*UDC 547.556.3 : 591.133.2*

# 23. Morizo Ishidate and Yoshiyuki Hashimoto: The Metabolism of *p*-Dimethylaminoazobenzene and Related Compounds. I. Metabolites of *p*-Dimethylaminoazobenzene in Dog Urine.

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The metabolism of *p*-dimethylaminoazobenzene (DAB), a representative carcinogenic aminoazo dye which produces hepatoma in a rat, has been studied by many investigators and reviewed by Miller and Miller.[1] However, a little has been reported on a conjugated form of urinary metabolites of DAB and difference in the metabolism among different animals which do and do not develop hepatoma by DAB.

From these points, examination was first made on the urinary metabolites of DAB in a dog. In connection with this, urinary metabolites of *p*-aminoazobenzene (AB) and 4'-hydroxy-4-aminoazobenzene (4'-OH-AB) were also examined.

## Methods

Three female mongrel dogs, having body weight of 10~15 kg., were used after vaginal operation so as to facilitate collection of the urine. Each animal was fed a constant diet of mixed cereals and fish. Aminoazo dye (DAB: 100~150 mg., AB: 200 mg., 4'-OH-AB: 200 mg.) was finely powdered in a mortar, packed in capsule, and administered orally. For examination of hydrolyzed products of urinary metabolite, the 24-hr. urine was collected in a bottle containing xylene and the solution was filtered through a cotton plug. For examination of a conjugated form of the metabolites, 3- to 7-hr. urine was collected directly from the bladder with a catheter and used after lyophilization or without any treatment.

**Paper Chromatography**—For the detection of hydrolyzed components, paper chromatography was carried out by a descending method, and for the conjugated components, by an ascending method, using Toyo Roshi No. 52 (2×40 cm.) for the one dimensional, or No. 51 and No. 51A (40×40 cm.) for the two dimensional method. Solvent systems were (A) PrOH-BuOH-$H_2O$ (2:3:5)[2] and (B) BuOH-AcOH-$H_2O$ (4:1:5).

## Materials

Melting points were measured on a Kofler micro hot-stage. DAB and AB were commercial products and recrystallized twice from EtOH. 4'-OH-AB was synthesized from N-acetyl-*p*-phenylenediamine and phenol. These aminoazo dyes were alumina chromatographically pure. For spectroscopic determination and paper chromatographic identification, the following compounds were prepared:

**3-Hydroxy-4-aminoazobenzene (3-OH-AB)**[3]—This compound was prepared by the demethylation of 3-methoxy-4-aminoazobenzene by the method of Jacobson and Hönigsberger.[4] 3-Methoxy-4-aminoazobenzene (1 g.) and $AlCl_3$ (6 g.) were mixed in a mortar and heated at 100~140° in an oil bath. After cool, 2*N* HCl was added to the mixture in small portions with stirring. The dark red substance was neutralized with $NH_4OH$ solution and extracted several times with $Et_2O$. The $Et_2O$ solution was washed with some water and extracted with 10% NaOH. The extract, containing some yellow solid, was acidified with HCl solution and again made alkaline with $NaHCO_3$. The product was collected as brown orange plates (0.6 g.) and recrystallized from toluene with activated charcoal to brown orange needles, m.p. 174~175°. *Anal.* Calcd. for $C_{12}H_{11}ON_3$: C, 67.59; H, 5.20; N, 19.71. Found: C, 67.72; H, 5.37; N, 19.90.

This dye was acetylated with $Ac_2O$ and pyridine, and the diacetate was obtained as long yellow needles, m.p. 181~182°, after the recrystallization from benzene. *Anal.* Calcd. for $C_{16}H_{15}O_3N_3$: C, 64.63; H, 5.09. Found: C, 64.68; H. 4.89.

**3-Hydroxy-4-methylaminoazobenzene (3-OH-MAB)**—One mole of 3-OH-AB was refluxed with 1

* Hongo, Tokyo (石舘守三, 橋本嘉幸).

