Hydroxyindole Intermediates in the Process of Melanogenesis: A Mass Spectrometric Study

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Studies on the enzymatic formation of polymers from hydroxyindoles are described.

FAB analysis of the reaction mixtures followed by collisional spectroscopy of the most abundant ionic species was applied to analyze products obtained from the reaction catalyzed by tyrosinase on 4- or 5-hydroxyindole at different times.

Results suggest that moieties of the precursors are still present in the oligomeric systems. Among the reaction products dimeric and trimeric compounds were detected by Fast Atom Bombardment and their structure assignment was achieved by collision spectroscopy.

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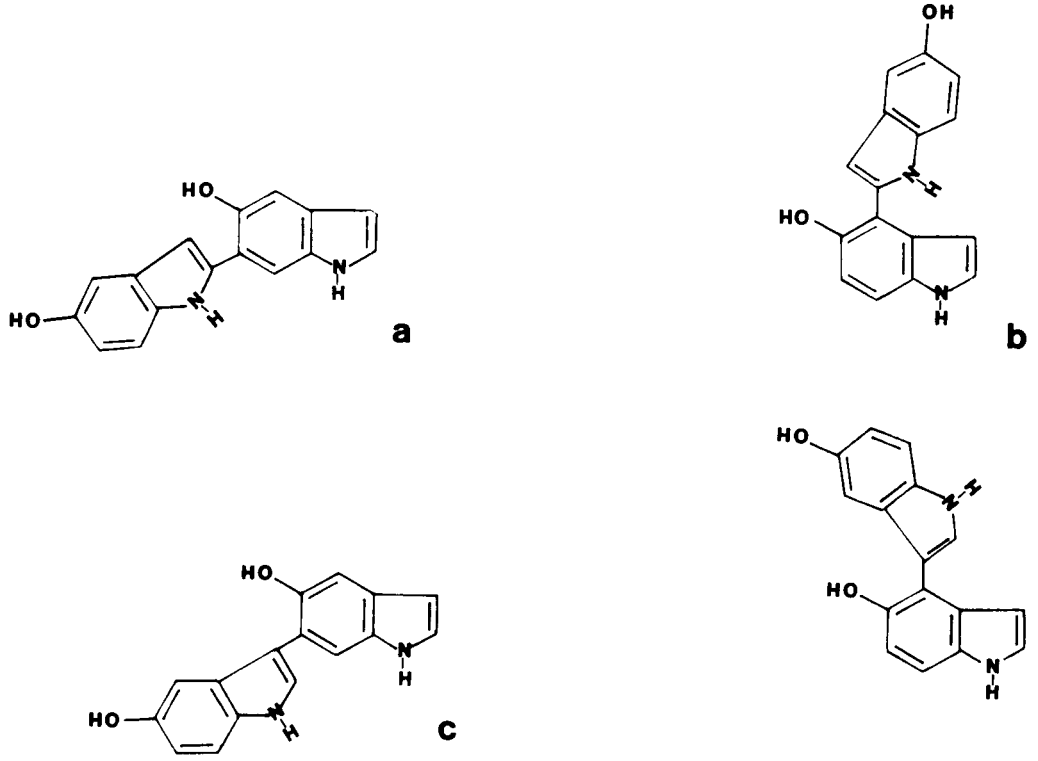
Introduction.

Our knowledge of the melanin structure is still rather scarce, although an enormous amount of work has been carried out on the chemistry of these biopolymers [1a,b].

It is generally accepted that tyrosine "via Dopa" is the physiological precursor of melanins. However, Chen and Chavin [2], using various labelled amino acids as substrates for tyrosinase from *Carassius auratus L.*, observed that, besides tyrosine, other amino aromatic acids, mainly tryptophan, could be incorporated into melanins.

