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119. Keijiro Samejima, Zenzo Tamura, and Morizo Ishidate: Metabolism of 4-Dimethylaminoazobenzene and Related Compounds. IV.*1 Metabolites of *o*-Aminoazotoluene in Rat Bile.

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Biliary metabolites of *o*-aminoazotoluene (OAT), *i.e.* 4-amino-3,2′-dimethylazobenzene, were investigated in rat and identified as N-glucuronide of OAT, N-glucuronide of OAT-derivative whose 2′-methyl group was oxidized hydroxymethyl, conjugates of 4′-hydroxylated OAT with sulfuric acid or glucuronic acid, similar conjugates of 4′-hydroxylated and N-acetylated OAT, and a double conjugate of 4′-hydroxylated OAT with N-glucuronic acid and O-sulfuric acid.

The method for determination of OAT-NG, a main metabolite, in which an internal standard substance was adopted to thin-layer chromatography, was examined and gave good results. Similar methods were also examined for determination of other minor metabolites.

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Previous work[1] in this series has shown the metabolic fate of 4-dimethylaminoazobenzene (DAB) in the bile of a living rat.

The present work deals with biliary metabolites of *o*-aminoazotoluene (OAT), *i.e.* 4-amino-3,2′-dimethylazobenzene, in the rat, and a method for determination of its main metabolite.

o-Aminoazotoluene was found by Sasaki and Yoshida[2] to induce liver tumors in rats, and this was the first proof of chemical carcinogen. Since that, the carcinogenic activity of OAT has been established with rat, dog and mouse. Comparatively more studies have been made on that for mouse, since OAT is very active in this species.[3~5] It was recognized that OAT induced hepatoma more easily in female mice than in males, while the livers of male rats were more susceptible to the carcinogenic action of DAB than those of females. Studies of the carcinogenic activity of isomeric aminoazotoluenes[6] and OAT derivatives such as N-methylated[7] or N-acetylated OAT[8] on rat and mouse have already been carried out by many other investigators to show that OAT itself is the most effective carcinogen.

One way to explain the complexity of azo dye carcinogenesis, concerning species or sex linked difference of animals and a variety of structure of azo dyes, is an investigation of metabolic features of these dyes. Although great majority of work has been done on the metabolism of DAB,[9~12] little attention has been paid to that of OAT, because it is less easily able to induce tumors on the rat and because "the

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presence of the methyl substituents in the aromatic rings makes it more difficult to prepare derivatives or potential metabolites."[13] The only one metabolite so far identified from the urine of rabbits administered OAT was 2-methyl-N,N'-diacetyl-*p*-phenylenediamine[14] formed by reductive fission of the azo bond. The experiments described below deal with OAT metabolites retaining azo bond in rat bile.

Materials and Methods

Animals——Donryu male and female rats weighing about 250~300 g. were used. They were fed the usual cube diet (CE-2).

Administration of OAT and Collection of Bile——Fifteen milligrams of OAT (commercial source), which was purified on alumina column with solvent system of petr. benzin and benzene, and recrystallized two times from petr. benzin, m.p. 105~106°, was dissolved in one ml. of olive oil. The OAT-olive oil solution was injected into a stomach of rat through a catheter. The rat was operated as previously described,[2] and the bile was collected for 24 hr. by the method of Ishidate and Watanabe.[15] An equal mixture of the intact bile and *n*-PrOH was successfully concentrated under reduced pressure without bubbling over.

Enzymatic Hydrolysis——Glucuronides were incubated with 0.01 ml. of β-glucuronidase of step 4 by Fishman, *et al.*,[16] in 10 ml. of 0.067*N* acetate buffer (pH 5.0) at 38° for 2 hr. Sulfates were incubated with 5 mg. of Takadiastase obtained from Sankyo Co., Ltd. in 10 ml. of 0.08*N* acetate buffer (pH 5.5) at 38° for 2 hr.

Paper Chromatography——Paper chromatography was carried out with Toyo Roshi No. 51A filter paper (2 × 40, 40 × 40 cm.), by the ascending development with solvent systems (I) PrOH-BuOH-H_2O(2:3:5) and (II) BuOH-AcOH-H_2O(4:1:5).

Thin-layer Chromatography[17]——The glass plate (5 × 20 cm.) was covered with Silica gel G or H (Merk Co., Ltd.). Thin-layer chromatography (TLC) was carried out with solvent systems of benzene, benzene-acetone (3:1), benzene-acetone (5:1), benzene-AcOEt (1:2), benzene-AcOEt (1:1), $CHCl_3$-MeOH-Me_2CO (10:3:6).

Staining Reagents——2*N*-HCl, Ehrlich's reagent, Gibbs' reagent, *p*-dimethylaminocinnamaldehyde (*p*-DCA) were properly used as spraying reagents for paper or thin-layer chromatogram.

Apparatus——The spectrophotometer of Hitachi EPU-2 Cary Medel 11 was used for all colorimetric and spectral determinations. The infrared spectra were obtained by Koken DS-301 or Hitachi EPI-S2 according to the method of KBr micro tablet. Toyo Roshi Co. Type C was used for paper electrophoresis.

