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Studies on Carcinogenic Azo Dyes. V.[1] The NIH Shift during the Aryl Hydroxylation of 3′-Methyl-4-(dimethylamino)azobenzene and 3-Methylacetanilide by the Rat[2]

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The NIH shift during the aryl hydroxylations of the specifically deuterated or tritiated 3′-methyl-4-(dimethylamino)azobenzene (3′-Me-DAB) and 3-methylacetanilide by a rat *in vivo* or *in vitro* has been investigated. 3′-Me-4′-OH-DAB was excreted in the bile and 3-methyl-4-hydroxyacetanilide in urine when 3′-Me-DAB[4′-^{2}H] was administered orally and 3-methylacetanilide[4–^{2}H, 4–^{3}H, or 5–^{2}H] intraperitoneally to rats. In *in vitro* experiments, 3′–Me–DAB[4′–^{2}H or 4′–^{3}H] was incubated at 37° in an aerobic conditions for 1 hr with liver homogenates and 3-methylacetanilide[4–^{2}H, 4–^{3}H, or 5–^{2}H] with liver microsomes. Retention of heavy hydrogen in the 4′- or 4-hydroxylated metabolite was determined by mass spectrometry or radioactivity counting.

The NIH shift was observed also during these hydroxylations. In *in vitro* hydroxylation of 4′-labeled 3′–Me–DAB, tritium was retained 94.1±2.7% and deuterium 43.7±3.4%. Therefore, isotope effect in retention was great in this reaction. In the case of 3-methylacetanilide, such isotope effect was not observed, and heavy hydrogen was retained 19—23% in the 4-hydroxylated metabolite *in vivo* or *in vitro*. Isotopic hydrogens at the position adjacent to hydroxylation in both substrates were stable under the employed conditions. It was demonstrated that 3-methylacetanilide belongs to class I substrate and 3′–Me–DAB to class II because of the effect of substituent on their retention of isotopic hydrogen.

Introduction

The enzymic hydroxylation of aromatic substrates specifically labeled with tritium or deuterium has been demonstrated to be accompanied by a migration of the heavy isotope from the site of hydroxylation to an adjacent position in the aromatic ring. This so-called NIH shift, the migration of a substituent (isotopic hydrogen, halogen, alkyl) observed in the metabolism of many aromatic substrates to phenols, is brought about by external monooxygenases from animal, bacterial, fungal, and plant sources.[4] The NIH shift may thus be regarded as a fundamental phenomenon of enzymic reactions. From the detailed studies of this phenomenon, Daly, *et al.*[5] proposed a mechanism for aryl hydroxylation in which arene oxides formed from substrates isomerize to phenols *via* enolization of the keto-tautomer, a transient intermediate.

Our previous papers[1,6] reported that 3′-methyl-4′-hydroxy-4-(dimethylamino)azobenzene (3′-Me-4′-OH–DAB), 3-methylacetanilide, and 3-methyl-4-hydroxyacetanilide were identified as the metabolites of 3′-methyl-4-(dimethylamino)azobenzene (3′-Me-DAB) by the liver homogenates of a rat, mouse, or hamster, and that tritium was retained in 3′-Me-4′-OH–DAB produced by the incubation of 3′-Me-DAB[4′-^{3}H] with the rat liver homogenate.[7] In the

1) Part IV: K. Toyoshi, Y. Mori, and S. Baba, *Yakugaku Zasshi*, **93**, 1554 (1973).
2) This was presented at the 6th Symposium on Drug Metabolism and Action, Tokyo, November 1974.
3) Location: *a) Mitahora higashi 5-6-1, Gifu; b) Horinouchi 1432-1, Hachioji, Tokyo.*
4) D. Reed, J. Vimmerstedt, D. Jerina, and J. Daly, *Arch. Biochem. Biophys.*, **154**, 642 (1973).
5) J. Daly, D. Jerina, and B. Witkop, *Experientia*, **28**, 1129 (1972).
6) S. Baba, Y. Mori, and K. Toyoshi, *Yakugaku Zasshi*, **92**, 1364 (1972).
7) Y. Mori, K. Toyoshi, and S. Baba, *Chem. Pharm. Bull.* (Tokyo), **21**, 2577 (1973).

present work, the NIH shift during the conversions of ^2H- or ^3H-3-methylacetanilide to 3-methyl-4-hydroxyacetanilide and of 3′-Me-DAB[4′-^2H] to 3′-Me-4′-OH-DAB in the metabolism by the rat *in vivo* or *in vitro* was examined.

