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22. Morizo Ishidate and Yoshiyuki Hashimoto: Metabolism of 4-Dimethylaminoazobenzene and Related Compounds. II. Metabolites of 4-Dimethylaminoazobenzene and 4-Aminoazobenzene in Rat Urine.

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In a previous work,[1] the metabolites of 4-dimethylaminoazobenzene (DAB) in the dog urine were studied and the conjugates of 3-hydroxy-4-aminoazobenzene (3-OH-AB) and 3-hydroxy-4-methylaminoazobenzene were detected as the new metabolites of DAB. The present paper describes the metabolites of DAB and 4-aminoazobenzene (AB) in rat urine.

It is well known that most of DAB administered to a rat, which is susceptible to the carcinogenic action of DAB, is excreted in the urine as the conjugated compounds of *p*-phenylenediamine, *p*-aminophenol, *o*-aminophenol, and aniline, following demethylation, hydroxylation, and reductive cleavage of azo linkage.[2] Among the metabolites which still remains the azo forms, 4-methylaminoazobenzene (MAB), AB, 4′-hydroxy-4-aminoazobenzene (4′-OH-AB), and 2′-OH-AB[3] were detected from the urine. However, identification of the last substance was rather indirect and the conjugated forms of these metabolites have not yet been studied. In the present work, the metabolites of DAB and AB which had azo forms were mainly examined, since it would be necessary to examine more thoroughly the fate of DAB in rat to elucidate the carcinogenic action of DAB in rat and to know the metabolic different species of animal.

Experimental

Methods——Wister strain male rats weighing about 150～200 g., which were fed the usual solid diet, were used. Oral administration of 10 mg. of DAB or 15 mg. of AB per rat each dissolved in 1 cc. of olive oil was given by stomach tube to a rat. The rats were housed in a metabolism cage and the urine was collected for 24 hr.

Paper chromatography and paper electrophoresis were carried out with Toyo Roshi No. 51 filter paper, by the ascending development with the solvent systems of PrOH–BuOH–H_2O (2:3:2) (solvent A) and BuOH–AcOH–H_2O (4:1:2) (solvent B), and on No. 51 paper using 0.05*M* AcOH–AcONa buffer-solution of pH 5.0 at 500 v., respectively. In order to separate the metabolites, cellulose powder zone electrophoresis was carried out with Toyo Roshi cellulose powder A and after the electrophoresis each corresponding part was taken out, packed into a column, and eluted with MeOH.

Materials——For the identification of metabolites of DAB in the urine, it is necessary to prepare many conjugated forms of aminoazobenzene derivatives and since they are usually conjugate of glucuronic acid, sulfuric acid and acetic acid, a several of these were newly synthesized in addition to the previously reported sulfates. Sulfates were prepared by the usual method described by Boyland, *et al.*[4] As the presence of a dihydroxy compound of aminoazobenzene was surmised, two compounds of this type were synthesized. The structure of these compounds is shown in Chart 1.

3-Acetoxy-4′-hydroxy-4-acetamidoazobenzene (I)——3.4 g. (0.012 mole) of 3-acetoxy-4-acetamidoazobenzene[1] in EtOH was reduced using Raney Ni W-1 as the catalyst. After filtration of the catalyst, EtOH was distilled off *in vacuo* in N_2 atmosphere. The residual oily substance was dissolved in 5 cc. of conc. HCl and 20 cc. of water and diazotized in the usual way with 1.57 g. (0.024 mole) of $NaNO_2$. After addition of a small amount of urea, the diazotized solution was poured into a solution of 3.0 g. (0.03 mole) of phenol in 50 cc. of 10% Na_2CO_3, below 5°. The yellow precipitate was

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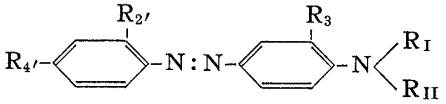
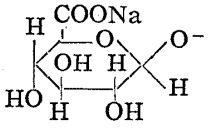
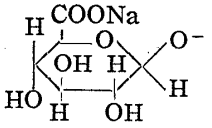
1) This Bulletin, **7**, 108 (1959).
2) J. A. Miller, E. C. Miller: Advances in Cancer Res., **1**, 366 (1953).
3) *Idem*: Cancer Research, **7**, 39 (1947).
4) E. Boyland, D. Manson: J. Chem. Soc., **1958**, 533.



(II) 3,4′-diOH-AB: R_3, $R_{4'}$; OH
(IV) 2′,4′-diOH-AB: $R_{2'}$, $R_{4'}$; OH
(V) 4′-OSNa-AB·N·Ac: R_I; CH_3CO-, $R_{4'}$; $NaSO_3O$-
(VI) 4′-OH-AB·N·SNa: R_I; $NaSO_3$, $R_{4'}$; OH



(VIII) 4′-OGNa-DAB: R_I, R_{II}; CH_3, $R_{4'}$;



(X) 4′-OGNa-MAB: R_I; CH_3, $R_{4'}$;

The other R are all H.

