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186. Mitsuo Watanabe and Morizo Ishidate*1 : Metabolism of 4-Dimethylaminoazobenzene and Related Compounds. V. *2 Quantitative Analysis of Biliary and Urinary Metabolites of 4-Dimethylaminoazobenzene in Rat.

(*Tokyo Biochemical Research Institute*1)*)

1. An available method of determination of biliary and urinary metabolites of DAB in rat was devised.

2. Twelve metabolites of DAB (DAB, MAB, AB, 4'-OG-DAB, 4'-OG-MAB, 4'-OG-AB, 4'-OG-AB-NAc, 4'-OS-DAB, 4'-OS-MAB, 4'-OS-AB and 4'-OS-AB-NAc in bile, and PAP-OS in urine) in rats administered with DAB were determined.

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In the 2nd report[1] of this series, urinary metabolites of 4-dimethylaminoazobenzene (DAB) in rat were investigated, and excretion of metabolites formed by reduction of azo bond, N-demethylation (followed by N-acetylation) and ring hydroxylation (followed by conjugation) was observed. In the 3rd report[2] it was found that metabolites retaining azo bond were mainly excreted in bile, while metabolites formed by cleavege of azo bond were excreted in urine. Furthermore, to investigate the metabolites in the turn of DAB feeding, separation and quantitative determination of the biliary metabolites*3 (AB, 4'-OG-DAB, 4'-OG-MAB, 4'-OG-AB, 4'-OS-DAB, 4'-OS-MAB and 4'-OS-AB) were carried out. However, in the experiment there was found very large fluctuation in the data.

The purpose of this investigation is to find more reliable procedure of the quantitative analysis of biliary and urinary metabolites of DAB and an available method was devised.

Experimental

Animals——Donryu male rats weighing about 250~300 g. They were bred in air-conditioned room (temperature; 25° ± 0.5°, humidity; 55%).

Reagents——DAB (commercially available) was purified on alumina column with benzene and recrystallized from EtOH, m.p. 117°. DAB-amine N-oxide was supplied by Dr. Hiroshi Terayama. Other metabolites had been prepared in the preceding work. DAB emulsion was prepared by mixing the equal volumes of normal rat bile and olive oil solution of DAB by sonic oscillator.

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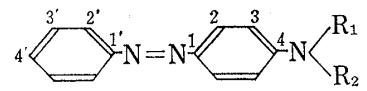
*1 3-41-8 Takada, Toshima-ku, Tokyo (渡辺光夫，石館守三).

*2 A part of this work was reported at 22nd Annual Meeting of the Japanese Cancer Association (1963). Part IV. This Bulletin, **15**, 964 (1967).

*3 

R_1, R_2=H 4-aminoazobenzene (AB).
R_1=H, R_2=CH_3 4-methylaminoazobenzene (MAB).
R_1, R_2=CH_3 4-dimethylaminoazobenzene (DAB).
R_1=H, R_2=$COCH_3$ 4-acetamidoazobenzene (AB-NAc).
OG=O-glucuronide.
OS=O-sulfate.

1) M. Ishidate, Y. Hashimoto : This Bulletin, **10**, 125 (1962).
2) M. Ishidate, Z. Tamura, K. Samejima : *Ibid.*, **11**, 1014 (1963).

As β-glucuronidase, ketodase (beef-liver-β-glucuronidase, 5×10^3 unit/ml., Warner Chilcott) was used.

Antirodential paint,*4 containing 5% of cycloheximide (Naramycin, Tanabe Seiyaku Co.), was supplied from Totoku Electric Wire Co.

Silica gel (Kieselgel unter 0.08 mm. für Chromatographie) was obtained from E. Merck AG.

Tentative Analytical Procedure fot Evaluation of Methods——For evaluation of the methods of administration of DAB and collection of bile, a simplified analytical procedure of metabolites was chosen as shown in Chart 1.