Experimental

Infrared (IR) spectra were measured with a Jasco IRA-1, ultraviolet (UV) spectra with a Hitachi 181, nuclear magnetic resonance (NMR) spectra with a JEOL JNM4H-100, mass spectra with a Hitachi RMU7L or a Shimadzu LKB-9000, and radioactivity was counted by a liquid scintillation spectrometer, Aloka LSC-651.

Substrates——^2H- or ^3H-3′-Me-DAB was prepared according to the method described in the previous report.[8] 3-Methylacetanilide[4-^2H, 4-^3H, or 5-^2H] was prepared by the reduction of 4-bromo- or 5-bromo-3-methylacetanilide with deuterium or tritium gas in dioxane containing Pd on charcoal and the equivalent of $(C_2H_5)_3N$. The specific radioactivity of 3-methylacetanilide[4-^3H] was 4.14 mCi/mmol. Radiochemical purity of the tritiated substrate was confirmed by comparing the behavior on thin-layer chromatography (scanning with Aloka TLC-101) with authentic samples. Specificity of labeling was ascertained in each case by IR and NMR analyses of the corresponding deuterated compound. Deuterium content of the substrates was determined by comparison of the molecular ion in mass spectra for each derivative with that of nonlabeled compound.

***In Vivo* Metabolism**——Male Wistar rats weighing 180—230 g were administered orally 0.3 mmol of 3′-Me-DAB[4′-^2H] or intraperitoneally 0.2 mmol of ^2H- or ^3H-3-methylacetanilide (^3H: 17.34 μCi/mmol)/kg in 1 ml cottonseed oil or aqueous emulsions solubilized with Tween 80. Bile or urine was collected for 24 hr after the administration of the substrate. The bile or urine was adjusted to pH 5.0 with acetate buffer and incubated with β-glucuronidase-aryl sulfatase (IBF, 100000 U.F. units) for 24 hr at 37°. Products were extracted several times with $CH_3COOC_2H_5$ or $(C_2H_5)_2O$. The combined extract was dried over Na_2SO_4 and concentrated *in vacuo*. The hydroxylated products were isolated on silica gel plates (20×20 cm, Wakogel B-10 or B-5F) by developing sequentially with the systems of benzene–petroleum benzine (2: 1), benzene–acetone (7: 1), and benzene–acetone (7: 3). The products were identified by mp measurement, elemental analysis of the product obtained from the unlabeled substrate under the same conditions, and by comparison of their spectroscopic data with those of the authentic samples.[6] Special care was taken to ensure that all other isomers of product were completely removed before the determination of the deuterium or tritium retention. The deuterium content in the phenolic products was determined by mass spectrometry using a direct probe inlet system on either the Hitachi RMU7L or Shimadzu LKB-9000 mass spectrometer. Deuterium retention in each product was calculated by dividing its deuterium content after making appropriate correction of the molecular ion for the natural abundance of heavier isotopes by that of the substrate, and the tritium retention in the products by dividing its specific radioactivity by that of the substrate. The position of deuterium in 3-methyl-4-hydroxyacetanilide produced from deuterated 3-methylacetanilide was determined by IR and NMR analyses.