1) J. A. Miller, E. C. Miller: Advances in Cancer Res., **1**, 366 (1953).
2) S. R. M. Bushby, A. J. Woiwod: Biochem. J., **63**, 406 (1956).
3) Among similar compounds only 3-hydroxy-4-dimethylaminoazobenzene has been synthesized by J. A. Miller, *et al* (Cancer Res., **17**, 387(1957)).
4) P. B. Jacobson, F. Hönigsberger: Ber., **36**, 4093 (1903).

mole of $CH_3I$ in MeOH for 3 hrs. After evaporation of the solvent, the reaction mixture was neutralized with $NaHCO_3$ solution and extracted with benzene. The benzene solution was poured through an alumina column and eluted with MeOH. The eluate from the second orange band was evaporated to dryness under reduced pressure in $H_2$ gas stream and a brown oil was obtained. Its spectra in 2*N* HCl (Fig. 2a) and in *N* NaOH agreed with those of the compound obtained by the hydrolysis of potassium 5-phenylazo-2-methylaminophenyl sulfate (3-$KSO_3O$-MAB).[5]

**3-Methoxy-4-methylaminoazobenzene (3-$CH_3O$-MAB)**—The diazo solution obtained by diazotization of 5.9 g. of aniline in 14 cc. of conc. HCl and 8 cc. of water with 4.4 g. of $NaNO_2$, was poured into a solution of 8.7 g. of N-methyl-*o*-anisidine in a mixture of 5 cc. of conc. HCl and 4 cc. of water, with ice-cooling. This mixture was shaken for a moment and NaOAc (4.4 g.) was added to it. After standing the solution in a refrigerator overnight, a dark red precipitate was collected, dissolved in EtOH, and the solution was extracted with $Et_2O$ after addition of $NH_4OH$. $Et_2O$ was evaporated, the residue was dissolved in petr. benzine, and the solution was poured through an alumina column, which was eluted with petr. benzine-benzene (5:1). This solidified on addition of some EtOH and recrystallized from petr. benzine to orange needles, m.p. 74°. *Anal.* Calcd. for $C_{14}H_{15}ON_3$: C, 69.69; H, 6.27; N, 17.42. Found: C, 69.95; H, 6.12; N, 17.69.

**2′-Methoxy-4-methylaminoazobenzene (2′-$CH_3O$-MAB)**—*o*-Anisidine (6.5 g.) in a mixture of water (50 cc.) and conc. HCl (17 cc.) was diazotized with $NaNO_2$ (4 g.) in $H_2O$ (25 cc.). This solution was poured into the solution of N-methylaniline (5.6 g.) dissolved in excess HCl solution, and NaOAc solution was added. The diazoamino compound (4.7 g.) so obtained was mixed with N-methylaniline hydrochloride (2.4 g.) and N-methylaniline (7.1 g.), and the mixture was warmed at 45° for 5 hrs. After standing at room temperature overnight, the mixture was poured into a dil. AcOH solution, the separated oily substance was dissolved in EtOH, and some water was added. After several hrs., reddish brown column (1.0 g.) separated and were recrystallized from petr. benzine to red plates, m.p. 90~90°. *Anal.* Calcd. for $C_{14}H_{15}ON_3$: C, 69.69; H, 6.27; N, 17.42. Found: C, 69.33, H, 6.48; N, 17.69.

**Potassium 5-Phenylazo-2-aminophenyl Sulfate (3-$KSO_3O$-AB)**—3-OH-AB (0.4 g) was reduced to a fine powder and suspended in a mixture of $CS_2$ (10 cc.) and dimethylaniline (5 cc.). Chlorosulfonic acid (0.5 g.) was added to this mixture, the mixture was allowed to stand overnight, excess of 10% $K_2CO_3$ was added, and washed with $Et_2O$. The separated yellow solid was filtered off and the filtrate was evaporated to small volume. Yellow crystalline substance separated, the solid and crystals were combined, dissolved in MeOH, and purified by alumina chromatography. Yellow plates were obtained by evaporation of MeOH eluate and recrystallized from 90% EtOH to yellow plates. *Anal.* Calcd. for $C_{12}H_{10}O_4N_3SK$: N, 12.69. Found: N, 12.52.