Incubating L-Dopa with tyrosinase from *Sepia officinalis* in the presence of labelled compounds such as tryptophan, tyrosine, L-phenylalanine, hystidine and phenol, Nicolaus [1a,b] also showed that other substances can be incorporated into melanins.

Scheme 1. Possible dimeric moieties arising from 5-hydroxyindole-tyrosinase reaction.



The role of tryptophan and its metabolites in melanogenesis has been demonstrated "*in vitro*" and "*in vivo*" in our previous papers [3-9] which revealed the incorporation of labelled tryptophan into the melanin of mouse Harding-Passey melanoma almost equal to that obtained with labelled tyrosine. Furthermore, other hydroxyindole metabolites may be considered as precursors of melanins. We recently undertook a series of systematic researches on the biogenesis of melanins by mass spectrometry, applying fast atom bombardment (FAB) and collisional spectroscopy to the characterization of hydroxyindoles [10-12] and to the kinetics of the dopamine-tyrosinase reaction [13].

Experiments carried by continuous flow FAB on the tyrosine-tyrosinase reaction mixture were unsuccessful, due to the formation of precipitates which blocked the 100μ i.d. capillary line. Hence, an off-line approach was adopted, which consisted of stopping the reaction at different times with diazomethane and then applying FAB analysis. This approach revealed various dimethoxyindoles, thereby proving the involvement of dihydroxyindoles in the process of melanogenesis [13]. In order to further verify such involvement, we carried out the same reaction catalysed by tyrosinase using 4- or 5-hydroxyindole (compounds **1** and **2**, respectively) as substrates.

The analytical approach employed was the same as that described above, *i.e.* FAB analysis of the enzyme reaction mixture at different times and collisional spectroscopy of the ionic species. The first set of data provided information on the kinetics of the reaction, while the second approach can give precious information on the structure of products.

EXPERIMENTAL

Enzymatic Reaction.

The enzyme activity of mushroom tyrosinase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase; EC 1.14.18.1) (Sigma) was studied on two different substrates: 4- and 5-hydroxyindoles (Sigma). Tyrosinase activity was 2,000-3,000 units per mg of solid.

Thirty ml solutions of 4- or 5-hydroxyindole (1 mg/ml) in double-distilled water saturated with oxygen for 15 minutes received 10,000 units of tyrosinase and were introduced in the Amicon Multi-Cell System (Model MMCA) equipped with a Diaflo XM 50 membrane.

Fractions were ultra-filtered, under an oxygen stream, to eliminate the enzyme at different reaction times of 15, 30, 90, 180, 300, 420 and 1400 minutes at room temperature and immediately lyophilized. Before lyophilizing, the process of the reaction was followed by means of thin layer chromatography.

Aliquots of the samples (100 μl) at each time were chromatographed (silica gel F-254 2 mm, Merck) and eluted with a solvent system composed of cyclohexane, chloroform, methanol and acetic acid (4:3.5:1.5:1). Components were detected by a uv lamp and the Ehrlich test.

Mass Spectrometry.

All mass spectrometric measurements were performed on a VG ZAB 2F instrument [14] operating in FAB conditions [15a,b].

Glycerol solutions of the reaction mixtures were bombarded by 8 keV Xe atoms. Collision experiments [16] were performed by colliding 8 keV preselected ions with nitrogen in the collision cell placed in the second field-free region. Nitrogen pressure was such as to reduce the main beam intensity to 60% of its usual value.

Parent ion spectroscopy was obtained by B^2/E linked scans [17].

Results and Discussion.

