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172. Morizo Ishidate,*1 Zenzo Tamura, and Keijiro Samejima : Metabolism of 4-Dimethylaminoazobenzene and Related Compounds. III.*2,2) Metabolites of 4-Dimethylaminoazobenzene in Rat Bile and Influence of DAB Feeding on their Amounts

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Many reports have been published on the metabolism of 4-dimethylaminoazobenzene (DAB), a carcinogenic aminoazo dye, and the classical works were reviewed by Miller and Miller.[3] Some new metabolites of this dye were also found in our laboratory.[1,2] Majority of the previous work concerned with urinary metabolites and changes by liver slice or homogenate. The finding of the excretion of DAB metabolites, conjugated and nonconjugated aminoazo dyes, in rat bile by the liver perfusion method[4] promoted us to study the metabolic fate of DAB in the bile of a living rat. On the basis of this finding, a method for their separatory determination was investigated and then observations were made on the changes of their amount during the course of carcinogenesis in the liver of rats fed with DAB diet.

Experimental Methods

Animals——Donryu*4 male rats were used.

Materials——DAB and AB (commercially available) were purified on alumina column with solvent system petr. benzin and benzene adequately. As the authentic samples, the following substances were used: (in abbreviated formulae*5) 4′-OH-AB, 4′-OH-MAB, 4′CH-DAB, 4′-OS-AB, 4′-OS-MAB, 4′-OS-DAB, 4′-OG-AB, 4′-OG-MAB, 4′-OG-DAB, 3,4′-dihydroxy-AB, and 2′,4′-dihydroxy-AB (OS : sulfate, OG: glucuronide). Most of these compounds were the same as those prepared and used in the work[1,2] previously.

Enzyme Preparation——β-Glucuronidase of the step-4 by Fishman, *et al.*[5] was used for the hydro lysis of glucuronides. Diastase obtained from Sankyo Co., Ltd. was used without further purification for the hydrolysis of sulfates.

Paper Chromatography——Paper chromatography was carried out with Toyo Roshi No. 51 filter paper, by the ascending development with the solvent system PrOH-BuOH-H_2O (2:3:5) (upper layer). Ten ml. of Me_2CO was mixed with 2 ml. of bile. After centrifugation, 6 ml. of PrOH was added to the supernatant. The resulting solution was evaporated to dryness under reduced pressure in N_2 atmosphere. The residual solid was dissolved in 0.5 ml. of H_2O and spotted on the filter paper.

Color Reactions——As aminoazo dyes were yellow in neutral or alkaline media and generally changed to red in acid media, 2*N* HCl was used as a spraying reagent. For the detection of primary

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*2 A Part of this work was communicated in this Bulletin, **10**, 75 (1962). Part II : *Ibid.*, **10**, 125 (1962).

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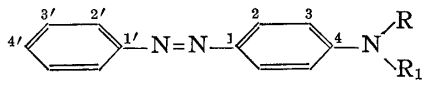
*4 Donryu rats were separated from Japanese common albino rats in 1950 by Dr. R. Sato and have been kept and inbred in the Central Laboratory for Experimental Animals. Specific character of these rats is that they keep native high sensitivity against the transplantation with Yoshida sarcoma cells. Transplontation rate of Yoshida sarcoma is more than 99.8% in these animals, while less than 96% in common Japanese rats.

*5 

R, R_1 : H 4-Aminoazobenzene (AB)
R : H, R_1 : CH_3 4-Methylaminoazobenzene (MAB)
R, R_1 : CH_3 4-Dimethylaminoazobenzene (DAB)

1) Part I. M. Ishidate, Y. Hashimoto : This Bulletin, **7**, 108 (1959).
2) Part II. *Idem* : *Ibid.*, **10**, 125 (1962).
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amines, Ehrlich's reagent was used. The Gibbs' reagent was used for the detection of *o*-aminophenol derivatives.[2)]

Silica Gel Partition Chromatography——The following systems gave good results for the separation of hydroxylated aminoazo dyes. Silica gel[*6]-H_2O (2:1) was used as a stationary phase and benzene-benzin-H_2O (3:2:2) (upper layer) as the moving phase, followed by benzene saturated with H_2O for the separation of monohydroxy aminoazo dyes (especially 4'-hydroxy derivatives). For the separation of dihydroxy-aminoazo dyes, 3,4'-dihydroxy-AB, and 2',4'-dihydroxy-AB, isopropyl Et_2O-benzin-H_2O (2:2:1) was used successfully as a solvent system.

