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Studies on Carcinogenic Azo Dyes. VI.[1] Effect of Factors Influencing Drug Metabolism on the NIH Shift during Hydroxylations of 4-Dimethylamino-3′-methylazobenzene and 3-Methylacetanilide[2]

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The NIH shift during aryl hydroxylations of 4-dimethylamino-3′-methylazobenzene (3′-Me-DAB) and 3-methylacetanilide under various conditions has been investigated. 4-Hydroxy-3-methylacetanilide was obtained from 3-methylacetanilide (4-^{2}H, or 4-^{3}H) by rat *in vivo* metabolism or by incubation with hepatic microsomal preparations of rat, mouse, hamster, or rabbit, and 3′-Me-4′-hydroxy-DAB by incubation of 3′-Me-DAB (4′-^{2}H, or 4′-^{3}H) with the liver homogenates. Retention of heavy hydrogen in the hydroxylated products was determined by mass spectrometry or liquid scintillation counting.

The degree of retention of isotopic hydrogen during hydroxylation of the labeled 3-methylacetanilide was affected by the pH of incubation media, the species variation, the sex of animal used as the source of microsomal preparations, and the pretreatment of animal with inducing agents. Pretreatment of rat or mouse with phenobarbital caused an increase in the retention of tritium in 4-hydroxy-3-methylacetanilide produced from 3-methylacetanilide-4-^{3}H, while pretreatment with 3-methylcholanthrene or 3,4-benzpyrene caused a decrease in the retention. Pretreatment of rat with the carcinogenic azo dye, 3′-Me-DAB, did not affect the NIH shift during the *in vivo* hydroxylation of 3-methylacetanilide-4-^{2}H.

The effect of induction on the retention of tritium or deuterium is unique to 3-methylacetanilide(4-^{2}H, or 4-^{3}H), and is not observed during hydroxylation of 3′-Me-DAB(4′-^{2}H, or 4′-^{3}H) in which the tritium retention is also independent on the pH of incubation media and the species.

Introduction

It was extensively recognized[4] that the activities of drug-metabolizing enzymes are influenced by the sex of animal, the species variation, and pretreatment of animal with various inducers. The induction of drug-metabolizing enzymes with phenobarbital, 3,4-benzpyrene, 3-methylcholanthrene, and other agents has received much attention in recent years because of implications in various fields. And the administration of the polycyclic hydrocarbons to rats caused the formation of a new form of microsomal hemoprotein, cytochrome P_1-450, which appears to differ from cytochrome P-450 in its biochemical and physical properties.[4,5] The NIH shift was also extensively recognized during the enzymic aryl hydroxylation reaction, which was much affected by these inducing agents, by the detailed studies making use of many aromatic substrates which were specifically labeled with tritium or deuterium and the various enzyme sources.[6]

1) Part V: Y. Mori, K. Toyoshi, and S. Baba, *Chem. Pharm. Bull.* (Tokyo), accepted.
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) Kitashinjuku 3-20-1, Shinjuku-ku, Tokyo.*
4) R. Kato, P. Vassanelli, G. Frontino, and E. Chiesara, *Biochem. Pharm.*, **13**, 1037 (1964); "Microsomes and Drug Oxidations," ed. by J.R. Gillette, A.H. Conney, G.J. Mannering, G.J. Cosmides, R.W. Eastabrook, and J.R. Fouts, Academic Press, New York, 1969; "Biological Hydroxylation Mechanisms," ed. by G.S. Boyd and R.M.S. Smellie, Academic Press, 1972.
5) C.J. Parli and G.J. Mannering, *Molecular Pharmacology*, **6**, 178 (1970).
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In the previous papers, we have reported that 4-dimethylamino-4′-hydroxy-3′-methylazobenzene(3′-Me-4′-OH-DAB), 3-methylacetanilide, and 4-hydroxy-3-methylacetanilide were identified as the metabolites of 4-dimethylamino-3′-methylazobenzene(3′-Me-DAB),[7] and that the NIH shift was observed also during the conversion of 3′-Me-DAB(4′-^{2}H, or 4′-^{3}H) or 3-methylacetanilide(4-^{2}H, or 4-^{3}H) to 3′-Me-4′-OH-DAB or 4-hydroxy-3-methylacetanilide, respectively, by rat *in vivo* or *in vitro*.[1] It was of interest for authors to investigate effects of the induction of microsomal enzymes and other factors on the NIH shift during these aryl hydroxylations. It was reported that the extent of deuterium retention in the 4-hydroxylated product from acetanilide-4-^{2}H is dependent on the pH of the microsomal incubation medium, on the animal source of the microsomes, on the presence or absence of acetone in the medium, and on pretreatment of animals with phenobarbital or polycyclic hydrocarbons.[6,8] In this paper, therefore, effects of several factors known to influence drug metabolism and the carcinogenic 3′-Me-DAB pretreatment on the hydroxylations of 3′-Me-DAB and 3-methylacetanilide, and on their NIH shift were studied.

