RETENTION OF DEUTERIUM IN p-TYROSINE FORMED ENZYMATICALLY FROM p-DEUTEROPHENYLALANINE

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Radioisotope assay methods which depend upon the production of tritiated water from the tritium at the position of hydroxylation have been successfully used in the study of tyrosine hydroxylase (Nagatsu, Levitt and Udenfriend, 1964), proline hydroxylase (Stone and Meister, 1962; Hutton, Tappel and Udenfriend, in press), and tyrosinase (Pomerantz, 1965). Attempts to devise a similar assay for the adaptive phenylalanine hydroxylase of Pseudomonas sp. (ATCC 11299A) (Guroff and Ito, 1963; Guroff and Ito, 1965) using commercial p-tritiophenylalanine were unsuccessful. Unexpectedly, in the case of the Pseudomonas enzyme, although large amounts of tyrosine were produced, little or no tritiated water appeared. Preliminary experiments indicated that the tyrosine still contained large amounts of tritium.

In order to investigate this problem further, p-deuterophenylalanine was synthesized by catalytic reduction of p-bromo-DL-phenylalanine with pure deuterium. The deuterophenylalanine was purified by column chromatography and was shown by nuclear magnetic resonance spectroscopy of its dichromate oxidation product (Kollmann, 1928), benzoic acid, to contain deuterium in the para

position only (Fig. 1). The n.m.r. spectrum (AA'BB' pair of doublets) was typical of a p-substituted benzoic acid.

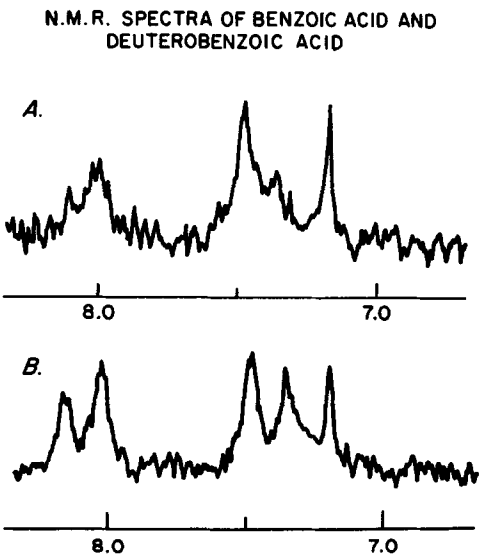

Fig. 1. N.M.R. spectra of A) benzoic acid obtained from phenylalanine by chromic acid oxidation and B) p-deuterobenzoic acid from deuterophenylalanine by chromic acid oxidation. The peak furthest to the right belongs to chloroform which was used as a reference.

The p-deuterophenylalanine was used as substrate for the Pseudomonas phenylalanine hydroxylase and the product was assayed using the nitrosonaphthol method (Waalkes and Udenfriend, 1957). Although an apparent isotope effect was observed (Table I), large amounts of p-tyrosine were produced from the fully deuterated material.

The enzymatically formed tyrosine from several reaction mixtures was isolated by paper chromatography in n-butanol, acetic

Table I

Action of Bacterial Phenylalanine Hydroxylase on Phenylalanine and p-Deuterophenylalanine

Incubation with:	Tyrosine formed
	mμmoles
p-deutero-DL-phenylalanine	149
L-phenylalanine	442

Incubations contained bacterial phenylalanine hydroxylase[1], 58 μg of protein, Fe^{++}, 250 mμmoles, DPNH, 1 μmole, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, 0.15 μmole (in 0.1 M mercaptoethanol) and 1 μmole of L-phenylalanine or p-deutero-DL-phenylalanine. Total volume: 0.25 ml. Incubation: 60', 30°, in air. Under these conditions maximal rates are obtained with L-phenylalanine.

acid, H_2O (12:3:5). The amino acid was eluted and compared in an MS-9 mass spectrometer with synthetic tyrosine and with tyrosine produced by incubation with nondeuterated phenylalanine. The product formed from deuterophenylalanine was found to be a mixture of deuterotyrosine (60 to 80%) and tyrosine (Fig. 2). Tyrosine itself gives a characteristic fragment of molecular weight 107 corresponding to the p-hydroxybenzyl residue. The analogous fragment from the product of deuterophenylalanine had a molecular weight of 108. The same results were obtained when the enzyme incubations were treated with dinitrofluorobenzene and the amino acid derivatives extracted and examined directly by mass spectroscopy instead of being separated by paper chromatography.

[1] Guroff, G. and Reifsnyder, C.A., details to be published.

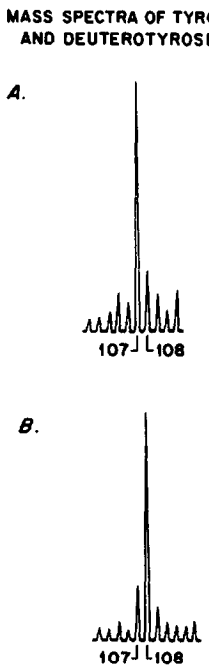

Fig. 2. Mass spectra (340°) of A) tyrosine produced by hydroxylation of phenylalanine and B) tyrosine produced by hydroxylation of p-deuterophenylalanine.

Retention of such substantial amounts of deuterium after hydroxylation in the para position can only be explained if the original p-substituent of the phenylalanine migrated to another position on the ring during the process of enzymatic hydroxylation. Preliminary experiments with p-tritiophenylalanine confirmed the retention of the p-substituent in the molecule and suggested that it migrated to the meta position since tritiotyrosine prepared with the bacterial phenylalanine hydroxylase from p-tritiophenylalanine (Nuclear-Chicago) released substantial amounts of tritium upon reaction with purified adrenal tyrosine hydroxylase[2] (Nagatsu,

[2]We wish to thank Dr. M. Levitt for performing these experiments.

Levitt and Udenfriend, 1964).

It appears, then, that the mechanism of phenylalanine hydroxylation involves migration of the para-substituent to the meta position. Data obtained in these laboratories over the past several years suggest that a similar migration occurs during hydroxylation of the appropriate substrates with the aromatic hydroxylases of liver microsomes, the tryptophan hydroxylase of mouse mast tumor cells, and the phenylalanine hydroxylase from rat liver. Indeed, it is possible that the complex oxidations catalyzed by p-hydroxyphenylpyruvate oxidase also involve a migration of this type.

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