***In Vitro* Metabolism**——Incubation of ^2H- or ^3H-3′-Me-DAB with the rat liver homogenate for 1 hr and identification of the phenolic product were carried out as described previously.[6] ^2H- or ^3H-3-methylacetanilide was incubated with a crude microsomal preparation at 37° in an aerobic condition for 1 hr. A crude microsome fraction from the rat liver was prepared by homogenization of 1 part of the liver in 3 volumes of cold 1.15% KCl and centrifugation at 9000×*g* for 30 min at 0°. The incubation mixture contained 1 μmol of ^2H- or ^3H-3-methylacetanilide in 0.05 ml C_2H_5OH, 250 μmol of Tris buffer (pH 7.4), 30 μmol of nicotinamide, 3 μmol of nicotineamide adenine dinucleotide phosphate, 10 μmol of glucose 6-phosphate, and 2 ml of microsome suspension (corresponding to 0.65 g liver), in a total volume of 5.2 ml. The phenolic product was extracted with benzene–acetone (1: 1) mixture, which was then concentrated under a gentle stream of nitrogen. 3-Methyl-4-hydroxyacetanilide was isolated from the extract by TLC (Wakogel B-5F) as described in the *in vivo* metabolism. In the case of a tritium sample the product was quantitated by colorimetric assay, carrier was then added, and the material recrystallized to constant specific activity. Deuterium or tritium retention in the hydroxylated products obtained from several incubations was determined as described in the *in vivo* studies.

Results and Discussion

Confirmation of Labeled Position

In the present work, position of deuterium or tritium in the aromatic ring is of primary importance and must be fully confirmed before starting other experiments. For example,

8) S. Baba, Y. Mori, M. Iwao, and S. Iwahara, *Yakugaku Zasshi*, **89**, 1158 (1969).

Table I. Deuterated Substrates Synthesized

Substrate	Deuterium content
3-Methylacetanilide[4-^{2}H]	0.82±0.02
3-Methylacetanilide[5-^{2}H]	0.87±0.02
3′-Methyl-4-(dimethylamino)-azobenzene[4′-^{2}H]	0.78±0.03
3′-Methyl-4-(dimethylamino)-azobenzene[5′-^{2}H]	0.87

The values are the number (mean±S.D.) of deuterium atoms per molecule after making appropriate corrections for the natural abundance of heavier isotopes. The method of synthesis consisted of deuterolysis of the corresponding brominated precursor as described in the Experimental Section.

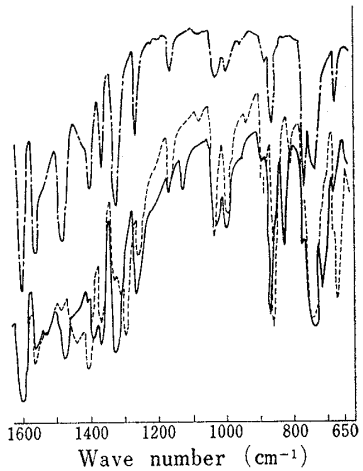


Fig. 1. Infrared Absorption Spectra of 3-Methylacetanilide and ^{2}H-3-Methylacetanilide (KBr)

———: 3-methylacetanilide
———: 3-methylacetanilide[4-^{2}H]
---------: 3-methylacetanilide[5-^{2}H]

hydrogenolysis of *p*-O-tosyloxyacetanilide over Raney nickel in the presence of deuterium or tritium led to acetanilide with substantial randomization of the label throughout the ring.[9] Specificity of the labeling in the substrates was, therefore, ascertained from IR and NMR spectra of the corresponding deuterated compounds, even though it was reported[10] that many compounds deuterated by the employed labeling method are judged to be free of the random labeling (<2%).

Establishment of the labeled position in ^{2}H-3′-Me-DAB was shown in our previous report.[7] Reduction of bromo-3-methylacetanilide with deuterium gas as described above produced a monodeuterated 3-methylacetanilide (M^+, 150 *m/e*), since the ratio of 151 peak to M^+ peak was not more than the isotopic abundance and the extent of deuterium substitution was about 85% of the theoretical, as shown in Table I.

