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Aryl Hydroxylation of Aminoazo Dyes and 3-Acetaminotoluene by Several Model Systems[1)]

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Enzymic hydroxylation of 3′-methyl-4-(dimethylamino)azobenzene[4′-^{2}H, 4′-^{3}H, or 5′-^{3}H] or 3′-methyl-4-(methylamino)azobenzene[4′-^{2}H] and the reactions of deuterated aminoazo dyes or 3-acetaminotoluene with several model hydroxylating systems were investigated in order to elucidate the NIH shift in these hydroxylations.

The values of retention of isotopic hydrogen (43%) were closely comparable in all hydroxylated metabolites produced from 4′-deuterated aminoazo dyes. Accordingly, it was confirmed that the enzymic hydroxylation of aminoazo dyes or 3-acetaminotoluene showed the characteristic NIH shift in each substrate.

The retention values during hydroxylation of these compounds by photolysis of hydrogen peroxide, the Udenfriend or Hamilton system, and *m*-Cl-perbenzoic acid were lower in any cases than those by photolysis of pyridine-N-oxide. Deuterium retention during these hydroxylations with pyridine-N-oxide was the same level as those obtained with rat *in vivo* and *in vitro*. Deuterium retention during all the oxidations of aminoazo dyes with the model systems used was higher than those of 3-acetaminotoluene. These results indicate the close analogy between the NIH shift recognized in the enzyme systems and that in the model hydroxylating systems.

Keywords——model hydroxylating systems; NIH shift; isotope effect in retention; carcinogenic aminoazo dyes; mass spectrometry

The NIH shift, the intramolecular migration of aromatic ring substituents during the metabolism of aromatic xenobiotics or cellular components to phenols, may be considered as a fundamental phenomenon associated with the action of monooxygenases.[3)] Such migrations are so characteristic of the enzyme reactions that oxidants which do not produce them may no longer be regarded as meaningful models for monooxygenases. Detailed studies on model hydroxylating systems which exhibit the NIH shift should permit further elucidation of the more complex enzymic oxidations.[4)]

In the previous paper, we have reported that the NIH shift was observed also during the enzymic conversion of tritiated 3′-methyl-4-(dimethylamino)azobenzene (3′-Me-DAB) or 3-acetaminotoluene to 3′-Me-4′-OH-DAB or 3-acetamino-6-hydroxytoluene, respectively.[5)] It was of interest for authors whether the NIH shift is similarly observed in the aryl hydroxylation of aminoazo dyes or 3-acetaminotoluene by model systems. Therefore, this paper deals with the NIH shift during the enzymic hydroxylation of 3′-Me-DAB[4′-^{2}H, 4′-^{3}H, or 5′-^{3}H] or 3′-methyl-4-(methylamino)azobenzene[4′-^{2}H] (3′-Me-MAB) and the chemical hydroxylation of deuterated aminoazo dyes or 3-acetaminotoluene by several model systems.

Table I shows the retention of isotopic hydrogen during 4′-hydroxylation of specifically labeled 3′-Me-DAB or 3′-Me-MAB by a rat. The retention values in all hydroxylated metabolites obtained from 4′-deuterated substrates were closely comparable. It is well known

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Table I. Retention of Isotopic Hydrogen during Aryl Hydroxylation of specifically Labeled Aminoazo Dyes by a Rat

Substrate	Product	% retention of heavy isotope[a]	
		In vivo	*In vitro*
3′-Me-DAB			
4′-^{2}H	4′-OH	44.5±0.6	43.7±3.4[b]
	4′-OH–MAB	45.4±1.9	—
4′-^{3}H	4′-OH	86.4±3.5	94.1±2.7[b]
5′-^{3}H	4′-OH	102.9±2.2	96.0±4.0[b]
3′-Me-MAB			
4′-^{2}H	4′-OH	39.2	42.5±1.0
	4′-OH–AB[c]	42.5	—

a) Deuterium content was determined by mass spectrometry and tritium by assay and liquid scintillation counting. Deviations from the mean are present for experiments which were repeated at least four times.
b) Data from the previous study.[5]
c) AB=4-aminoazobenzene.

that the magnitude of the retention was strongly affected by the nature of the substituent.[6] However, the retention values during the conversion of 3′-Me-DAB[4′-^{2}H] or 3′-Me-MAB[4′-^{2}H] to 4′-OH-aminoazo dyes with tertiary, secondary, or primary amino group were almost equal; this result indicates that the NIH shift in these hydroxylations is independent of the nature of substituent.

