CHEMICAL & PHARMACEUTICAL BULLETIN

Vol. 21, No. 12 December 1973

Regular Articles

[Chem. Pharm. Bull. 21(12)2577—2584(1973)]

UDC 547.556.31.04.09 : 615.277.4.011.5

Studies on Carcinogenic Azo Dyes. III.[1] Retention of Tritium during Enzymatic Hydroxylation of 3'-Methyl-4-dimethylaminoazobenzene(4'-^{3}H, or 5'-^{3}H) or Chemical Substitutions of the Related Compound, and the Effect of the Repeated Administration of This Azo Dye on the Retention of Tritium[2]

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(Received November, 6, 1972)

This paper is intended to report the retention of tritium during enzymatic hydroxylation at the 4' position of 3'-methyl-4-dimethylaminoazobenzene[4'-^{3}H, or 5'-^{3}H](3'-Me-DAB) or during nonenzymatic substitutions at the 4 position of 3-methylacetanilide(4-^{3}H, or 5-^{3}H), and the effect of the repeated administration of carcinogenic 3'-Me-DAB upon the aryl hydroxylation activity against 3'-Me-DAB(5'-^{3}H) and retention of tritium in the hydroxylated product.

The enzymically formed 3'-methyl-4'-hydroxy-4-dimethylaminoazobenzene from 3'-Me-DAB(4'-^{3}H) retained tritium 94% on an average. Accordingly, it may be proved that the NIH shift also occurs in the enzymatic hydroxylation of 3'-Me-DAB(4'-^{3}H). The chemically produced 4-hydroxy-, 4,6-dibromo-, and 4-nitro-3-methylacetanilide from 3-methylacetanilide(4-^{3}H) retained tritium approximately 65% in each case.

Since no significant effect of feeding 0.06% 3'-Me-DAB for 1—4 weeks on the aryl hydroxylation activity against 3'-Me-DAB(5'-^{3}H) and retention of tritium in the hydroxylated product from 3'-Me-DAB(4'-^{3}H) was observed, it may be suggested that the aryl hydroxylase activity of rat liver against 3'-Me-DAB dose not be qualitatively changed during the early stage of the carcinogenesis by 3'-Me-DAB.

Introduction

It was discovered at the National Institutes of Health (U.S.A.) that tritium or deuterium migrate intramolecularly to the adjacent position in the aromatic ring when the labelled position is enzymically hydroxylated.[4] Migrations of chloro, bromo, and methyl substituents also occur. The intramolecular migration of aromatic ring substituents, which occurs during enzymatic hydroxylation by mixed-function oxidases, provides a new criterion for evaluating chemical model systems capable of introducing the hydroxyl moiety into aromatic rings.

1) Part II: S. Baba, Y. Mori, and K. Toyoshi, *Yakugaku Zasshi*, **92**, 1364 (1972).
2) This was partly presented at the 92nd Annual Meeting of Pharmaceutical Society of Japan, Osaka, April, 1972.
3) Location: *a*) *Mitahora 492-36, Gifu*; *b*) *Kitashinjuku 3-20-1, Shinjuku-ku, Tokyo.*
4) G. Guroff, J.W. Daly, D. Jerina, B. Witkop, and S. Udenfriend, *Science*, **157**, 1524 (1967).

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Such migrations, commonly referred to as the NIH shift, generally occur in the enzyme reactions[4] and consequently, nonezymatic hydroxylating systems which do not produce them may no longer be considered as meaningful models for mixed-function oxidases. Using the various hydroxylating systems, Daly, *et al.* revealed that such migrations occur also in certain nonenzymatic reactions.[5]

In a previous report,[1] we investigated the metabolism of the carcinogenic azo dye, 3′-methyl-4-dimethylaminoazobenzene(3′-Me-DAB) with rat liver by use of the tracer method, and the hydroxylated products at the 4′ position were identified among the metabolites. Using the two tritiated 3′-Me-DAB *i.e.*, 3′-Me-DAB(4′-^{3}H) and 3′-Me-DAB(5′-^{3}H), we studied whether tritium migration does occur during enzymatic hydroxylation, nonenzymatic hydroxylation, and such substitution reactions as bromination and nitration.

It was reported that the administration of the carcinogenic 3-methylcholanthrene or benzpyrene, an inducer of drug metabolizing enzymes, caused a decrease in the retention of deuterium in 4-hydroxyacetanilide enzymically produced from 4-deuteroacetanilide.[6] Then, at the early stage of the carcinogenesis by 3′-Me-DAB,[7] the effect of the repeated administration of 3′-Me-DAB upon the aryl hydroxylation activity against 3′-Me-DAB(5′-^{3}H) and retention of tritium in the hydroxylated product from 3′-Me-DAB(4′-^{3}H) was also studied.