Determination of Remain of DAB in Alimentary Tract——The stomach and intestine of the rat were minced into small pieces and the minced organs were kept in AcOEt for 24 hr. DAB in the AcOEt layer was extracted with 4*N* HCl and determined colorimetrically at 525 mμ.

Spectrophotometry——Hitachi spectrophotometer (EPU-2) was used for colorimetric determinations.

Results

I. Methods of Administration of DAB and Collection of Bile

1) Disadvantage of the Previous Method

Fifteen milligrams of DAB in 1 ml. of olive oil was administered into a stomach of each rat through a catheter. After 1 hr. the rat was anesthesized with intraperitoneal injection of 0.5% pentobarbital sodium and a polyethylene tube was inserted into a bile duct. The bile was collected under continuous anesthesia using pentobarbital. The results are shown in Table I.

Table I. Quantitative Analysis of Metabolites of DAB by the Previous Method

	Remain of DAB		Excretion of biliary metabolites				
	in stomach (%)	in intestine (%)	AB (%)	4′-OR-DAB (%)	4′-OR-MAB (%)	4′-OR-AB (%)	total (%)
1	3.0	25.0	2.20	0.27	0.42	0.75	3.64
2	8.9	26.7	1.80	0.66	1.42	1.31	5.19
3	5.4	38.2	1.59	0.35	0.66	1.27	3.87
4	10.3	33.4	2.99	0.46	1.34	1.36	6.15
5	48.5	7.7	0.36	0.09	0.20	0.52	1.17
6	20.0	41.6	0.74	0.21	0.51	1.17	2.63
7	54.4	32.4	0.18	0.01	0.17	0.61	0.97

Percentage shows molar ratio of metabolites to administered DAB (15 mg.).

As shown in Table I clearly the results obtained by this method seems to be unreliable because excretion of DAB metabolites showed large fluctuation. The fluctuation seems to be caused by fluctuation of absorption of DAB from an alimentary tract. To avoid this fluctuation, the animals were starved for 24 or 48 hours prior to DAB administration, or fed the diet only either during daytime or during night. Intraduodenal administration was also attempted. However, the results obtained by the above modified methods did not afford more satisfactory results than the previous method.

2) Improved Method

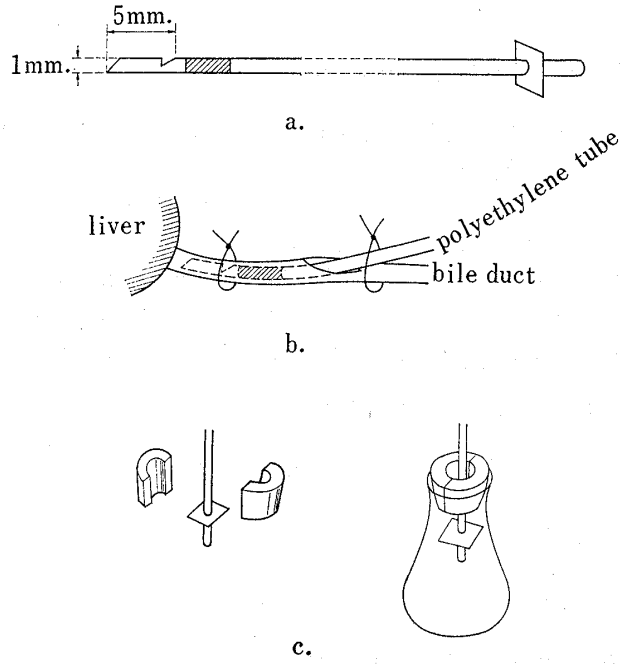
It is undesirable to perform continuous anesthesia, for there is possibility that anesthesia may affect the absorption and metabolism of DAB and henceforth cause the fluctuation in the results. Therefore, following experiments were carried out without continuous anesthesia.

*4 Commercial paint contains 0.25% of Naramycin. The paint used in this report was prepared specially by Totoku Electric Wire Co.