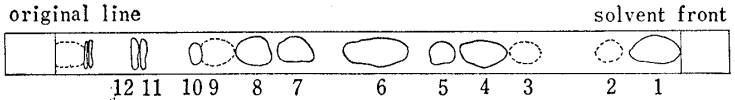
Apparatus——Hitachi spectrophotometer (EPU-2) was used for all colorimetric and spectral determination, and Toyo Roshi Co. Type C for paper electrophoresis.

Results

1. Metabolites of DAB in Rat Bile

Fifteen milligram of DAB dissolved in one ml. of olive oil was injected into a stomach of rat through a catheter. After 1 hour, the rat was anesthesized with pentobarbital sodium[*7] and a polyethylene tube with external diameter of 1 mm. was inserted into the bile duct of anesthesized rat by surgical operation. The bile was collected for the following 5 hours and subjected to the paper chromatography, which revealed more than seven kinds of aminoazo dyes (Fig. 1). Thereafter, DAB-bile was fractionated as follows : 2 ml. of DAB-bile and 50 ml. of 0.1*N*-acetate buffer (pH 5.5) were mixed,

Fig. 1. Paper Chromatogram of DAB-Bile and Color Reaction of Each Spot



Spot No. / Reagent	12	11	10	9	8	7	6	5	4	3	2	1
Without reagent	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	N	Y
2*N* HCl	c P	d P	R O	N	c P	d P	R O	c P	d P	?	N	R O
Ehrlich	c P	d P	O	Y	c P	d P	O	c P	d P	O	Y	O

Y : yellow, R : red, O : orange, P : purple, N : colorless, c : clear, d : dark

and shaken with 50 ml. of ether. After removal of the ether layer (F-I), the buffer solution was incubated with 25 mg. of Diastase for 2 hours at 38° and extracted with 50 ml. of ether (F-II). The residual buffer solution was further incubated with β-glucuronidase (0.05 ml.) for 2 hours at 38° and extracted with 50 ml. of ether (F-III). Each fraction was evaporated to dryness in a reduced pressure in nitrogen atmosphere, after confirmation of the presence or absence of a substance extractable with 10% NaOH.

F-I (Unconjugated)——Azo dyes in this fraction were not extracted with 10% NaOH and it was thought that they had no hydroxyl group. Color reactions (red orange by 2*N* HCl and orange by the Ehrlich reagent) showed the presence of AB. Alumina column chromatography of the substance mixed with authentic AB, as described under Experimental, showed only one band. Absorption spectra of this substance, after purification on alumina also agreed with AB (UV : λ_{max}^{MeOH} 384 mμ). Accordingly AB was a main azo dye in F-I.

F-II (Sulfates)——Azo dyes in this fraction were almost completely extracted with 10% NaOH, and were assumed to be hydroxy-aminoazo dyes which were originally conjugated with sulfuric acid. This fraction was subjected to silica gel partition chromatography and afforded three kinds of azo dyes (f1, f2, and f3). The color reactions on the paper with 2*N* HCl and the Ehrlich reagent, paper electrophoretic behavior

*6 Kieselgel unter 0.08 mm. für Chromatographie obtained from E. Merck AG.
*7 Nembutal (Abbott Lab.).

Table I. Paper Electrophoretic Behaviors of Fractions f1—f2 and f3, compared with Authentic Samples

Substances	Color in pH 13 glycine buffer	Migration distance (cm.)
f1	orange	5.0
4′-OH-DAB	〃	5.0
f2	orange yellow	6.0
4′-OH-MAB	〃	6.0
f3	yellow	7.0
4′-OH-AB	〃	7.0

0.05*N* glycine-NaCl-NaOH buffer, 400 v. 13 mA., 4 hr.