Experimental

Infrared (IR) spectra were obtained on a Jasco model IRD 301, ultraviolet (UV) spectra on a Hitachi model 101, nuclear magnetic resonance (NMR) spectra on a JEOL model JNM4H-100, and mass spectra on a Hitachi model RMU7L. Thin-layer chromatography (TLC) was performed on silica gel (Wakogel B-5F, or B-10) plates and scanned with Aloka TLC-2B.

Materials——According to the method described in the previous report,[8] 3′-Me-DAB(4′-^{3}H) and 3′-Me-DAB(5′-^{3}H) were prepared by coupling dimethylaniline with diazonium salts of 3-toluidine(4-^{3}H) and 3-toluidine(5-^{3}H), respectively, which were obtained by tritiation of 4-bromo- and 5-bromo-3-toluidine, respectively, in ethanol over palladium black. Radiochemical purity of ^{3}H-3′-Me-DAB was confirmed by comparing the behaviors in TLC and paper chromatography (PPC) (scanning with Aloka TRM-1) with those of the authentic sample.[8] Specificity of labelled position in ^{3}H-3′-Me-DAB was ascertained in each case by IR and NMR spectra of its starting material of coupling reaction, ^{2}H-3-toluidine and of ^{2}H-3′-Me-DAB. 3-Toluidine(4-^{2}H, or 5-^{3}H) was prepared by a similar reduction of the corresponding bromo compound with deuterium gas in monodeuteroethanol, and 3′-Me-DAB(4′-^{2}H, or 5′-^{2}H) was similarly obtained by coupling dimethylaniline with diazonium salt of 3-toluidine(4-^{2}H, or 5-^{2}H). And deuterium contents were determined by mass spectrometry. 3-Methylacetanilide(4-^{3}H, or 5-^{3}H), which was obtained chemically by hydrogenation and acetylation or enzymically by rat liver[1] from 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H), was used as a substrate for chemical substitutions. The specific ^{3}H activity/mmole of the employed substrate was as follows: 3′-Me-DAB(4′-^{3}H), 85.80 mCi; 3′-Me-DAB(5′-^{3}H), 17.25 mCi; and 3-methylacetanilide(4-^{3}H, or 5-^{3}H), 0.5—30 μCi.

Determination of Radioactivity——Radioactivity assay by liquid scintillation counter (Aloka LSC-502) was carried out according to the method described in the previous report.[1] To improve accuracy in determining quenched samples, special care was taken in cases of the sample having color or chemical quenching to make the quenching level nearly equal. The specific radioactivity of such colored samples as 3′-Me-DAB, 3′-methyl-4′-hydroxy-4-dimethylaminoazobenzene(3′-Me-4′-OH-DAB) was determined by both colorimetry and liquid scintillation spectrometry.

Enzymatic Hydroxylation——Wistar male rats weighing between 150—200 g were used. 3′-Me-DAB-(4′-^{3}H, or 5′-^{3}H) was incubated with the rat liver homogenate at 37° in air for 1—2 hours, and the phenolic metabolites were separated on silica gel plate and identified by reverse isotope dilution analysis as previously reported.[1] 3′-Me-4′-OH-DAB obtained from several runs was gathered togather and its specific ^{3}H activity was determined.

5) J. Daly, D. Jerina, W. Landis, B. Witkop, and S. Udenfriend, *J. Am. Chem. Soc.*, **89**, 3347 (1967); D. Jerina, G. Guroff, and J. Daly, *Arch. Biochem. Biophys.*, **124**, 612 (1968); D.M. Jerina, J.W. Daly, and B. Witkop, *Biochemistry*, **10**, 366 (1971).
6) J. Daly, D. Jerina, J. Farnsworth, and G. Guroff, *Arch. Biochem. Biophys.*, **131**, 238 (1969).
7) P.E. Hughes, *Chem.-Biol. Interactions*, **1**, 301 (1969).
8) S. Baba, Y. Mori, M. Iwao, and S. Iwahara, *Yakugaku Zasshi*, **89**, 1158 (1969).

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Nonenzymatic Substitutions——Retention of tritium during chemical substitution reactions of 3-methylacetanilide(4-^{3}H, or 5-^{3}H) was studied as the model reaction for chemical substitutions at the 4′ position of 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H). The product prepared in each chemical reaction was identified by mp measurement, elemental analysis, and comparison of its spectroscopic data with those of the authentic sample.

