylamino-2-aminobenzoic acid	(4AA2ABA)		9.8
2,4-Diacetylaminobenzoic acid	(2,4-DAABA)		27.3

a) A, 10 mM sodium potassium buffer (pH 7.4)- acetonitrile (85 : 15 v/v); B, 2 mM tetra-*n*-butylammonium bromide in water-acetonitrile (85 : 15 v/v).

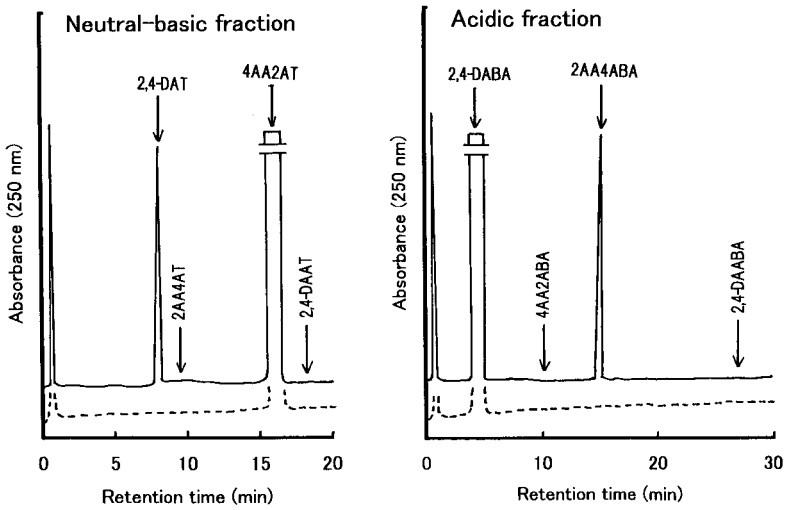

Fig. 1. High-Performance Liquid Chromatograms of Neutral-Basic Fraction and Acidic Fraction from Incubation of 4AA2AT with Male Wistar Rat Liver Cytosol Fraction for 40 min in the Presence of 2,4-DABA

Mobile phase A was used for the neutral-basic fraction; mobile phase B was used for the acidic fraction. (——), Sample; (------), control. Arrows show the retention times of the authentic compounds. 4AA2AT, 4-acetylamino-2-aminotoluene; 2AA4AT, 2-acetylamino-4-aminotoluene; 2,4-DAAT, 2,4-diacetylaminotoluene; 2,4-DABA, 2,4-diaminobenzoic acid; 2AA4ABA, 2-acetylamino-4-aminobenzoic acid; 4AA2ABA, 4-acetylamino-2-aminobenzoic acid; 2,4-DAABA, 2,4-diacetylaminobenzoic acid.

HPLC operating conditions were as follows: flow rate, 1 ml/min; UV monitor, 250 nm; column temperature, ambient. Under these conditions, the authentic compounds were separated (Table 1). Detection of products was carried out by the co-chromatography of samples and controls with authentic compounds. The limit of detection for each compound was 0.1 μg/ml. Quantities of products were determined from standard curves plotted as peak areas. A linear relationship between the amount of compound and peak area was found over the range of 0.2–200 μg/ml (data not shown). The amount of products formed was expressed as nanomoles of the product per milligram of protein.

RESULTS

As shown in Fig. 1, 2,4-DAT and 2AA4ABA were detected after incubation of 4AA2AT with the liver cytosol fraction in the presence of 2,4-DABA. However, no 2AA4AT, 4AA2ABA, or 2,4-DAABA was detectable after incubation. In addition, the incubation of 2,4-DAAT with the cytosol fraction in the presence of 2,4-DABA yielded 2AA4AT and 2AA4ABA (Table 2). No 4AA2AT, 4AA2ABA, or 2,4-DAABA was detected after incubation under the present conditions.