When bile is collected without anesthesia, a polyethylene tube is often bitten by the rat. Millburn[3] and Radomski,[4] *et al.* have reported a method of collecting bile by keeping the rat in a small cage so as to prevent movement. Van Zyl[5] and Boyland[6] devised a method to collect rat bile from a tube inserted through the nape. However, these techniques are rather complicated or effects of stress caused by restricting the animal may not be avoided. In this report, a simple method of collecting rat bile with neither continuous anesthesian or rstericton was adopted using rat repelent, cycloheximide.[7]

Neither oral administration nor intraduodenal injection of the olive oil solution of DAB gave satisfactory results, because the complete absorption of DAB was impossible, while intraduodenal administration of DAB emulsified in rat bile gave better results. After all these trials, the procedure adopted finally is as follows.

Antirodential paint containing 5% of cycloheximide was painted on a polyethylene tube with 1 mm. diamter. As shown in Fig. 1a, the tube was hurt*6 with a razor and marked with ink and a piece of celluloid plate was fixed near the other end. Stump of 2 cm. was set between a metallic cage (18×18×20 cm.) and a funnel for the collection of urine. A rat was anesthesized with ether and a tube painted with antirodential paint was inserted into the bile duct. The tube and the bile duct were tied with strings at two points as shown in Fig. 1b.



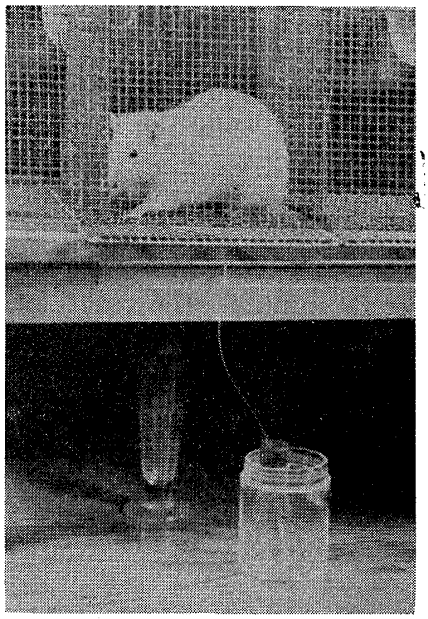


Fig. 1. Apparatus for Collection of Bile. d.

A half ml. of the DAB emulsion was injected into the duodenum. Then 0.2 ml. of Ringer's solution containing 10% of Mycirin*6 was injected into a peritoneal cavity and the abdominal wall was sutured.

*5 When enzymes were added to bile directly, recovery was not complete. Addition of enzymes to effluent from DEAE cellulose column gave satisfied result.

*6 Procain penicillin G 300,000 unit, Penicilline G-Potassium 100,000 unit and dehydrostreptomycin 0.5 g. were contained in 1 g. It was purchased from Meiji Seika Co.

3) P. Millburn, R. L. Smith, R. T. Williams: Biochem. J., **90**, 5p (1964).
4) J. L. Radomski, T. L. Mellinger: J. Pharm. Exptl. Therap., **136**, 259 (1962).
5) A. Van Zyl: J. Endocrin., **16**, 213 (1953).
6) E. Boyland, S. Ramsay, P. Sims: Biochem. J., **78**, 376 (1960).
7) T. Okuda, K. Ashino, Y. Egawa, S. Harigaya, M. Suzuki: Yakugaku Zasshi, **79**, 193 (1959).

The rat was put into a cage and the end of the tube was inserted into a 30 ml. flask (Fig. 1c and 1d) to collect 20~25 ml. of bile during 24 hours. The bile thus collected was analysed by the method described in Chart 1.