(1) Chemical Hydroxylation: For nonenzymatic hydroxylation, the Fenton's reagent described by Udenfriend, *et al.*[9] was slightly modified. Each flask contained, in a final volume of 147 ml, 1.2 mmoles of 3-methylacetanilide(4-^{3}H, or 5-^{3}H), 47 ml of 0.05M L-ascorbic acid, 32 ml of 0.05M disodium ethylenediaminetetraacetate (EDTA), 6 ml of 0.05M ferrous sulfate, 60 ml of phosphate buffer (pH 6.1), and 2 ml of H_2O_2 (30%). The ferrous sulfate and EDTA were combined and dissolved in the phosphate buffer immediately before addition of the other components. After 2 hrs' stirring at room temperature, the unreacted substrate and products were extracted with ethyl acetate several times. The combined extracts were dried over Na_2SO_4 and concentrated before separation by chromatography. 3-Methyl-4-hydroxyacetanilide[10] was purified on silica gel plates by developing alternately with benzene–acetone (7: 1, by vol.) and with those (7: 3), usually 5 times. Special care was taken to ensure that all other hydroxylated products were completely removed before determination of specific radioactivity. Retention of tritium in 3-methyl-4-hydroxyacetanilide was calculated by dividing its specific radioactivity by that of the substrate.

(2) Bromination: 4 mmoles of 3-methylacetanilide (4-^{3}H, or 5-^{3}H) dissolved in 90% acetic acid was brominated to 3-methyl-4,6-dibromoacetanilide[11] by addition of 8 mmoles of bromine in one portion in an ice bath. The reaction mixture was poured into ice water and the crude dibromo compound was filtrated, recrystallized from ethanol to constant specific radioactivity (5 times). Retention of tritium in dibrominated product was determined in each case by comparison of the specific radioactivity with that of the substrate.

(3) Nitration: 5 mmoles of 3-methylacetanilide (4-^{3}H, or 5-^{3}H) was slowly added to 2.5 ml of nitric acid (*d* 1.50) at 0—10° in an ice bath. After 1 hrs' stirring, the reaction mixture was poured into ice water and the precipitate was filtrated. This was charged on silica gel column (Wakogel C-100) and eluated with benzene–acetone (7: 1, by vol.) to four fractions. The second yellow fraction was evaporated under reduced pressure to give a crude 3-methyl-4-nitroacetanilide,[12] which was recrystallized from water to constant specific radioactivity, usually 4 or 5 times.

Effect of the Repeated Administration of 3′-Me-DAB upon the Aryl Hydroxylation Activity and ^{3}H Retention in the Hydroxylated Product——Wistar male rats, weighing between 100—150 g at the start of the experiment were used. One group of rats were received the standard diet,[13] served as a control, and the others were fed on the standard diet containing 0.06% 3′-Me-DAB.[13] The diets and water in each group were given *ad libitum* throughout the experimental course.

(1) Assay of the Hepatic Aryl Hydroxylation Activity against 3′-Me-DAB (5′-^{3}H): In each experimental group, the animals were killed by decapitation between 1—4 weeks after the start of giving the diet. The liver was homogenized in 1.15% KCl with a Potter homogenizer having a teflon pestle in crushed ice, and 20% liver homogenate thus prepared was used as the enzyme solution. A reaction mixture contained 0.6 ml of 1.15% KCl, 0.1 ml of 0.1M $MgCl_2$, 0.2 ml of 0.6M nicotinamide, 0.5 ml of 0.1M phosphate buffer (pH 7.4), 0.1 ml of 0.1M glucose-6-phosphate, 0.1 ml of 0.1% NADP, 0.1 ml of 0.1% NAD, 1 ml of the homogenate, and 0.05 ml of the ethanol solution of 140 μg 3′-Me-DAB (5′-^{3}H), which was added last. The total volume of the reaction mixture was 2.75 ml. The incubation flask was gently shaken at 37° in air for 15 min and separation and identification of the hydroxylated products were performed as previously reported.[1] The rate of aryl hydroxylation was expressed in term of mμmoles of 3′-Me-4′-OH-DAB produced/200 mg liver/15 min.

(2) Measurement of Tritium Retention in 3′-Me-4′-OH-DAB produced from 3′-Me-DAB (4′-^{3}H): According to the procedure mentioned above, the liver of rat administered 3′-Me-DAB for 31 days was homogenized in 1.15% KCl and incubated with 3′-Me-DAB (4′-^{3}H) at 37° in air for 1—2 hours. Retention of tritium in 3′-Me-4′-OH-DAB formed from several incubations was determined by comparison of the specific radioactivity with that of 3′-Me-DAB (4′-^{3}H).

Result and Discussions

Confirmation of Label Position

When a specific position of aromatic ring is labelled with tritium or deuterium, its label position must be fully ascertained. For example, hydrogenolysis of *p*-O-tosyloxyacetanilide

9) S. Udenfriend, P.Z. Nirenberg, J. Daly, G. Guroff, C. Chidsey, and B. Witkop, *Arch. Biochem. Biophys.*, **120**, 413 (1967).
10) W.A. Jacob and M. Heidelberger, *J. Am. Chem. Soc.*, **41**, 1453 (1919).
11) S.C.J. Olivier, *Rec. Trav. Chim.* **44**, 1109 (1925).
12) N.V. Subba Rao and C.V. Ratnam, *J. Sci. Industr. Res.*, **15B**, 410 (1956).
13) Commercial product of the Oriental Yeas Industrial Company, Ltd, Tokyo, Japan.

