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Absence of isotope effects in the microsomal hydroxylation of acetanilide*

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THE IMPORTANT role played by the hydroxylating enzymes present in liver microsomes in the detoxication of foreign organic compounds is established. In a mechanism study of the conversion of acetanilide to *p*-hydroxyacetanilide, with rabbit liver microsomes, it was shown[1] that molecular oxygen is utilized by the microsomes and is incorporated as the hydroxyl group in *p*-hydroxyacetanilide.

The present study was undertaken to determine whether carbon–hydrogen bond breaking is rate determining in the microsomal hydroxylation of acetanilide. For this purpose *p*-tritioacetanilide was incubated with rabbit liver microsomes and the unreacted acetanilide was isolated and examined for tritium enrichment. In addition, the rate of hydroxylation of pentadeuterioacetanilide was compared with that of unlabeled acetanilide. Prompted by the reports by Guroff *et al.*[2, 3] that the labels are retained in the product after the enzymic parahydroxylation of *p*-tritio- and *p*-deuteriophenylalanine, we also examined the extent of retention of the *para*-tritium in *p*-hydroxyacetanilide after the enzymic hydroxylation of *p*-tritioacetanilide.

EXPERIMENTAL

Acetanilide-4′-H^3_1 (*p*-tritioacetanilide) was prepared by decomposing *p*-aminophenyl-lithium, prepared by the procedure of Gilman and Stuckwisch,[4] with water enriched with tritium (0·25 mc/g). The aniline-NH^3_2-4-H^3_1 was isolated by preparative gas-phase chromatography and then acetylated.

To assess the per cent of tritium in the *para*-position of the synthesized tritioacetanilide, the compound was brominated in acetic acid and the sp. act. of the purified *p*-bromoacetanilide was

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ascertained.* By this procedure, it was established that 41 per cent of the label in the tritioacetanilide was actually in the *para*-position. This was taken into account in calculating the degree of retention of *para*-tritium in *p*-hydroxyacetanilide.

Pentadeuterioacetanilide was prepared from pentadeuteriontrobenzene purchased from Stohler Isotope Chemicals, Quebec, Canada. The reduction of nitrobenzene to form aniline was carried out as described by Fieser.[5] The NMR spectrum of the final product, pentadeuterioacetanilide, revealed no detectable aromatic carbon–proton bond.

Rabbits were pretreated with sodium phenobarbital (75 mg/kg/day) for 3 days. The liver microsomal and postmicrosomal supernatant fractions were prepared according to a procedure previously published.[6]

The incubation mixture was extracted twice with ether after adding 2 g K_2HPO_4. The combined ether extract was evaporated to dryness. The residue was taken up in a small volume of ethanol and applied as a streak on a 5 × 20 cm or a 20 × 20 cm thin-layer plate, coated with silica gel GF254. The plates were developed with 5% methanol in ether. Acetanilide and *p*-hydroxyacetanilide were eluted with ether; the former was assayed either by an ultraviolet spectrophotometric method (242 mμ) or by the method of Brodie and Axelrod,[7] and the latter was assayed by the method of Mitoma and Udenfriend[8] or by the method of Ninomiya.[9] Radioactivity was determined by a Nuclear-Chicago scintillation counter.

RESULTS

The isotope effect of substituting tritium for hydrogen in the *para*-position of acetanilide was investigated by examining the re-isolated *p*-tritioacetanilide for tritium enrichment. As shown in Table 1, the sp. act. of *p*-tritioacetanilide after part of it was allowed to hydroxylate were identical with those that did not undergo any reaction. The ratio, k_H/k_T, was virtually unity, which indicated the absence of a primary kinetic isotope effect in the microsomal hydroxylation reaction.

TABLE 1. ISOTOPE ENRICHMENT EXPERIMENT WITH *p*-TRITIOACETANILIDE*

Expt. no.		*p*-Tritioacetanilide (cpm/μmole)	k_H/k_T
1	Control	2375	0·995
	Experimental	2364	
2	Control	2274	0·999
	Experimental	2271	
3	Control	2266	0·977
	Experimental	2214	

* Incubation beakers consisted of 0·5 ml Tris buffer, 0·2 M, pH 8·0; nicotinamide, 15 μmole; NADP, 0·25 μmole; acetanilide-4′-H^3_1, 4 μmole; glucose 6-phosphate, 40 μmole; glucose 6-phosphate dehydrogenase, 7 units; ATP, 5 μmole; NADH, 5 μmole; and 0·3 ml of microsome preparation in a final volume of 2·4 ml. The incubation was performed at 37° in air for 1 hr.

Each figure is an average of three incubations. The control samples were treated similarly to the experimental samples, except that the rabbit liver microsomes were heat denatured. The per cent hydroxylation was 27 per cent.