**Potassium 4-(4′-Aminophenylazo)phenyl Sulfate (4′-$KSO_3O$-AB)**—This compound was synthesized from 1 mole of 4′-OH-AB and 1 mole of chlorosulfonic acid in $CS_2$ and dimethylaniline, by the same procedure as that for 3-$KSO_3O$-AB. The yellow powder obtained was purified through an alumina column. Long yellow needles were obtained after evaporation of an eluate and recrystallized from 90% PrOH. *Anal.* Calcd. for $C_{12}H_{10}O_4N_3SK$: N, 12.69. Found: N, 12.78.

These sulfates gave paper chromatographically one spot.

## Experimentals and Results

**Nature of DAB-Urine**—The pH of a 24-hr. DAB-urine was 6.0~6.5 and the upper xylene layer was yellow. The urine from the bladder was yellow orange, pH 7.2~8.0.

**Components in the Hydrolyzed DAB-Urine**—Stevenson, *et al.*[6] had fractionated DAB-urine of rat and obtained *p*-aminophenol, *p*-phenylenediamine, and their acetylated derivatives. In the present case, the method was modified for the fractionation. The 24-hr. urine was collected from 2 dogs administered 100-mg. dose of DAB and extracted twice with benzene. The extract was combined with the upper xylene layer and this fraction was designated as F-1.

The urine was acidified to pH 1 with conc. HCl and extracted with $Et_2O$. Then brought to pH 8 with solid $NaHCO_3$, and again continuously extracted for 10 hrs. with $Et_2O$ (F-2). The urine was reacidified to pH 1 and boiled for 1 hr., brought to pH 8, and extracted with $Et_2O$ (F-3).

The method of Stevenson, *et al.* terminated at this stage but for the hydrolysis of glucuronides, the urine was acidified further to 3*N* HCl concentration with conc. HCl and boiled for 1 hr. Then the solution was brought to pH 8 with $NaHCO_3$ and extracted with $Et_2O$ (F-4). These fractions were separated into amine and aminophenol fractions and the fractionated substances were determined by paper chromatography with solvent B. The color of the spots was given by spraying Ehrlich's reagent (0.5% EtOH solution of *p*-dimethylaminobenzaldehyde containing 1 cc. of conc. HCl/100 cc.), salicylaldehyde reagent[7] (1% EtOH solution of salicylaldehyde containing 5% AcOH), ammoniacal $AgNO_3$, or 0.1% 2-(2-diethyl-

5) This compound was synthesized by H. Hanaki (unreported).
6) E. S. Stevenson, *et al.*: Cancer Res., **2**, 160 (1942).
7) D. Robinson, J. N. Smith, R. T. Williams: Biochem. J., **50**, 228 (1951).

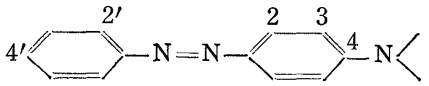
aminoethyl)-1-naphthylamine, Tsuda's reagent) in EtOH after spraying a mixture of 0.1*N* HCl and 0.1*N* $NaNO_2$. From the resulting chromatograms, following substances were detected from each fraction: *o*-aminophenol (Rf. 0.76) and *p*-aminophenol (0.68) from F-3 and F-4: *p*-phenylenediamine (0.52) from F-3, and aniline (0.86) from F-2 and F-3 fractions.

*o*-Aminophenol in F-3 (corresponding to sulfate fraction) was larger in amount than the other substances. *p*-Phenylenediamine in F-3 was also characterized by the color change from dark green to violet by 5% $FeCl_3$ solution.

F-1 fraction was dissolved in petr. benzine after evaporation of the solvent, adsorbed on alumina column, and then eluted with petr. benzine-benzene (2:1). The two yellow bands were separated and from each eluate, two components were obtained, which were identified by alumina chromatography as AB and MAB. Their absorption spectra in 50% ethanolic *N* HCl were also identified (AB: $\lambda_{max}$ 320, 500 mμ; MAB; 320, 510 mμ). AB and MAB were also detected in the 3- to 7-hr. DAB-urine. DAB was not detected in either of the cases.

