THE PRODUCTION OF META-CHLOROTYROSINE FROM PARA-CHLOROPHENYLALANINE BY PHENYLALANINE HYDROXYLASE

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It has recently been shown that the hydroxylation of para-deuterophenylalanine by bacterial phenylalanine hydroxylase leads to deuterated tyrosine (Guroff, Reifsnyder, and Daly, 1966). Further studies using para-tritiophenylalanine and either bacterial or mammalian phenylalanine hydroxylase have proved that the original para-substituent migrated to the meta-position of the tyrosine which had been formed (Guroff et al., 1966). Similar migrations of deuterium and tritium have now been shown to occur during tryptophan hydroxylation by the mast-cell tryptophan-5-hydroxylase (Renson et al., 1966) and during acetanilide hydroxylation by liver microsomes[1].

Consideration of published findings on the hydroxylation of para-halogenated phenylalanines with purified liver phenylalanine hydroxylase (Kaufman, 1961) suggested to us that migrations of halogen atoms might also occur. In these experiments it was found that when para-halogenated phenylalanines were used as substrates, the amount of TPNH consumed was much greater than the amount of tyrosine formed. Since tyrosine had been assayed by the highly

[1] Udenfriend, S., Zaltzman-Nirenberg, P., Daly, J., Guroff, G., and Witkop, B., in preparation.

specific nitrosonaphthol procedure (Waalkes and Udenfriend, 1957) it was conceivable that most of the TPNH was utilized in forming an additional, undetected hydroxylated product. By analogy with the metabolism of the para-deutero- and para-tritiophenylalanines it was postulated that the metabolism of para-halogenated phenylalanines could lead to the production of meta-halogenated tyrosine. The studies presented here concerning the action of bacterial phenylalanine hydroxylase on para-chlorophenylalanine show that this is indeed the case. Similar observations have been made using liver phenylalanine hydroxylase.

Para-chloro- and meta-chloro-DL-phenylalanines were obtained from Chas. Pfizer and Co., Inc. Meta-chloro-L-tyrosine was provided by Warner-Lambert Research Institute. Para-chloro-m-hydroxy-DL-phenylalanine (para-chloro-meta-tyrosine) was prepared from 4-chloro-3-hydroxybenzaldehyde (Hodgson and Beard, 1926) by O-benzylation, reduction to the benzyl alcohol with lithium aluminum hydride and conversion to the benzyl chloride with thionyl chloride. The benzyl chloride was reacted with diethylacetamidomalonate, the product was hydrolyzed with a concentrated hydrochloric acid/acetic acid mixture and the amino acid was purified by gradient elution from a Dowex-50 column.

A 15- to 20-fold purified[2] preparation of Pseudomonas phenylalanine hydroxylase (Guroff and Ito, 1965) was used for these studies. Preliminary experiments were done using descending paper chromatography in 2-propanol:NH_3:H_2O (80:10:10) to examine the re-

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

action mixtures. For positive identification and quantitation the enzymatic products were separated using a 20 cm column of PA-35 resin at pH 5.28 and 50° on a Beckman Amino Acid Analyzer Model 120 C.

Initial studies showed that the action of bacterial phenylalanine hydroxylase on para-chloro-DL-phenylalanine led to the production of a material which had an R_f characteristic of chlorotyrosine (~0.15), as well as to the production of tyrosine (~0.40). When aliquots of the reaction mixtures were placed on the Amino Acid Analyzer (Fig 1) it was clearly shown that the major product was indeed meta-chlorotyrosine. In addition the chromatograms confirmed that tyrosine was also a product of the reaction.

Control experiments demonstrated that the production of meta-chlorotyrosine was dependent upon the presence of both the reduced pteridine cofactor and the enzyme. The purity of the substrate, para-chlorophenylalanine, was verified using the Amino Acid Analyzer. This material did not contain any of the isomeric meta-chlorophenylalanine which is easily separated from the para-isomer.