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with Raney nickel in the presence of detuerium or tritium led to acetanilide with substantial randomization of the label throughout the ring.[5] So, it is necessary to confirm the label position of tritiated 3′-Me-DAB before the start of the experiments. It may be a confirmation method to substitute the labelled hydrogen with a substituent chemically. But, we recognized previously that the isotope effect or label migration was attended during such chemical substitutions.[8] So specificities of labelling in ^{3}H-3-toluidine and ^{3}H-3′-Me-DAB were ascertained physicochemically with IR and NMR spectra of deuterated 3-toluidine and 3′-Me-DAB prepared by the same method as tritium compounds.

The reduction of bromo-3-toluidine with deuterium as described in the Meterial Section led to the production of menodeuterated 3-toluidine (parent peak, 108 *m/e*), because the ratio of 109 peak is not more than that of the isotopic abundance.[14] And the extent of deuterium substitution was calculated about 70% to the theoretical as shown by mass spectroscopy.

The IR spectrum of 3-toluidine(4-^{2}H, or 5-^{2}H) was peculiar for each deuterated compound as shown in Fig. 1 and any contamination with each other was not detected; *i.e.*, the spectrum of 3-toluidine(4-^{2}H) is characteristic in the region of 990, 1130, 1270, and 1485 cm^{-1} and that of 3-toluidine(5-^{2}H) in the region of 850, 1290, and 1466 cm^{-1}. The NMR spectrum of 3-toluidine in CCl_4 was shown in Fig. 2A and assigned as follows: 6.27 ppm (1H, doublet, at position 6), 6.30 ppm (1H, singlet, at position 2), 6.42 ppm (1H, doublet, at position 4), 6.90 ppm (1H, triplet, at position 5). In the spectrum of 3-toluidine(5-^{2}H), the signals of 4- and 6-ring protons are changed to singlets respectively (Fig. 2B) and in that of 3-toluidine(4-^{2}H) the signal of 5-proton changed to doublet (Fig. 2C). And broadening of the 4- and 6-singlet of B and in the 5-doublet of C is typical of *ortho*-coupling with deuterium. Triplet at 6.90 ppm in B or C and doublet at 6.42 ppm in C are attributed to the residual signals of the undeuterated material. The IR and NMR (Figures. 1 and 2) of each deuterated 3-toluidine showed that the deuterium was exclusively present in the desired position. Therefore, tritium in 3-toluidine(4-^{3}H, or 5-^{3}H) prepared by the same hydrogenolysis as that for the deuterium compound was proved to be located at the only desired position.

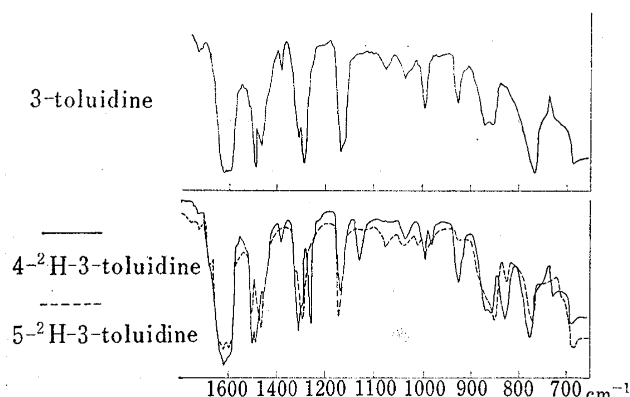

Fig. 1. Infrared Absorption Spectra of 3-Toluidine and ^{2}H-3-Toluidines (Liquid Film)

The NMR spectrum of 3′-Me-DAB(4′-^{2}H, or 5′-^{2}H) in $CDCl_3$, which was prepared from 3-toluidine(4-^{2}H, or 5-^{2}H) as described in the Material Section, was peculiar for each deuterated compound as shown in Fig. 3. It was shown that the deuterium in 3-toluidine(4-^{2}H, or 5-^{2}H) was not migrated during this coupling reaction, and that 3′-Me-DAB(4′-^{2}H, or 5′-^{2}H) was deuterated exclusively at the disired position. Accordingly, it was proved that 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H) prepared by the same coupling reaction from 3-toludine (4-^{3}H, or 5-^{3}H) was tritiated in each at the only desired position.

Enzymatic Hydroxylation

When 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H) was incubated with the rat liver homogenate, 3′-Me-4′-OH-DAB was produced in approximately 3% yield[1] after complete separation from the

14) J.H. Beynon, "Mass Spectrometry and Its Application to Organic Chemistry," Elsevier, Amsterdam, 1960.