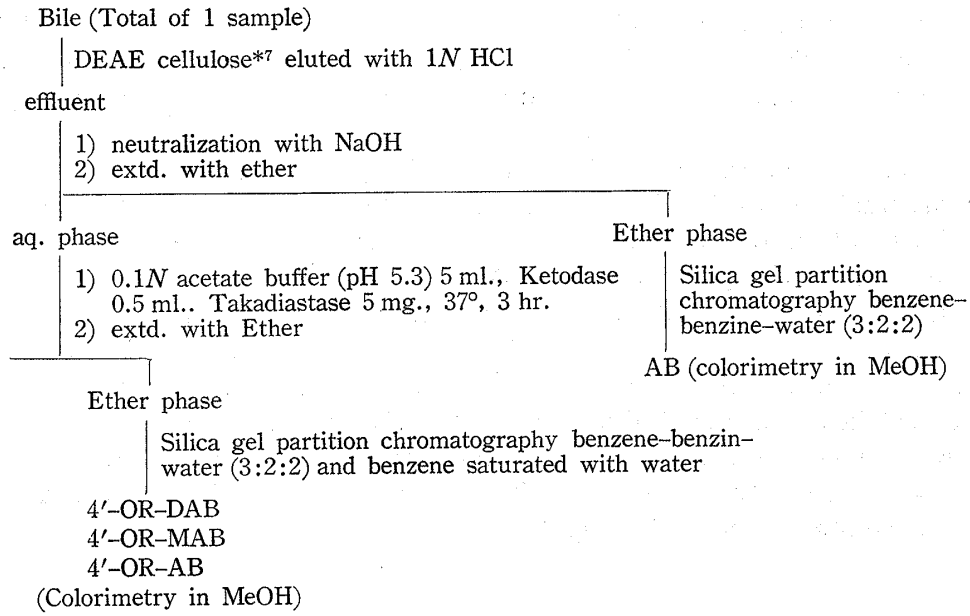


Chart 1. Method for Determination of Biliary Metabolites of DAB

A main urinary metabolite, *p*-aminophenol-O-sulfate (PAP-OS), was determined as follows. The collected urine was passed through a column of anion exchange resin, Dowex I-XI Cl. After washing the column by 20 ml. of 1*N* hydrochloric acid and determined by an indophenol reaction.[8] Five ml. of the effluent was heated at 100° for 15 minutes. Then 5 ml. of 0.1% potassium ferricyanide in an aqueous solution, 0.5 ml. of 5% phenol in an aqueous solution and 1 ml. of 28% ammonia water were added. After 30 minutes, the reaction mixture was filtrated and the amount of PAP-OS in

Table II. The Residual Amounts of DAB in Intestine

4 hr. (%)	6 hr. (%)	15 hr. (%)
36.8	3.5	0
30.8	6.5	0
	7.2	

Each rat was administered with 5 mg. of DAB intraduodenally.

Table III. Variation of Excretion of Biliary Metabolites of DAB

	0~3 hr. (%)	3~6 hr. (%)	6~15 hr. (%)	15~24 hr. (%)	total (%)
AB	1.0	1.27	0.14	0	2.42
4′-OR-DAB	0.26	0.45	0.10	0	0.81
4′-OR-MAB	0.30	0.33	0.19	0	0.82
4′-OR-AB	0.44	0.95	0.12	0	0.54

Percentage shows molar ratio of metabolites to administered DAB (5 mg.).

*7 When the tube and bile duct is tied at this point, the rat cannot draw out the tube.

8) J. N. Smith, R. T. Williams: Biochem. J., **44**, 242 (1949).

the filtrate was determined colorimetrically at 607 mμ. Metabolites retaining azo bond, which exist in very small amount in urine, did not affect the assay because they were not eluted by 1*N* hydrochloric acid from the column.

As shown in Table Ⅱ and Ⅲ, most of DAB administered was absorbed within 6 hours and excretion of metabolites in bile ended within 15 hours.

Nine rats were devided into 3 groups. To each animal in a group, 3.4, 4.0 and 5.0 mg. of DAB per rat was administered respectively. Biles and urines were collected during 24 hours after the dye administration and used for the assay of metabolites. Then the rat was killed and DAB in stomach and intestine was examined. Usually no remain of DAB in alimentary tract was detected.