Preparation of Compound

4-Acetamido-4'-hydroxy-2',3-dimethylazobenzene (I)——4-Nitro-*o*-acetotoluidine (m.p. 208°) which was prepared by the method given in the literature was reduced in EtOH using Pd-C as the catalyst. After filtration of the catalyst, EtOH was distilled off *in vacuo* in N_2 atmosphere. The residual substance, 4-amino-*o*-acetotoluidide (m.p. 143°) (1.0 mole), was diazotized in the usual manner. The diazotized solution was then poured into a solution of *m*-cresol (1.2 mole) in water and AcONa. After the mixture was allowed to stand overnight in a refrigerator, dark brown crystals were precipitated, which were collected, washed with water and recrystallized from EtOH-water. m.p. 210°. *Anal.* Calcd. for $C_{16}H_{17}O_2N_3$: C, 67.82; H, 6.05; N, 14.83. Found: C, 67.58; H, 5.94; N, 14.60. I was dissolved in 2*N* NaOH and the solution was refluxed in N_2 atmosphere for about 1 hr. The reaction mixture was cooled and neutralized carefully with 2*N* HCl. The yellow substance, 4-amino-4'-hydroxy-2',3-dimethylazobenzene (II), was then precipitated, which was recrystallized from EtOH-water to yellow plates. m.p. 158°. *Anal.* Calcd. for $C_{14}H_{15}ON_3$: C, 69.69; H, 6.27; N, 17.42. Found: C, 69.50; H, 6.06; N, 17.17.

4-Amino-5-methoxy-2',3-dimethylazobenzene (III)——The diazo solution obtained by diazotization of 0.3 g. of *o*-toluidine in 0.96 ml. of conc. HCl and 3.5 ml. of water with 0.2 g. of $NaNO_2$, after addition of a small amount of ammonium sulfamate, was poured into a solution of 0.38 g. of 2-amino-3-methylanisole which was prepared by a method similar to that described by Hodgson[18] (m.p. 30°), in 0.5 ml. of AcOH with ice cooling. 0.5 g. of AcONa was added to the mixture. The reaction mixture was allowed to stand

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16) W. H. Fishman, P. Bernfeld: Methods in Enzymology, **1**, 262 (1955).
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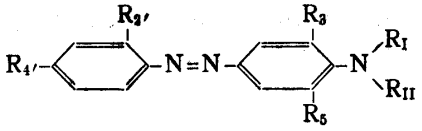
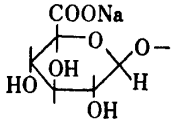
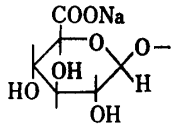


	Abbreviated formulae	R_I	R_{II}	$R_{2'}$	$R_{4'}$	R_3	R_5
	OAT	H	H	CH_3	H	CH_3	H
I	4'OH-OAT-NAc	H	CH_3CO	CH_3	OH	CH_3	H
II	4'OH-OAT	H	H	CH_3	OH	CH_3	H
III	5MeO-OAT	H	H	CH_3	H	CH_3	OCH_3
IV	5MeO-OAT-NAc	H	CH_3CO	CH_3	H	CH_3	OCH_3
V	2'CH_2OH, 3CH_3-AB	H	H	CH_2OH	H	CH_3	H
VI	2'COOH, 3CH_3-AB	H	H	COOH	H	CH_3	H
VII	4NO_2, 2',3-diMe-AzB	O	O	CH_3	H	CH_3	H
VIII	OAT-NG	H		CH_3	H	CH_3	H
IX	4'OS-OAT	H	H	CH_3	OSO_3Na	CH_3	H
X	4'OS-OAT-NG	H		CH_3	OSO_3Na	CH_3	H
	AB	H	H	H	H	H	H
	DAB	CH_3	CH_3	H	H	H	H

two days in a refrigerator. Dark brown crystals were precipitated, which were then collected, washed with water and recrystallized from EtOH–water to give brown columns. m.p. 67°. *Anal.* Calcd. for $C_{15}H_{17}ON_3$: C, 70.56; H, 6.71; N, 16.46. Found: C, 70.37; H, 6.39; N, 16.56. III was treated with Ac_2O to obtain 4-acetamido-5-methoxy-2',3-dimethylazobenzene (IV) which was recrystallized from MeOH. m.p. 178°. *Anal.* Calcd. for $C_{17}H_{19}O_2N_3$: C, 68.66; H, 6.44; N, 14.13. Found: C, 69.35; H, 6.87; N, 14.79.

4-Amino-2'-hydroxymethyl-3-methylazobenzene (V)——*o*-Nitrobenzylalcohol was reduced with Na_2S to obtain *o*-aminobenzylalcohol (m.p. 85°). The diazo solution, obtained by diazotization of 0.308 g. of *o*-aminobenzylalcohol in 0.6 ml. of conc. HCl and 6.5 ml. of water with 0.2 g. of $NaNO_2$, was poured into a solution of 0.625 g. of sodium *o*-toluidinomethanesulfonate and 1.025 g. of AcONa with ice cooling (pH about 5.5). The reaction mixture was kept in the refrigerator for two days, and a tan precipitate was collected. The precipitate was dissolved in 50 ml. of 2*N* NaOH, and an equal volume of toluene was added to the alkaline solution. This was heated at 100° for about ten minutes, then the hydrolyzed product removed to the toluene layer. The organic layer was separated, washed with water, and toluene was distilled off *in vacuo* in N_2 atmosphere. The oily substance obtained was chromatographed over alumina using solvent systems of benzene–acetone (2:1) and AcOEt. After removal of organic solvent from main azo dye fraction orange yellow columns were obtained, which were recrystallized from EtOH–water. m.p. 109°. *Anal.* Calcd. for $C_{14}H_{15}ON_3$: C, 69.69; H, 6.27; N, 17.42. Found: C, 70.12; H, 6.46; N, 17.11.