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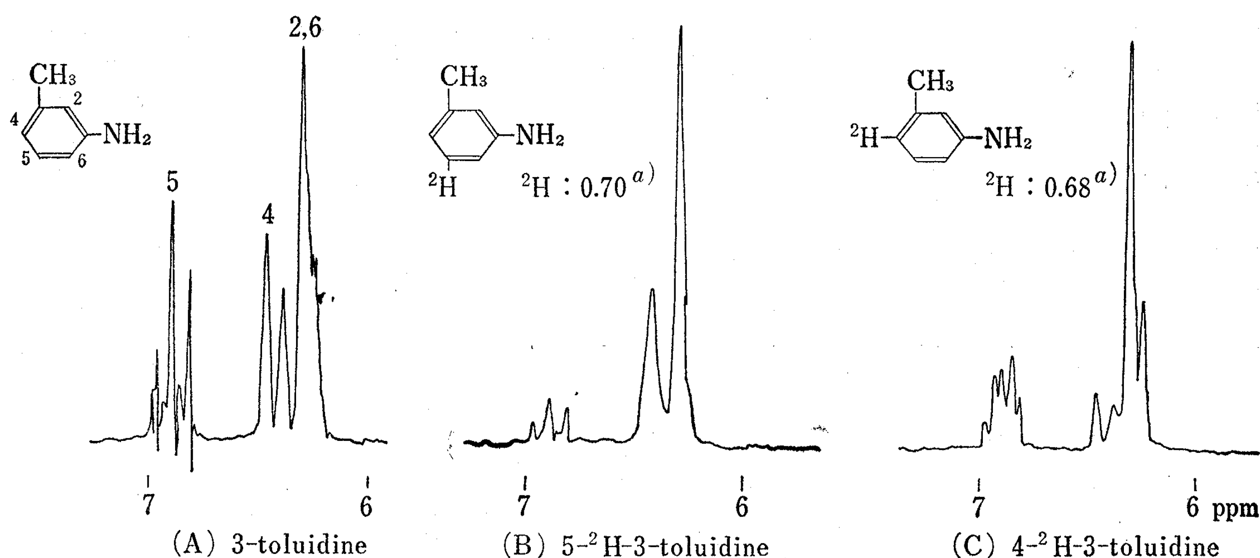

Fig. 2. NMR Spectra of 3-Toluidine and ^{2}H-3-Toluidine (CCl_4)

a) The numbers indicate the number of deuterium atoms per molecule.

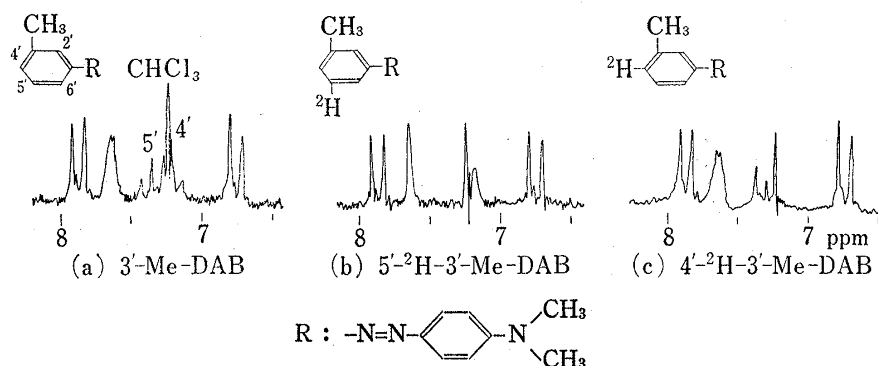

Fig. 3. NMR Spectra of 3′-Me-DAB and ^{2}H-3′-Me-DAB ($CDCl_3$)

substrate and other metabolites. Both 3′-Me-4′-OH-DAB obtained from each substrate retained tritium, independently of labelled position of substrate, 90—100% of that originally present in each substrate (Table I). No primary isotope effect was observed in the hydroxylation of ^{3}H-3′-Me-DAB with the rat liver, since the specific radioactivity of unreacted 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H) remained constant. To determine whether the tritium in the substrate and in the produce was stable under the reaction conditions, the recovered 3′-Me-DAB-(4′-^{3}H, or 5′-^{3}H) and the produced 3′-Me-4′-OH-DAB were rechromatographed and reincubated. But the specific radioactivities of the 3′-Me-4′-OH-DAB and the residual unreacted 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H) remained constant. Accordingly, it was proved that the tritium labelled or retained is stable under these experimental conditions.

