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# 5-HYDROXYDOPA, A NEW COMPOUND IN THE RAPER–MASON SCHEME OF MELANOGENESIS

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*Abstract.* Oxidation of tyrosine or dopa by tyrosinase leads to the formation of 5-OH-dopa, a compound previously unknown in melanogenesis. 5-OH-dopa, first detected as an unknown compound on HPLC of the dopa-tyrosinase incubate, was purified by $Al_2O_3$-adsorption, semipreparative HPLC, and ion-exchange chromatography. 5-OH-dopa was identified by comparison with the authentic compound in several chromatographic systems, by electrochemical oxidation studies and by mass spectrometry.

*Key words:* Dopa; Tyrosinase; Melanin; 5-Hydroxydopa

The formation of melanin by enzymatic oxidation of tyrosine has been extensively studied by Raper (13). He detected the following intermediary substances: dopa, 5,6-dihydroxyindole, and 5,6-dihydroxyindole-2-carboxylic acid. Dopa was isolated as a lead salt, and the two indoles as their dimethyl ethers. Raper found that in an initial stage of melanin formation a red colour appeared which he ascribed to the formation of dopachrome. He regarded tyrosinase as being responsible for the reactions up to dopaquinone, but considered the subsequent stages to be non-enzymatic (12).

Mason studied the oxidation of dopa spectroscopically (9), and observed a red stage with absorption maxima at 305 and 475 nm, a purple stage with maxima at 300 and 540, and a final stage with general absorption. These early studies led to a concept of melanogenesis often described as the Raper–Mason scheme, according to which melanin is a polymer of indole-5,6-quinone. However, later studies by several research groups have yielded evidence that melanin produced by the enzymatic oxidation of tyrosine is a much less homogeneous polymer than was originally thought.

Many of the compounds illustrated in Fig. 1 seem to copolymerize with the indoles (10). In vivo the polymers may become still more complicated by incorporation of other molecules, e.g. cysteine (14).

Many attempts to define intermediates in melanogenesis have been hampered by the instability of these compounds. Recently developed techniques for studying catecholic compounds using high pressure liquid chromatography (HPLC) and electrochemical detection have created new possibilities in this field, however (5, 6). We now report the detection of a previously unknown intermediate of melanogenesis in the Raper–Mason scheme, 5-hydroxy-dopa. We first observed the compound as a peak in HPLC chromatograms of incubates of dopa with tyrosinase which did not correspond to any of the known intermediary substances in melanogenesis.

## MATERIAL AND METHODS

The chemicals used were dopa (Merck), 5-OH-dopa (Hoffman–La Roche, Basle), 6-OH-dopa (Labkemi AB, Gothenburg), and tyrosinase (Sigma, 4000 U/mg). All-glass triple-distilled water was used throughout.

4 mg dopa was incubated with 0.08 mg tyrosinase in 24 ml 0.1 M phosphate buffer (pH 6.5) for 15 min at 22°C. The incubation was interrupted by addition of 4 M perchloric acid to a final concentration of 0.4 M. 400 mg $Al_2O_3$, 200 mg EDTA, and 10 mg sodium metabisulphite was then added. The pH was adjusted to 7.5 with 2 M sodium carbonate. The alumina was spun down at 4000 r.p.m. and washed twice with distilled waterbefore elution with 3 ml 0.1 M HCl. 0.1 ml of the eluate was analysed by HPLC, and an unknown compound with a retention time of 9 min was found under the chromatographic conditions described below. The remaining eluate was concentrated to about 1 ml, which was purified on a semipreparative HPLC column.