Tritium was retained to higher degree (about 2 times) than deuterium during the *in vivo* hydroxylation of 4′-labeled 3′-Me-DAB in agreement with the *in vitro* results. Consequently, it was confirmed that the NIH shift recognized in the formation of 3′-Me-4′-OH-DAB occurred with the great isotope effect in retention. On the other hand, we reported that the retention of isotopic hydrogen during the *in vivo* or *in vitro* hydroxylation of 3-acetaminotoluene[6-$^{2(3)}$H] was about 20% and no isotope effect in retention was observed in this NIH shift, but the retention values were depend on the factors influencing drug metabolism and vary from 11 to 47%.[7]

These substrates which showed the characteristic NIH shift in the enzymic hydroxylation were oxidized by several model systems as shown in Table II and Table III.

Table II. Aryl Hydroxylation of 3-Acetaminotoluene [6-^{2}H] by Several Oxidants

Oxidizing system	Yield (%)	Deuterium retention (%)
Pyridine-N-oxide, ***hv***	0.03	26.5±0.9
H_2O_2, ***hv***	0.04	9.8±1.9
Fe(II), ascorbate, H_2O_2	3.9	8.4±1.1
Fe(III), catechol, H_2O_2	5.6	16.0±2.9
m-Cl-perbenzoic acid	3.0	11.0±1.8

The retention values in 3-acetamino-6-hydroxytoluene obtained from 3-acetaminotoluene-[6-^{2}H] were approximately similar to those observed during hydroxylation of 3-acetaminotoluene[6-^{3}H] with these model systems used[8]; no isotope effect in retention was recognized in the manner as the enzymic reaction. The NMR spectrum of the hydroxylated product

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Table III. Deuterium Retention (%) during 4′-Hydroxylation of 3′-Me-DAB[4′-^{2}H] or 3′-Me-MAB[4′-^{2}H] by Several Oxidants

Oxidizing system	3′-Me-4′-OH–DAB	3′-Me-4′-OH–MAB
Pyridine-N-oxide, *hv*	51.1±2.3	59.7±1.4
H_2O_2, *hv*	28.3±4.1	25.3±0.7
Fe(II), ascorbate, H_2O_2	31.6±1.2	40.7±0.2
Fe(III), catechol, H_2O_2	36.8±5.1	—
m-Cl-perbenzoic acid	22.1±1.0	—

obtained by the oxidation with *m*-Cl-perbenzoic acid, the Udenfriend or Hamilton system which catalyzed the aryl hydroxylation in higher yield than other systems (Table II) was measured in order to determine the position of deuterium retained. As shown in Fig. 1, integration of the signals gave 2 protons for the 2,4 (doublet), and 0.85—0.95 protons for the 5 doublet, indicating 5—15% deuterium content. Accordingly, it was suggested that deuterium retained in the product was present at 5 position.

In the reactions of 3′-Me-DAB[4′-^{2}H] or 3′-Me-MAB[4′-^{2}H] with the model systems used, any systems catalyzed the oxidative N-demethylation of 3′-Me-DAB or 3′-Me-MAB rather than the aryl hydroxylation; this is in agreement with the results of the enzymic reaction.[9] Similar result in the oxidation of DAB with Fenton's reagent was already reported by Ishidate *et al.*[10]

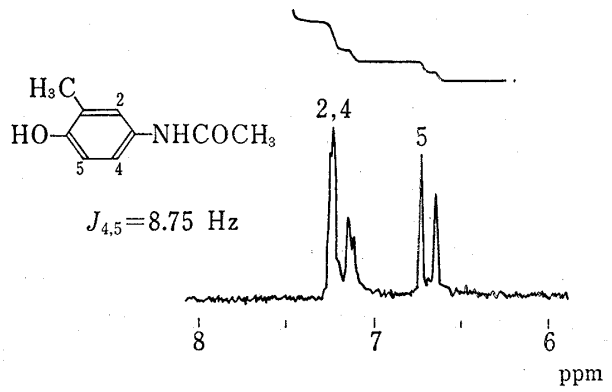

Fig. 1. NMR Spectrum of 3-Acetamino-6-hydroxytoluene produced from 3-Acetaminotoluene[6-^{2}H] by Oxidant [$(CD_3)_2SO$]