**Paper Chromatography of Synthesized Sulfate of Hydroxyaminoazo Dyes**—Rf values and color reactions are listed in Table I. The ascending method was used with Toyo Roshi No. 52 paper.

TABLE I. Rf Values and Color Reactions of Hydroxyaminobenzene Sulfates




| Compound | Solvent system | | Color given by | |
|---|---|---|---|---|
| | A | B | 2*N* HCl | Ehrlich |
| 3-$KSO_3O$-AB | 0.66 | 0.69 | pink | pink orange |
| 3-$KSO_3O$-MAB | 0.77 | 0.77 | pink | pink |
| 4'-$KSO_3O$-AB | 0.62 | 0.63 | pink | orange |

**Spectral Data of Synthetic Hydroxyaminoazo Compounds**—The absorption maxima of the dyes in 2*N* HCl are shown in Table II together with the ratio of the absorbance in the ultraviolet region (A band) to that in visible region (C band).

TABLE II. Spectral Data of Synthetic Hydroxyaminoazo Dyes

| Compound | Absorption maxima in | | $\varepsilon_C/\varepsilon_A$ |
|---|---|---|---|
| | A band (mμ) | C band (mμ) | |
| 4'-OH-AB | 350 | 470 | 0.32 |
| 4'-OH-MAB | 350 | 530 | 1.7 |
| 4'-OH-DAB | 350 | 545 | 1.2 |
| 2'-OH-AB | 325 | no peak | |
| 2'-OH-DAB | 320 | 535~540 | 0.28 |
| 2'-MeO-MAB | 325 | 525 | 3.0 |
| 3-OH-AB | 320 | 505 | 1.5 |
| 3-OH-MAB | 320~330 | 505~510 | 10.0 |
| 3-MeO-AB | 320~325 | 505 | 3.2 |
| 3-MeO-MAB | 315~330 | 505~510 | 16.0 |

**Two-dimensional Paper Chromatography of Aminoazo Dye-Urine**—Three to 7-hr. DAB-, AB-, or 4'-OH-AB-urine was spotted on Toyo Roshi No. 51 (40×40 cm.) paper and two-dimensional paper chromatography was carried out, developed first with solvent A and then with solvent B or solvent A. These chromatograms are shown in Fig. 1. In the chromatogram of DAB-urine, spots Nos. I and II were deep yellow, followed by Nos. III and V, and No. IV, No. VI were rather faint yellow. In that of the AB-urine, the order of color intensity was Nos. I, II, and III, and in the 4'-OH-AB urine, No. I was deep yellow and No. II and III were faint yellow. All these spots colored yellow and changed to red on spraying of 2*N* HCl. This shows that these spots will be aminoazobenzene derivatives. In the case of Fig. 1, C, although all the spots were arrested diagonally to the paper, the spot corresponding to No. V separated into two spots and one of the spots (No. V') existed on the upper side of diagonal line of the paper. Detail about this spot will be described in a later section.

**Separation of Each Component**—The urine was spotted on a line to Toyo Roshi No. 51A (40×40 cm.) and paper chromatography was carried out with solvent A. The corresponding yellow bands were cut off and the strips were extracted with MeOH. After concentration of MeOH, each residual solution was again spotted on a line on a fresh paper and chromatographed with solvent B. From these procedures, MeOH

