THE PRODUCTION OF META-TRITIOTYROSINE FROM p-TRITIO-PHENYLALANINE BY PHENYLALANINE HYDROXYLASE

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Recent work in these laboratories has shown that the para-hydroxylation of para-deuterophenylalanine with bacterial phenylalanine hydroxylase yields tyrosine which contains substantial amounts of deuterium (Guroff, Reifsnyder, and Daly, 1966). This indicated that the original para-substituent migrated to another position in the molecule during the hydroxylation. In order to investigate this phenomenon more fully and, specifically, to locate the position in the molecule to which the para-substituent had migrated, further experiments have been carried out using para-tritio-L-phenylalanine (Nuclear-Chicago Corporation). This material was prepared by catalytic replacement of the chlorine in para-chlorophenylalanine by tritium using $Pd/CaCO_3$ catalyst in alcoholic potash. This method, when used in the preparation of deuterophenylalanine from para-bromophenylalanine yielded phenylalanine with isotope exclusively in the para-position (Guroff, Reifsnyder, and Daly, 1966).

The para-tritiophenylalanine was purified by paper chromatography in 2-propanol:NH_3:H_2O (8:1:1) in order to remove, among

other things, any trace of contaminating radioactive tyrosine. The para-tritiophenylalanine was used as substrate for either the purified[1], activated phenylalanine hydroxylase from Pseudomonas sp. (Guroff and Ito, 1965) or a preparation of rat liver phenylalanine hydroxylase purified through the first ammonium sulfate fractionation (Kaufman, S., 1962). Incubations were carried out for 120 min at 30° in air in the presence of 6,7-dimethyltetrahydropteridine 0.15 μmole, Tris buffer, pH 7.3, 25 μmoles, carrier L-phenylalanine, 1 μmole, and water in a final volume of 0.25 ml.

Following incubation several such mixtures were heated at 100° for 2 min. After centrifugation the supernatant fractions were chromatographed in 2-propanol:NH_3:H_2O (8:1:1). The tyrosine was eluted from the papers and its specific activity estimated by means of scintillation counting and the nitrosonaphthol method (Waalkes and Udenfriend, 1957). In each case approximately 40% of the phenylalanine substrate was converted to tyrosine. With both enzymes the specific activity of the tyrosine produced was found to be 70% of that of the phenylalanine substrate.

The tyrosine obtained by paper chromatography was incubated for 30 min at 37° with purified bovine adrenal tyrosine hydroxylase (Nagatsu, Levitt, and Udenfriend, 1964a) in the presence of dimethyltetrahydropteridine, mercaptoethanol, and phosphate buffer, pH 6.5. After incubation half the mixture was used for isolation of the product, DOPA (Nagatsu, Levitt, and Udenfriend, 1964b), while the other half was used for assay of the tritiated water

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[1] Guroff, G. and Rhoads, C. A., in preparation.

produced in the reaction (Nagatsu, Levitt, and Udenfriend, 1964c). The data show (Table I) that almost equal amounts of radioactivity were found in the DOPA and in the water. The same result was obtained with 3,5-ditritiotyrosine (New England Nuclear Corporation) used as a control. The radioactivity in DOPA and water are probably somewhat closer than shown in the table since the recovery of DOPA by this procedure has been estimated at 85% while that of water is at least 90%.

Table I

Action of tyrosine hydroxylase on tyrosine produced by enzymatic hydroxylation of p-tritio-L-phenylalanine

Source of tyrosine	Specific activity	Radioactivity (dpm) in:	
		THO	DOPA
	dpm/mμmole		
L-tyrosine from incubations with Pseudomonas enzyme	2100	62,500 62,500	49,500 53,700
L-tyrosine from incubations with liver enzyme	1800	63,100 62,900	54,900 56,400
L-tyrosine-3,5-T (New England Nuclear Corp.)	6000	223,000 238,000	201,750 225,000

The mixtures were incubated for 30 min at 37° with 1.5 mg enzyme protein. Heated controls were about 5% of the above values and incubations without dimethyltetrahydropteridine 1-2%. All counting was done between 7 and 10% efficiency. Values are presented uncorrected for recovery.

Tyrosine hydroxylase has been shown to act exclusively on para-tyrosine and to produce DOPA (Nagatsu, Levitt, and Udenfriend, 1964c). If the tyrosine contained tritium in one of the meta-positions, equal amounts of radioactive DOPA and THO should be produced. That is, excluding isotope effects, the enzyme should

remove tritium from half the molecules producing radioactive water and remove hydrogen from the other half leaving radioactive DOPA. If the tritium was in any other position such equivalence could not be found. The control ditritio-tyrosine also gave equivalence since in this case both the water produced and the DOPA formed from a single molecule of tyrosine are radioactive.

Although the data from the tyrosine hydroxylase experiments are clear and localize the tritium from the para-position of phenylalanine in the meta-position(s) of tyrosine a second and independent approach to this problem was undertaken. Iodination of a phenol, such as tyrosine, is known to occur exclusively in the meta-positions to form 3,5-diiodotyrosine (Oswald, 1909). Accordingly, the tyrosine produced in the phenylalanine hydroxylase reactions was treated with N-iodosuccinimide under mild conditions. Separate experiments on the iodination reaction have shown that, as with iodine itself, the reaction of tyrosine with N-iodosuccinimide under the conditions used in this work gave at least an 80% yield of 3,5-diiodotyrosine. At the end of the reaction the mixtures were acidified and passed over short Dowex-50 columns to absorb the amino acid. The tritium released by iodination was estimated by counting the tritiated water passing through the column. The data show (Table II) that most of the radioactivity in the tyrosine was released by iodination. Again 3,5-ditritiotyrosine, used as a control, behaved in an identical fashion to the enzymatically produced tyrosine.