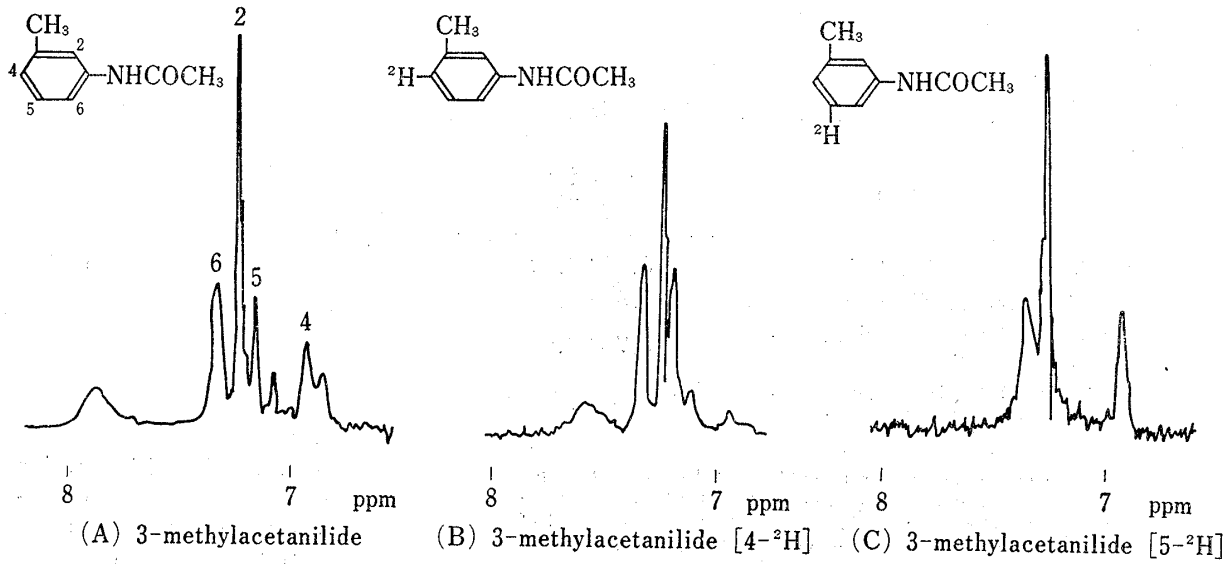
The IR spectrum of 3-methylacetanilide[4-^{2}H or 5-^{2}H] was peculiar to each deuterated compound as shown in Fig. 1 and any contamination was not detected; *i.e.*, the spectrum of 3-methylacetanilide[4-^{2}H] is characteristic in the regions of 720, 840, 1135, and 1395 cm^{-1}, and that of 3-methylacetanilide[5-^{2}H] in the regions of 680, 820, 865, 1300, and 1445 cm^{-1}. The NMR spectrum of 3-methylacetanilide in $CDCl_3$ is shown in Fig. 2, and assigned as follows: 6.86 ppm (1H, doublet, at position 4), 7.14 ppm (1H, at position 5), 7.24 ppm (1H, singlet, at position 2), 7.28 ppm (1H, doublet, at position 6). In the spectrum of 3-methylacetanilide-[4-^{2}H], the signal of 5-ring proton changed to a doublet (Fig. 2B) and in that of 3-methylacetanilide[5-^{2}H], the signals of 4- and 6-ring protons changed to singlets (Fig. 2C). Signals at 6.86 ppm in B and at 7.14 ppm in C were attributed to the undeuterated materials.

IR and NMR spectra of each deuterated substrate proved that the deuterium was exclusively located in the desired position on the ring.

Migration of Deuterium during 4-Hydroxylation of 3-Methylacetanilide[4-^{2}H] by a Rat *in Vivo*