Pyridine-N-oxide photolysis is of great value as a mechanistic model for enzymic oxidation, since this system is capable of many oxidation reactions typical of mixed function oxidases and exhibits the NIH shift in which the retention values are similar to those observed during hydroxylation of several substrates with microsomes.[11] Deuterium retention during hydroxylations of 3-acetaminotoluene[6-^{2}H] and 4′-deuterated aminoazo dyes with this system was also the same level as those obtained with rat *in vivo* and *in vitro*.[5] On the other hand, the retention values during aryl hydroxylation of aminoazo dyes and 3-acetaminotoluene by photolysis of hydrogen peroxide, the Udenfriend or Hamilton system and *m*-Cl-perbenzoic acid were lower in any cases than those by photolysis of pyridine-N-oxide. However, it should be noted that the hydroxylation of aminoazo dyes by any model systems employed showed higher retention than that of 3–acetaminotoluene; these results indicate the close analogy between the NIH shift recognized in the enzyme systems and that in the model systems.

Experimental

Labeled Compounds——3′-Me-DAB, 3′-Me-MAB, and 3-acetaminotoluene were labeled at the specific position with tritium or deuterium by the method described in the previous report.[5]

Enzymic Hydroxylation——Male Wistar rats weighing 180—200 g were orally administered 0.3 mmol/kg of labeled 3′-Me-DAB or 3′-Me-MAB in 1 ml of cottonseed oil. Bile was collected for 24 hr after the administration. ^{2}H-3′-Me-MAB was incubated with 9000 *g* supernatant fraction of liver homogenate at 37°

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for 30 min. The phenolic metabolites extracted from the hydrolyzed bile or incubation mixture were isolated by thin-layer chromatography (TLC).[5]

Chemical Hydroxylation——Oxidation of ^{2}H-3′-Me-DAB, 3′-Me-MAB, or 3-acetaminotoluene with such model systems as photolysis of pyridine-N-oxide or hydrogen peroxide, *m*-Cl-perbenzoic acid, and the Udenfriend or Hamilton system were carried out as described in the previous report,[8] except for the use of *tert*-BuOH as solvent of aminoazo dyes.

Retention of Isotopic Hydrogen in Hydroxylated Products——Percentage retention of deuterium or tritium in each product isolated with TLC was calculated by dividing its deuterium content determined by mass spectrometry or its specific radioactivity by that of the substrate.[5,7,8]

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Studies on Gas–Liquid Chromatography Separative Determination of 4′-Chloro-5-methoxy-3-biphenylylacetic Acid and Its Metabolites in Urine

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The amounts of 4′-Chloro-5-methoxy-3-biphenylylacetic acid (DKA-9) and its metabolites (DKA-9G, DKA-24, DKA-24G, DKA-24OG and DKA-24S, see Chart 1) in human urine could be determined separately by measuring the amounts of DKA-9 and DKA-24 generated after hydrolizing under various conditions with gas–liquid chromatography.

Keywords——gaschromatography; anti-inflammatory drug; separative determination; deconjugation; metabolite; derivatization

4′-Chloro-5-methoxy-3-biphenylylacetic acid (DKA-9) is a potent anti-inflammatory and analgesic agent developed in our laboratories.[2] Its biopharmaceutical behaviors, the ADME studies, in various animal species and human were already studied[3–5] and as shown in Chart 1, DKA-9 was mainly metabolized through two major pathways, glucuronide formation and demethylation followed by sulfate formation and about 73% of the dose was excreted in urine as metabolites in 24 hours after oral administration in human.

In order to design the dosage form and dosage schedule of a drug, the drug behaviors in body must be elucidated pharmacokinetically. And for these studies, the precise analytical method of the drug and its metabolites is necessary.

The present report describes about the separative determination of DKA-9 and its metabolites in human urine by measuring the amount of DKA-9 and 4′-chloro-5-hydroxy-3-biphenylylacetic acid (DKA-24)[6] with gas–liquid chromatography (GLC) after hydrolizing glucuronides and sulfate under various conditions.

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