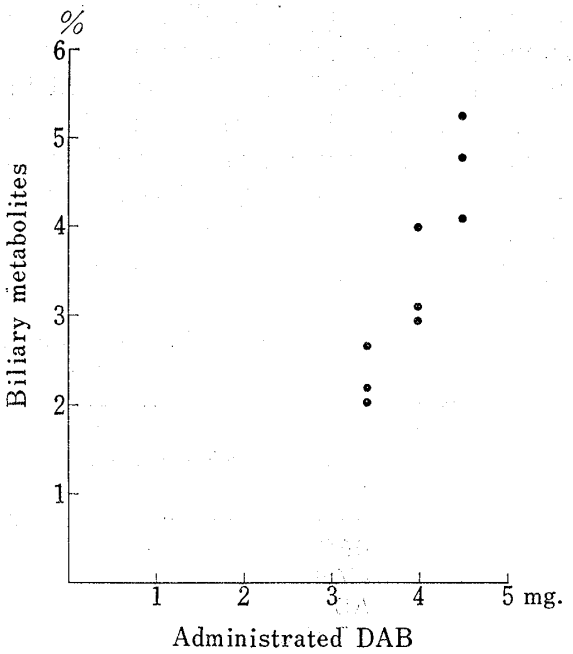


Fig. 2. Correlation between Biliary Metabolites and Administered DAB.

The results are shown in Table Ⅳ. It was also found that by increasing the amount of DAB administered the amounts of metabolites excreted in bile increased as shown in Fig. 2.

Table Ⅳ. Quantitative Analysis of Metabolites of DAB by the Improved Method

Administered DAB (mg.)	Excretion of Metabolites (%) in urine PAP-OS	in bile AB	4′-OR-DAB	4′-OR-MAB	4′-OR-AB	Total of biliary metabolites
3.4	50.5	0.80	0.40	0.70	0.77	2.67
	49.1	0.66	0.30	0.60	0.63	2.19
	48.4	0.62	0.25	0.55	0.62	2.04
4	40.5	1.20	0.24	0.73	0.79	2.96
	39.9	1.34	0.64	1.06	0.96	4.00
	60.3	1.04	0.36	1.01	0.70	3.11
4.5	51.0	2.29	0.62	1.08	1.27	5.26
	49.2	1.86	0.50	1.00	1.41	4.77
	39.5	1.81	0.44	0.80	1.03	4.08

Percentage shows molar ratio of metabolites to administered DAB.

II. Analytical Methods of Metabolites of DAB

1) Separation of Metabolites by Thin-layer Chromatography

Thin-layer chromatography was adopted for the separation of metabolites.

Thin-layer (0.5 mm.) of Silica gel : Gyps (5:1), which had been heated at 110° for 30 minutes, was used. Samples dissolved in ether were spotted on the thin-layer plate. Rfs[*8] of metabolites related to DAB are shown in Table Ⅴ. Solvent A was used for

[*8] Referred to report by Hashimoto and Samejima.[9]

9) Y. Hashimoto, K. Samejima : Yakugaku Zasshi, **86**, 451 (1966).

the separation of 4′-OH-DAB, 4′-OH-MAB, 4′-OH-AB and 4′-OH-AB-NAc, while solvent B was used for the separation of DAB, MAB and AB. An inner standard method[10] was adopted using DAB as a standard in the case of solvent A, and 2,2′-dimethyl DAB in the case of solvent B. Materials comprising yellowish band on the thin-layer chromatogram were extracted with methanol and the amounts of DAB, MAB, AB, 4′-OH-DAB, 4′-OH-MAB, 4′-OH-AB, 4′-OH-AB-NAc and 2,2-dimethyl-DAB were determined colorimetrically at 404, 398, 384, 404, 398, 384, 362 and 414 mμ respectively. The results of recovery tests are shown in Table Ⅵ.