The FAB spectra of the reaction mixtures originated from enzyme activity after 420 minutes on compounds **1** and **2**, always show the presence of easily detectable ionic species at m/z 264 and 395, formally corresponding to di-

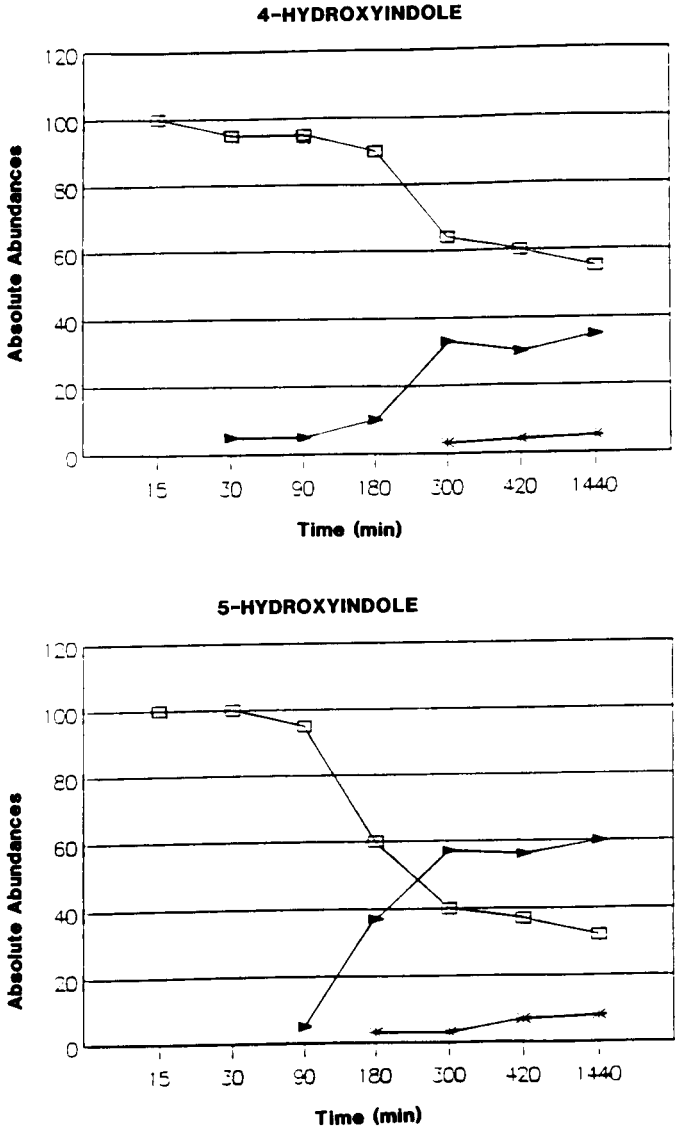


Figure 1. Absolute abundance of ionic species at m/z 133 (□), 264 (▲) and 395 (*) versus time obtained from reaction between 4- or 5-hydroxyindole.

meric and trimeric moieties of the hydroxyindole precursor.

The production of such dimeric and trimeric species was studied by analysis of FAB spectra of the reaction mixtures between hydroxyindole and tyrosinase at incubation times of 15, 30, 90, 180, 300, 420 and 1400 minutes. Figure 1 shows the absolute abundance of ionic species at m/z 133, 264 and 395 versus time, produced by the enzyme reaction of compounds **1** and **2**. In particular, for 4-hydroxyindole (Figure 1) dimeric compounds were observed after 30 minutes and the production of the trimeric moiety was not favoured, as ions at m/z 395 were detectable only after 300 minutes. From this time onwards, the production of dimeric moieties reached a steady-state, proving their intermediacy in the production of the trimeric moieties.

For 5-hydroxyindole (Figure 1) slower kinetics was observed: the dimeric species appeared after 180 minutes. This difference in kinetics seems to indicate 4-hydroxyindole as the preferential intermediate in the polymerization of hydroxyindoles to products similar to melanins.

After 420 minutes quite high concentrations of ions at m/z 264 and 395 were observed.