Experimental

Procedures for preparation of the deuterated or tritiated substrates have been described in the earlier paper of this series. All deuterated substrates contained 80—90% deuterium in each desired position on the ring. The labeled position was established by infrared (IR) and nuclear magnetic resonance (NMR) analyses of the corresponding deuterated compound.[1,9]

Young adult animals were used throughout the experiment. Male or female Wistar rats weighed from 180 to 200 g, male or female dd mice 25 g, male rabbits 2.5 kg, and male Golden hamsters from 100 to 120 g. Male rats or mice were pretreated with either phenobarbital (80 mg/kg, one injection daily) for 3—4 days or with 3,4-benzpyrene (20 mg/kg in cottonseed oil, one injection daily) or 3-methylcholanthrene (40 mg/kg in cottonseed oil, one injection daily) for 2 days. For *in vivo* studies, the labeled substrate was administered 24 hr after the last injection of inducing agent. 0.2 mmole of 3-methylacetanilide (4-^{2}H, or 4-^{3}H) was administered intraperitoneally to male rats and the urine was collected for 24 hr and treated with β-glucuronidase-aryl sulfatase as previously described.[1]

In vitro hydroxylations of 4- or 4′-labeled 3-methylacetanilide or 3′-Me-DAB were carried out for 1 hr in aerobic conditions with either microsome suspension or whole homogenates.[1] Substrates were added as ethanol solutions (0.05 ml). Hydroxylated products from *in vivo* or *in vitro* studies were extracted into ethyl acetate or benzene–acetone mixture. The combined extracts were dried and concentrated to a small volume *in vacuo* or under a gentle stream of nitrogen. The phenolic products were separated and isolated by thin-layer chromatography (TLC), and the deuterium content of the product was determined by mass spectrometry using a direct probe inlet system on either a Hitachi RMU7L or a Shimadzu LKB-9000 mass spectrometer. The isolation and the determination of deuterium or tritium retention in the hydroxylated products have been described in earlier papers.[1,7,9]

Results

Experiments on *in Vivo* Hydroxylation of 3-Methylacetanilide

When 3-methylacetanilide(4-^{2}H, or 4-^{3}H) was administered intraperitoneally to rats pretreated with phenobarbital, 3-methylcholanthrene, or 3,4-benzpyrene, 4-hydroxy-3-methylacetanilide was produced in urine in 10—20% yield after the several purifications on TLC. The 4-hydroxylated product was identified by mp measurement, elementary analysis of the product from the unlabeled substrate under the same conditions, and comparison of its spectroscopic data with those of the authentic sample.

The retentions of either tritium or deuterium in 4-hydroxy-3-methylacetanilide obtained from the *in vivo* hydroxylation of 3-methylacetanilide-4-^{3}H or 3-methylacetanilide-4-^{2}H were found to be affected by pretreatment of animal with inducing agents. These data are

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8) J. Daly, D. Jerina, J. Farnsworth, and G. Guroff, *Arch. Biochem. Biophys.*, **131**, 238 (1969).
9) Y. Mori, K. Toyoshi, and S. Baba, *Chem. Pharm. Bull.* (Tokyo), **21**, 2577 (1973).