The production of 2,4-DAT and 2AA4ABA (Fig. 2A) increased with longer incubation periods,

Table 2. Products from the Incubation of 2,4-DAT and its Acetylated Derivatives in the Presence and Absence of 2,4-DABA or Acetyl-CoA Detected by HPLC

Incubation System	*N*-Deacetylation Product	Acetylation Product
4AA2AT + 2,4-DABA	2,4-DAT	2AA4ABA
4AA2AT alone	2,4-DAT	
2,4-DAAT + 2,4-DABA	2AA4AT	2AA4ABA
2,4-DAAT alone	2AA4AT	
2AA4AT + 2,4-DABA	n.d.	n.d.
2AA4AT alone	n.d.	
2,4-DAT + Acetyl-CoA		4AA2AT
2AA4AT + Acetyl-CoA	n.d.	2,4-DAAT
4AA2AT + Acetyl-CoA	n.d.	2,4-DAAT

n.d., not detectable.

as did that of 2AA4AT and 2AA4ABA (Fig. 2B). This finding indicates that the cytosol catalyzes the transfers of acetyl moieties from 4AA2AT and 2,4-DAAT to 2,4-DABA, producing 2,4-DAT, 2AA4AT, and 2AA4ABA. However, no metabolite was detected after incubation of 2AA4AT in the presence of 2,4-DABA.

The incubation of 4AA2AT and 2,4-DAAT with cytosol in the absence of 2,4-DABA yielded 2,4-DAT and 2AA4AT, respectively (Table 2). The production of 2,4-DAT (Fig. 2A) and 2AA4AT (Fig. 2B) also increased with longer incubation periods, indicating that the cytosol catalyzes the *N*-deacetylations of 4AA2AT and 2,4-DAAT to produce 2,4-DAT and 2AA4AT. Thus the production of 2,4-DAT (Fig. 2A) and 2AA4AT (Fig. 2B) depends either on the *N*-deacetylation of 4AA2AT and 2,4-DAAT or on the acetyl transfers from 4AA2AT and 2,4-DAAT to 2,4-DABA. 2AA4AT was inactive in the cytosolic *N*-deacetylation.

Since the rate of production of 2,4-DAT and 2AA4AT is less than in the incubation with 2,4-DABA than without it (Figs. 2A and 2B), the effect of 2,4-DABA concentration in the incubations of 4AA2AT and 2,4-DAAT was examined. As shown in Figs. 3A and 3B, the production of 2,4-DAT and 2AA4AT decreased with increasing 2,4-DABA concentrations up to 10 mM, whereas the production of 2AA4ABA rose as the 2,4-DABA concentration increased. This finding, together with the results shown in Fig. 2, indicates that 2,4-DABA acts as an inhibitor of *N*-deacetylation and it also acts as an acceptor for the acetyl transfers.

2,4-DAT (0.5 μmol) was rapidly converted to 4AA2AT by the cytosol in the presence of acetyl-CoA (4 μmol) (Fig. 4). The formation of 4AA2AT reached a plateau at 30 min, but 2AA4AT and 2,4-DAAT were not detected in the incubation. This finding was in agreement with the results of Glinsukon *et al.*,[10] who showed that 2,4-DAT is selectively *N*-acetylated to 4AA2AT by Fischer rat liver cytosol in the presence of acetyl-CoA, but that no 2AA4AT or 2,4-DAAT was detectable during incubation, and that 4AA2AT and 2AA4AT were converted to 2,4-

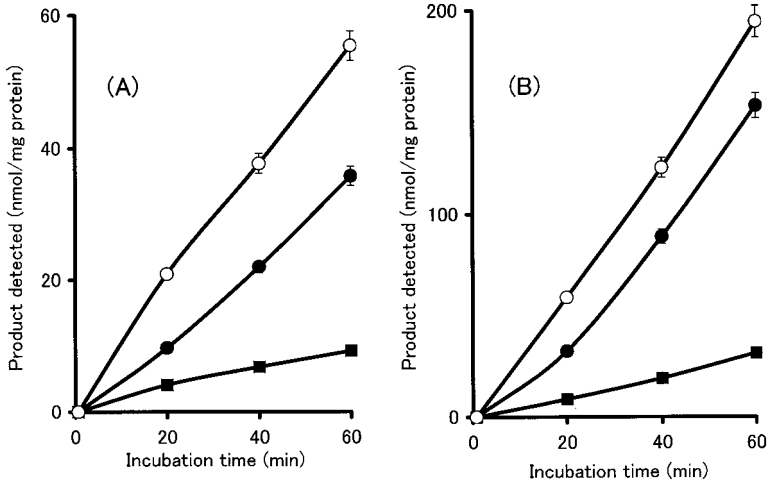

Fig. 2. Time Courses of Metabolism of 4AA2AT (A) and of 2,4-DAAT (B) by Male Wistar Rat Liver Cytosol Fraction in the Presence and Absence of 2,4-DABA