These experiments show that large amounts of the para-tritium of the substrate phenylalanine migrated to the neighboring position(s) of the ring (meta to the alanine side chain) during the

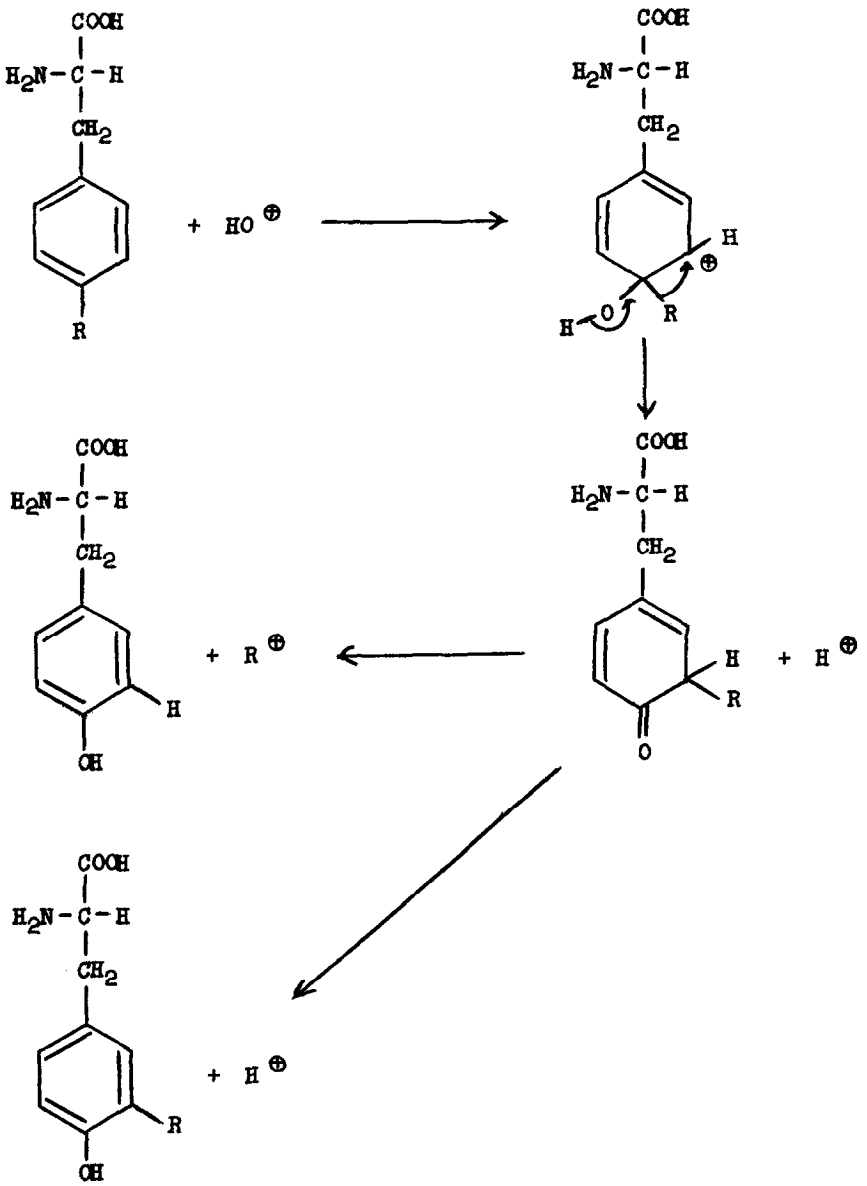
Table II

Action of N-iodosuccinimide on tyrosine produced by enzymatic hydroxylation of p-tritio-L-phenylalanine

Source of tyrosine	Radioactivity of tyrosine (dpm)	Released by iodination (dpm)	%
L-tyrosine from incubations with Pseudomonas enzyme	136,200	111,100	82
L-tyrosine from incubations with liver enzyme	128,800	107,200	83
L-tyrosine-3,5-T (New England Nuclear Corp.)	77,000	62,700	81

The reaction mixtures contained tyrosine, Tris buffer, pH 7.3, 25 μmoles, mercaptoethanol, 3 μmoles, and water to a final volume of 0.75 ml. N-iodosuccinimide (0.2 ml of a 1% solution in water) was added and allowed to react for 5 min at 0°. Then 0.05 ml of 30% trichloroacetic acid was added and the mixtures passed over 1 ml columns of Dowex-50. Each column was washed with 1 ml of water and the combined eluate and wash were counted in a scintillation counter at efficiencies of between 7 and 10%. Less than 100 cpm passed through the columns from incubations in which the iodinating agent was omitted.

formation of tyrosine. On the basis of these and other experiments now in progress a working hypothesis concerning the mechanism of hydroxylation has been formulated and is shown below. This mechanism assumes that the hydroxylation is electrophilic. When R is deuterium or tritium a large proportion of radioactive product is found since the strength of the carbon-tritium or carbon-deuterium bond is substantially greater than that of the carbon-hydrogen bond.



R = H, D or T

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