In principle, looking at the complexity of the reaction mixture under study, which reflects to the complexity of the related FAB spectra, it could be assumed that such ions were fragmentation products of other molecular

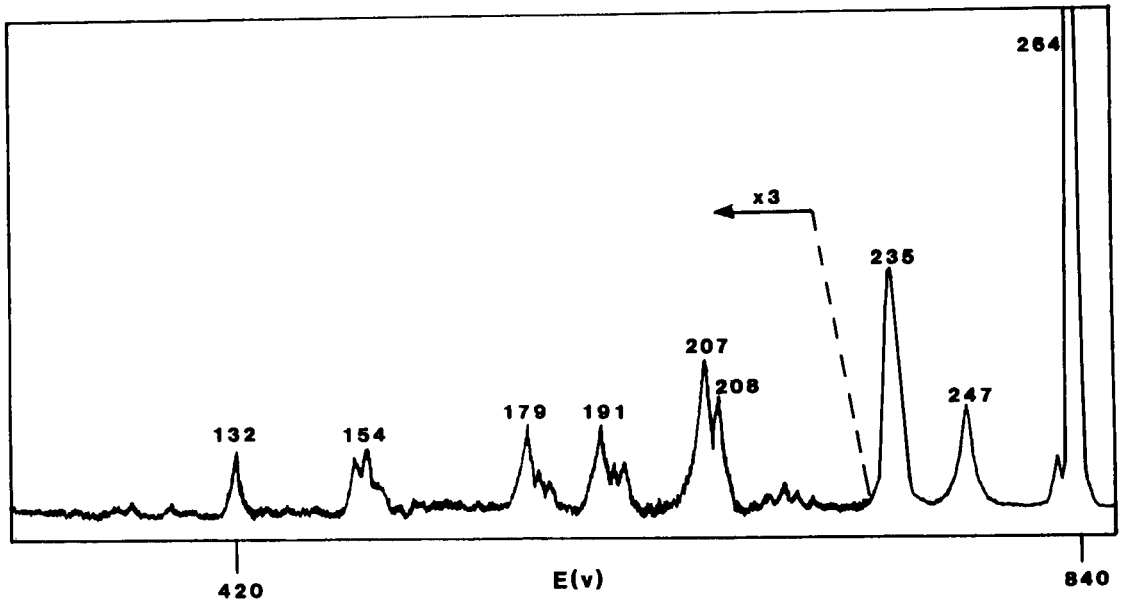


Figure 2. Collision spectrum of ion at m/z 264 for 5-hydroxyindole.

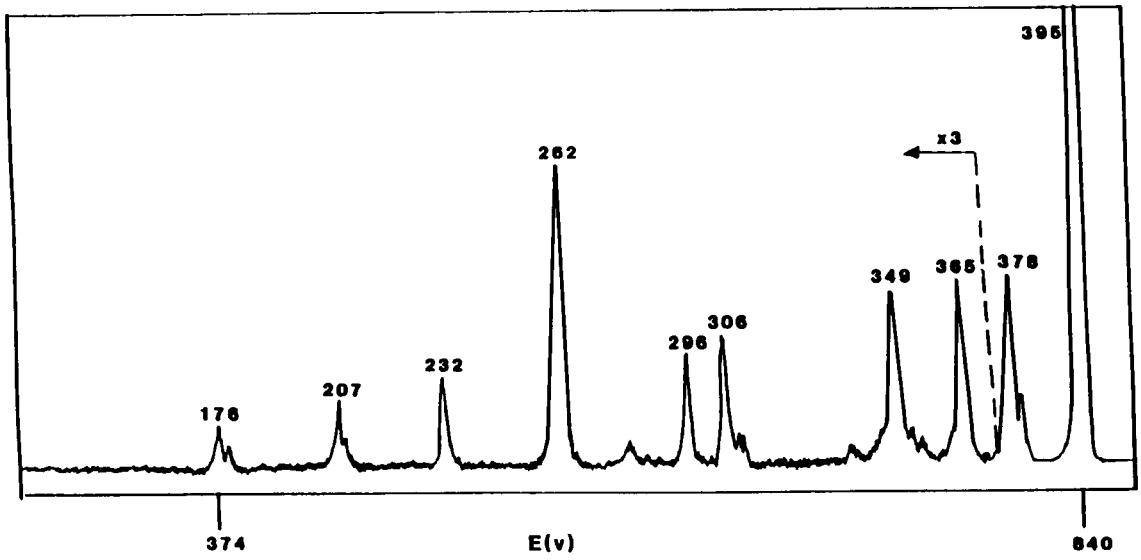


Figure 3. Collision spectrum of ion at m/z 395 for 5-hydroxyindole.