Table I. Effect of Induction on the Retention of Tritium or Deuterium in 4-Hydroxylated Product Derived from 3-Methylacetanilide (4-^{2}H, or 4-^{3}H) by Rat *in Vivo* Metabolism[a)]

Pretreatment	Percentage retention[b)] of	
	Tritium	Deuterium
None	18.6±0.8	19.1±0.9
Phenobarbital	21.4±0.9	20.5±1.0
3-Methylcholanthrene	12.9±0.3	12.2±0.4
3,4-Benzpyrene	14.4±0.1	12.6±0.5

a) Rats were received the substrate 24 hr after the last injection of inducing agents as described in Experimental. 4-Hydroxy-3-methylacetanilide was excreted in urine as a major metabolite of 3-methylacetanilide.
b) Deuterium contents were determined by mass spectrometry and tritium contents by assay and liquid scintillation counting as previously described.[1)] Deviations from the mean are presented for experiments which were repeated at least three times.

presented in Table I. No apparent effects of the phenobarbital pretreatment on the degree of retention of isotopic hydrogen in the product were observed. Consistently lower retention values of isotopic hydrogen were obtained in the hydroxylations by rat pretreated with 3-methylcholanthrene or 3,4-benzpyrene than those by untreated rat. Isotope effect in retentions, however, was not recognized in normal rat and in any case of pretreatments of rat with inducing agents.

3′-Me-DAB is as highly hydrophobic and carcinogenic compound as the polycyclic hydrocarbons. So, in this experiment effect of pretreatment of rat with 3′-Me-DAB on the retention of deuterium in 4-hydroxy-3-methylacetanilide was also studied. Rats were fed with the diet containing 0.06% 3′-Me-DAB for 1 month (received approximately 380 mg of 3′-Me-DAB/rat), and 3-methylacetanilide-4-^{2}H was intraperitoneally administered 24 hr after the last feeding. Deuterium retention in the 4-hydroxylated product, however, was 19.7±0.7%, indicating that this NIH shift was not affected by 3′-Me-DAB pretreatment.

In Vitro Hydroxylation of 3-Methylacetanilide

In the *in vitro* metabolism of 3-methylacetanilide by rat, mouse, hamster, or rabbit, effect of the pretreatment of animal with inducer, presence of acetone in the medium, or sex of animal on the retention of isotopic hydrogen in the 4-hydroxylated product was studied. When 3-methylacetanilide(4-^{2}H, or 4-^{3}H) was incubated with the microsomal system, 4-hydroxy-3-methylacetanilide was produced as the major metabolite, and neither *ortho*- nor *meta*-hydroxylated products to acetamido group could be detected on TLC. The relative hydroxylation activities in the hepatic microsomes of rat, mouse, hasmter, and rabbit in terms of μmoles of 4-hydroxy-3-methylacetanilide formed/g liver/hr are plotted against pH of the incubation media in Fig. 1.

The aryl hydroxylation activities of male rat and mouse were greatly enhanced by pretreatment with inducing agents. In the case of rat, polycyclic hydrocarbon pretreatments have a preference to phenobarbital for the induction of the aryl hydroxylation activity. The activities in the range of pH used with male and female rats were comparable, but those of female mouse show apparently higher than male under the employed conditions despite the reports[4)] that the drug-metabolizing activities of male animals is generally higher than female. As compared with other animal used, the hydroxylation activities of hamster and rabbit were higher than those of mouse and rat. Accordingly, it was found that this hydroxylation activity was dependent on the species and sex of the animal, and affected by pretreatment of animals with inducing agent. However, no primary isotope effect was observed in the aryl hydroxylation of 3-methylacetanilide, since the amounts of the 4-hydroxylated product (μmoles/g liver/hr) were the same in the nonlabeled, deuterated, and tritiated rings.

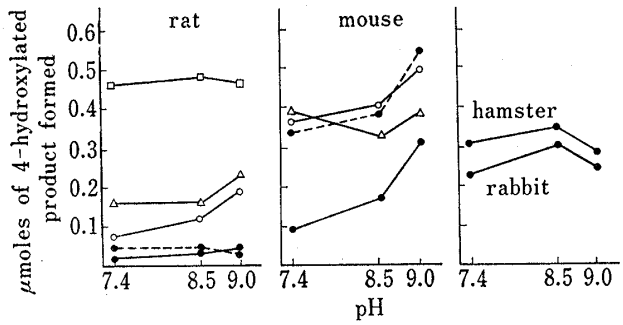

Fig. 1. Metabolism of 3-Methylacetanilide with Hepatic Microsomes

Incubations carried out with 2 ml of microsome suspension corresponding to 0.65 g liver and with 1 μmole of substrate at 37° for 1 hr. The pH's of incubation medium were adjusted with 0.1 mole Tris buffer.