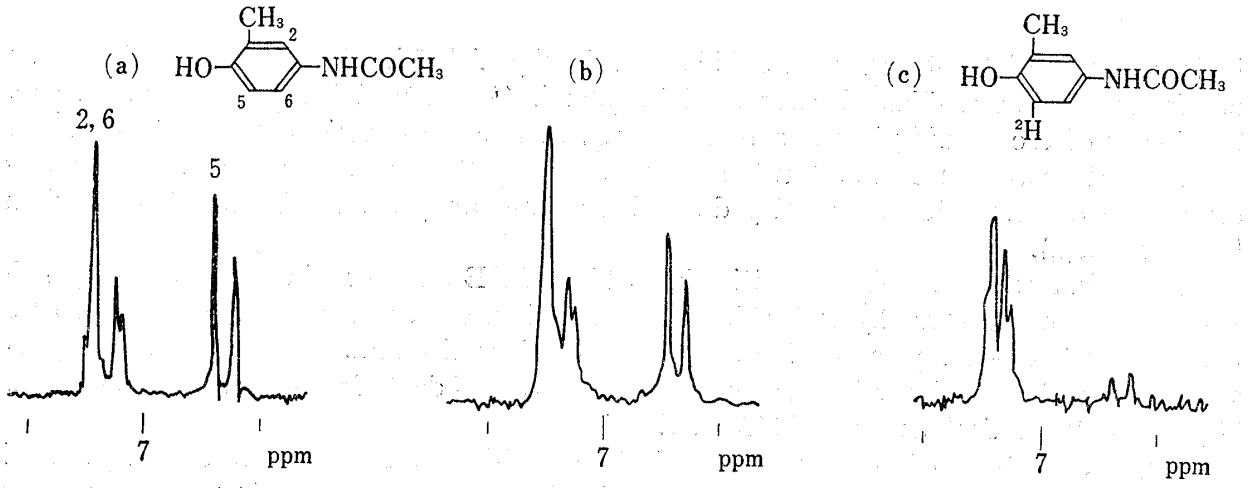
When 3-methylacetanilide was administered intraperitoneally to rats, 3-methyl-4-hydroxyacetanilide and its conjugated product were excreted in urine. The NMR spectrum of the product in $(CD_3)_2SO$ is shown in Fig. 3, and assigned as follows: 6.66 ppm (1H, doublet, at position 5), 7.18 ppm (1H, doublet, at position 6), 7.22 ppm (1H, singlet, at position 2). In

9) D. Jerina, J. Daly, W. Landis, B. Witkop, and S. Udenfriend, *J. Am. Chem. Soc.*, **89**, 3347 (1967).
10) D.M. Jerina, J.W. Daly, and B. Witkop, *Biochemistry*, **10**, 366 (1971).



(A) 3-methylacetanilide (B) 3-methylacetanilide [4-^{2}H] (C) 3-methylacetanilide [5-^{2}H]

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



(b) 3-methyl-4-hydroxyacetanilide from 3-methylacetanilide [4-^{2}H]

Fig. 3. NMR Spectra of 3-Methyl-4-hydroxyacetanilide Produced from 3-Methylacetanilide-[4- or 5-^{2}H] [$(CD_3)_2SO$]

the spectrum of the product obtained from 3-methylacetanilide[4-^{2}H] (Fig. 3b), broadening of the doublet signal at position 6 in Fig. 3b is typical of *ortho*-coupling to deuterium and integration of b (relative to the three protons of the methyl group of the product) gave 2 protons for the 2, 6 (doublet), and 0.87 protons for the 5 doublet, indicating approximately 13% deuterium content and 16% retention of deuterium. This was confirmed by mass spectrometry which showed 19.1±0.9% retention of deuterium. Fig. 3c shows that the deuterium initially present at the position adjacent to the hydroxyl group (at 5 position) was stable under these conditions. The signal at 6.66 ppm in c is attributed to the doublet at position 5 (0.15 proton), which was initially contaminated in the substrate (Table I). The IR spectrum of 4-hydroxylated product from 3-methylacetanilide[4-^{2}H] has the absorption at 1245 cm^{-1} which is characteristic of the product from 3-methylacetanilide[5-^{2}H].

Examination of this product by IR, NMR, and mass spectrometry confirmed the presence of deuterium and demonstrated that it was in the position adjacent to the hydroxyl and *meta* to the acetylated amine substituent. Accordingly, it was proved that the NIH shift also occurs during 4-hydroxylation of 3-methylacetanilide[4-^{2}H].