Table II. Ultraviolet Absorptions of AB, 4′-OH-DAB, 4′-OH-MAB, and 4′-OH-AB

Substances	UV λ_{max}^{MeOH} mμ	Molar extinction coefficient (ε)
AB	384	2.38×10^4
4′-OH-DAB	404	2.95×10^4
4′-OH-MAB	398	2.87×10^4
4′-OH-AB	384	2.80×10^4

(Table I), and absorption spectra in neutral media (cf. Table II) of f1, f2, and f3, were identical with those of authentic 4′-OH-DAB, 4′-OH-MAB, and 4′-OH-AB, respectively. Accordingly the presence of aminoazo dyes conjugated with sulfuric acid was certain in the bile.

F-III (Glucuronides)——The results of qualitative analysis of this fraction were completely the same as those of F-II. Accordingly the presence of these three 4′-hydroxy-azo dyes conjugated with glucuronic acid was certain in the bile.

For the purpose of the identification of each spot on the paper chromatogram in Fig. 1, the following experiments were carried out. When the ether-insoluble fraction was subjected to paper chromatography after treatment of bile with Diastase, spots No. 4, 5, and 6 disappeared, while spots No. 7, 8, and 10 disappeared by treatment with β-glucuronidase; and in each case spot No. 1 increased. Therefore these spots were certain to be of sulfates and glucuronides. Each band in the sheet paper chromatography of the bile was extracted, subjected again to paper chromatography or to paper electrophoresis, and compared with an authentic sample. As a result, the main metabolites in the bile were identified as shown in Table III.

Table III. Main Metabolites in Bile

Spot No.	Metabolite identified	Spot No.	Metabolite identified
1	AB	7	4′-OG-DAB
2	unknown	8	4′-OG-MAB
3	〃	9	urea
4	4′-OS-DAB	10	4′-OG-AB
5	4′-OS-MAB	11	unknown
6	4′-OS-AB	12	〃

2. Quantitative Analyses of the Metabolites

Separation of Unconjugated Dyes, Glucuronides and Sulfates——DEAE-cellulose (0.8 g.), obtained from Brown Co., Ltd., was treated with *N* HCl to change into the Cl-type and packed into a column 12 mm. in diameter. The column was washed with distilled water until chloride ion could no longer be detected. On the column, was adsorbed 5 ml. of bile. By elution with 0.002*N* HCl (120 ml.), only unconjugated aminoazo dyes were eluted, and with 0.01*N* HCl (40 ml.) glucuronides in one band, then similarly with 0.04*N* HCl (80 ml.) sulfates were eluted. Recovery of 4′-OG-AB and 4′-OS-AB was respectively 105% and 96%.

Enzymatic Hydrolysis of the Conjugates——Examination of conditions necessary for quantitative hydrolysis of glucuronides or sulfates was made by the colorimetric deter-

mination of 4′-OH-DAB liberated from the substrate 4′-OG-DAB or 4′-OS-DAB by the action of β-glucuronidase or Diastase, and adequated conditions were determined as follows : Glucuronides incubated with 0.01 ml. of β-glucuronidase (step 4) and 10 ml. of *N*/15 acetate buffer (pH 5.0) at 38° for 2 hours. Sulfates incubated with 5 mg. of Diastase and 10 ml. of 0.08*N* acetate buffer (pH 5.5) at 38° for 2 hours.