4-Amino-2'-carboxyl-3-methylazobenzene (VI)——Anthranilic acid was diazotized in the usual manner and the diazotized solution was poured into a solution of sodium *o*-toluidinomethanesulfonate in water and AcONa. After the mixture was allowed to stand for two days in a refrigerator, a tan precipitate was collected. The precipitate dissolved in 2*N* NaOH was heated on boiling water bath for 20 minutes, then the alkaline solution was cooled and neutralized with 2*N* HCl until slight acidity (pH 3.0). The contents were extracted with AcOEt. After several washing of the AcOEt layer with water, the organic solvent was distilled off *in vacuo* in N_2 atmosphere. The residual brown powder was recrystallized from AcOH–H_2O. m.p. 225° (decomp.). *Anal.* Calcd. for $C_{14}H_{13}O_2N_3$: C, 65.87; H, 5.13; N, 16.46. Found: C, 65.78; H, 5.33; N, 16.50.

4-Nitro-2',3-dimethylazobenzene (VII)——2-Nitro-5-nitrosotoluene, which was synthesized by the method given in the literature, was heated with *o*-toluidine in AcOH at 100° for about an hour. The contents were extracted with benzene, and the extracts were washed with water, 2*N* HCl, water, 2*N* alkaline, water alternately and benzene removed in a reduced pressure. The residue was chromatographed over alumina using the solvent system of benzin–benzene (1:1). The first eluting azo dye fraction was crystallized. m.p. 107°. *Anal.* Calcd. for $C_{14}H_{13}O_2N_3$: C, 65.87; H, 5.13; N, 16.46. Found: C, 66.06; H, 5.16; N, 16.53

1-Deoxy-1-(4-*o*-tolylazo-*o*-toluidino)-*α*-D-glucopyranuronic Acid Sodium Salt (VIII)——1.2 g. of OAT dissolved in 14 ml. of acetone and 0.4 g. of sodium glucuronate dissolved in 10 ml. of ethyleneglycol, were mixed and a catalytic amount of acetic acid (1 drop of 30% AcOH) was added to the mixture. After slight warming of the mixture for about 10 minutes at 40°, it was allowed to stand at room temperature for 2 days. By addition of about 100 ml. of acetone to the reaction mixture, crystals were precipitated. The crystals collected by centrifugation were washed with acetone several times till OAT was not recognized in the acetone layer. The acetone-washed crystals were then dissolved in small amount of water and reprecipitated from aqueous acetone. m.p. 180~185°(decomp.). *Anal.* Calcd. for $C_{20}H_{22}O_6N_3Na$: C, 56.70; H, 5.20; N, 9.95. Found : C, 55.63; H, 5.89; N, 9.15. This was easily decomposed to OAT and sodium glucuronate in an acid pH.

4-Amino-4'-hydroxy-2',3-dimethylazobenzene 4'-O-ester with Sodium Sulfate (IX)——A mixture of 2 g. of II, 58 ml. of CS_2, and 29 ml. of dimethylaniline was cooled and stirred, and 1 ml. of $ClSO_3H$ was added to the mixture. It was allowed to stand at room temperature for 2 days. The red oily precipitate was collected, and dissolved in water. The solution was neutralized with $NaHCO_3$, extracted with *n*-BuOH. The extract was washed with small amount of water, and *n*-BuOH was removed under reduced pressure in N_2 atmosphere. The residue was dissolved in 0.1*N* HCl, and the solution was heated at 100° for 15 min. for the hydrolysis of *N*-sulfate, neutralized with $NaHCO_3$, and extracted with ether. The water layer was extracted with *n*-BuOH and *n*-BuOH was removed similarly. The residue was purified over alumina using MeOH. *Anal.* Calcd. for $C_{14}H_{14}O_4N_3SNa$: C, 48.98; H, 4.08. Found : C, 48.25; H, 4.50. This was easily hydrolized to 4'-OH-OAT with Takadiastase.

1-Deoxy-1-[4-(5-sulfoöxy-*o*-tolylazo)-*o*-toluidino]-*α*-D-glucopyranuronic Acid Disodium Salt (X)——0.1 g. of IX and 0.13 g. of sodium glucuronate were dissolved in the mixture of 4 ml. of ethylene glycol and 5 ml. of acetone. To the solution catalytic amount of 30% AcOH (1 drop) was added, and the reaction mixture was allowed to stand at room temperature (20~25°) for 1 day. After addition of about 50 ml. of acetone, the precipitate was collected, washed with acetone to remove small amount of ethyleneglycol and dissolved in about 10 ml. of water. The aqueous solution was then submitted to ion exchange column chromatography of TEAE cellulose (Brown Co., Ltd.) by elution with various concentration of NaCl. The fraction eluted with *N* NaCl was collected, water removed under slight alkaline medium in a reduced pressure. The concentrated aqueous solution was submitted to Sephadex G-50 (medium) column using 0.1% NH_4OH as an eluting solvent. The fraction containing X was purely separated from those of NaCl and IX by this column. During the concentration of this fraction in a reduced pressure adding PrOH, crystals were precipitated. m.p. 170~220°(gradually decomp.). *Anal.* Calcd. for $C_{20}H_{21}O_{10}N_3SNa_2$: N, 7.76. Found: N, 7.13.

Results

1. Metabolites of OAT in Rat Bile

Paper chromatograms of OAT-bile——The concentrated OAT-bile was submitted to paper chromatography with two solvent systems I and II. As shown in Fig. 1, the chromatograms indicated the existence of some acid labile metabolites in OAT-bile, since the main metabolite of Rf 0.5 in the neutral solvent system I was migrated to the solvent front in the acid solvent system II.