A Model 6000 A (Waters Ass., Milford, Mass.) high-pressure liquid chromatograph was used with a Model-7120 100-$\mu$l sample valve injector (Rheodyne, Berkeley, Calif.) and a Model LC-10 electrochemical detector (Bioanalytical Systems, West Lafayette, Ind.). The detector potential was set at +0.75 V vs. an Ag/AgCl reference electrode. The electrode was prepared from CPO graphite material. For analytical purpose Nucleosil $C_{18}$ (5 $\mu$m, Macherey, Nagel & Co., Düren, G. F. R.) was used as column-packing material, and the columns were 200×4.6 mm. The mobile phase contained 0.5 g butanesulphonic

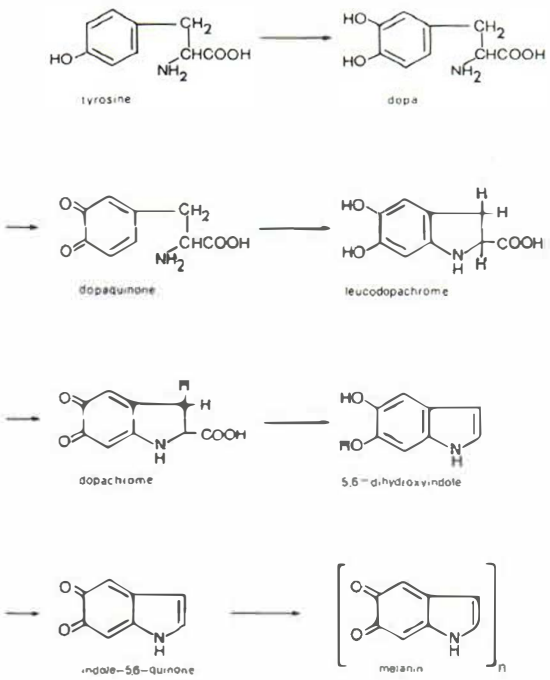


*Fig. 1.* The Raper–Mason scheme.

acid, 2.5 g methanesulphonic acid, and 2.9 g phosphoric acid per litre of water. The pH was adjusted to 1.75 with 5 M NaOH. Isocratic elution was used and the flow rate was 1.5 ml per min.

For semipreparative liquid chromatography a prepacked Microbondapack (Waters Ass.) ODS column was used, and the injector was equipped with a 600 $\mu$l loop. The flow rate was 2 ml/min. The analysis was monitored with a Varian Vari-Chrom UV-detector working at 280 nm. Fractions containing the unidentified compound were collected and loaded on a strong cation-exchange resin (Dowex 50W-X4, 50×5 mm in $H^+$-form). The column was washed twice with 5 ml distilled water, and then eluted with 1 M HCL. 2-ml fractions were collected. Fractions 3 and 4 containing the unknown compound were pooled and further analysed with HPLC using the following eluents:

I. 2.5 g Methanesulphonic acid, 0.5 g butanesulphonic acid and 2.9 g phosphoric acid per litre water. The pH was adjusted to 1.75 with 5 M NaOH.

II. 2.5 g Methanesulphonic acid, 0.5 g butanesulphonic acid and 2.9 g phosphoric acid per litre water. The pH was adjusted to 2.80.

III. 2.5 g Methanesulphonic acid, 0.5 g butanesulphonic acid and 2.9 g phosphoric acid per litre 5% methanolic aqueous solution. The pH was adjusted to 1.75.

The oxidation properties of the unknown substance, 5-OH-dopa, and dopa were evaluated by changing the working potential of the electrochemical detector in 0.1 V steps from 0 to 1.0 V vs. Ag/AgCl reference electrode, and running a liquid chromatogram for each substance at each potential. The height of the peak for each substance in the chromatogram then showed to what extent the substance had been oxidized.

For the GC-MS analysis a sample was evaporated to dryness after purification on alumina, semipreparative HPLC, and ion exchange chromatography. The residue was treated with 150 $\mu$l 3 M HCl in methanol under nitrogen atmosphere at 100°C for 15 min in a sealed tube. After this esterification procedure the methanol-HCl was evaporated at room temperature using a stream of nitrogen. 30 $\mu$l pentafluoropropionic anhydride (PFPA) was added, and the sample was heated under nitrogen atmosphere at 60°C for 30 min in a sealed tube. The PFPA was then evaporated to a small volume, and the residue dissolved in 20 $\mu$l 1% PFPA in ethyl acetate.

The mass spectroscopic analyses were performed with an LKB 2091 mass spectrometer. The gas chromatography was carried out with the aid of a glass column (9 ft×2 mm) packed with 3% OV-17 on Chromosorb W, operating at a temperature of 145°C. The ionization energy was 70 eV.