Chart 1.

collected, washed with dil. HCl, and then water, and dried. To remove the by-product (4-hydroxyazobenzene), the precipitate was washed with 50 cc. of EtOH and the residual yellow substance was recrystallized from hot EtOH to yellow needles (0.55 g.), m.p. 175~176°. *Anal.* Calcd. for $C_{16}H_{15}O_4N_3$: C, 61.33; H, 4.83; N, 13.41. Found: C, 61.39; H, 5.02; N, 13.37.

3,4′-Dihydroxy-4-aminoazobenzene (3,4′-diOH-AB) (II)——A suspension of 0.3 g. of (I) in 5 cc. of 10% NaOH was warmed on a boiling bath in N_2 atmosphere for 2 hr. and the solution was neutralized with dil. HCl. The yellow precipitate was collected, washed with some water, and recrystallized from hot water to yellow brown columns, m.p. 183°(decomp.). *Anal.* Calcd. for $C_{12}H_{11}O_2N_3$: C, 62.87; H, 4.84; N, 18.33. Found: C, 63.02; H, 4.71; N, 18.18. (II) is easily soluble in EtOH and MeOH, soluble in water, Et_2O, and AcOEt, and sparingly soluble in benzene.

2′,4′-Diacetoxy-4-acetamidoazobenzene (III)——7.3 g. of *p*-aminoacetanilide, suspended in 15 cc. of conc. HCl and 70 cc. of water was diazotized in the usual way with 3.5 g. of $NaNO_2$ and the solution was poured into a solution of 6.0 g. of resorcinol in an excess of 10% Na_2CO_3 with ice cooling. The precipitate was collected, dried, and washed with 100 cc. of EtOH. The EtOH-soluble fraction was acetylated with Ac_2O and AcONa on a boiling water bath. The acetate was purified by alumina chromatography using AcOEt and $CHCl_3$ as the solvent. Orange solid was obtained from the eluate and recrystallized from hot EtOH to orange needles, m.p. 175~176°. *Anal.* Calcd. for $C_{18}H_{17}O_5N_3$: C, 60.84; H, 4.82; N, 11.83. Found: C, 60.60; H, 4.73; N, 11.50.

2′,4′-Dihydroxy-4-aminoazobenzene[*2] **(2′,4′-diOH-AB) (IV)**——(III) was deacetylated by the same method for (I) and the red precipitate so obtained was recrystallized from 40% hydr. EtOH to blood-red plates, m.p. 193.5°(decomp.), easily soluble in EtOH. *Anal.* Calcd. for $C_{12}H_{11}O_2N_3$: C, 62.87; H, 4.84; N, 18.33. Found: C, 62.80; H, 4.89; N, 18.28.

Sodium 4′-(4-Acetamidophenylazo)phenyl sulfate (4′-OSNa-AB·N·Ac) (V)——2.0 g. of 4′-hydroxy-4-acetamidoazobenzene was added to a solution of 15 cc. of CS_2, 15 cc. of pyridine and 2.0 cc. of $ClSO_3H$. The solution was allowed to stand at room temperature overnight and poured into 10% Na_2CO_3. After several washing of the solution with AcOEt, fine yellow columns separated from the aqueous layer, which were washed with Et_2O and recrystallized from hot water to orange needles (2.0 g.), which gradually decomposed from 142°. *Anal.* Calcd. for $C_{14}H_{12}O_5N_3NaS$: N, 11.76. Found: N, 11.75.

Sodium 4′-(4-Hydroxyphenylazo)phenyl sulfamate (4′-OH-AB·N·SNa) (VI)——A solution of 2.5 g. of 4′-OH-AB dissolved in 7 cc. of pyridine and 1.5 cc. of $ClSO_3H$ was allowed to stand at room temperature overnight and the solution was poured into a solution of 25 cc. of water and 2.7 g. of NaOH. The yellow precipitate was collected, washed with Et_2O, and recrystallized from hot water to yellow-brown needles (1.7 g.). This substance lost its crystal water after drying over $CaCl_2$ at 90° for 6 hr. *Anal.* Calcd. for $C_{12}H_{10}O_4N_3NaS$: N, 13.33. Found: N, 13.27.

On reduction with $NaHSO_3$ in NaOH solution, (VI) gave *p*-aminophenol and sodium *p*-aminophenylsulfamate.

Methyl [4′-(*p*-Dimethylaminophenylazo)phenyltri-O-acetylglucosid]uronate (VII)—— 0.26 g. of methyl (*p*-aminophenyl tri-O-acetyl-β-D-glucosid)uronate,[*3] dissolved in 0.15 cc. of conc. HCl and 2 cc.