On spraying with N,2-6-trichloro-p-benzoquinoneimine and exposure to NH_3 fumes paper chromatograms revealed a light blue spot overlapping but not superimposable on the ninhydrin positive area due to meta-chlorotyrosine. A blue color with the quinoneimine reagent is characteristic of meta-tyrosines (Gibbs, 1927) and the spot probably represents para-chloro-meta-tyrosine. On the Amino Acid Analyzer synthetic para-chloro-meta-tyrosine was separated completely from the major product meta-chlorotyrosine. It was eluted from the columns after meta-chlorotyrosine and exactly where a small

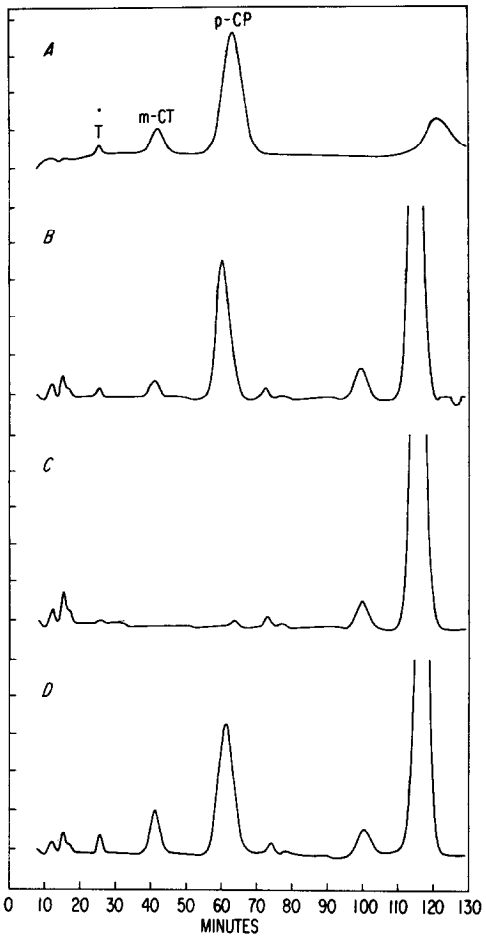

Fig. 1. Chromatograms of aliquots from various reaction mixtures.
A. Standard mixture. T - tyrosine (2 mμmoles); m-CT - meta-chlorotyrosine (10 mμmoles); p-CP - para-chlorophenylalanine (100 mμmoles).
B. Enzyme incubation with para-chlorophenylalanine as substrate.
C. Enzyme incubation with no substrate.
D. Same as B but with 10 mμmoles of meta-chlorotyrosine added for co-chromatography.
Incubation mixtures contained enzyme (approx. 1 mg of protein), ferrous ammonium sulfate, 250 mμmoles, Tris, pH 7.3, 25 μmoles, DPNH, 1 μmole, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, 0.15 μmole (in 0.03 ml of 0.1 M mercaptoethanol), and para-chlorophenylalanine, 1 μmole in a final volume of 0.25 ml. The mixtures were incubated for 120 min at 30° and then heated for 1 min at 100°; the mixtures were filtered and 0.025 ml used for amino acid chromatography.

additional peak was observed due to the metabolism of para-chlorophenylalanine by the enzyme. This peak was more evident in experiments in which larger aliquots of the mixtures were chromatographed.

Thus three products were produced by the action of bacterial phenylalanine hydroxylase on para-chlorophenylalanine. The structures and approximate proportions of these products are shown in Fig 2. It can be seen that the major product was meta-chlorotyro-