Chromatographic Separation of the Hydrolysates——Four grams of silica gel containing 2 ml. of water was packed into a column (diameter, 13 mm.) with benzene-benzin-water. The substance hydrolyzed by an enzyme was applied to the column and separated by elution with the same solvent. AB, 4′-OH-DAB, 4′-OH-MAB and 4′-OH-AB were eluted successibly by the techniques described above. Each fraction was evaporated under reduced pressure in nitrogen atmosphere, dissolved in MeOH and diluted to an aliquot volume. Absorption spectrum of each of these samples was the same as that of each authentic sample. Recovery of these substances (AB 99%, 4′-OH-DAB 98%, 4′-OH-MAB 98%, and 4′-OH-AB 91%) showed a tendency to decrease according to their elution volume, but was not less than 90% of the amount taken. The overall method for the determination is summarized in Chart 1.

3. Influence of DAB feeding on the Amount of Metabolites During DAB feeding

Care of Animals——One hundred male Donryu rats, 7 weeks old, were kept in metal cages, each of which contained a group of 5 animals. Food and water were made available *ad libitum*. One half of them (control rats) were fed on a basal diet[*8] and the other half (DAB feeding rats) on a DAB diet[*9] for 144 days. Five control rats and five DAB rats were used in one experiment and the diet of the latter was replaced by the basal one for 2 days before the experiment, since excretion of the metabolites

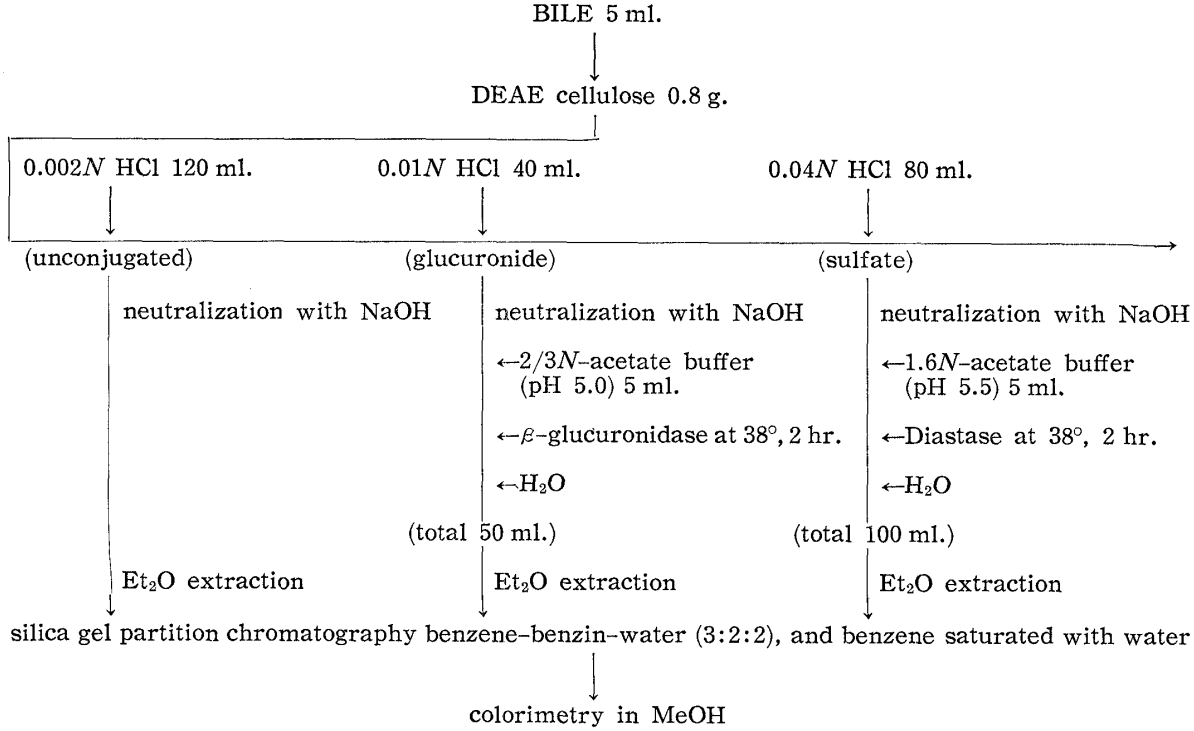


Chart 1. Method for Separatory Determination of seven kinds of DAB Metabolites

[*8] Composition of the basal diet was as follows: Crude protein 24%, carbohydrate 51%, crude fat 3.5%, salt mixture 9%, vegetable fiber 4.5%, and water 8%.