Table Ⅴ. Rf Values of Related Compounds of DAB on Thin-layer Chromatography

Developing solvent	Rf	
	A	B
DAB	0.95	0.64
MAB	0.94	0.41
AB	0.83	0.19
AB-NAc	0.23	0
4′-OH-DAB	0.54	0
4′-OH-MAB	0.39	0
4′-OH-AB	0.25	0
4-OH-AB-NAc	0.09	0
3-OH-AB	0.37	0
2,2′-dimethyl-4-dimethylaminoazobenzene	0.99	0.91

Solvent A Benzene : Acetone (7:1)
Solvent B Benzene : Benzine (4:1)
Plate Kieselgel : Gyps (5:1)

Table Ⅵ. Recovery of Related Compounds of DAB in Thin-layer Chromatography

	30 (%)	60 (%)
AB	100.3	100.0
4′-OH-DAB	96.1	95.9
4′-OH-MAB	100.2	100.5
4′-OH-AB	98.4	95.8
4′-OH-AB-NAc	104.0	105.0

2) Detection of 3-OH-AB

3-OH-AB could not be separated from 4′-OH-MAB by Silica gel partition or thin-layer chromatography. Detection of 3-OH-AB was carried out as follows.

A band of 4′-OH-MAB on the plate of thin-layer was extracted with methanol. The methanol was evaporated to dryness and 0.5 ml. of 2*N* hydrochloric acid and 1 ml. of aqueous solution of 0.5% sodium nitrite were added. After 5 minutes the sample was heated at 100 for 5 minutes. The reaction mixture was shaken with ether and absorbancy of upper layer at 450 mμ was measured with spectrophotometer.

3) Analysis of Biliary Metabolites

The analysis of biliary metabolites was carried out as described in Chart 2. From the elute through the DEAE cellulose column, DAB, MAB and AB were extracted with

10) K. Samejima, Z. Tamura, M. Ishidate : This Bulletin, **15**, 964 (1967).

ether. Then, O-glucuronides and O-sulfates were hydrolysed by β-glucuronidase and Takadiastase, and liberated dyes were extracted with ether. Ether was evaporated from the extracts, dyes were separated by means of thin-layer chromatography and assays as described above.