[*2] This compound was reported by R. Meldola (Soc., **47**, 657 (1885)), but it would be 4′,6′-bis(4-aminophenylazo)resorcinol, considering its physical properties.

[*3] This compound was prepared from the nitro compound synthesized in this laboratory.

of water was diazotized in the usual way with 0.035 g. of $NaNO_2$ in 0.5 cc. of water. After addition of 0.063 cc. of dimethylaniline in 1.0 cc. of AcOH, 0.1 g. of AcONa was added to it and the solution was kept for 1 hr. in an ice bath. The yellow precipitate formed was collected, washed with water, dried, and recrystallized from EtOH to yellow needles (0.17 g.), m.p. 204°. *Anal.* Calcd. for $C_{27}H_{31}C_{10}N_3$: C, 58.20; H, 5.57; N, 7.54. Found: C, 58.12; H, 5.58; N, 7.68.

Sodium [4′-(4-Dimethylaminophenylazo)phenyl glucosid]uronate (4′-OGNa-DAB) (VIII)——0.20 g. of (VII) was suspended in 0.2% MeONa-MeOH and the crystals turned to a powder-like yellow deposit after a while. After allowing to stand at room temperature for 3 hr., the deposit was collected, washed with benzene, and 0.15 g. of yellow powder was obtained. This was recrystallized from hot water to long yellow needles, m.p. over 260°. *Anal.* Calcd. for $C_{20}H_{22}O_7N_3Na$: C, 54.54; H, 5.22; N, 9.54. Found: C, 54.72; H, 5.12; N, 9.69.

Methyl [4′-(4-Methylaminophenylazo)phenyl tri-O-acetylglucosid]uronate (IX)——1.05 g. of methyl (*p*-aminophenyl tri-O-acetyl-β-D-glucosid)uronate was diazotized in the same way for (VIII). After addition of 0.25 cc. of methylaniline dissolved in dil. HCl, 1.0 g. of AcONa was added to the diazotized solution and the solution was kept for 1 hr. in an ice bath. The yellow precipitate was collected, washed with water, and dried. Recrystallization from benzene-petr. benzine gave the diazoamino compound (X) as pale yellow plates, m.p. 167~168°. *Anal.* Calcd. for $C_{26}H_{29}O_{10}N_3$: C, 57.43; H, 5.38; N, 7.73. Found: C, 57.50; H, 5.65; N, 7.40.

To 1.0 g. of (X), 1.5 cc. of methylaniline and 0.25 g. of methylaniline hydrochloride were added and the mixture was kept at 45° for 4 hr. with frequent stirring, then at room temperature overnight. A dark red deposit precipitate formed on addition of excess 10% HCl was collected and filtered from the mixture. The precipitate was then neutralized with 5% Na_2CO_3, and the crude base was collected, washed with water, and dried to 0.6 g. of yellow powder. It was recrystallized several times from benzene-petr. benzine with a small amount of activated charcoal to long yellow needles (IX), m.p. 167~168.5°. *Anal.* Calcd. for $C_{26}H_{29}O_{10}N_3$: C, 57.43; H, 5.38; N, 7.73. Found: C, 57.57; H, 5.27; N, 7.85.

Sodium [4′-(4-Methylaminophenylazo]phenyl glucosid]uronate (4′-OGNa-MAB) (XI)——(IX) was deacetylated by the same procedure for (VII), and the yellow-brown powder was obtained but it could not be crystallized.

Sodium [1-(N-Methyl-*p*-phenylazoanilino)-1-deoxyglucopyranosid]uronate (MAB·N·GNa) (XII)——0.1g. of methyl [1-(N-methyl-*p*-phenylazoanilino)-1-deoxy-2,3,4-tri-O-acetylglucopyranosid]uronate[5] was suspended in 3 cc. of 4% MeONa-MeOH. The original crystals dissolved after a while. After allowing it to stand at room temperature overnight, alumina chromatography was carried out using MeOH followed by MeOH-H_2O (1:1) as the solvent.

The material eluted with MeOH. The eluate from the second band was collected and the solvent was distilled off *in vacuo* in N_2 atmosphere. Orange-yellow plates obtained were dried over $CaCl_2$ at 90° for 3 hr. and melted above 200°(decomp.). *Anal.* Calcd. for $C_{19}H_{20}O_6N_3Na$: N, 10.27. Found: N, 9.97.

This compound is soluble in water, MeOH, and EtOH, insoluble in Me_2CO, Et_2O and benzene. In an acid pH, it easily decomposed to MAB and glucuronic acid. This compound was examined by zinc uranyl acetate after laying in ashes and was found to be Na salt.