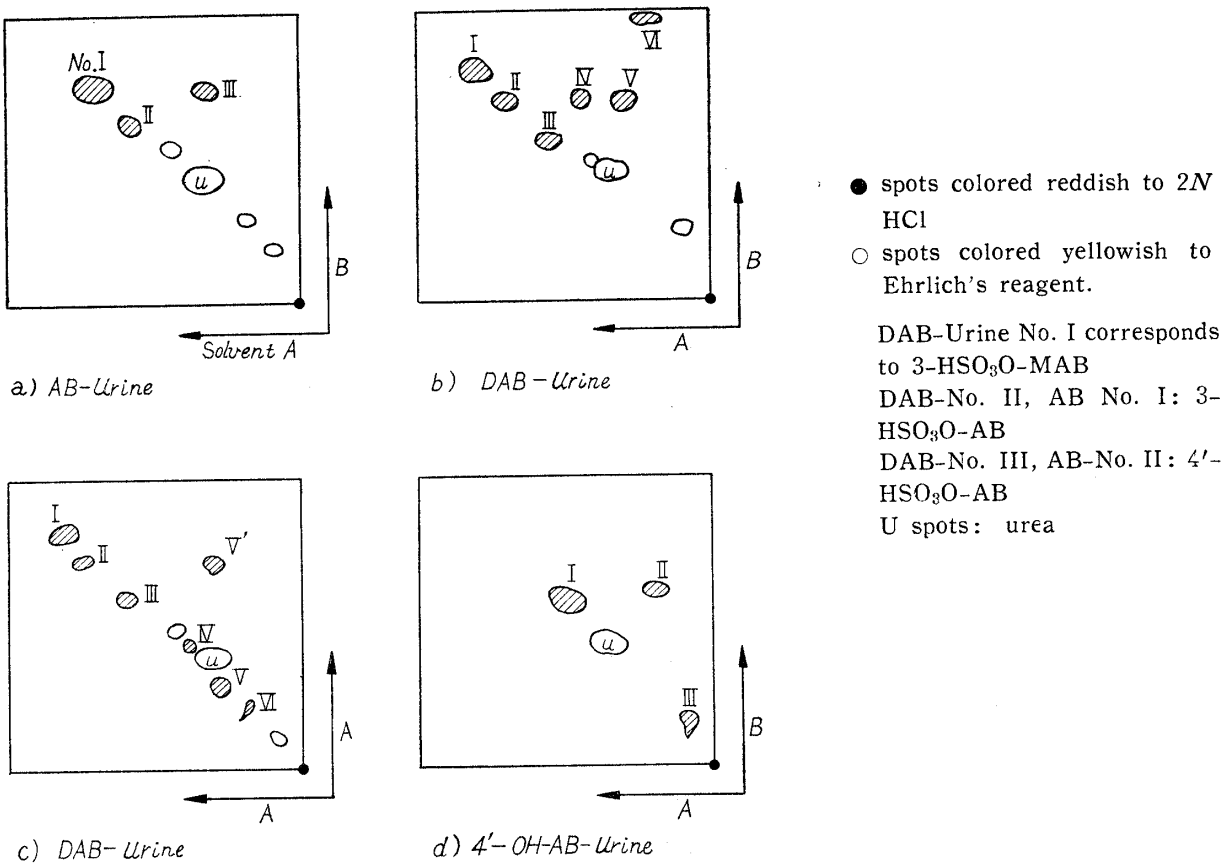



Fig. 1. Two-dimensional Chromatograms of Aminoazo Dye-Urine

solutions corresponding to DAB Nos. I, II, III, and V, AB Nos. I, II, and III, and 4'-OH-AB No. I were obtained.

These spot components were compared by paper chromatography with the synthesized aminoazobenzene derivatives of conjugated form (the above-described sulfates, and sodium AB N-glucosiduronate and sodium MAB N-glucosiduronate, and 4'-OH-DAB, 4'-OH-MAB, and 4'-OH-AB O-glucosiduronic acids.[8] As a result, the component of DAB No. I was identified with 3-$KSO_3O$-MAB, DAB Nos. II and V, AB Nos. I and III with 3-$KSO_3O$-AB, DAB No. III, AB No. II, and 4'-OH-AB No. I with 4'-$KSO_3O$-AB through their Rf values by solvent A and B.

**Hydrolysis of the Spot Components**—All these spot components were completely hydrolyzed by warming with *N* HCl for 15 mins. on a boiling water bath and were transferred into benzene layer when the solution was basified with $NaHCO_3$ and shaken with benzene. If the yellow benzene solution was extracted with 10% NaOH, the yellow component transferred into the aqueous layer which colored orange. The residual solution left after extraction with benzene became turbid when 5% $BaCl_2$ solution was added after acidification with HCl solution. From these facts, these spot components were regarded as O-sulfate conjugates of hydroxylated aminoazobenzenes.