The kinetic experiments on pentadeuterioacetanilide were carried out to rule out the possibility that, before the hydroxylation reaction took place, the *para*-tritium may have been removed by a valence bond isomerization that is observed under photochemical conditions to isomerize substituted benzenes.[10] The data obtained with pentadeuterioacetanilide were no different from those with tritioacetanilide (Table 2).

* We thank Dr. John Daly of National Institutes of Health for suggesting this experiment.

Guroff *et al.* reported that large amounts of the *para*-tritium or deuterium of phenylalanine migrated to the neighboring positions (*meta* to the side chain) during hydroxylation and were retained by the product, tyrosine.[2, 3] A similar migration was observed during hydroxylation of acetanilide by the liver microsomal hydroxylase system.*

TABLE 2. LACK OF DEUTERIUM ISOTOPE EFFECT IN THE HYDROXYLATION OF PENTADEUTERIOACETANILIDE*

Expt. no.		Per cent reaction	*p*-Hydroxyacetanilide (μmole formed/beaker)	k_H/k_D
1	Control	18·1	0·73 ± 0·14	1·11
	Experimental	16·3	0·65 ± 0·10	
2	Control	19·6	0·78 ± 0·05	1·03
	Experimental	19·1	0·76 ± 0·05	
3	Control	14·0	0·56 ± 0·02	0·99
	Experimental	14·1	0·57 ± 0·10	

* The experimental conditions were the same as described under Table 3 except that 4 μmole pentadeuterioacetanilide was used as the substrate. Equivalent amounts of unlabeled and deuterioacetanilide were weighed out fresh for each experiment.
Each figure is the average ± S.D. from 5 separate incubations.

TABLE 3. RETENTION OF TRITIUM BY THE PRODUCT*

Per cent reaction	Sp. act. (cpm/μmole)		Retention of tritium (Percent)	
	Substrate (A)	Product (B)	B/A	Corrected†
20	2096 ± 104	1858 ± 105	88·6	72·6

* Incubation beakers consisted of 1·0 ml of Tris buffer, 0·2 M, pH 8·0; nicotinamide, 15 μmole; NADP, 0·25 μmole; glucose 6-phosphate, 25 μmole; $MgCl_2$, 20 μmole; NADH, 5 μmole; acetanilide-4′-H^3, 4 μmole; and 0·5 ml each of microsomes and supernatant in a final volume of 3·5 ml. The incubation was performed at 37° in air for ½ hr.
The figure is the average ± S.D. of 10 separate incubations.

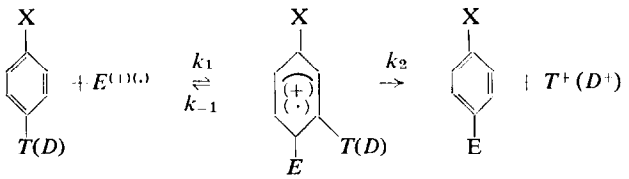
† Corrected retention of the *para*-tritium $= \frac{(B) - 0{\cdot}59\,(A)}{0{\cdot}41\,(A)} \times 100.$

In agreement with their observation, we found substantial amounts of the *para*-tritium to be retained by *p*-hydroxyacetanilide (Table 3).

DISCUSSION

Melander[11] has reviewed the use of tritium substitution on aromatic nuclei for determining rate effects on aromatic substitution processes. He has concluded that electrophilic and radical substitutions in aromatic nuclei proceed by a two-step mechanism. In reactions which show no isotope effects, such as nitration of toluene, k_1 is rate determining. Small isotope effects have been observed in the sulfonation of bromobenzene where k_2 is rate determining.

* Personal communication from Dr. S. Udenfriend, NIH.



The results reported here seemed to indicate that, in the hydroxylation of acetanilide, carbon–tritium bond breaking is not the rate determining step and that either the attachment of the molecular oxygen to the *para*-position or fixation of oxygen by the enzyme is the slow step in this reaction.

The extensive retention of the *para*-tritium in the product can be explained by analogy to the findings of Guroff *et al.* with respect to phenylalanine hydroxylation that the *para*-tritium migrated to the neighboring positions during hydroxylation and that the tritium is preferentially retained at the new position because of the greater strength of the C–T bond over that of the C–H bond.

It is of interest that among the microsome-catalyzed oxidative reactions, we have observed definite kinetic isotope effects in the oxidation of 3′-deuterio-5-butyl-5-ethylbarbituric acid (Neonal) to 3-ethyl-5-(3′-hydroxybutyl)-barbituric acid and of CD_3-*o*-nitroanisole to form *o*-nitrophenol.[12] Similar to the aromatic hydroxylation reaction, the microsome-catalyzed oxidation of CD_3-tolbutamide to form hydroxymethyltolbutamide was not affected by the insertion of a heavy isotope at the site of oxidation.[13]

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