## RESULTS

On HPLC analysis of tyrosine–tyrosinase incubates, a peak was observed that did not correspond to any of the known intermediates in the Raper–Mason scheme. This peak was more pronounced in incubates of dopa-tyrosinase. The compound responsible for the peak could be adsorbed onto alumina in the same way as dopa; this is compatible with the presence of a compound having two hydroxyl groups in *cis*-position on vicinal carbon atoms. On chromatography on a strong cation-exchange resin the substance behaved as a neutral amino acid similar to dopa.

The substance had a shorter retention time than dopa in all tested reversed-phase HPLC-systems, as could be expected for a substance more polar than dopa. 6-Hydroxydopa is one such a polar compound, and it has been suggested as a possible intermediate in mealnogenesis. Authentic 6-hydroxydopa was quite distinct from our unknown substance when analysed by HPLC, however.

HPLC analysis at different working potentials showed that our unknown substance was oxidized at a lower potential than dopa, and in two steps (Fig. 2). The first oxidation step corresponds to the generation of a *o*-kinon derivative from a corresponding catechol. Under our conditions dopa then resisted further oxidation. The unknown substance, however, became further oxidized at a higher potential. This characteristic oxidation in two steps is an electrochemical property which we have previously observed for 6-OH-dopa and 5-OH-dopa

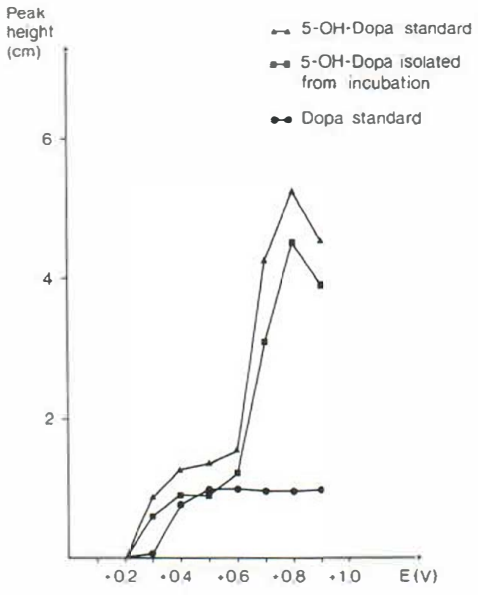


*Fig. 2.* Peak heights in HPLC chromatograms at different oxidation potentials of the electrochemical detector.

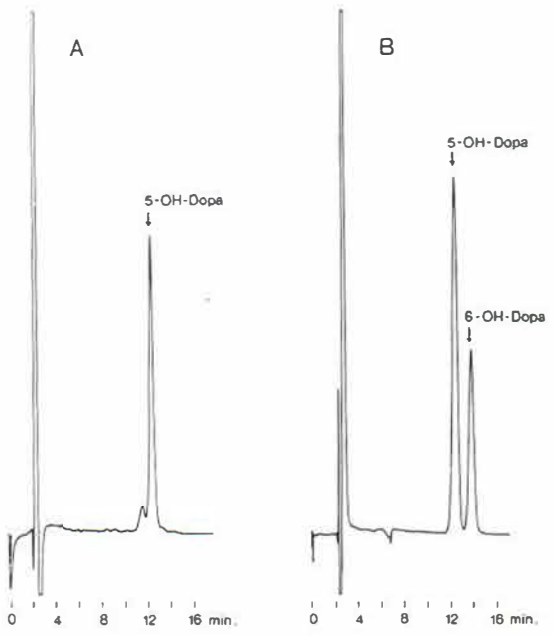


*Fig. 3.* (A) Chromatogram of purified dopa-tyrosinase incubate and (B) chromatogram of a standard solution of 5-OH-dopa and 6-OH-dopa. Column: Nucleosil $C_{18}$ (5 $\mu$m). Eluent: 2.5 g methanesulphonic acid, 0.5 g butanesulphonic acid, 2.9 g phosphoric acid per litre water, pH 1.75 with NaOH.

both with this HPLC technique and with conventional cyclic voltametry.