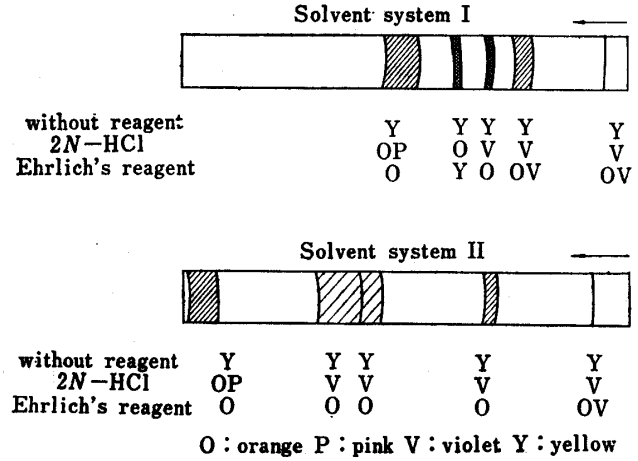


Fig. 1. Paper Chromatograms of OAT Bile and Color Reactions

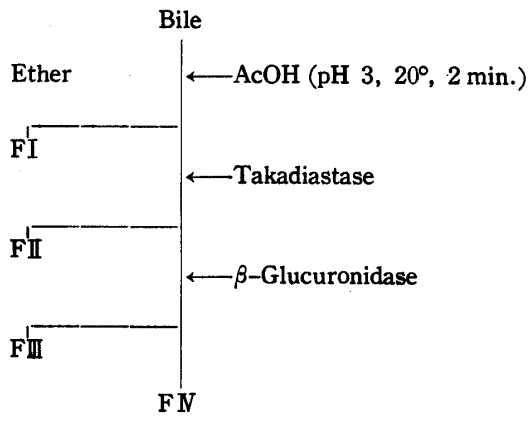


Chart 1. Diagram of Separation of Hydrolyzed Metabolites in OAT Bile

Hydrolyzed metabolites of OAT——Metabolites in OAT-bile were systematically hydrolyzed to unconjugated forms according to the usual scheme (Chart 1) and each has been identified as follows.

F-I——Azo dyes in this ether fraction which must have been acid labile forms, had no phenolic hydroxyl group, since they were not extracted with 10% NaOH. After removal of ether, the residual azo dyes were dissolved in benzene, and the solution was submitted to alumina chromatography. When an appropriate volume of benzene was passed through the column, an orange-yellow band consisting of the major azo dye moved downward. After elution of the first band, the second yellow band of a trace of azo dye was eluted by the solvent system of benzene–acetone (2:1). The first main azo dye was rechromatographed over alumina and crystallized after removal of solvent. The melting point and the ultraviolet absorption spectra of the crystalline azo dye were identical to those of OAT (m.p. 102°, λ_{max}^{EtOH} (mμ) 390, $\lambda_{max}^{HCl\text{-}EtOH}$ (mμ) 325, 400). The second minor azo dye was submitted to thin-layer chromatography with benzene–acetone (5:1). The Rf-value of 0.4 suggested an oxidation product of OAT. The most probable form in this case was a product having alcoholic hydroxyl group which would be occurred by the oxidation of either 2′- or 3-methyl of OAT. The ultraviolet spectra of this substance were very similar to those of OAT as shown in Fig. 2. The effective procedure to decide which methyl group was oxidized was the reductive fission of the azo bond with sodium hydrosulfite in alkaline solution.[17] The produced aniline part and phenylenediamine part were identified as *o*-aminobenzylalcohol and 2-methyl-*p*-phenylenediamine respectively by thin-layer chromatography (Fig. 3), although there was a trace of *o*-toluidine. Color reactions, ultraviolet spectra, and thin-layer chromatographic behavior of this isolate were also identical to the corresponding properties of synthetic 2′-CH_2OH, 3-CH_3-AB.

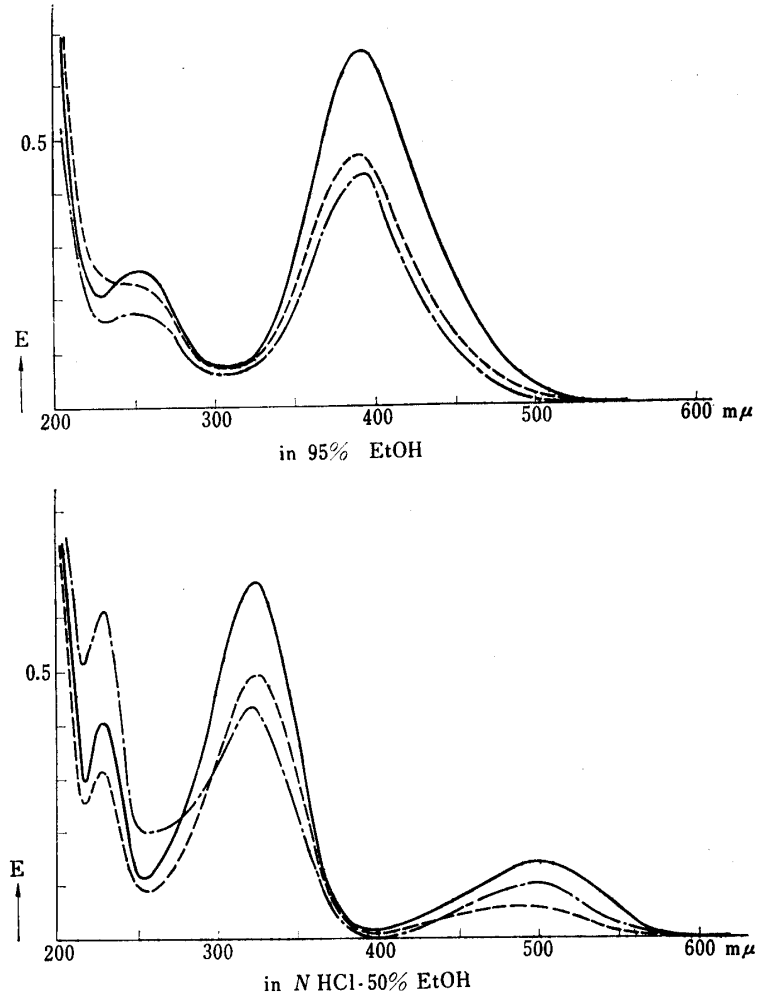


Fig. 2. Ultraviolet Spectra of the Purified Minor Azo Dye, 2′-CH_2OH, 3-CH_3-AB and OAT