[*9] DAB diet was made by an addition of DAB, dissolved in olive oil, in a proportion of 0.06% to the powder of basal diet and then made into cubes.

of DAB was almost negligible after 2 days. Examination of metabolites was made nine times, at the begining of the dye feeding (June 19, 1961), and after 1, 3, 6, 11, 16, 21, 26 and 31 weeks.

Collection of Bile for Determination——Each rat was treated as described in section1. The bile excreted during the first one hour was discarded, because of comparably small content of DAB metabolites. The bile of following 5 hours was collected for analysis. The biles from 5 control rats and from 5 DAB rats were respectively

Table IV. Result of Quantitative Analysis

	Control groups								
Weeks	0	1	3	6	11	16	21	26	31
Total body weight (g.)	865	935	885	1150	1265	1255	1410	1505	1580
Total bile (ml.)	14.3	17.5	14.6	16.2	15.8	14.4	12.1	14.8	19.1
4′-OG-DAB (16^{-7}mole)	5.1	3.6	7.9	2.7	2.7	6.4	6.6	5.8	10.6
4′-OG-MAB	10.3	11.6	18.4	9.6	10.8	24.8	22.6	16.0	35.0
4′-OG-AB	0.8	0.9	1.5	1.3	1.0	2.1	2.2	2.0	5.6
4′-OS-DAB	22.7	22.0	7.0	3.1	4.2	11.8	7.1	5.2	9.0
4′-OS-MAB	7.1	8.7	4.5	2.5	3.3	7.0	5.0	3.7	6.5
4′-OS-AB	7.8	14.7	7.2	5.9	11.6	16.0	16.8	14.7	25.1
AB	33.6	38.3	44.1	20.8	31.1	72.0	52.0	50.0	79.5
Total	87.4	99.8	90.6	45.9	64.7	140.1	112.3	97.4	171.3
	DAB feeding groups								
Total body weight (g.)	860	800	950	1025	1165	1275	1325	1315	
Total bile (ml.)	20.0	20.9	21.5	26.3	22.1	25.1	18.4	12.1	
4′-OG-DAB (10^{-7}mole)	7.6	24.8	8.7	19.5	4.2	14.3	3.0	0.9	
4′-OG-MAB	23.3	49.7	18.7	36.6	11.6	25.5	7.5	1.8	
4′-OG-AB	1.3	3.7	2.0	4.0	1.1	1.3	0.8	0.0	
4′-OS-DAB	12.4	14.9	11.0	24.8	9.0	23.5	12.9	2.6	
4′-OS-MAB	5.9	7.5	5.1	9.0	5.1	7.9	5.8	1.2	
4′-OS-AB	6.7	10.0	4.5	11.7	7.7	7.7	8.2	1.7	
AB	72.4	65.1	36.0	77.1	18.8	70.3	26.9	5.6	
Total	129.6	175.7	86.0	182.7	57.5	151.5	65.1	13.8	

Each is a combined value of five rats.

Table. V. State of Rat Liver by Microscopic Examination

Weeks	Conditions of liver	Degeneration					Cholangio-fibrosis					Slight pro-liferation					Liver cell adenoma					Liver cancer				
	Rat No.	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1	Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Feeding	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Feeding	−	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	Control	+	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Feeding	+	+	+	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
11	Control	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Feeding	+	+	++	+	+	+	+	+	++	+	+	+	++	+	+	−	−	−	−	−	−	−	−	−	−
16	Control	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Feeding	++	+	++	++	++	++	+++	+	++	+	++	+	++	+	++	−	−	−	−	−	−	?	II	−	−
21	Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Feeding	+	+	++	++	++	−	+	+	+	+	−	+	+	++	++	−	−	−	−	−	−	−	−	−	−
26	Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Feeding	++	++	+	+	++	−	+	−	+	−	+	+	+	+	+	−	−	+	+	−	I II	I II	I	II	II
31	Control	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	Feeding	++	++	++	++	++	+	−	−	−	+	++	+	+	+	+	+	+	+	+	+	?	I	I	I II	I II

I : Liver cancer type I II : Liver cancer type II

combined. Results of quantitative analysis of the bile according to the above described method (Chart 1) are summarized in Table IV. States of the liver was examined through a microscope after collection of the bile (Table V).