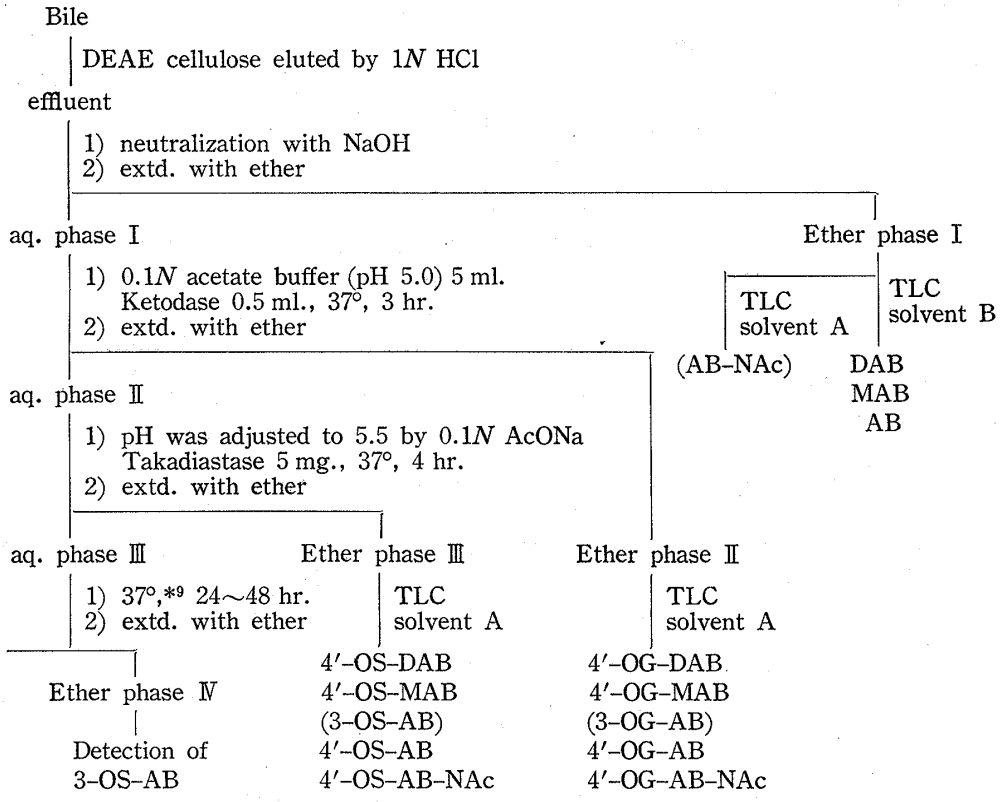


Chart 2. Method of Determination of Biliary Metabolites of DAB
TLC means Thin-layer Chromatography.

Recoveries of 30γ of AB, 4'-OG-DAB, 4'-OS-DAB, 4'-OS-MAB and 4'-OS-AB dissolved in 20 ml. of rat bile were 96.0, 94.8, 96.5, 101.0 and 98.4%, respectively.

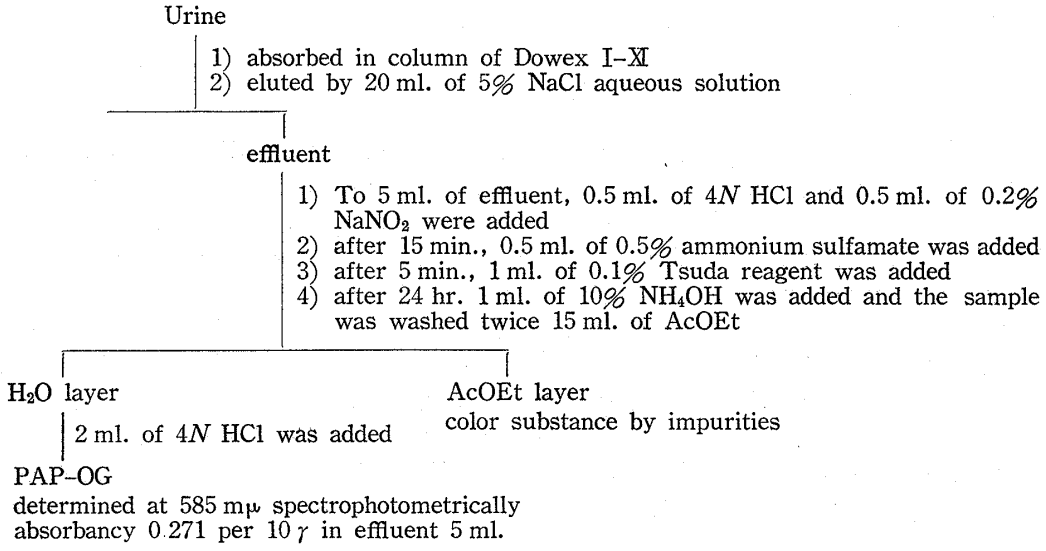


Chart 3. Detection of PAP-OG

*9 As 3-OS-AB is hard to be hydrolysed by enzyme, incubation of 24~48 hr. is needed for complete hydrolysis.

Detection of DAB-amine N-oxide was carried out as follows. Aqueous phase I was extracted with chloroform. The chloroform layer was evaporated to dryness. The residue dissolved in a small volume of chloroform. The sample was spotted on silica gel thin-layer and developed by chloroform : acetone : methanol (10:6:3). Rf of DAB-amine N-oxide was 0.6.

4) **Analysis of Urinary Metabolites**

The determination of PAP-OS was carried out by the method described above. The detection of *p*-aminophenol-O-glucuronide(PAP-OG) was carried out as described in Chart 3.

III. Quantitative Study on Metabolites of DAB in Rat

Five mg. of DAB was administered to a rat and bile and urine were collected for 24 hours. The determination of biliary and urinary metabolites was carried out by the method described in this paper. The results are shown in Table Ⅶ. 3-OG-AB, 3-OS-AB, AB-NAc and DAB-amine N-oxide in bile and PAP-OG in urine could not be detected (sensitivities : 3-OG-AB, 3-OS-AB, AB-NAc and DAB-amino N-oxide 5 μg. PAP-OG 20~30 μg./rat. day).