We now investigated the possibility that the unknown compound might be 5-OH-dopa formed by nucleophilic 1.6-addition of water to dopaquinone by comparing the unknown compound with authentic 5-OH-dopa. Our unknown substance behaved in the same way as 5-OH-dopa in the isolation procedure, i.e. it became adsorbed onto alumina, and chromatographed on the semipreparative HPLC and on the ion-exchange column in the same way. The eluate containing our unknown substance purified by alumina adsorption, semipreparative HPLC, and ion-exchange chromatography showed only one minor impurity on HPLC analysis. The purified substance was compared with 5-OH-dopa in several HPLC systems (Figs. 3 and 4), and proved identical.

GC-MS analysis of the unknown substance was performed after esterification using methanol-HCl followed by an acylation reaction with pentafluoropropionic anhydride. This derived compound showed a distinct peak in the gas chromatogram, with a retention time of 4.5 min typical of the derivative of 5-OH-dopa prepared in the same way.

The mass spectrum of the MeOH-PFPA derivative of the unknown substance is shown in Fig. 5*a*. No molecular ion was detected, but the fragmentation pattern tallied exactly with that of 5-OH-dopa (Fig. 5*b*) processed in the same way as the unknown substance. It should be noted that under the ionizing conditions used, no molecular ion of 5-OH-dopa was observed. Fig. 6 shows the probable origins of the most important fragments for the identification of the 5-OH-dopa derivative.

The quantity of 5-OH-dopa present in our incubate after 15 min was 30 $\mu$g. Large amounts of dopa then remained unoxidized. No attempts were made to determine the quantity of 5-OH-dopa which had already been oxidized at that time. To exclude the possibility that 5-OH-dopa might be formed by our isolation procedure, 0.1 ml of a dopa-tyrosinase incubate was injected directly onto the HPLC column. Under these conditions too the 5-OH-dopa peak was present in the chromatogram.

No peak corresponding to 6-OH-dopa was detected in the incubate after 15 minutes' incubation. When authentic 6-OP-dopa was added to the incubate just before injection onto the HPLC column its peak appeared clearly separated from that of 5-OH-dopa.

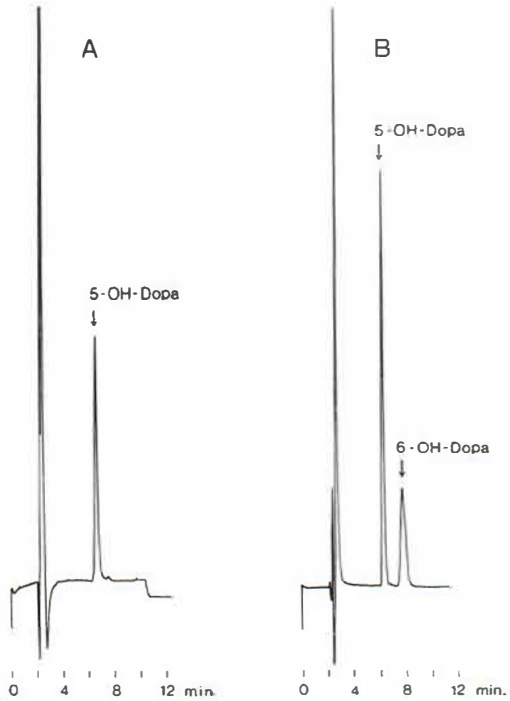


*Fig. 4.* (A) Chromatogram of purified dopa-tyrosinase incubate and (B) chromatogram of a standard solution of 5-OH-dopa and 6-OH-dopa. Column: Nucleosil $C_{18}$ (5 $\mu$m). Eluent: 2.5 g methanesulphonic acid, 0.5 g butanesulphonic acid, and 2.9 g phosphoric acid per litre water, pH 2.80.

## DISCUSSION

We have demonstrated a new compound, 5-hydroxydopa, as an intermediate in the tyrosinase-catalysed production of melanin from tyrosine or dopa. Authentic 5-OH-dopa was used for the identification of the enzymatically formed 5-hydroxydopa by several methods.