Discussion

It was interesting that many kinds of DAB metabolites retaining azo bond were excreted in the bile, while DAB metabolites formed by reductive fission of the azo bond, such as aniline, *p*-aminophenol, *o*-aminophenol, phenylenebiamine etc. were mainly excreted in the urine or in the dialyzed solution of liver perfusion. A few reports had already been published on such selectivity of rat liver. Mellinger, *et al.*[6] investigated the absorption fate and excretion in rats of the water soluble azo dyes, such as FD & C Red No. 4 and FD & C Yellow No. 6.

They pointed out that, when these were administered by a stomach tube to rats in doses of 50 mg., no dye was recovered, but the products of reductive fission appeared in the urine and feces, and that when the dyes were administered through the portal system to rats, 73~93% was recovered in the bile, 0~20% in the urine. Ryan, *et al.*[7] examined the biliary excretion of 16 kinds of sulfonated azo dyes after intravenous injection. The present experiment concerned the biliary excretion of DAB metabolites. The presence of a considerable amount of glucuronides, shown in Table III, is to be taken into account, because it was proved in early observations in our laboratory that the main form of conjugation in rat urine was a sulfate and glucuronide could not be detected. There have been several reports about the excretion of glucuronides in the bile. One of them using chloramphenicol[8] showed that chloramphenicol glucuronide was excreted in the bile of lower animals and was readily hydrolysed by intestinal bacteria (β-glucuronidase) to chloramphenicol which might be reabsorbed or reduced by the bacteria. In this connection, the metabolic fate of 4′-OG-DAB and 4′-OS-DAB, which are DAB metabolites and whose dimethylamino radical remained unchanged, was examined by the technique of liver perfusion. Among the results obtained, the behavior of 4′-OG-DAB was interesting, because it was mainly excreted unchanged into the bile again while it was thought hydrolysed during transfering from portal vein to bile duct through the liver cells which contained much β-glucuronidase or demethylase, and only a small amount of it changed by reductive fission of the azo bond and excreted in the dialysate. On the other hand, 4′-OS-DAB was completely changed to other metabolites.

The effect of DAB feeding is discussed below, on the basis of results shown in Table IV. Since the values given in the Table show a large fluctuation, it is difficult to interpret them as they are. Some reasons may be considered for this fluctuation. One of them may be the method of anesthesia and of bile collection. As mentioned above, pentobarbital sodium was employed and its administration was controlled by observing the conditions of rats, and there was no large fluctuation in the amount of bile from each rat. The total amount of seven kinds of metabolites excreted into the bile during 5 hours was only a few percent of the amount given, although even such a small amount may have a significance, since, for example, the values from both control and DAB feeding groups always showed the following relation : 4′-OG-DAB$\leqq$4′-OS-DAB, 4′-OG-MAB$>$4′-OS-MAB, 4′-OG-AB$<$4′-OS-AB. The other fluctuations to be caused by season and by the age of rats can be canceled by taking the ratio of the