Tritium retention during enzymatic hydroxylation at the 4′ position of 3′-Me-DAB-(4′-^{3}H) was found to be similar to that observed during the hydroxylation of a variety of specifically labelled aromatic substrates,[15–17] despite the fact that 3′-Me-DAB(4′-^{3}H) has only one position (5′ position) available for the tritium to migrate to. According to the influence of ring substituents, aromatic compounds are devided into 2 classes.[15] Substrates belonging to the class I exhibit lower retentions, involved the compounds with ionizable substituents, for example phenols and anilines. Tritium retention in 3′-Me-4′-OH-DAB

15) J. Daly, D. Jerina, and B. Witkop, *Arch. Biochem. Biophys.*, **128**, 517 (1968).
16) J.W. Daly, *Atomlight*, **68**, 1 (1969).
17) J. Daly, G. Guroff, D. Jerina, S. Udenfriend, and B. Witkop, *Advan. Chem. Ser.*, **1968**, No. 77, 279.

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was 94% on an average and this magnitude of tritium retention belongs to higher class among other substrates[15] to be hydroxylated. Its high retention may be attributed to that this substrate belongs to the class II, in which the substrate do not have the ionizable hydrogen and so, in which no delocalization of charge can take place in a cationoid intermediate during aromatic hydroxylation postulated by Daly, *et al.*[15,16]

It was shown[4] that no significant loss of tritium observed during the hydroxylation of substrates labelled adjacent position or positions to be hydroxylated. The similar results were obtained in the hydroxylation at 4′ position of 3′-Me-DAB(5′-^{3}H). So, these results may support the proposed mechanism[4] of NIH shift that, in case of the hydroxylation of ^{3}H-3′-Me-DAB, 3′-Me-DAB(4′-^{3}H) and (5′-^{3}H) would be hydroxylated at the 4′ position through the same transition intermediate.

Nonenzymatic Substitution

It was very difficult to hydroxylate or substitute chemically the 4′ position of 3′-Me-DAB, so, for nonenzymatic substitutions 3-methylacetanilide(4-^{3}H, or 5-^{3}H) prepared chemically or enzymically from 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H) was used as the model substrate (Fig. 4). The specific radioactivity (mCi/mmole) of 3-methylacetanilide(4-^{3}H, or 5-^{3}H) prepared thus coincided completely with that of 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H). The NMR spectrum of 3-methylacetanilide (4-^{2}H, or 5-^{2}H) in $CDCl_3$, which was obtained from 3′-Me-DAB(4′-^{2}H, or 5′-^{2}H) by the same method as the tritium compound, showed that the deuterium was exclusively present in the desired position as shown in Fig. 5. Therefore, it was proved that neither loss nor migration of tritium is accompanied during preparation of the model substrate from 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H).

The chemical yield after several purifications in hydroxylation, dibromination, or nitration of 3-methylacetanilide was 7%, 40%, or 30% respectively (Fig. 4). ^{3}H Retention in

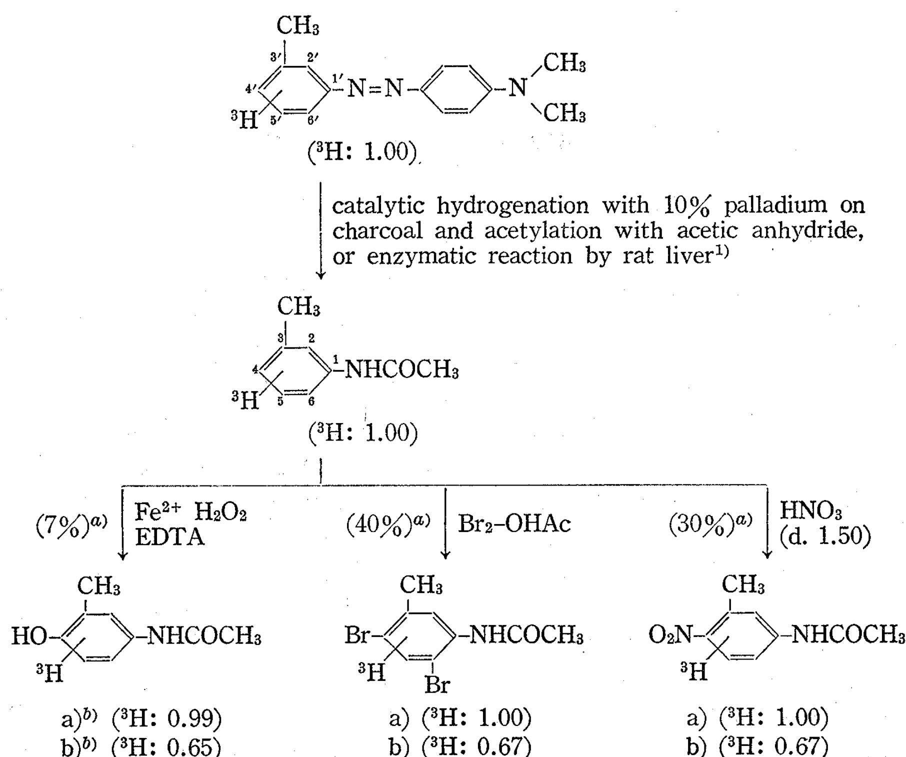

Fig. 4. Tritium Retention during Preparation of the Model Substrate from 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H) and in the Products from 3-Methylacetanilide (4-^{3}H, or 5-^{3}H) by Nonenzymatic Substitutions

a) Chemical yield in each substitution reaction after several purifications.
b) The a) is retention of tritium in each product from 3-methylacetanilide (5-^{3}H) and b) is from 3-methylacetanilide (4-^{3}H).