The incubation was carried out at 37°C. Each point represents the mean ± S.D. of three samples. (A), 2,4-DAT (●) and 2AA4ABA (■) produced during incubation of 4AA2AT in the presence of 2,4-DABA, and 2,4-DAT (○) produced during incubation of 4AA2AT in the absence of 2,4-DABA. (B), 2AA4AT (●) and 2AA4ABA (■) produced during incubation of 2,4-DAAT in the presence of 2,4-DABA, and 2AA4AT (○) produced during incubation of 2,4-DAAT in the absence of 2,4-DABA.

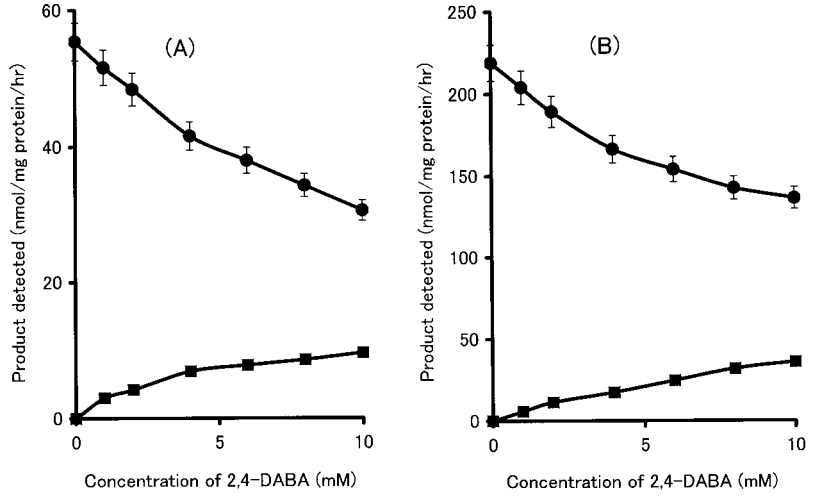

Fig. 3. Effect of 2,4-DABA Concentration on the Incubation of 4AA2AT (A) and 2,4-DAAT (B) with Male Wistar Rat Liver Cytosol Fraction

The incubation was carried out at 37°C for 60 min. Each point represents the mean ± S.D. of three samples. (A), 2,4-DAT (●) and 2AA4ABA (■) produced from the incubation of 4AA2AT. (B), 2AA4AT (●) and 2AA4ABA (■) produced from the incubation of 2,4-DAAT.

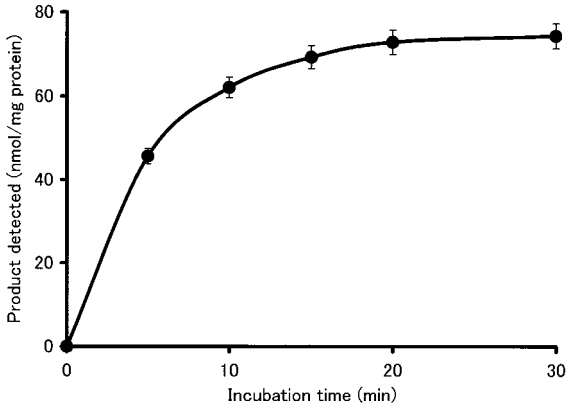

Fig. 4. Time Course of Metabolism of 2,4-DAT by Male Wistar Rat Liver Cytosol Fraction in the Presence of Acetyl-CoA

The incubation was carried out at 37°C. ●, 4AA2AT produced during incubation of 2,4-DAT. Each point represents the mean ± S.D. of three samples.