Table Ⅶ. Biliary and Urinary Metabolites of DAB in Rats

	DAB (%)	MAB (%)	AB (%)	4′-OG-DAB (%)	4′-OG-MAB (%)	4′-OG-AB (%)	4′-OG-AB-NAc (%)
1	0.13	0.14	2.30	0.08	0.10	0.09	0.52
2	0	0.08	1.90	0.10	0.20	0.12	0.39
3	0	0.29	3.20	0.11	0.28	0.16	0.67

	4′-OS-DAB (%)	4′-OS-MAB (%)	4′-OS-AB (%)	4′-OS-AB-NAc (%)	Total of biliary metabolites	PAP-OS in urine	Body weight (g.)
1	0.52	0.39	0.79	1.01	6.07	48.0	362
2	0.37	0.26	0.55	0.75	4.72	40.0	343
3	0.67	0.73	1.23	1.45	8.79	48.3	382

Percentage shows molar ratio metabolites to administered DAB (5 mg.).

Discussion

When DAB is orally given to a rat, the amounts of metabolites retaining azo bond excreted in bile is 4~5% of administered DAB, while the amounts of metabolites excreted in urine is only 0.1~0.2%. This fact may support the hypothesis presented by Williams[9,11] suggesting that the metabolites having molecular weight of 150 and more, and having polar radical are mainly excreted in bile.

Biliary metabolites can be reabsorbed from intestine and remetabolized. By perfusion of rat liver, 4′-OS-DAB was shown to be remetabolized, while 4′-OG-DAB was excreted in bile without being changed.[2] However, when 4′-OS-DAB and 4′-OG-DAB were administered orally, both of them were metabolized.[12] The O-glucuronide may be absorbed after hydrolysis of the conjugation. Examination of biliary metabolites is suitable for studying the pattern of primary metabolites as well as metabolic function of liver.

11) R. T. Williams : Reports of the 10th Anniversary Symposium on Glucuronic Acid, p. 1 (1964).
12) Y. Hashimoto, H. Sudo : Proceedings of the 83rd Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, November 1963, p. 156 (1963).

It was reported in the 3rd report[2] of this series, that major substance of the biliary metabolites from DAB was AB and that, among 4′-hydroxylated metabolites, the order of quantities excreted in bile was as follows, 4′-OG-DAB≧4′-OS-DAB, 4′-OG-MAB>4′-OS-MAB and 4′-OG-AB<4′-OS-AB. However, the data presented there by using the previous method were not satisfactory as discussed before. The method adopted in this paper seems to be more valuable for the quantitative analysis of metabolites, because it is more simple, the fluctuation of data is in small magnitude and possible effect due to continuous anesthesia is removed. The results in this paper showed that 4′-OG-AB-NAc and 4′-OS-AB-NAc, which were not determined in preceding paper, were main metabolites among the 4′-hydroxylated compounds. This fact indicates that large parts of the demethylated substances are N-acetylated. The amount of 4′-OG-MAB excreted in bile is smaller than 4′-OS-MAB. This results is in contradiction to the preceding report.[2] DAB-amine N-oxide and 3-hydroxylated compounds, which were supposed to be proximate carcinogens in the DAB metabolites, could not be detected by the present analytical method.

The influence of barbital, which shows the retarding effect[13] on liver carcinogenesis by DAB, to the metabolites will be reported.[14]

The authors wish to express their deep gratitude to Prof. Dr. Z. Tamura, University of Tokyo, for his kind leading. They are also grateful to Dr. H. Terayama for his kind supply of DAB-amine N-oxide and to Totoku Electric Wire Co. for the donation of the antirodential paint.

13) M. Ishidate : Acta Union Internationate Contre le Cancer, **20**, 909 (1964).
14) M. Ishidate, M. Watanabe, S. Odashima : Gann, 58, 267 (1967).