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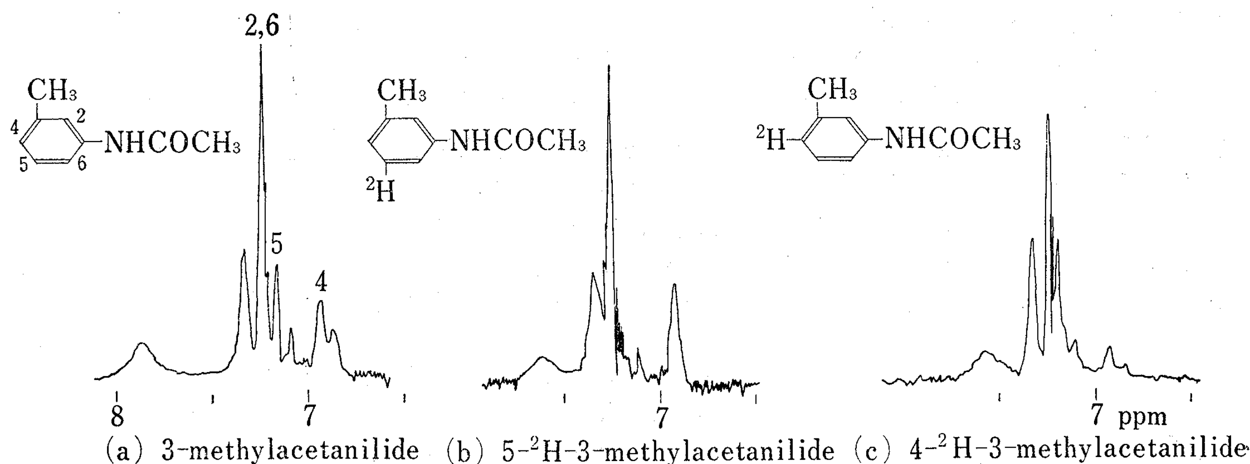

(a) 3-methylacetanilide (b) 5-^{2}H-3-methylacetanilide (c) 4-^{2}H-3-methylacetanilide

Fig. 5. NMR Spectra of 3-Methylacetanilide and ^{2}H-3-Methylacetanilide ($CDCl_3$)

4-hydroxy-, 4,6-dibromo-, and 4-nitro-3-methylacetanilide obtained from 3-methylacetanilide-(5-^{3}H) were approximately 100% as shown in Fig. 4, so the tritium attached to benzene ring was proved to be stable under the conditions of these chemical substitutions. The products, which were obtained from 3-methylacetanilide(4-^{3}H) by substitutions at 4 position with hydroxyl, bromo, or nitro group, retained tritium 65.2%, 67.2%, or 67.2% on the average respectivly (Fig. 4). The results were recognized also in the case when the enzymically produced 3-methylacetanilide(4-^{3}H, or 5-^{3}H) from 3′-Me-DAB(4′-^{3}H, or 5′-^{3}H) was used as a substrate for nonenzymatic reaction.

It is an unexpected result that more than half of ^{3}H was retained in such chemical reaction as the labelled position was substituted. Intramolecular migration of tritium or deuterium during nonenzymatic hydroxylation was observed in case of chemical hydroxylation by peroxytrifluoroacetic acid, peroxyacetic acid, *m*-chloroperoxybenzoic acid,[5] or photolysis of aromatic-N-oxide[18] as an oxidant. But the present study may be first observation that tritium was retained in the product of hydroxylation by the modified Fenton's reagent (generating OH-radical)[19,20] or of the electrophilic substitution such as halogenation[4,19] and nitration.[5] Taking into consideration of dibromination experiment (Fig. 4), it may be seen that the tritium retained should be located in the adjacent position (5 position) to substitution. But now, by use of 3-methylacetanilide(4-^{2}H, or 5-^{2}H) as a substrate, the nonenzymatic sub-

Table I. ^{3}H Retention in the Products of Various Reactions

Substrate	System	Product	Retention[a]
3′-Me-DAB(5′-^{3}H)	enzymatic	4′-OH	96.0
3′-Me-DAB(4′-^{3}H)	hydroxylation	4′-OH	94.1
3-Methylacetanilide (5-^{3}H)	Fe^{2+}, H_2O_2, EDTA	4-OH	98.8
	dibromination	4,6-diBr	99.8
	nitration	4-NO_2	99.6
3-Methylacetanilide (4-H^3)	Fe^{2+}, H_2O_2, EDTA	4-OH	65.2
	dibromination	4,6-diBr	67.2
	nitration	4-NO_2	67.2

a) retention % $= \frac{\text{specific radioactivity of product (mCi/mole)}}{\text{specific radioactivity of substrate}} \times 100$

18) D.M. Jerina, J.W. Daly, and D.R. Boyd, *Tetrahedron Letters*, **1970**, 457.
19) V. Ullrich and H. Staudinger, "Microsomes, Drug, Oxidi. Pro. Symp.," Academic Press, New York, 1969, p. 199.
20) J.W. Daly and D.M. Jerina, *Biochim. Biophys. Acta*, **208**, 340 (1970).