——— 2′-CH_2OH, 3-CH_3-AB, – – – OAT, —·—·— purified minor azo dye

F-II——Azo dyes in this ether fraction, which were originally conjugated with sulfuric acid were almost completely extracted with 10% NaOH. The phenolic azo dyes were subjected to thin-layer chromatography to reveal two main spots of Rf 0.3

and 0.6. The lower was negative to Ehrlich's reagent and hydrolyzed with acid or alkaline to remove to the higher which was positive to Ehrlich's reagent. This suggested that the lower was *N*-acetylated product of the higher. The azo bond of the higher azo dye of Rf 0.6 was reduced with sodium hydrosulfite as described above. After the produced aminophenol part and phenylenediamine part were separated, each was identified as 4-amino-3-methylphenol or

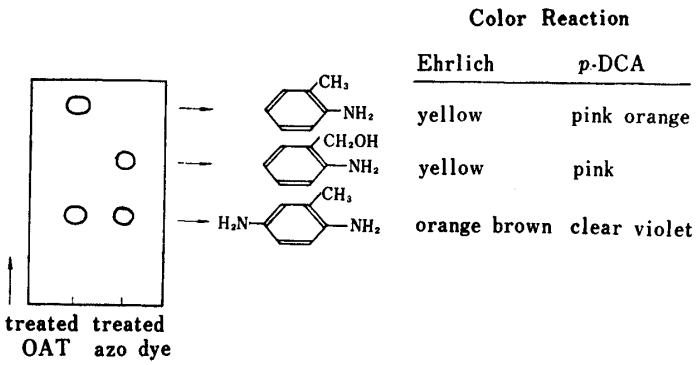


Fig. 3. Thin-layer Chromatogram of the Reduction Products of the Purified Minor Azo Dye or OAT, and Their Color Reactions

Solvent system: Benzene-AcOEt (1:2)

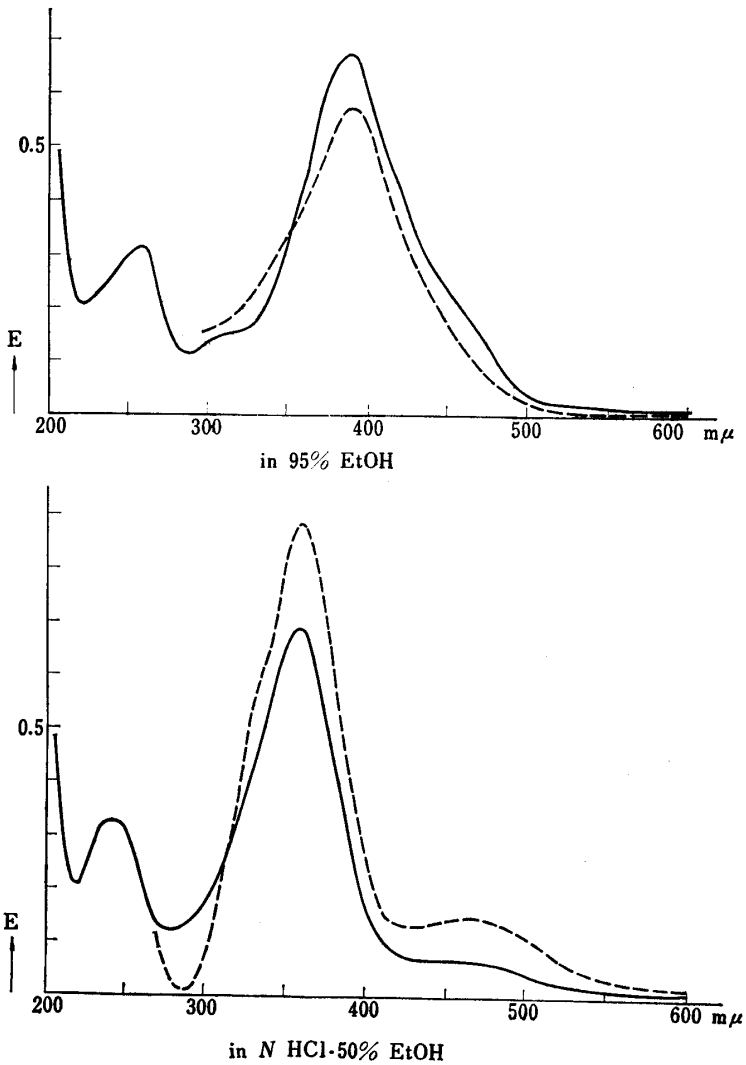


Fig. 4. The Ultraviolet Spectra of the Purified Azo Dye of Rf 0.6 and 4′-OH-OAT

——— 4′-OH-OAT, ····· purified azo dye of Rf 0.6

2-methyl-*p*-phenylenediamine respectively by thin-layer chromatography using solvent system of benzene-acetone (2:1). Accordingly the higher azo dye was 4′-OH-OAT and the lower one was 4′-OH-OAT-NAc. Color reactions, ultraviolet spectra (Fig. 4) and thin-layer chromatographic behavior of these azo dyes were respectively identical to the corresponding properties of synthetic 4′-OH-OAT and 4′-OH-OAT-NAc.

F-III——Azo dyes in this ether fraction, which were originally conjugated with glucuronic acid were extracted with 10% NaOH. The identified compounds of this fraction were completely the same as those of F-II.