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7) A. J. Ryan, S. E. Wright : J. Pharm. & Pharmacol., **13**, 492 (1961).
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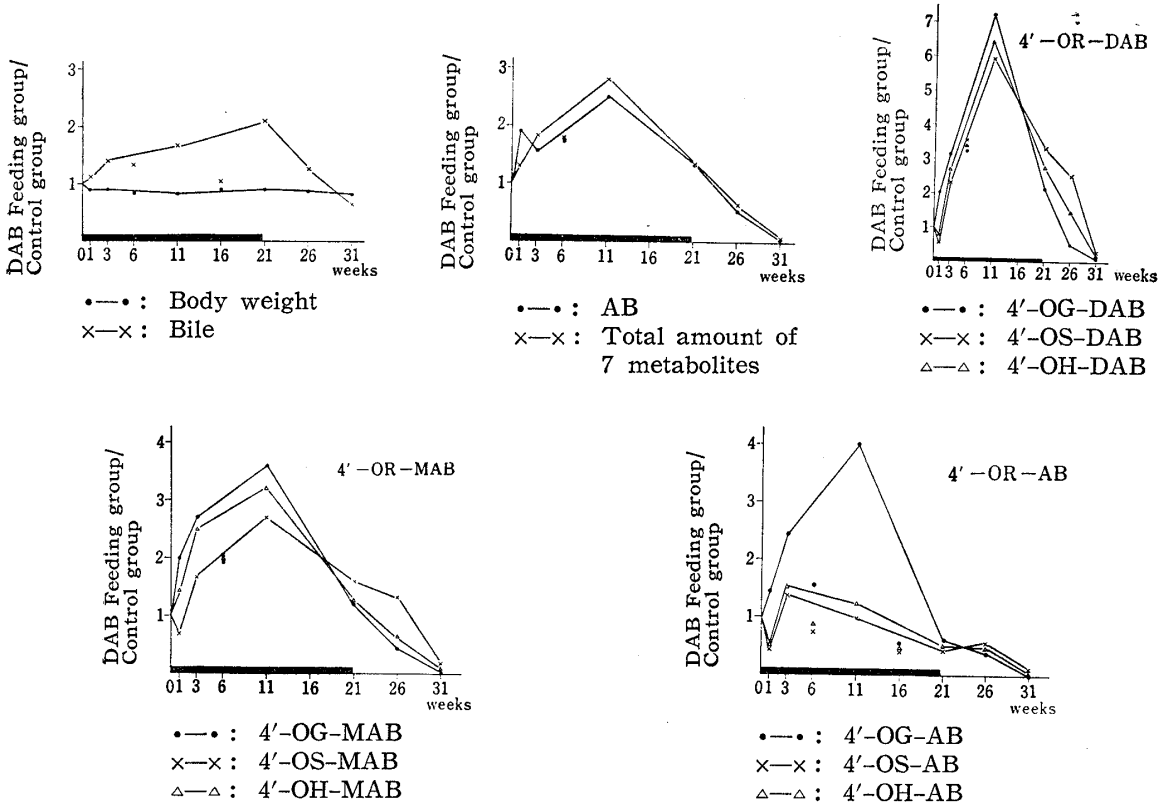


Fig. 2. The Ratio of Each Metabolite of DAB feeding Group to That of Control Group

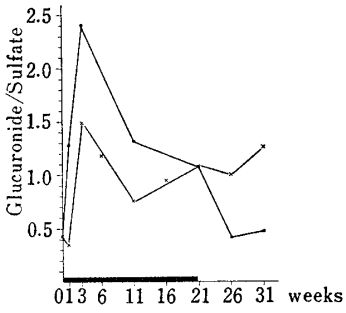
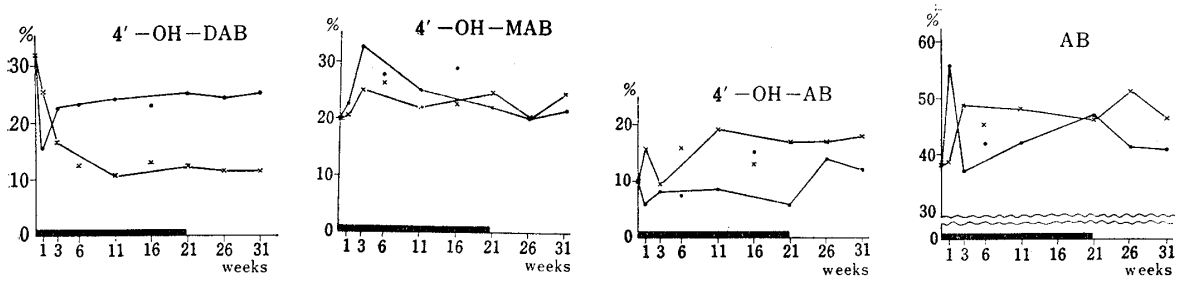