(VIII) and (XI) were easily hydrolyzed on incubation with β-glucuronidase in acetate buffer of pH 5.

Sodium 4′-(4-dimethylaminophenylazo)phenyl sulfate (4′-OSNa-DAB), sodium 4′-(4-methylaminophenylazo)phenyl sulfate (4′-OSNa-MAB) and dipotassium 4′-(4-sulfoaminophenylazo)phenyl sulfate (4′-OSK-AB·N·Sk) were prepared from the corresponding hydroxyaminoazo dyes by the method of Boyland, *et al.*

Results

Nature of Urine——The color of the DAB-urine was brownish yellow and colored reddish to HCl. The urine contained a small amount of white precipitate and the pH of the urine was 5.8~6.6 (that of normal rats was 5.0~6.6). The nature of the AB-urine was similar to that of the DAB-urine. The white precipitate obtained from the both urine was recrystallized from EtOH to white columns, m.p. 315° which were identified as *p*-acetamidoacetanilide by a mixed melting point determination and paper chromatography after hydrolyzation.

Components in the Hydrolyzed Urine——Following acid hydrolysis by the same procedure as described in the previous paper, *p*-aminophenol, *o*-aminophenol, and *p*-pheny-

5) M. Ishidate, S. Takitani, T. Kishi: This Bulletin, **7**, 291 (1959).

lenediamine were detected from the DAB-urine. In this case the quantity of *p*-aminophenol was much larger than that of *p*-aminophenol and this result was reverse of the case of a dog.

Paper Chromatograms of AB- and DAB-Urine——Two-dimensional paper chromatography of AB- and DAB-urine was carried out using the solvents A and B, and the chromatograms are shown in Fig. 1.

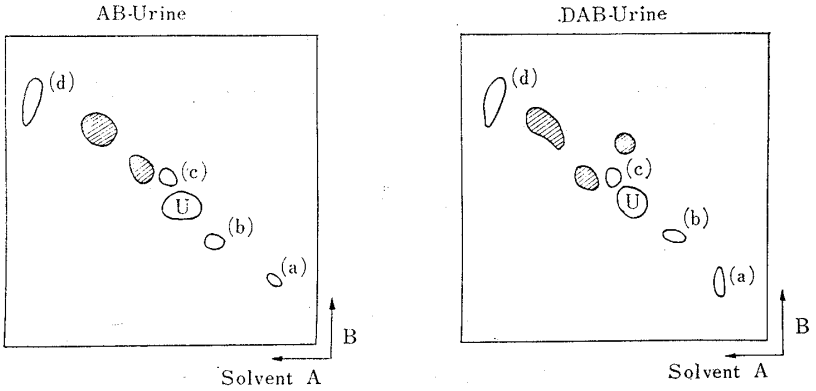


Fig. 1. Two-dimensional Paper Chromatograms of AB and DAB Urine

Shaded spots colored reddish to 2*N* HCl (azo dyes)
(a) corresponds to *p*-aminophenylglucosiduronic acid.
(b) corresponds to *p*-aminophenylsulfonic acid, (a) and (b) colored yellowish to the Ehrlich reagent.
(c) spot colored bluish to 2*N* HCl.
(d) corresponds to *p*-acetamidoacetanilide, colors orangish to the Ehrlich reagent after a while.
(U) spots of urea.

On the both chromatograms, the spots corresponding to *p*-aminophenyl glucuronide (Spot a), *p*-aminophenyl sulfate (b), and *p*-acetamidoacetanilide (d) appeared besides the spots of azo dyes. Spot C colored bluish some time after spraying 2*N* HCl (the same spot also appeared on the chromatogram of urine of normal rats). Spot U is that of urea.

Separation of Metabolites in AB- and DAB-Urine which have kept Aminoazo-Forms——130 cc. of AB-urine from 25 rats administered a total of 375 mg. of AB, 23 cc. of DAB-urine from 8 rats administered a total of 80 mg. of DAB, and 95 cc. of DAB-urine from 20 rats administered a total of 200 mg. of DAB were collected, respectively. Each urine was fractionated as follows; The urine was continually extracted with Et_2O for 6 hours and the ether-soluble substances (F-I) were fractionated into neutral, basic (F-I-1), and phenolic basic (F-I-2) fractions using 10% NaOH. Each fraction was then purified over alumina column. The residual urine from ether-extraction was extracted with 200 cc. of BuOH in 3 portions. By this procedure almost all of the azo compounds were extracted in the BuOH layer, BuOH was then distilled off *in vacuo* in N_2 atmosphere. The residual yellowish brown substance was dissolved in a mixture of MeOH-$CHCl_3$ (1:1), poured into an alumina column, and eluted successively with MeOH-$CHCl_3$ (1:1, 2:1, 4:1), MeOH, and MeOH-H_2O (2:1). The fractions obtained from the developed yellow bands were designated as AB-F-II (1~5) and DAB-F-II (1~13). After purification by paper chromatography, cellulose powder zone electrophoresis and alumina chromatography, the aminoazo component in each fraction was compared with synthetic substance by their Rf values on paper chromatography (Fig. 2, those of the synthetic aminoazo dyes are listed in Table I), the migration distances on paper chromatography (Fig. 2, Table I), and the absorption spectra (Table II). In addition, amount of each azo dye was calcu-