F-IV——The proof indicating the existence of 4′,5-diOH-OAT was obtained in this fraction, but it was not certified.

Conjugated metabolites of OAT——The conjugation of the phenolic metabolites, 4′-OH-OAT and 4′-OH-OAT-NAc, with sulfuric acid or glucuronic acid is described in F-II or F-III. The following results are concerned with other forms of conjugation.

Isolation of OAT-N-glucuronide——As the existence of OAT-N-glucuronide (OAT-NG) was suggested from various observations such as the paper chromatogram (Fig. 1) which showed the existence of some acid labile metabolites, a major amount of OAT in F-I and of glucuronic acid which was detected by furfural method[19] from the aqueous layer, and the paper electrophoretic behavior (Fig. 5), the isolation of OAT-NG was tried as follows. About 5 ml. of bile was diluted to 50 ml. with 0.001*N* NaOH and applied on a TEAE cellulose column, 0.8 g., 12 mm. in diameter and equilibrated with 0.001*N* NaOH. By the elution with 0.1*N* NaCl in 0.001*N* NaOH, the fraction of OAT-NG was successfully eluted. The eluate was concentrated with an addition of *n*-propanol, applied to Sephadex G-50 (medium) column and eluted with 0.1% NH_4OH to separate from NaCl. The perfectly desalted fraction of OAT-NG was carefully evaporated to dryness in a reduced pressure with *n*-propanol. The residual substance was subjected to the following partition chromatography. Silica gel-celite (9:1) saturated with the lower phase of isopropylether-ethanol-water (1:1:1) was packed into a column with the upper phase. The residual substance dissolved in a small amount of the upper phase was applied to the column and separated by elution with the same solvent. The eluate containing OAT-NG was collected. Organic solvent was evaporated under reduced pressure and water was removed by lyophilization. The infrared spectra of the azo dye isolated and synthetic OAT-NG were superimposed as shown in Fig. 6 (micro IR method using KBr).

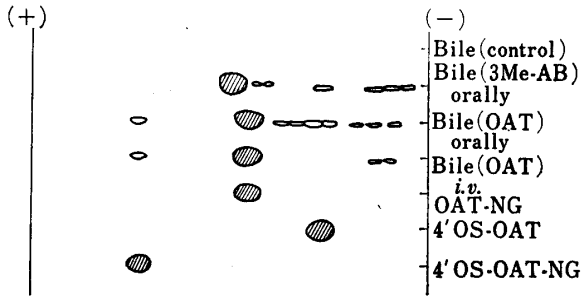


Fig. 5. Paper Electrophoresis of Biliary Metabolites and Authentic Samples
0.07*M* Acetate buffer (pH 6.2), 20 volt/cm., 6 hr.

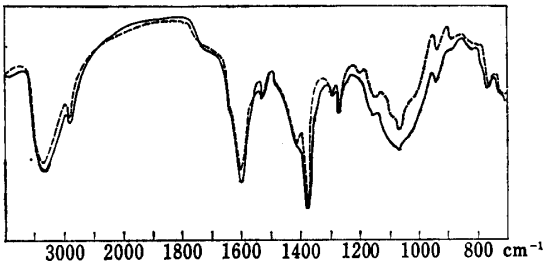


Fig. 6. Infrared Spectra of Isolated OAT-NG and Authentic OAT-NG (in KBr)
——— Isolated OAT-NG,
- - - - - - Authentic OAT-NG

Identification of other conjugated metabolites——The minor in the neutral solvent system I (Fig. 1) was cut out from the paper chromatogram, and the azo dye was extracted with 0.1% NH_4OH solution. The extract was adsorbed on TEAE cellulose column. When the concentration of eluting salt was up to 0.5*N*, a yellow band gradually moved downward and away from another smaller amount of yellow band. The faster moving azo dye fraction was collected and desalted through Sephadex G-50 (medium) column as described above. When the desalted azo dye was rechromatographed with solvent system I, two bands of Rf 0.2 and 0.3 were revealed. The higher one was extracted with 0.1% NH_4OH solution. After treatment of the extract with acetic acid, the obtained azo dye was identical to synthetic 4′-OS-OAT, and glucuronic acid was also detected paper chromatographically. This suggested the higher band of Rf 0.3 was 4′-OS-OAT-NG, a double conjugated metabolite. The paper elctrophoretic behavior of the higher one was also identical to that of synthetic 4′-OS-OAT-NG (Fig. 5).

19) M. Ishidate, S. Owari, T. Kinoshita : Yakugaku Zasshi, **80**, 1433 (1960).

2. Quantitative Analysis of the Main Metabolites

Method for determination of OAT-NG——As OAT-NG was easily hydrolyzed to OAT in a mild acidic medium, the determination of the hydrolyzed OAT was adequate to this purpose. The point of this method is that an internal standard substance was adopted to thin-layer chromatography.