Fig. 3. The Ratio of the Amount of Glucuronide to That of Sulfate

•—• : DAB feeding group
×—× : Control group



•—• : DAB feeding group ×—× : Control group

Fig. 4. Comparison between Control Group and DAB Feeding One about each Percentage of 4′-OH-DAB, 4′-OH-MAB, 4′-OH-AB, or AB to their Total Amount

▬▬ represents DAB feeding period.

feeding group to the control. As a result of this treatment, a distinct tendency was recognized as shown in Fig. 2. There was also an individual difference as shown in Table V. Particularly, 3 rats in the control group showed hepatic degeneration in the sixth week and some rats in the feeding group of the sixteenth week showed abnormal changes. Therefore, the observed values of these two stages were omitted from plots of line graph in Figs. 2, 3, and 4. It can be easily recognized from Fig. 2 that, in any case, the values increase untill about the eleventh week and then death results by cancer. This tendency is particularly remarkable in 4′-OR-DAB, and becomes smaller in the order of 4′-OR-MAB and 4′-OR-AB. While the conjugation of glucuronic acid is markedly increased in the first stage, that of sulfuric acid is comparatively lower. This can be also infared from Fig. 3 which shows the ratio of the amount of glucuronide to that of sulfate. Fig. 4 decrease till illustrates the ratio of the amount of 4′-OH-DAB, 4′-OH-MAB, 4′-OH-AB, or AB to the total amount of seven kinds of metabolites, and clearly shows that the percentage of 4′-OH-DAB in the feeding groups is always greater and on the contrary that of 4′-OH-AB is smaller, from third week till the last.

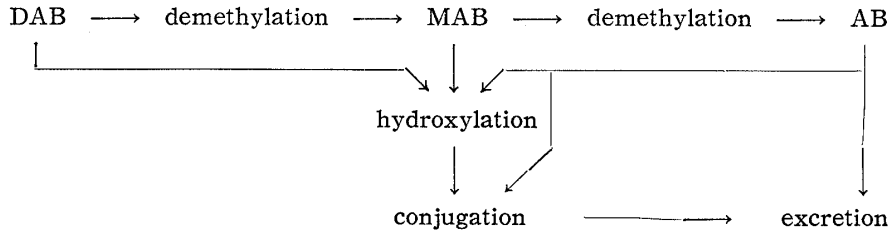


Chart 2. Excretory Mechanism of DAB Metabolites retaining Azo Bond

Chart 2 shows the well-recognized excretory mechanism of DAB metabolites retaining the azo bond. Since the hydroxylated substances are immediately conjugated and excreted, what regulates the route of this detoxication system is probably demethylation or hydroxylation. Although Fig. 4 seems to suggest decrease of demethylation in the DAB feeding groups, a more suitable explanation for the results shown in Figs. 2 and 4 is possible by considering the notable promotion of hydroxylation to the aminoazo dyes over the whole stages and reduction of excretory functions in later stages.

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Summary

The biliary excretion of DAB metabolites was first examined by paper chromatography, colorimetry, and by other methods. Presence of a selectivity in rat liver was recognized such as the main excretion of DAB metabolites retaining the azo bond into the bile and seven kinds of DAB metabolites (AB, 4′-OS-DAB, 4′-OS-MAB, 4′-OS-AB, 4′-OG-MAB, 4′-OG-MAB, 4′-OG-AB) were identified.

Second, influence of DAB feeding on the amount of these seven kinds of DAB metabolites was examined by the method of newly devised separatory determination using DEAE ion-exchanger and other methods. The most appropriate explanation for the results obtained was the promotion of hydroxylation in the first and middle stages of DAB feeding, although there were yet several problems.

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