The new compound and 5-OH-dopa were chromatographed in many different systems, with identical results. The electro-chemical oxidation took place in two characteristic steps for both the new compound and 5-OH-dopa. Gas chromatography and mass spectrometry of derivatives of the purified substance showed a fragmentation pattern that made final identification possible.

Earlier studies have produced evidence of the formation of a trihydroxy derivative of phenylalanin in melanin biosynthesis (7), and a methylated derivative of 2,4,5-trihydroxydopa (6-hydroxydopa) has been identified as a product of the micro-

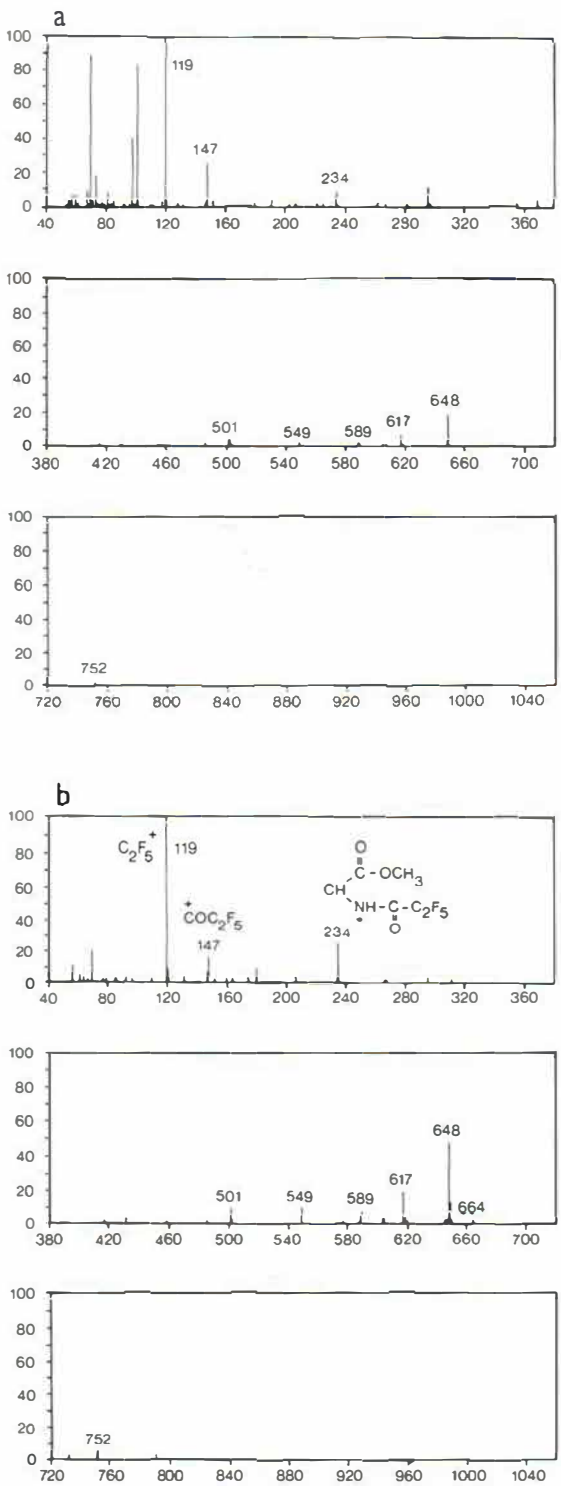


*Fig. 5.* Mass spectrum of the MeOH-PFPA derivative (*a*) of the compound isolated from a dopa-tyrosinase incubate and (*b*) of 5-OH-dopa.