The spectra of these hydrolyzed substances in 2*N* HCl, compared with those of the synthesized hydroxyaminoazobenzenes are shown in Fig. 2. The hydrolyzed component of DAB No. I was identified with 3-OH-MAB, those of DAB No. II and V, and AB No. I and III with 3-OH-AB, and those of DAB No. III, AB No. II, and 4'-OH-AB No. I with 4'-OH-AB.

**DAB No. V or AB No. III Spot**—Boyland, *et al.*[9] had inferred the existence of N-glucosiduronate of 2-amino-1-naphthyl hydrogen sulfate in the urine of rabbit dosed with 2-naphthylamine. A similar phenomenon could be observed in the case of metabolites of DAB in the dog urine.

DAB- or AB-urine of the dog was chromatographed with solvent A, the spot of Rf 0.4 was cut off, it was extracted with MeOH, and again submitted to paper chromatography with solvent A. Besides the original spot, a spot of Rf 0.7 appeared, while in the chromatogram with solvent B, only one spot of Rf 0.7 was shown. Further, DAB No. II or AB No. I component was dissolved in some water, excess sodium

8) These compounds were synthesized by the present authors (unreported).
9) E. Boyland, D. Manson, S. F. D. Orr: Biochem. J., **65**, 417 (1957).

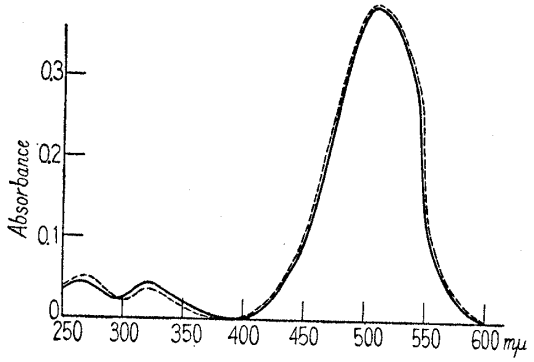
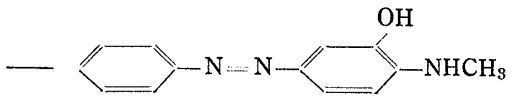
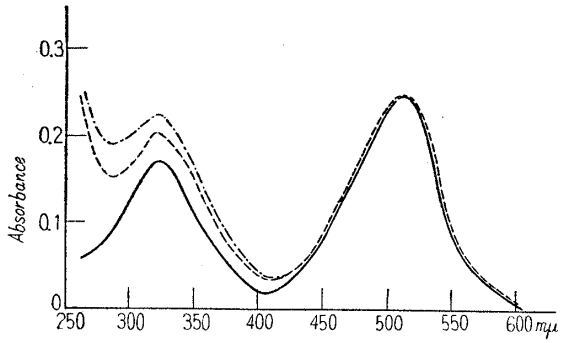



Fig. 2. Absorption Spectra of Hydrolyzed Component of Azo-Spot and Synthesized Hydroxyaminoazobenzene in 2*N* HCl

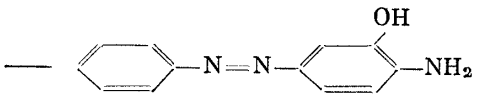
a)




---- DAB-urine No. I

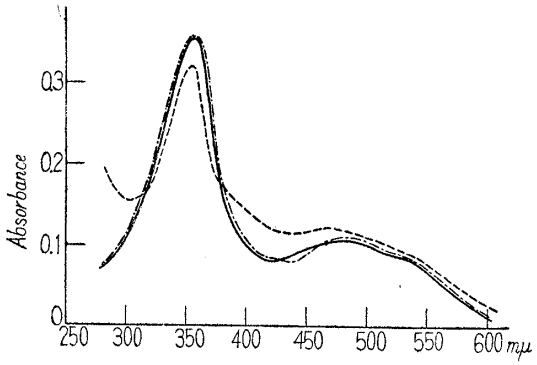



b)