E_{IS}^{t} : the total absorbance of added internal standard substance (IS) (λ_{max}^{IS}, 10 ml. of 95% EtOH)

E_{S}^{t} : the total absorbance of sample (S) (λ_{max}^{S}, 10 ml. of 95% EtOH)

E_{IS}^{obs} : the absorbance of internal standard substance extracted from TLC (λ_{max}^{IS}, 10 ml. of 95% EtOH)

E_{S}^{obs} : the absorbance of samle extracted from TLC (λ_{max}^{S}, 10 ml. of 95% EtOH)

$$E_{S}^{t}=\frac{E_{IS}^{t}}{E_{IS}^{obs}}\times E_{S}^{obs}$$

The standard method for determination was established as follows : a mixture of 1 ml. of OAT bile and 1 ml. of DAB solution dissolved in 2*N* HCl as an internal standard substance was left at room temperature for 1 hour in a 25 ml. test tube with stopper. After this hydrolyzing procedure of OAT-NG, 1.5 ml. of 2*N* NaOH was added to the reaction mixture, and with water the final volume was made up to 10 ml. The dilute alkaline solution was extracted with 10 ml. of ether. The ether was washed with 10 ml. of water and the ether virtually removed under reduced pressure in N_2 atmosphere. The condensed solution was applied in a line on a glass plate (5×20 cm.) covered with silica gel H, and thin-layer chromatography was carried out with benzene. The Rf values of DAB and OAT were 0.7 and 0.4 respectively. Each band was removed with a spatula and collected in a suction glass tube,[20] and DAB or OAT was eluted with 95% EtOH to an aliquot volume for colorimetry which was carried out with each absorption maximum (λ_{max}) (Table I). The reliability of this method was satisfactorily assured in both recovery and reproducibility as shown in Table II. An example of the application of this method to rat bile was shown in Table III. In the experiment several doses of OAT were added to rats, and each amount of OAT excreted as OAT-NG was measured.

Application of TLC method to the other OAT metabolites——2′-CH_2OH, 3-CH_3-AB which was identified in FI could be determined on the same sample solution used in OAT determination. After development with benzene, the dried plate was subjected again to thin-layer chromatography using the solvent system of benzene-ethylacetate (1:1), in which 2′-CH_2OH, 3-CH_3-AB was developed showing the Rf value of 0.7, until the solvent front approached to the line of OAT with a distance of about 1 cm. Amount of 2′-CH_2OH, 3-CH_3-AB was determined colorimetrically using DAB as an internal standard. The reproducibility of synthetic 2′-CH_2OH, 3-CH_3-AB and of a bile sample was shown in Table IV and V respectively. In the case of the latter, a marked error was observed owing to a smaller amount and a contamination of some biliary pigments.

The determination of 4′-OH-OAT or 4′-OH-OAT-NAc was also possible using 4′-OH-DAB as an internal standard with the solvent system of benzene-acetone (5:1).

20) T. Nambara, R. Imai, S. Sakurai : Yakugaku Zasshi, **84**, 680 (1964).

Table I. Ultraviolet Absorptions of OAT, 2′-CH_2OH, 3-CH_3-AB, 4′-OH-OAT and 4′-OH-OAT-NAC

	max (mμ) (in 95% EtOH)	Molar extinction coefficient (ε)
OAT	390	2.42×10^4
2′-CH_2OH, 3-CH_3-AB	393	2.36×10^4
4′-OH-OAT	390	2.58×10^4
4′-OH-OAT-NAc	365	3.12×10^4

Table II.

a) Recovery Test of Authentic OAT

Exptl. No.	E^{obs}_{OAT}	E^{obs}_{DAB}	$E^{obs}_{OAT}/E^{obs}_{DAB}$	E^{t}_{OAT}/E^{t}_{DAB}
1	0.586	0.228	2.57	
2	1.314	0.521	2.52	
3	1.550	0.605	2.56	
Average			2.55	2.56

b) Recovery Test of Authentic OAT-NG

Exptl. No.	E^{obs}_{OAT}	E^{obs}_{DAB}	$E^{obs}_{OAT}/E^{obs}_{DAB}$	E^{t}_{OAT}/E^{t}_{DAB}
1	0.340	0.611	0.557	
2	0.399	0.690	0.564	
3	0.330	0.587	0.562	
Average			0.561	0.558

(measured as OAT)

c) Reproducibility of Values of OAT/DAB obtained by the Treatment of OAT Bile with the Standard Method

Exptl. No.	1	2	3	4	5
E^{obs}_{OAT}	0.740	0.593	0.687	0.700	0.745
E^{obs}_{DAB}	0.463	0.374	0.431	0.442	0.460
$E^{obs}_{OAT}/E^{obs}_{DAB}$	1.598	1.586	1.594	1.584	1.620

Table III. Determination of OAT-NG excreted in Rat Bile

Rat (♀) No.	1	2	3	4	5	6	7
OAT added (mg.)	5	5	5	10	10	10	20
Body weight (g.)	240	220	190	240	240	253	260
Bile excreted (ml.)	8.0	12.3	12.9	7.3	12.5	22.8	20.8
OAT excreted as OAT-NG (mg.)	0.28	0.32	0.25	1.00	1.25	1.52	3.95

Table IV. Reproducibility of Authentic 2′-CH_2OH, 3-CH_3-AB

Exptl. No.	$E^{obs}_{2'h}$ [a]	E^{obs}_{DAB}	$E^{obs}_{2'h}/E^{obs}_{DAB}$
1	0.079	0.248	0.319
2	0.084	0.262	0.321
3	0.146	0.467	0.313

a) 2′h: 2′-CH_2OH, 3-CH_3-AB

Table V. Reproducibility of Biliary 2′-CH_2OH, 3-CH_3-AB and OAT

Exptl. No.	$E^{obs}_{2'h}$ [a]	E^{obs}_{OAT}	E^{obs}_{DAB}	$E^{obs}_{2'h}/E^{obs}_{DAB}$	$E^{obs}_{OAT}/E^{obs}_{DAB}$
1	0.055	0.940	0.351	0.157	2.68
2	0.061	0.930	0.342	0.178	2.72
3	0.066	0.974	0.361	0.183	2.70

a) 2′h: 2′-CH_2OH, 3-CH_3-AB

Discussion

It has been known that azo dyes were excreted in the bile of experimental animals.[21~24] The previous paper[1] of this series showed that the administration of DAB to rats resulted in the biliary excretion of DAB metabolites retaining the azo bond whereas the products formed by reductive cleavage of the azo bond were mainly excreted in the urine. In addition a large quantity of the glucuronides of hydroxylated aminoazo dyes were excreted in the bile although such compounds were not detected in the urine. These results concerning DAB were also true in the case of OAT. Recently Williams[25] suggested that molecular size and conjugation, especially glucuronic acid conjugation, played a role in biliary excretion, and for easy biliary excretion, the water-soluble polar group must be attached to a molecule of a larger molecular weight than 150. It is of interest in connection with Williams' hypothesis that OAT-NG was excreted in the bile as a main metabolite of OAT.