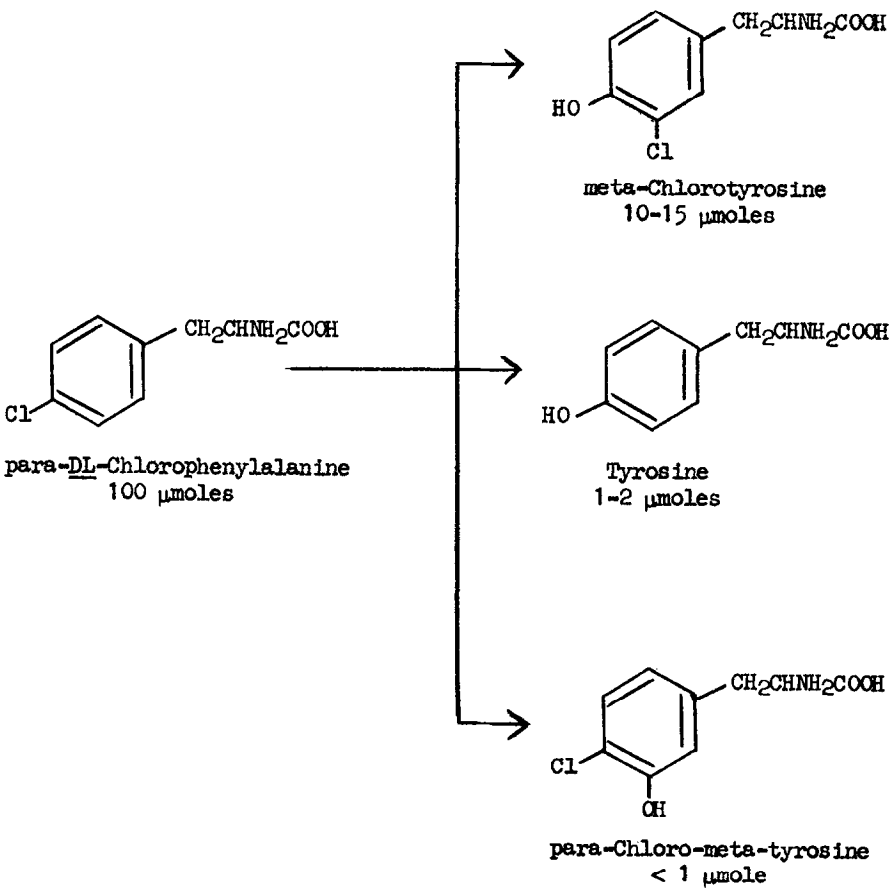

Fig. 2. Products formed through the action of bacterial phenylalanine hydroxylase on para-chlorophenylalanine.

sine. About one-tenth as much tyrosine was produced and a trace of para-chloro-meta-tyrosine could be detected. Experiments with purified rat liver phenylalanine hydroxylase showed that meta-chlorotyrosine was also the major product of its action on para-chlorophenylalanine although the liver enzyme was much less active with chlorophenylalanine as substrate than was the bacterial enzyme.

Analogous experiments have indicated that meta-bromotyrosine is produced from para-bromophenylalanine by phenylalanine hydroxylase. On the other hand no fluorinated tyrosines have yet been detected after the action of either bacterial or liver phenylalanine hydroxylase on para-fluorophenylalanine. In this case tyrosine appears to be the only product formed by both enzymes.

The formation of meta-chlorotyrosine from para-chlorophenylalanine is consistent with the previous observations (1,2) on the fate of deuterium or tritium in the para-position and extends the range of these observations to include the migration of substituents other than hydrogen and its isotopes. The working hypothesis presented in a previous communication (2) can be amplified to explain the formation of all the products found in these experiments. The meta-chlorotyrosine could arise by migration and retention of chlorine, the tyrosine by migration and loss of chlorine. Although other explanations are at present equally plausible, para-chloro-meta-tyrosine could result from an initial attack of hydroxyl in the para-position and its subsequent migration to the meta-position.

A shift of a chlorine atom has previously been observed in the

formation of a minor product of hydroxylation of 2,4-dichlorophenoxyacetic acid by Aspergillus niger (Faulkner and Woodcock, 1965). Presumably this shift occurs by a similar mechanism. Whatever the mechanism these experiments represent an unusual pathway for the metabolism of halogenated compounds and strengthen the possibility that migrations of the type shown here are of general metabolic importance.

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