normal animal
male: —●—
female: ---●---

pretreatment of male animal with
phenobarbital: —○—
3,4-benzpyrene: —△—
3-methylcholanthrene: —□—

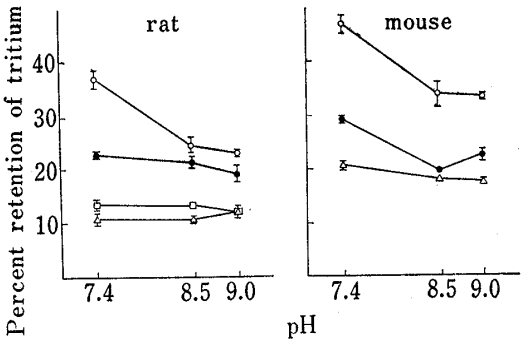

Fig. 2. Retention of Tritium in 4-Hydroxylated Product after Microsomal Hydroxylation of 3-Methylacetanilide-4-^{3}H

(●), control; (○), pretreated with phenobarbital; (△), pretreated with 3,4-benzpyrene; and (□), pretreated with 3-methylcholanthrene.

Incubations were carried out as described in Fig. 1. Standard deviations from the mean are reported for experiments which were repeated at least four times.

The effect of pretreatment of animals with inducing agents on the tritium retentions in the 4-hydroxylated product obtained from several incubations of 3-methylacetanilide-4-^{3}H with liver microsome preparations was presented in Fig. 2 along with the effect of pH of incubation medium. The degree of tritium retention in the *in vitro* hydroxylation was found to be also affected by pretreatment of animals with each inducer. Pretreatment of rat with phenobarbital caused an increase in the retention, especially at pH 7.4. Such great effect was not observed in the *in vivo* reaction (Table I). On the other hand, pretreatment of rat with 3,4-benzpyrene or 3-methylcholanthrene caused a decrease in the retention, and the tritium retentions were independent on the pH of incubation media. The values with the *in vivo* experiments (Table I) may be compared to corresponding retentions of 18.9, 23.3, 11.8, and 12.3% obtained at pH 9.0 *in vitro* for control, phenobarbital, 3-methylcholanthrene, and 3,4-benzpyrene inductions. The effects of inductions on the deuterium retention in this reaction with rat liver microsomes at pH 7.4 were also studied by use of the deuterated substrate. The values of deuterium retention obtained for control, phenobarbital, and 3,4-benzpyrene pretreatments of rat were respectively, 21±1, 29±2, and 16±1%. These results are indicatting that the similar effects were observed in the studies with the deuterated substrate as with the tritiated substrate at pH 7.4 (Fig. 2), and that in the NIH shift with 3-methylacetanilide substrate no isotope effect in retentions was recognized also in the *in vitro* studies at pH 7.4, except for about 10% low retention of deuterium in the case of phenobarbital induction.

The tritium retentions in this reaction with mouse liver microsomes were similary affected by pretreatment with phenobarbital or 3,4-benzpyrene as with rat liver microsomes (Fig. 2). The pH of incubation medium also affects the tritium retention in the 4-hydroxylated product using rat or mouse liver microsomes from control animals and animals pretreated with inducing agents. The retention values obtained with mouse are much more dependent on the pH rather than are the retentions found using rat liver microsomes.

As a factor influencing aryl hydroxylation activities, the effect of addition of acetone to the incubation mixture on this reaction was studied, for it was recognized[4,10] that the addition of acetone to an incubation mixture increases in the activity of aryl hydroxylases in a few substrates, and that both Km and V_{max} values for aniline hydroxylation are affected

10) M. Ikeda and H. Ohtsuji, in Proc. the 2nd Symposium on Drug Metabolism and Action, Kyoto, 1970, p. 63

with acetone. When 3-methylacetanilide-4-^{3}H was incubated with rat or mouse liver microsomes containing 0.2 ml of acetone (3.7%), the conversion to 4-hydroxy-3-methylacetanilide was apparently enhanced, though the relative increase was different in each case as shown in Table II.