It has been well known that administration of aromatic amine to an experimental animal caused an increased excretion of glucuronic acid in the urine. The first discovery of acid labile aromatic amine N-glucuronide in the urine of rabbit administered aniline was done by Smith and Williams.[26] A few attempts were carried out to isolate such acid labile N-glucuronides from the urine of rabbit administered with 4,4′-diaminodiphenyl sulphone[27] and β-naphthylamine.[28] When DAB or 4′X-AB (X:F or Cl) was administered to dogs or rats respectively, N-glucuronide was observed paper chromatographically.[11,29] Comparing with these technics, those used for isolation of OAT-NG will offer more general method for isolating an acid labile aromatic amine N-glucuronide.

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26) J. N. Smith, R. T. Williams: *Ibid.*, **44**, 242 (1949).
27) S. R. M. Bushby, A. J. Woiwod: *Ibid.*, **63**, 406 (1956).
28) E. Boyland, D. Manson, S. F. D. Orr: *Ibid.*, **65**, 417 (1957).
29) M. Ishidate, Y. Hashimoto: Presented at the 83rd Annual Meeting of Pharmaceutical Society of Japan, Abstracts of papers, p. 155 (Nov. 1963, Tokyo).

The mechanism of N-glucuronide formation has not been clarified. There have been known two hypotheses in the formation of aromatic amine N-glucuronide, namely enzymatic[30,31] and nonenzymatic. It is probable that OAT-NG is formed by an enzymatic reaction, since biliary excretion of OAT-NG occurs immediately after administration of OAT to rat, while no conjugation is observed from the mixture of OAT and glucuronic acid dissolved in rat bile.

One of the main biliary metabolites of DAB was AB which was not conjugated but excreted as it was,[1] while OAT was mainly excreted as OAT-NG. These behaviors of AB and OAT suggest that there may be some relationships between the chemical structure of an aromatic amine and the formation of its N-glucuronide. The 3-methyl group of OAT may play an important role for formation of glucuronic acid conjugate of 4-amino group, since the rat administered with 4-amino-3-methylazobenzene (3-Me-AB) excreted mainly as 3-Me-AB-N-glucuronide (Fig. 5), while the rat administered AB did not excrete any N-glucuronide but mainly 4′-hydroxylated and O-conjugated metabolites. In the case of 4-aminodiphenyl,[32] the *o*-methyl derivative increased the carcinogenic activity. Thus it would be interesting to study the influence of *o*-substituents of an aromatic amine.

2′-Methyl group of OAT plays also an interesting role. A small amount of 2′-hydroxymethyl-3-methyl-AB-N-glucuronide was detected from the bile. It is well known as a detoxication mechanism that methyl group of aromatic ring is oxidized to carboxylic acid through hydroxymethyl and aldehyde. 2′-CH_2OH, 3-CH_3-AB is probably an intermediate of the oxidation. Rats administered 2′-CH_2OH, 3-CH_3-AB excreted 2′-COOH, 3-CH_3-AB-NG and larger amount of N-glucuronide of unaltered 2′-CH_2OH, 3-CH_3-AB, which showed that N-glucuronidation had predominantly occurred prior to oxidation of 2′-hydroxymethyl group. No evidence was obtained about oxidation of 3-methyl group of OAT. Such difference between 2′- and 3-methyl groups against oxidation reminds us of the larger alterations in carcinogenic activity caused by the introduction of a ring monomethyl group into DAB.[10] Carcinogenic activity of the 3′-methyl derivative of DAB was known to be about twice as active as DAB, and the activities of the 2′-methyl and 4′-methyl derivatives were one third to one half and less than one sixth respectively, and 2-methyl and 3-methyl derivatives were both inactive. This shows that the introduction of a methyl group to the ring with the dimethylamino group loses carcinogenicity, while that to the other ring maintains the carcinogenic activity of DAB. Therefore, further studies must be done for metabolites of 3′-methyl-DAB and 3,3′-dimethyl-AB.

A hydroxymethyl group is unstable in biological systems, and there are a few reports about isolation of metabolites possess such a group. Akagi[33] reported that rabbits which received a single oral dose of 2-methyl-3-*o*-tolyl-4(3*H*)-quinazoline excreted 2-methyl-3-*o*-hydroxymethylphenyl-4(3*H*)-quinazolinone as a major metabolite from the urine. And Francis,[34] using 2,3-dimethylquinoxaline-1,4-dioxide which has no antibacterial activity, isolated 2-hydroxymethyl-3-quinoxaline-1,4-dioxide which is higher active against *Salmonella dublin* and *Clostridium welchii* from sheep urine. This result proposes a working hypothesis that a hydroxymethyl group has some physiological activities against organisms.

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Considerable evidences are available indicating that N-hydroxy metabolites are proximate ones in carcinogenesis of aromatic amines. Miller[35] found AB-N-OH from the urine of experimental animals which received AB. Though N-hydroxylated metabolite of OAT was not identified in this work, Terayama[36] isolated dimer of OAT from rat liver administered OAT, which might prove indirectly the existence of OAT-N-OH as an intermediate. Further investigations are necessary for elucidation of the mechanism of OAT-carcinogenesis.

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