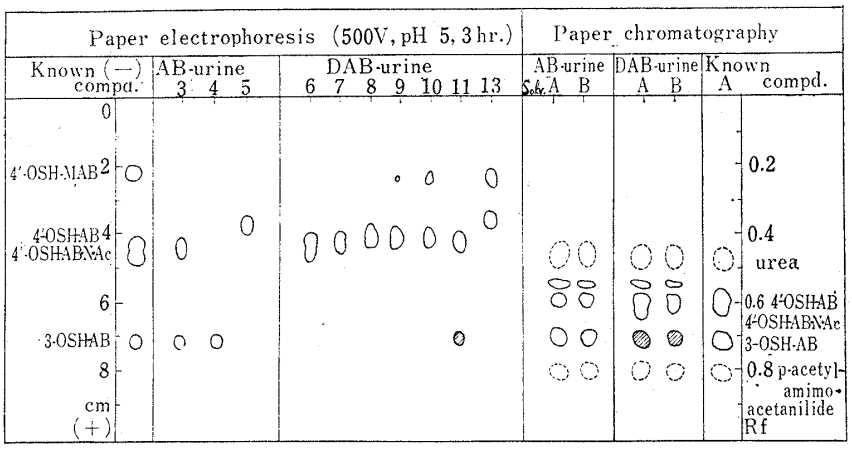


Fig. 2. Paper Electrophoretic Behavior and Paper Chromatograms of F-II Fractions of AB and DAB Urine

Paper electrophoresis and paper chromatography were carried out under the same conditions as the Table I. Solid lines represent spots which color to 2*N* HCl and shaded spots show the faint spots (c-spot; cf. Fig. 1).

Table I. Paper Electrophoretic and Paper Chromatographic Behavior of Synthetic Conjugated Dyes

Compounds	Migration toward anode (cm./3 hr.) pH					Rf Value Solvent	
	4	5	6	7	8	A	B
4'-OSK-AB	5.0	4.4	4.1	4.5	4.2	0.57	0.57
4'-OSK-MAB	2.7	2.4	2.3	2.2	2.2	0.65	0.60
4'-OSK-DAB	2.5	2.5	2.2	2.2	2.2	0.73	0.65
4'-OH-AB·N·SNa		3.9				0.53	0.47
4'-OSNa-AB·N·Ac		4.7				0.57	0.52
4'-OSK-AB·N·SK	12.2	12.7	12.0	12.0	12.4	0.36	0.23
3-OSK-AB	7.8	7.2	6.9	6.2	6.1	0.70	0.70
3-OSK-MAB	6.7	6.6	5.4	5.4	5.6	0.76	0.76
3-OSK-DAB	4.9	7.4	7.1	6.7	6.7		0.84
AB·N·SK	7.7	7.4	6.8	6.3	6.3	0.61	0.61
4'-OGNa-MAB	3.8	4.1	3.7	3.2	3.6	0.47	0.70
4'-OGNa-DAB	3.2	3.8	3.4	2.8	3.3	0.48	0.71
AB·N·GNa[5]	(0.5)	6.9	6.4	5.5	5.5	0.46	(0.91)
MAB·N·GNa	(0.5)	6.9	6.4	5.5	5.5	0.46	(0.92)

Electrophoresis was carried out on Toyo Roshi No. 51 paper (35 cm.) at 500 v. (0.4～0.7 mA./cm.) at 18～20° with ice cooling, using 0.05*M* acetate buffer (pH 4～5) and 0.04 *M* phosphate buffer (pH 6～8).

Paper chromatography was carried out on the No. 51 paper by ascending development for 15 hr.

Parenthesized values show Rf values of the decomposed substances.

lated from the molar absorptivity of the synthetic azo dyes listed in Table II.

The results and data on fractions, methods of purification, number of azo compounds contained in the fraction, Rf values on paper chromatography by solvent A and B, migration distances on paper electrophoresis, absorptions maxima in 2*N* HCl before and after hydrolysis, and identified synthetic azo dyes, are listed in Table III (AB) and IV (DAB). The percentage ratio of each dye to the amount of dye administered are also shown in these Tables.