Retention of Isotopic Hydrogen during Hydroxylation of 3′-Me-DAB or 3-Methylacetanilide

When 3′-Me-DAB in a cottonseed oil was administered orally to rats, the 4′-hydroxylated metabolite was not detected at all in urine but excreted in bile. No primary isotope effect was observed in the aryl hydroxylation at the labeled position of deuterated or tritiated substrates.[5] Similarly, the rate of hydroxylation of 3′-Me-DAB and 3-methylacetanilide was the same in the nonlabeled, deuterated, and tritiated rings. Retention of deuterium or tritium in the hydroxylated metabolites is listed in Table II.

Table II. Retention of Isotopic Hydrogen during Aryl Hydroxylation of Specifically Labeled Substrates by a Rat

Substrate	Product	Retention of heavy isotope[a] (%)	
		in vivo	*in vitro*[b]
3′-Me-DAB[c]			
4′-^{2}H		34.1±0.4	43.7±3.4
4′-^{3}H	4′-OH	—	94.1±2.7[d]
5′-^{3}H		—	96.0±4.0[d]
3-Methylacetanilide			
4-^{2}H		19.1±0.9	20.7±1.1
4-^{3}H	4-OH	18.6±0.8	22.9±0.6
5-^{2}H		95.5±0.5	96.0±0.8

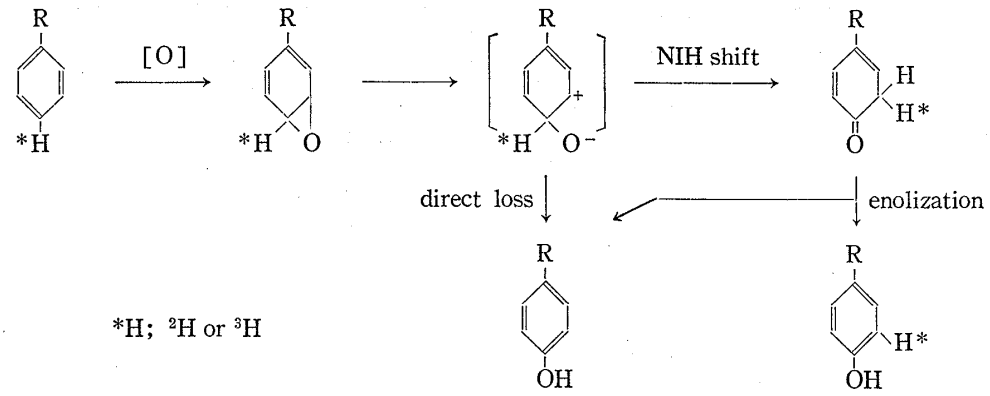
a) Deuterium content was determined by mass spectrometry and tritium content by assay and liquid scintillation counting. Derivations from the mean are presented for experiments which were repeated at least four times.
b) 3′-Me-DAB was incubated with a liver homogenate and 3-methylacetanilide with liver microsomes at pH 7.4.
c) 3′-Me-DAB=3′-methyl-4-(dimethylamino)azobenzene
d) data from the previous study[7]

Deuterium or tritium in each hydroxylated metabolite produced from 4′- or 4-labeled substrate was retained slightly higher in the *in vitro* system than *in vivo*. Since isotopic hydrogens adjacent to the position of hydroxylation were not lost during the conversion of 5′- or 5-labeled substrate to the 4′- or 4-hydroxylated product, it was proved that these are stable under the employed conditions. Tritium was retained to a higher degree (2.15 times) than deuterium during the hydroxylation of 4′-labeled 3′-Me-DAB, but there were no significant difference in retention between the isotopic hydrogens in the *in vivo* and *in vitro* formation of 3-methyl-4-hydroxyacetanilide.