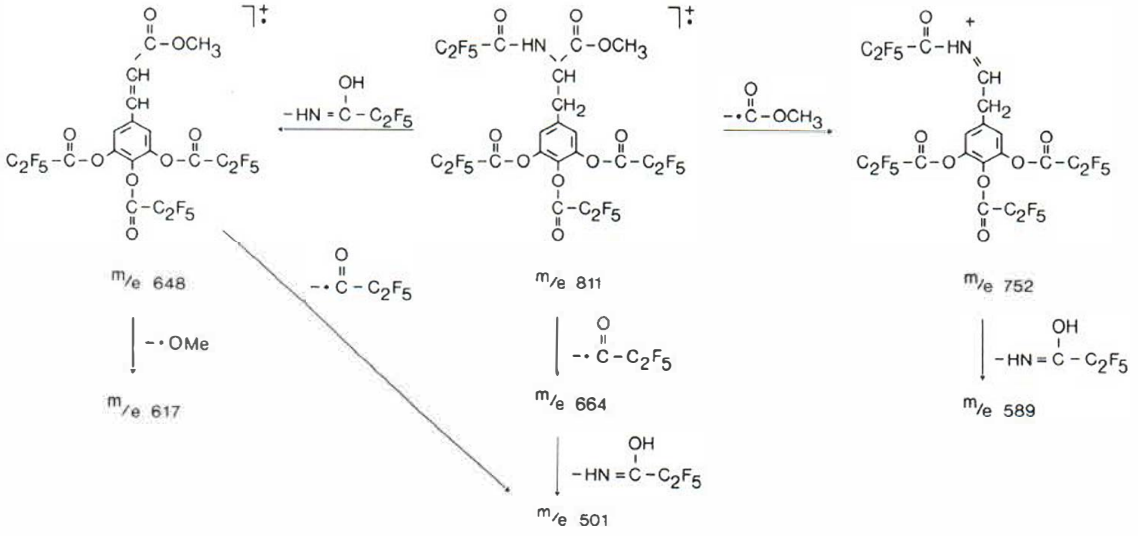


*Fig. 6.* Probable mass spectral fragmentation of the pentafluoropropionylderivative of the methylester of 5-OH-dopa.

organism *Microspira tyrosinatica* by direct comparison with an authentic sample (8).

The possible occurrence of 6-hydroxydopa as an intermediate in melanin synthesis was investigated by Swan in 1963 (18). It was discussed by Witkop (21), who in collaboration with Senoh et al. studied the formation of 2,4,5-trihydroxyphenethylamine from dopamine (15, 16, 17), and also by Dawson (1) who in collaboration with Tarpley described the hydroxylation of quinones in the catechol–tyrosinase reaction (2). Swan (19) found that on oxidation 6-hydroxydopa could produce a dopachrome-type product; but Graham et al. (4) concluded that although 6-hydroxydopa can be oxidized to dopachrome the 6-hydroxydopa pathway must be of minor importance in melanin synthesis by mushroom tyrosinase.

The HPLC system used by us makes it possible to separate 5-OH-dopa and 6-OH-dopa. Under our conditions of incubation no 6-OH-dopa was detected, and the quantities of this dopa derivative—if present—must be far below those of 5-OH-dopa.

5-OH-dopa has previously been synthesized and used experimentally as a precursor of the adrenergic false transmitter 5-OH-dopamine (20).

Our present finding of 5-OH-dopa as an intermediate in the enzymatic oxidation of tyrosine to melanin illustrates the possibilities of modern techniques to further elucidate reactions previously investigated in depth. It seems possible that 5-hydroxydopa is formed by nucleophilic addition of water to dopaquinone. Water is of course a weak nucleophile, but it occurs in high concentrations and in the absence of strong nucleophiles such as thiols, the addition of water seems to be of great importance.

Because of its comparatively low oxidation potential the 5-OH-dopa formed will be able to induce reduction of dopaquinone to dopa. Such a reaction may be part of the explanation for the regeneration of dopa due to reduction of dopaquinone during enzymatic oxidation of tyrosine. This dopaquinone reduction has generally been thought to be an effect of the oxidation of leukodopachrome to dopachrome (3).

Under in-vivo conditions, availability of stronger nucleophiles such as glutathione and cysteine will limit the formation of 5-OH-dopa. Glutathionyldopa and cysteinyldopa have been found in melanin-forming tissues (14). Model experiments with different levels of SH-containing compounds present during tyrosine oxidation will permit the definition of the biological role of 5-OH-dopa in melanin formation.

It can be assumed, however, that the instability of 5-OH-dopa will make its detection in melanin-forming tissues difficult. The demonstration of 5-OH-dopa will depend on the presence of

melanocytic redox systems with an even lower oxidation potential.

## ACKNOWLEDGEMENTS

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