Table II. Effect of Acetone on the Hydroxylation of 3-Methylacetanilide

Species	Pretreatment	% increase in hydroxylation with acetone[a]	% retention of tritium[b]		
			Normal (N)	Acetone (A)	A/N
Rat	none	275	23±1	21±2	0.91
	phenobarbital	361	38±1	24±1	0.63
Mouse	none	253	29±0	27±2	0.93
	phenobarbital	26	47±2	31±1	0.66
	3,4-benzpyrene	25	20±1	17±1	0.85

a) Incubations were carried out at pH 7.4 as described in Fig. 1, except for adding 0.2 ml of acetone (3.7%) to the incubation mixture.
b) Deviations from the mean are presented for experiments which were repeated at least four times.

No significant effect of acetone on the tritium retention was observed in the hydroxylations by liver microsomes of untreated rat or mouse. While, in the hydroxylations by those of animals pretreated with phenobarbital, incubations with acetone caused the almost same decrease in both animals with 34—37%, a value significantly lower than those by normal incubations. These are, however, unexpected results, for the converse results with acetanilide-4-^{2}H and no effect with anisole-4-^{2}H and 4-fluorobiphenyl-4′-^{2}H in which the hydroxylation activities were also enhanced by acetone were reported.[8] From these observations, it seems likely that the reaction of acetone to aryl hydroxylases is not a simple process.

Table III. Retention of Tritium in 4-hydroxy-3-Methylacetanilide after Microsomal Hydroxylation of 3-Methylacetanilide-4-^{3}H[a]

Species		Percent retention of tritium		
		pH 7.4	8.5	9.0
Rat	male	23±1	22±1	19±2
	female	17±2	15±2	15±0
Mouse	male	29±0	20±0	23±1
	female	34±1	25±1	26±1
Rabbit	male	30±3	20±6	21±2
Hamster	male	31±3	20±0	20.5

a) Incubations were carried out as described in Fig. 1.

Table III shows the effects of sex of animal and species variation on the tritium retention along with the pH of incubation medium. The sex of animal also had influence on the tritium retention. Comparing male animals with female, the retention values in the hydroxylations by male rat were about 5% higher, whereas those by male mouse showed approximately 5% lower at pH 7.4—9.0. So, it is difficult to interpret these data as a result of simple hormonal effect. A similar result that the deuterium retention during the hydroxylation of acetanilide-4-^{2}H by male rat was 5% higher than that by female rat was reported,[8] but there are little other examples. This reaction showed an apparent dependence on species only at pH 7.4 among male animals and at pH 7.4—9.0 between female rat and mouse (Table III). But at pH 8.5 and 9.0 among male animals no significant effect were observed.

Experiments on *in Vitro* Hydroxylation of 3′-Me-DAB

Effects of the similar factors influencing drug metabolism on the NIH shift during the hydroxylation of 3′-Me-DAB(4′-^{3}H, or 4′-^{2}H), showing higher retention,[1] were studied. Incubations carried out with 0.54 μmoles of the substrate and with 250 mg of the liver homo-

Table IV. Retention of Tritium during 4′-Hydroxylation of 4-Dimethylamino-3′-methylazobenzene-4-^{3}H by Liver Homogenates[a]

Species	Condition or pretreatment	A relative hydroxylation activity (%)[b]	Retention of tritium (%)
Rat	pH 7.4	100	94.1±2.7[c]
	pH 8.5	247	93.6±1.4
	pH 9.0	187	95.3±0.9
	pH 7.4		
	phenobartital	1124	95.4±0.4
	3-methylcholanthrene	196	94.1±1.3
	3,4-benzpyrene	153	94.6
	3′-Me-DAB	103	95.0±0.4[c]
Rabbit	pH 7.4	65	97.7±3.2
Hamster	pH 7.4	29	98.1±2.9
Mouse	pH 7.4	22	93.1

a) 0.54 μmoles of 3′-Me-DAB-4-^{3}H were incubated in air with 250 mg of liver homogenates at 37° for 1 hr, and the isolation and the determination of the product are described elsewhere.[7] Tritium retention in 3′-Me-4′-OH-DAB formed from several incubations was determined as described in Table I.

b) The values are percentages of the hydroxylation activity to as that of normal rat at pH 7.4 is 100.

c) Data from the previous study.[9]

genates at 37° for 1 hr in an aerobic condition. The hydroxylation activities were enhanced by pretreatment of inducers, especially by phenobarbital induction. The activities of rabbit, hamster, and mouse were lower than that of rat under the employed conditions.