The detailed data of these fractions were as follows :

AB-F-I-1——Azo dyes in this fraction was separated by alumina chromatography into two dyes. One was identified as AB. The other color reddish to 2*N* HCl, only after

Table II. Spectral Data of Synthetic Hydroxyaminoazo Dyes

Compound	Solvent: H_2O λ_{max} mμ ($\varepsilon \times 10^{-4}$)		N NaOH λ_{max} mμ ($\varepsilon \times 10^{-4}$)		$2N$ HCl λ_{max} mμ ($\varepsilon \times 10^{-4}$)	
4′-OH-AB			445(2.9)	Y	350(2.3)	470(0.68) O R
4′-OH-MAB			458(3.1)	Y	350(1.4)	530(2.1) P
4′-OH-DAB			463(3.2)	Y	350(1.4)	547(1.6) P
3-OH-AB			358(1.7) 467(2.8)	O	320(1.3)	505(1.9) R
3,4′-diOH-AB			462(2.2)	O	353(1.7)	480(0.81) O R
2′,4′-diOH-AB			477(2.8)	O	445(2.7)	Y
4′-OSK-AB	376(2.0)	Y			325(1.7)	502(0.82) P
4′-OSK-MAB						
4′-OSK-DAB	453(2.2)				325(0.96)	520(3.0) P
4′-OH-AB·N·SNa	365	Y			363	513 P
4′-OSNa-AB·N·Ac	348(1.9)	Y			348(2.4)	Y
3-OSK-AB	378(2.1)	Y			321(1.4)	505(2.2) P
4′-OGNa-DAB	450(2.0)	Y			338(1.2)	530(2.6) P

ε indicates molar absorptivity.
Capital letters show appearent color of the solution : Y, yellow, O, orange, P, pink, R, red.

Table III. The Metabolites of AB in the Rat Urine

Fraction No.		Methods of purification[a]	No. of azo compd.	Paper chromatography Rf values solvent A (B)	Paper electrophoresis (cm.) toward anode	Absorption max. in $2N$ HCl before hydrolysis (mμ)		Absorption max. in $2N$ HCl after hydrolysis (mμ)		Identified as	Percentage to AB given (%)
F-I	1	AC (petr. benzine, benzene)	2		0	320	500			AB	[non-conjugated] AB : 0.07
								320	500	AB·N·Ac	4′-OH-AB : 0.06
	2	AC (petr. benzine, iso-PrOH)	1		0	350	470			4′-OH-AB	
F-II	1	AC (petr. benzine, benzene)	1		0	320	500			AB	[conjugated] 4′-OH-AB : 0.01
	2	AC ($CHCl_3$, MeOH)	1		0	350	500			4′-OH-AB	3-OH-AB : 0.05
	3	PPC, CZE	2	0.58(0.60)	4.4	340	503	350	470	4′-OSH-AB / 4′-OSH-AB·N·Ac	3,4′-diOH-AB : 0.06
				0.70(0.70)	7.2	320	505	320	505	3-OSH-AB	
	4	PPC	1	0.71(0.71)	7.2	320	505	320	505	3-OSH-AB	
	5	PPC	1	0.57(0.59)	3.7	353	523	352	480	4′-OH, 3-OSH-AB	

a) AC, alumina chromatography; PPC, paper chromatography; CZE, cellulose powder zone electrophoresis.

hydrolysis, and the hydrolyzed substance was identified as AB. Therefore, this must be *p*-acetylaminoazobenzene, but the quantity was very small.

AB-F-II-3——It was found that this fraction contained two azo dyes. After seperation by paper chromatography, a compound with Rf value of 7.0 (solvent A) was identified as 3-OSH-AB from its physical properties. The compound of Rf 0.58 was surmised to be 4′-OSH-AB considering from the Rf values and orange color by the Ehrlich reagent. However, when this part of the fraction was hydrolyzed with $2N$ HCl, color of the solution changed from pink to blue and the blue color did not change to yellow even after neutralization. This phenomenon is due to a contaminant (Spot C in Fig. 1) which colors bluish on spraying of $2N$ HCl and reduces azo linkage on warming in an acid solution. But as this substance migrated much faster than the conjugated azo dyes on paper electrophoresis, a separation was carried out using cellulose powder zone electrophoresis. Fol-