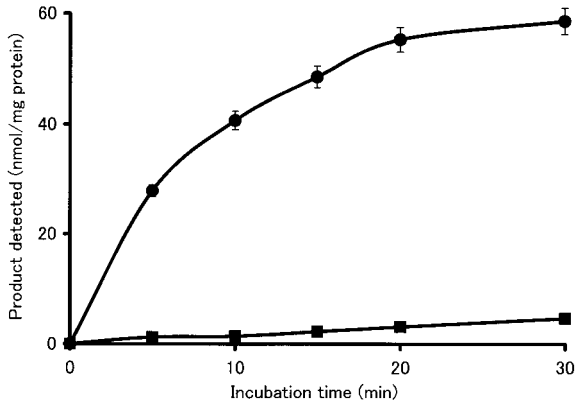

Fig. 5. Time Course of Metabolism of 2AA4AT and 4AA2AT by Male Wistar Rat Liver Cytosol Fraction in the Presence of Acetyl-CoA

The incubation was carried out at 37°C. ●, 2,4-DAAT produced from the incubation of 2AA4AT in the presence of acetyl-CoA; ■, 2,4-DAAT produced from the incubation of 4AA2AT in the presence of acetyl-CoA. Each point represents the mean ± S.D. of three samples.

DAAT by incubation of cytosol with acetyl-CoA (Fig. 5). The rate of production of 2,4-DAAT was considerably higher during incubation with 2AA4AT than with 4AA2AT, suggesting that 2AA4AT is the better substrate.

DISCUSSION

The present data indicate that 2,4-DAT is formed either by the cytosolic acetyl transfer between 4AA2AT and 2,4-DABA, or by the cytosolic *N*-deacetylation of 4AA2AT (Chart 1). The rate of production of 2,4-DAT during incubation with 2,4-DABA was higher than that of 2AA4ABA (Fig. 2A), indicating that the formation of 2,4-DAT depends more on the *N*-deacetylation of 4AA2AT than on the acetyl transfer between 4AA2AT and 2,4-DABA. In addition, the results shown in Fig. 3A indicate that the *N*-deacetylation of 4AA2AT is inhibited in the presence of 2,4-DABA. Since 2,4-DABA is thought to be formed by the oxidation of 2,4-DAT in the liver, this finding also suggests that the reproduction of

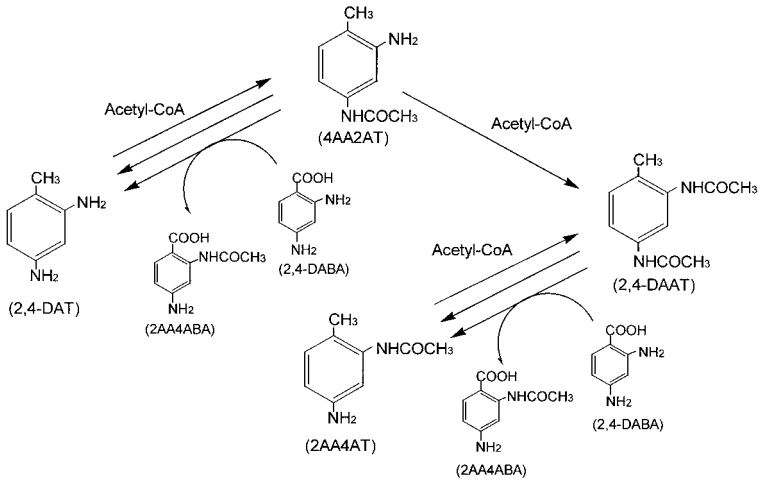

Chart 1. Proposed Metabolic Pathway of 2,4-DAT in Male Wistar Rat Liver Cytosol Fraction

2,4-DAT, 2,4-diaminotoluene; 2AA4AT, 2-acetylamino-4-aminotoluene; 4AA2AT, 4-acetylamino-2-aminotoluene; 2,4-DAAT, 2,4-diacetylaminotoluene; 2,4-DABA, 2,4-diaminobenzoic acid; 2AA4ABA, 2-acetylamino-4-aminobenzoic acid.

2,4-DAT caused by the *N*-deacetylation of 4AA2AT is stimulated by lowering the enzyme activity catalyzing the hepatic oxidation of 2,4-DAT.

The higher formation rate of 2AA4ABA during incubation with 2,4-DAAT (Fig. 2B) than with 4AA2AT (Fig. 2A) suggests that 2,4-DAAT is the better acetyl donor. The selective formation of 2AA4ABA in the acetyl transfers may due to the higher affinity of the *o*-amino group of 2,4-DABA for the enzyme catalyzing the acetyl transfers.