Pretreatment of rat with inducing agents under conditions which caused induction of the aryl hydroxylases did not result in a change in the tritium retention as shown in Table IV. Similarly, the deuterium retention in 3′-Me-4′-OH-DAB produced from 3′-Me-DAB-4′-^{2}H was not also affected by inductions. The values of deuterium retention obtained for control and 3,4-benzpyrene pretreatments of rat were respectively, 43.7±3.4 and 47.5±4.5%. And the tritium retention in the 4′-hydroxylated product was also not affected by the pH of incubation medium and species variation.

Discussion

3′-Me-DAB is a potent carcinogen to the liver of rat.[11] But its pretreatment of rat had not the inducing effect for the aryl hydroxylations of 3′-Me-DAB (Table IV) and 3-methylacetanilide, though the carcinogenic polycyclic hydrocarbons are known to be inducing agents for drug metabolizing enzymes. Moreover, the hepatic activities of aryl hydroxylation of 3′-Me-DAB with rat, mouse, and hamster were not affected by 3′-Me-DAB administrations for 1—7 weeks.[12] These results show that this amino-azo dye carcinogen may be involved in the fact that many polycyclic hydrocarbons which are potent carcinogens are poor inducing agents, and *vice versa*.[13] The NIH shift during the *in vitro* hydroxylation of 3′-Me-DAB-4′-^{3}H or the *in vivo* hydroxylation of 3-methylacetanilide-4-^{2}H was not also affected by pretreatment of 3′-Me-DAB for 1 month. Accordingly, it may be suggested that the aryl hydroxylase activities of rat for 3′-Me-DAB and 3-methylacetanilide were not qualitatively changed during the early stage of the carcinogenesis by 3′-Me-DAB.

Induction of the aryl hydroxylases for 3′-Me-DAB with phenobarbital or polycyclic hydrocarbon and other factors influencing drug metabolism did not affect the retention of

11) J.A. Miller, *Cancer Res.*, **30**, 559 (1970).
12) Y. Mori, K. Toyoshi, and S. Baba, *Chem. Pharm. Bull.* (Tokyo), submited.
13) J.C. Acros, A.H. Conney, and Ng. Buu-Hoi, *J. Biol. Chem.*, **236**, 1291 (1961).

isotopic hydrogen in 3′-Me-4′-OH-DAB formed from 3′-Me-DAB (4′-^{3}H, or 4′-^{2}H). Aromatic substrates have been classified into two groups depending on the effect of substituent on the NIH shift.[14] Taking this classification into consideration, it was found that the NIH shift during hydroxylations of 3′-Me-DAB, phenylalanine-4-^{3}H,[6] anisole-4-^{2}H, 4-fluorobiphenyl-4′-^{2}H, and chlorobenzene-4-^{2}H[8] which are all belonging to class II substrates is independent on such conditions as the pretreatment, species, and pH of the incubation medium, and these NIH shifts depend solely on the nature of substrate.

On the other hand, the NIH shift with 3-methylacetanilide (4-^{2}H, or 4-^{3}H) was much affected by several conditions. Pretreatment of rat or mouse with phenobarbital caused an increase in the retention of isotopic hydrogen in the product while pretreatment with 3,4-benzpyrene or 3-methylcholanthrene which causes the formation of a new hemoprotein in the liver[4,5] caused a decrease in the retention (Table I, Fig. 2). It was reported that the retention values for closely related acylanilines[6] are also dependent on the incubation conditions. It is, however, surprising to find that the NIH shift with 3-methylacetanilide substrate was such greatly dependent on every factors influencing drug metabolism. The observed variation of retentions of isotopic hydrogen during 3-methylacetanilide (4-^{2}H, or 4-^{3}H) hydroxylation may tend to support the concept of a multiplicity of drug-metabolizing enzymes. Chemical hydroxylations of specifically labeled 3-methylacetanilide with deuterium or tritium as a useful further guide for the enzymic hydroxylations are now under investigated.

14) J. Daly, D. Jerina, and B. Witkop, *Arch. Biochem. Biophys.*, **128**, 517 (1968).