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stitutions and enzymatic hydroxylation are under investigation in detail to determine where tritium retained during the present experiments is migrate to.

The results of enzymatic and nonenzymatic reactions were summarized in Table I.

Effect of the Repeated Administration of the Carcinogenic 3′-Me-DAB upon the Aryl Hydroxylation Activity and ^{3}H Retention in the Hydroxylated Product

The results are shown in Fig. 6. The activity of aryl hydroxylation, lying between 4 and 9 mμmoles/200 mg liver/15 min, was not apparently affected by the administration of 3′-Me-DAB. When the liver homogenate of rats fed 3′-Me-DAB for 31 days as an enzyme solution and 3′-Me-DAB(4′-^{3}H) as a substrate were used for enzymatic hydroxylation, ^{3}H retention of the obtained 3′-Me-4′-OH-DAB was approximately 95% as shown in Table II.

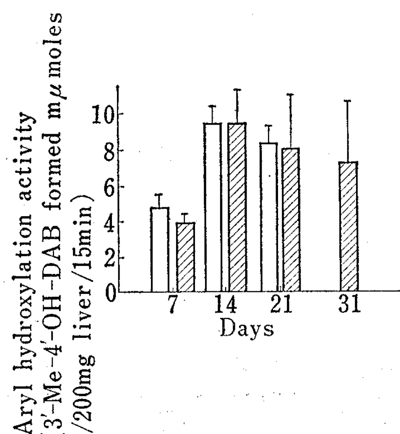

Fig. 6. Effect of Feeding 3′-Me-DAB on the Activity of Aryl Hydroxylation in the Rat Liver Homogenate
control diet: ▭
0.06% 3′-Me-DAB containing diet: ▨

It was reported by Hughes,[7] concerning the incidence of tumors after 0.06% 3′-Me-DAB for 1—6 weeks to Wistar male rats, that in rats fed dye for 3 weeks or less no tumors resulted while practically all rats fed dye for 5 or more weeks developed hepatic neoplasms. The present data demonstrated that no significant effect of feeding dye for 1—4 weeks on the aryl hydroxylation activity against 3′-Me-DAB (5′-^{3}H) was observed as shown in Fig. 6. This observation is in agreement with the previous work by Yamane, *et al.*[21] in which the aryl hydroxylation activity against DAB was measured after feeding 0.09% DAB for 1—21 weeks to Wistar female rats.

Table II. Effect of Feeding 3′-Me-DAB on the Retention of Tritium of 3′-Methyl-4′-hydroxy-4-dimethylaminoazobenzene produced by the Rat Liver Homogenate

Experiment	3′-Me-DAB(4′-^{3}H) (mCi/mmole)	3′-Me-4′-OH-DAB[a] (mCi/mmole)	Retention of ^{3}H in product (%)
a	85.74	81.13	94.62
b	85.74	81.53	95.09
c	85.74	81.67	95.25

The liver of rats fed 3′-Me-DAB for 31 days was homogenized in 1.15% KCl and incubated with 3′-Me-DAB(4′-^{3}H) as substrate according to the method described in experimental section.
a) 3′-Me-4′-OH-DAB formed from several incubations.

It was shown that the administration of the carcinogenic 3-methylcholanthrene or benzpyrene caused the formation of cytochrome P_1-450,[22] which differs from cytochrome P-450 in various points, and caused a decrease in the retention of deuterium in 4-hydroxyacetanilide produced from 4-deuteroacetanilide.[6] However, the retention of tritium in the 3′-Me-4′-OH-DAB produced from 3′-Me-DAB(4′-^{3}H) was not affected by the administration of 3′-Me-DAB under these experimental conditions (Table I and II). Accordingly, from these results it may be suggested that the aryl hydroxylase activity of rat liver against 3′-Me-DAB does not be qualitatively changed during the early stage of the carcinogenesis by 3′-Me-DAB.

21) Y. Yamane, K. Sakai, I. Uchiyama, M. Tabata, and A. Hanaki, *Chem. Pharm. Bull.* (Tokyo), **17**, 2488 (1969).
22) C.J. Parli and G.J. Mannering, *Molecular Pharmacology*, **6**, 178 (1970).

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