The isotope effect in retention and the fate of isotopic hydrogen adjacent to the position of hydroxylation are very important in the discussion of the mechanism of hydroxylation and the NIH shift. From extensive studies on the monooxygenase-catalyzed formation of phenols from a variety of other substrates, Daly, *et al.*[11] proposed a mechanism for aryl hydroxylation as the sequence of reactions as shown in Fig. 4. Evidence for the intermediate formation of a keto-tautomer was proved in naphthalene oxide, 1,2-dimethylnaphthalene 1,2-dioxide.[12]

A proposed arene oxide and keto intermediate which tautomerizes to the phenol with significant isotope effect require that tritium be retained to a greater extent than deuterium, and that retention of tritium and deuterium be the same, regardless of whether they are initially present at the ultimate position of the phenolic hydroxyl group or in the adjacent position. During the formation of the same phenolic metabolites from anisole, aniline, and phenylalanine, tritium was retained to a higher degree than deuterium.[5] The other mechanism

11) D.M. Jerina, H. Yagi, and J.W. Daly, *Heterocycles*, **1**, 267 (1973).
12) N. Kaubisch, J.W. Daly, and D.M. Jerina, *Biochemistry*, **11**, 3080 (1972).



Class I : R=OH, NH_2, $NHCOCH_3$, $NHSO_2C_6H_5$, $NHCOCF_3$
Class II: R=OCH_3, C_6H_5, CH_3, NO_2, CN, Cl, Br, F

Fig. 4. Postulated Intermediates Formed during Enzymic Hydroxylation and Their Relation to the NIH Shift[5,11]

leading to the direct loss of heavy hydrogen in the formation of phenol from arene oxide is also proposed (Fig. 4).

This simplified mechanism adequately rationalizes the NIH shift in the formation of 3′-Me-4′-OH-DAB similar to 1-naphthol, tyrosine, and 5-hydroxytryptophan,[5] but such has not been the case for hydroxylation of 3-methylacetanilide, in which the NIH shift occurred without the isotope effect in retention and the isotopic hydrogen was preferentially retained when it was initially in the adjacent position (Table II). Accordingly, this result may support the significance of an alternative path leading to the direct loss of heavy hydrogen in the NIH shift of 3-methylacetanilide.

The magnitude of the retention was strongly affected by the nature and position of the substituent (R). Substrates have been classified into two groups[13] as shown in Fig. 4 according to the kind of R present. Since 3′-Me-DAB does not have an ionizable hydrogen in R and the retention of isotopic hydrogen in the hydroxylated product is high (Table II), 3′-Me-DAB, therefore, belongs to class II. Examples of exhibiting high retention of tritium as in the hydroxylation of 3′-Me-DAB[4′-^{3}H] (>90%) are in the metabolism of phenylalanine, tryptophan, and amphetamine.[14] It was shown that acetanilide belongs to class I and toluene to class II (Fig. 4), and that the magnitude of deuterium retention during *ortho*-hydroxylation of toluene[2-^{2}H] was found to be similar to that observed during *para*-hydroxylation of toluene-[4-^{2}H], 43% retention.[15] As expected, retention of the isotopic hydrogen in 3-methyl-4-hydroxyacetanilide produced from 3-methylacetanilide[4-^{2}H or 4-^{3}H] corresponds to half value of the NIH shift of acetanilide[4-^{2}H or 4-^{3}H].[5] Moreover, the degree of retention of the isotopic hydrogen in this reaction depended on pH of the incubation medium.[16] Consequently, in the effect of substituent on the NIH shift during hydroxylation of 3-methylacetanilide, the acetamido group is predominant, and this substrate belongs to class I, though this compound has both the methyl and acetamido groups.

Acknowledgement The authors are grateful to Mr. H. Horie and Mr. H. Mita, Tokyo College of Pharmacy, for their help in the measurement of mass spectrometry.

13) J. Daly, D. Jerina, and B. Witkop, *Arch. Biochem. Biophys.*, **128**, 517 (1968).
14) G. Guroff, J. Daly, D. Jerina, J. Renson, B. Witkop, and S. Udenfriend, *Science*, **157**, 1524 (1967).
15) J. Daly and D. Jerina, *Arch. Biochem. Biophys.*, **134**, 266 (1969).
16) Y. Mori, K. Toyoshi, and S. Baba, *Chem. Pharm. Bull.* (Tokyo), **24**, 500 (1976).