species of higher molecular weight. However, parent ion spectroscopy on ions at m/z 264 and 395, obtained by B^2/E linked scan measurements, proved the complete lack of any precursors for such species, thus revealing their nature as real molecular ions.

It is quite difficult from the usual FAB spectra, to obtain any structural information on dimeric and trimeric species by study of their fragmentation patterns, since the complexity of the reaction products and the presence of the matrix lead to a high chemical background. Furthermore, in principle, the polymerization process may involve the loss of various hydrogen atoms of the reacting species. In other words, different structures can be assigned to polymeric species according to the linking of their monomeric units.

In order to investigate this aspect, we studied the FAB spectra of the products obtained from the reaction mixtures, after ultrafiltration, lyophilization and dissolution in deuterated methanol, so as to reveal the numbers of exchangeable hydrogens still present in the polymeric systems. The analyses showed shifts of 4 daltons for ion at m/z 264 and 6 daltons for ion at m/z 395. These results mean that the exchangeable hydrogens present in the hydroxyindole (*i.e.*, those of the NH and OH moieties) are still present in the oligomeric systems.

To gain further information on the structure of the oligomeric compounds, we undertook a series of collision spectroscopy experiments. The collision spectrum of ions at m/z 264 is reported in Figure 2.

The spectrum shows particularly favoured losses of CO and $OH^{\cdot}$, leading to ions at m/z 235 and 247 respectively. While CO loss is a fragmentation pathway commonly observed in phenol systems, $OH^{\cdot}$ loss is usually observed in them only when substituted in the *ortho* position. The presence of easily detectable ions at m/z 132, corresponding to the monomeric units, is to be emphasized.

The collision spectrum of ionic species at m/z 395, reported in Figure 3, is more complex. The loss of $OH^{\cdot}$ is still present, leading to the ion at m/z 378, while CO loss is not favoured in this case. New fragmentation routes due to $CH_2OH^{\cdot}$ loss (leading to ion at m/z 365) and to sequential losses of $OH^{\cdot}$ and CH_2O (leading to ion at m/z 349) become detectable. The most abundant collisionally induced fragmentation product is detected at m/z 262 and corresponds to the loss of a monomeric unit.

The data so far obtained can be summarized as follows:

i) By reaction of hydroxyindoles with tyrosinase, dimeric and trimeric moieties can be produced and easily detected by FAB mass spectrometry.

ii) Deuterium exchange experiments show that hydrogens linked to both nitrogen and hydroxyl oxygen are still present in the dimeric and trimeric structures. This means that the linking between the various monomeric units must be between two carbon atoms.

iii) Collision spectroscopy on dimeric moieties shows CO and OH losses. While the first fragmentation pathway is typical of molecules containing aromatic hydroxyl groups, the second is typical of *ortho*-substituted phenol systems. Different structures can consequently be proposed, *e.g.* **a-d** structures from 5-hydroxyindole (Scheme 1).

iv) Collision spectroscopy of trimeric moieties leads to more complicated results and the structures that can be proposed to increase in number.

v) Similar results were obtained for the products arising from reactions between 4-hydroxyindole and tyrosinase.

In conclusion, FAB mass spectrometry together with collision spectroscopy provides information on the structure assignment of oligomeric compounds which are obtained from the hydroxyindole-tyrosinase reaction and which may be intermediates in melanogenesis.

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