Table IV. The Metabolites of DAB in the Rat Urine

Fraction No.		Methods of purification[a]	No. of azo compd.	Paper chromatography Rf values solvent A (B)	Paper electrophoresis (cm.) toward anode	Absorption max. in 2N HCl before hydrolysis (mμ)		Absorption max. in 2N HCl after hydrolysis (mμ)		Identified as	Percentage to DAB given (%)
											[non-conjugated]
F-I	1	AC (petr. benzine, benzene)	1		0	320	500			AB	AB : 0.02
F-I	2	AC($CHCl_3$, MeOH)	2		0	350	480			4′-OH-AB	4′-OH-AB : 0.16
						350	495			—	
F-II	6	CZE	1	0.62(0.60)	4.4	348		350	470	4′-OSH-AB·N·Ac	[conjugated]
F-II	7	CZE, PPC	2	0.62(0.61)	4.3	350	480	350	470	4′-OSH-AB·N·Ac 4′-OSH-AB	4′-OH-AB : 0.35
F-II	8 9	CZE	2	0.58(0.60)	4.5	350	490	350	470	4′-OSH-AB·N·Ac 4′-OSH-AB	4′-OH-MAB : 0.002
F-II	10	CZE	2	0.53(0.56)	4.4	330	500	350	470	4′-OSH-AB	3,4′-diOH-AB : 0.05
					2.5	330	505	350	530	4′-OSH-MAB	
F-II	11	CZE, PPC	2	0.60(0.60)	4.4 7.2	320	505	350	480	4′-OSH-AB 3-OSH-AB	(3-OH-AB)
F-II	13	CZE	2	0.53(0.54)	3.7	350	520	350	480	4′-OH, 3-OSH-AB	
				0.64(0.64)	2.5	350	525	350	500	(4′-OH, 3-OSH-MAB)	

a) Same as in Table III.

lowing this procedure the dye in this part of the fraction was hydrolyzed to 4′-OH-AB on warming with 2*N* HCl on a boiling bath for 15 minutes. Further, as the absorbance of the hydrolyzed solution at λ_{max} at 470 mμ was much more intense than that of the original solution, it was considered that an amino group of the azo dye must have been acetylated or sulfonated. Therefore, further purification was carried out using paper chromatography and the results of physical properties such as Rf values, migration distance in paper electrophoresis and spectrum in 2*N* HCl showed that a part of this fraction contained 4′-OSH-AB·N·Ac besides 4′-OSH-AB.

AB-F-II-4——This fraction contained only one azo substance which was identified as 3-OSH-AB.

AB-F-II-5——This fraction contained one azo dye with Rf 0.57 which was detected from a dog urine and the spot on the paper chromatogram colored purplish to 2*N* HCl. This compound was hydrolyzed on warming with 2*N* HCl on a boiling bath for 15 minutes. or on incubation with Takadiastase in an acetate buffer of pH 5, but it was not hydrolyzed by β-glucuronidase. The hydrolyzed yellow substance was extractable with EtOAc

Table V. Color Changes of Hydroxyaminoazo Dyes on Reaction with the Gibbs Reagent

Compound	NH_3 Vapor	HCl Vapor
3-OH-AB	Green violet	does not color reddish
2′-OH-AB	Dark violet	does not color reddish
4′-OH-AB	Yellow	Red
3,4′-OH-AB	Green violet	does not color reddish
3-OSK-AB	Yellow	Red
4′-OSK-AB	Yellow	Red

One drop of a sample solution is placed on a filter paper, one drop of 0.5% benzene solution of the Gibbs reagent (2,6-dichloroquinonimide chloride) is placed on the same filter paper, and the paper is exposed to ammonia vapor for a few minutes. If *para*-position of a phenol group of the dye is vacant, the reaction occurs and the original yellow color of the dye disappears and the color does not change to red even after exposing it to hydrochloric acid vapor.

from the neutral solution but not with benzene which extracted all monohydroxyaminoazobenzene derivatives. It lost the red color in an acid state by reaction with Gibbs reagent on a filter paper*4 (Table V). The absorptions spectra of the hydrolyzed dye in 2*N* HCl and 1*N* NaOH (Fig. 3) were similar to that of 4'-OH-AB and 3-OH-AB, respectively.

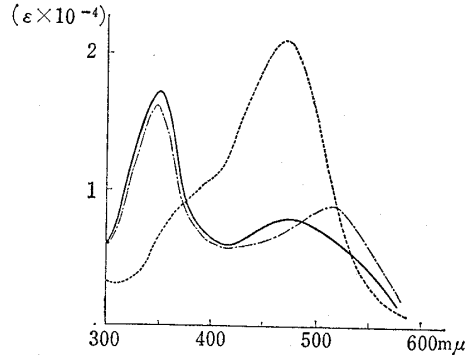
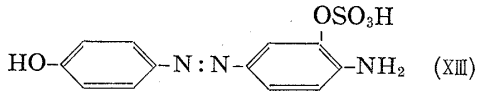


Fig. 3. Absorption Spectra of 3,4'-diOH-AB and the Conjugate in AB- and DAB-Urine

——— 3,4'-diOH-AB in 2*N* HCl.
····· 3,4'-diOH-AB in 1*N* NaOH.
—·—· the conjugate of 3,4'-diOH-AB in AB- and DAB-urine in 2*N* HCl.