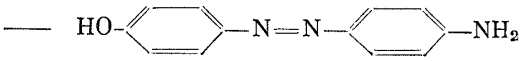



---- DAB-urine No. II (AB No. I)

-·-·- AB-urine No. III




c)



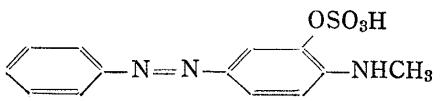

---- DAB-urine No. III (AB No. II)

-·-·- 4′-OH-AB-urine No. I

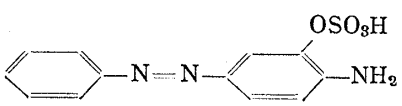
glucuronate added, and the solution was allowed to stand at a room temperature for 2 days. When the solution was submitted to paper chromatography with solvent A, a spot of Rf 0.4 appeared besides the original spot of Rf 0.7. Considered from these facts, it is supposed that the original component of DAB No. V or AB No. III will be the N-glucosiduronic acid of DAB No. II or AB No. I (3-$HSO_3O$-AB).

## Conclusion and Discussion

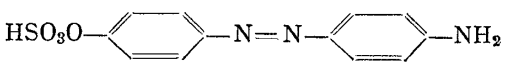
From the above-described data, it was concluded that the azo components which were found in the dog urine after administration of DAB were MAB, AB, 3-$HSO_3O$-MAB (a), 3-$HSO_3O$-AB (b), and 4′-$HSO_3O$-AB (c). The existence of 3-$HSO_3O$-AB N-glucosiduronate (d) was also surmised.



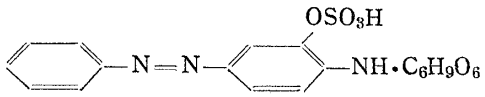

(a)




(b)




(c)




(d)

Other spots of azo dye components, DAB Nos. IV and VI, are supposed to be O-glucosiduronate and N-glucosiduronate of some aminoazobenzene derivative, but a reliable conclusion is not yet established.

*o*-Aminophenol, *p*-aminophenol, *p*-phenylenediamine, and aniline were detected in the DAB-urine hydrolyzed for 24 hrs.

Thus DAB administered to dog suffers N-demethylation, hydroxylation, and reduction of azo-linkage as in rat. About the hydroxylation of DAB administered to rat, it has been observed that the 4′-position suffers hydroxylation more favorably than the other positions[10] but, in the case of dog, the 3-position suffered hydroxylation rather than the 4′-position. This fact will be interesting compared with the Clayson's report[11] that 4-aminodiphenyl administered to dog suffers hydroxylation in the *ortho* position of its amino group.

Among the urinary metabolites produced by cleavage of azo linkage, *o*-aminophenol was larger in amount than *p*-aminophenol. This fact coincided with the case of the urinary metabolites of aniline and azobenzene (unreported).

The authors are grateful to Mr. E. Kimura and Miss N. Shinriki for the elemental analysis.

## Summary

The urinary metabolites of *p*-dimethylaminoazobenzene (DAB) in dog were studied, and in this connection, the metabolites of *p*-aminoazobenzene (AB) and 4′-hydroxy-4-aminoazobenzene (4′-OH-AB) were also examined. From the hydrolyzed 24-hr. urine after administration of DAB, *o*-aminophenol, *p*-aminophenol, aniline, *p*-phenylenediamine, AB, and *p*-methylaminoazobenzene (MAB) were detected. The amount of *o*-aminophenol was larger than that of the other amines.

By paper chromatography 5-phenylazo-2-aminophenyl hydrogen sulfate (3-$HSO_3O$-AB), 3-$HSO_3O$-MAB, and 4′-$HSO_3O$-AB were respectively identified from the 3- to 7-hr. DAB-urine. The presence of 3-$HSO_3O$-AB N-glucosiduronic acid in the DAB- and AB-urine was assumed from their paper chromatographic behavior.

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10) J. A. Miller, E. C. Miller: Cancer Res., **7**, 39 (1947).
11) L. Bradshaw, D. B. Clayson: Nature, **176**, 974 (1955).