2AA4ABA is formed either by the incubation of 4AA2AT or by the incubation of 2,4-DAAT in the presence of 2,4-DABA (Figs. 1 and 2), but it was not detected from urine of rats given 2,4-DAT.[7,8] It remains to be determined whether 2AA4ABA is converted to 2,4-DAABA by the acetyl-CoA-dependent *N*-acetylation. However, since the results shown in Figs. 4 and 5 indicate that the *p*-amino groups of 2,4-DAT and 2AA4AT are selectively *N*-acetylated by incubation with cytosol in the presence of acetyl-CoA, the absence of 2AA4ABA in the urine may due to the rapid *N*-acetylation of 2AA4ABA to 2,4-DAABA.

It was thought that the formation of 2,4-DAAT from 2,4-DAT proceeds via 4AA2AT, not via 2AA4AT (Chart 1), because the product formed from incubation of 2,4-DAT with acetyl-CoA was 4AA2AT alone (Fig. 3), and because 4AA2AT could be converted to 2,4-DAAT by incubation with acetyl-CoA (Fig. 4). In addition, the finding that 2AA4AT was produced during the incubation of 2,4-DAAT with cytosol in the presence and absence of 2,4-DABA (Table 2) indicates that the formation of 2AA4AT is responsible either for the *N*-deacetylation of 2,4-DAAT or for the acetyl transfer between 2,4-DAAT and 2,4-DABA (Chart 1).

It has been shown that the acetyl-CoA-dependent *N*-acetylations of aromatic amines proceed via a two-step process involving the transfer of an acetyl group from acetyl-CoA to the enzyme, forming an acetylated enzyme intermediate (step 1), followed by the transfer of an acetyl group from the acetylated enzyme intermediate to aromatic amines (step 2),[16] Thus it is possible to assume that the rapid formation of 4AA2AT in the *N*-acetylation of 2,4-DAT (Fig. 3) and the greater production of 2,4-DAAT in the *N*-acetylation of 2AA4AT are due to the higher affinities of 2,4-DAT and 2AA4AT for the acetylated enzyme intermediate.

The enzyme activity that catalyzes the *N*-deacetylation of various aromatic arylacetamides has been shown to be present mostly in the microsomal fraction of liver from various animal species, including rat and guinea pig,[17–20] while the enzyme activity catalyzing the *N*-deacetylation of 2,4-DAAT has been shown to be localized mainly in the cytosolic fraction of mouse livers.[21] Our preliminary experiment also revealed that the activity catalyzing the *N*-deacetylations of 2,4-DAAT and 4AA2AT is much lower in the incubation with the microsomal fraction than with the cytosolic fraction (data not shown). These observations suggest that the enzyme activity catalyzing the *N*-deacetylation of 4AA2AT and 2,4-DAAT is somewhat different from that catalyzing the *N*-deacetylation of arylacetamides such as 4-acetylaminobiphenyl and 2-acetylaminofluorene.

The activity catalyzing the deacetylation of hydroxamic acid has been shown to be eluted from Sephadex G-75 columns with activity catalyzing the transacetylation between hydroxamic acids and arylamines, suggesting that these two activities represent expression of the same enzyme.[12] 2,4-DAAT and 4AA2AT are the substrates for acetyl transfers and are also the substrates for *N*-deacetylation (Table 1). 2AA4AT is inactive for both acetyl transfer and *N*-deacetylation (Table 1). These observations suggest that the enzyme(s) catalyzing the acetyl transfer and *N*-deacetylation have similar substrate specificity. Thus it is likely that a systematic study including purification of the cytosolic enzyme(s) catalyzing the acetyl transfer and *N*-deacetylation would reveal the enzymatic similarity.

In conclusion, our results show that 2,4-DAAT produced during the metabolism of 2,4-DAT depends on the *N*-acetylation of 4AA2AT, and the formation of 2AA4AT depends either on the *N*-deacetylation of 2,4-DAAT or on the acetyltransfer of 2,4-DAAT. The results also suggest that the reproduction of 2,4-DAT caused either by the *N*-deacetylation of 4AA2AT or by the acetyl transfer of 4AA2AT occurs during the metabolism of 2,4-DAT. These results provide fundamental information for understanding the toxic action of 2,4-DAT and its detoxification.

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