These facts indicated that the hydrolyzed substance was 3,4'-diOH-AB and it was so identified with the synthetic one by its physical properties and the reaction with the Gibbs reagent. The original compound in F-II-5 is therefore consumed to be a monosulfate of 3,4'-diOH-AB (XIII), since it has a migration distance on paper electrophoresis similar to that of the monosulfate conjugates of aminoazo dyes and ultraviolet absorption maximum similar to that of 4'-OH-AB in 2*N* HCl and it is hydrolyzable by Takadiastase.



(XIII)

DAB-F-II-6——The following data indicated that the azo dye in this fraction was only 4'-OSH-AB·N·Ac. The absorption in 2*N* HCl was the same as that of synthetic 4'-OSNa-AB·N·Ac, the solution did not colored reddish until warmed, and the same phenomenon was also observed on a paper chromatogram. The other physical properties were also completely identical with those of the synthetic compound.

DAB-F-II-7～9——These fractions were shown to be a mixture of 4'-OSH-AB and 4'-AB·N·Ac like the part of AB-F-II-3 fraction.

DAB-F-II-10——The dyes in this fraction were separated into two substances, one of which was identified as 4'-OSH-AB and the other as 4'-OSH-MAB.

DAB-F-II-11——From this fraction, a spot corresponding to 3-OSH-AB was detected on paper electrophoresis but as the quantity was very small, further identification was impossible.

DAB-F-II-13——This fraction contained two azo dyes. One was identified as the dye in AB-F-II-5, 3-OSH, 4'-OH-AB (XIII), and the other, though it could not be identified, was assumed to be a sulfate of 3,4'-diOH-MAB, considering from its fraction on alumina chromatography and absorption spectra.

Conclusion and Discussion

As reported in the previous paper,[1] DAB administered to a dog is excreted in the urine as MAB, AB and conjugated forms of 3-OH-AB, 3-OH-MAB, 4'-OH-AB, and reduced amines such as *o*- and *p*-aminophenol, and *p*-phenylenediamine. In this case, it was

*4 This method was suggested by Dr. F. Feigl.

found that the quantity of *o*-hydroxyamino compounds was larger than that of *p*-hydroxyamino compounds. In the present series of experiments with rats, it was observed that AB administered to a rat was excreted in the urine as AB, O-sulfates of 4′-OH-AB, 4′-OH-AB·N·Ac, and 3-OH-AB, and *p*-acetamidoacetanilide and conjugated forms of *p*- and *o*-aminophenol. Further, as a new metabolite, the sulfate of 3,4′-diOH-AB was detected. These metabolites were also detected from the DAB-urine, in which the presence of sulfates of 4′-OH-MAB and 3,4′-diOH-MAB was assumed. In both cases, *p*-hydroxyamino compounds were excreted in the urine in larger quantities than the *ortho* compounds. Similar results have been observed in the metabolic experiments in rat with aniline,[6] *p*-aminodiphenyl, *p*-aminostilbene,[7] etc. However, it was interesting that appreciable amount of 3-OH-AB was detected from the AB-urine.

Detection of a rather large amount of non-conjugated 4′-OH-AB might be due to decomposition during the treatment, since its sulfate was rather easily hydrolyzed by acid and Takadiastase, as in *p*-aminophenyl sulfate.[8]

The quantity of azo compounds analyzed in this experiment did not show the exact percentage to the administered dyes, because the amount of total metabolites excreted in the urine for 24 hours would be lower than 50%, considering from the experiments using labeled DAB[9] and 3′-methyl-DAB.[10] From this point of view, more accurate quantitative analysis should be carried out using aniline-ring labeled DAB. In any case, the percentage of azo dyes in the urine was much smaller than that of the reduced substances such as *p*-aminophenol, *p*-phenylenediamine, and so on, and it seems not possible to consider that these azo dyes are directly responsible for carcinogenesis in a rat. However, it seems important that DAB administered to a rat was excreted in the urine in such manifold azo dye forms, considering that DAB might be resistant to detoxication by a rat and takes part in carcinogenesis in the course of the metabolism.

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Summary

The urinary metabolites of *p*-dimethylaminoazobenzene (DAB) and *p*-aminoazobenzene (AB) in a rat were examined. AB, ethereal sulfates of 4′-hydroxy-AB, N-acetyl-4′-hydroxy-AB, and 3-hydroxy-AB, *p*-acetamidoacetanilide, and conjugated forms of *p*- and *o*-aminophenol were detected from the AB-urine. As a new type of metabolite, ethereal sulfate of 3,4′-dihydroxy-AB was also detected from the urine. These metabolites were also detected from the DAB-urine, in which the presence of ethereal sulfates of 4′-hydroxy-4-methylaminoazobenzene was also assumed. For the identification of these metabolites, several sulfates and glucuronides of aminoazo dyes were newly prepared and their physical properties were examined.

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