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5,6-DIHYDROXYINDOLE-2-CARBOXYLIC ACID BY TREATMENT OF SEPIOMELANIN WITH SODIUM BOROHYDRIDE.

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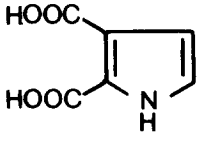
Abstract: Mild treatment of sepiomelanin and biosynthetic eumelanins with $NaBH_4$ in 0.1 N NaOH leads to the isolation of 5,6-dihydroxyindole-2-carboxylic acid (3), a component of structural interest which may account for most of the degradation products of melanins so far obtained.

Melanins are widely occurring natural pigments of high molecular weight which arise biogenetically from oxidative polymerization of dopa *via* 5,6-dihydroxyindole(s)[1]. Most of the present-day knowledge on the structure of these highly heterogeneous pigments comes from extensive oxidative degradations carried out on sepiomelanin[2] which have led to the identification of a series of pyrrolecarboxylic acids, notably 1 and 2 , arising probably from disruption of 5,6-dihydroxyindole units present in the polymer[3]. However, the fact that these products have been obtained in trace amounts under conditions which have recently been shown to give rise to artefacts[4] makes it uncertain as to how much importance should be attached to their formation as far as the fundamental structure of eumelanins is concerned.



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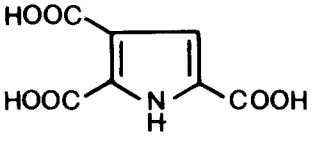

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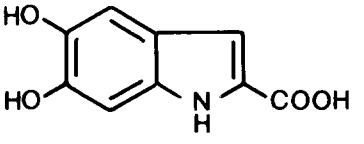

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In seeking for new chemical approaches to the structure of eumelanins we found that mild treatment of sepiomelanin at room temperature with excess sodium borohydride in 0.1 N NaOH for 20 hr , followed by acidification, yields an ethyl acetate-extractable fraction containing mainly a compound (λmax = 316 nm) with a strong blue fluorescence typical of 5,6-dihydroxyindoles. This was isolated by TLC on silica followed by preparative HPLC on a reverse phase column (0.05 M formic acid-methanol 8:2 v/v) and identified as 5,6-dihydroxyindole-2-carboxylic acid (3) by spectral analysis[5].Confirmation of its identity came from comparison of its spectral and analytical properties with those of an authentic sample prepared by a conventional procedure[6].

Noteworthy, 5,6-dihydroxyindole-2-carboxylic acid was previously obtained from sepiomelanin in exceedingly low amounts by alkaline fusion at 300°C[7], and the fact that it can also be obtained under mild non-degradative conditions is of interest in relation to its origin and structural significance.

As shown in Table 1, 3 could be obtained both from native sepiomelanin and from samples purified from accompanying protein according to described procedures.[8,9] Notably, in the case of samples purified according to Nicolaus[9], the "yields"of 3 are significantly lower, suggesting some alteration of the pigment granules caused by the prolonged acid digestion.

5,6-dihydroxyindole-2-carboxylic acid was also obtained by treatment with $NaBH_4$ of some biosynthetic melanins[10]. These include enzymic dopa melanin, prepared at pH 6.8 in the presence of tyrosinase and catalase[11], as well as autoxidative dopa melanin, prepared at pH 8 both in the presence and in the absence of catalase. As shown in Table 2, the yields of 3 vary significantly , probably owing to the different physical and chemical properties of the samples.

Noteworthy, each natural or biosynthetic melanin sample, when subjected to repeated similar reactions with $NaBH_4$, afforded additional small amounts of 3. A sample of native sepiomelanin thus subjected to this treatment four times gave the indole in 140 μg/g of melanin overall yield.

Further experiments were aimed at ascertaining whether the release of 5,6-dihydroxyindole--2-carboxylic acid by $NaBH_4$ results from chemical degradation or is simply due to mechanical removal of the compound incorporated as such into the pigment granule. Preliminarily, the possible occurrence of the indole into the whole ink of *Sepia officinalis* was carefully investigated. HPLC analysis of the melanin-free ink revealed the presence of some pyrrole--2,3,5-tricarboxylic acid (2) in variable amounts depending on the aging of the cephalopod ink [10] but no trace of 5,6-dihydroxyindole-2-carboxylic acid. The same result was obtained from prolonged extraction of a large sample of fresh collected sepiomelanin with aqueous dioxane or ethanol containing1% acetic acid, even after extensive sonication of the pigment and heating.

TABLE 1

Yields of 3 obtained by reaction of native and purified sepiomelanin with $NaBH_4$ (4 g/g of melanin) in 0.1 N NaOH for 20 hr at room temperature.

melanin sample	yield (μg/g of melanin)
NM	80
HPM	76
CPM	27

NM = native sepiomelanin extensively sonicated in deionized water.
HPM = sepiomelanin heated under vacuum in 6 N HCl for 1 hr at 120°C[8].
CPM = sepiomelanin digested with conc. HCl for 370 hr at room temperature[9].

TABLE 2

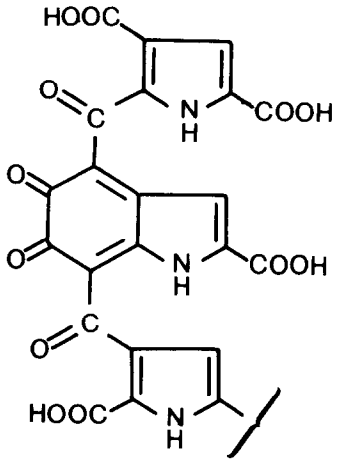
Yields of 3 obtained by reaction of biosynthetic dopa melanins[10] with $NaBH_4$ (4 g/g of melanin) in 0.1 N NaOH for 20 hr at room temperature.

melanin sample	yield (μg/g of melanin)
EDM	15
ADMC	37
ADM	70

EDM = enzymic dopa melanin prepared in 0.05 M phosphate buffer, pH 6.8, with 10 mg of tyrosinase and 5 mg of catalase/g of dopa.
ADMC = autoxidative dopa melanin prepared in 0.05 M phosphate buffer at pH 8 in the presence of catalase (10 mg/g of dopa).
ADM = autoxidative dopa melanin prepared as ADMC but in the absence of catalase.

Parallel experiments showed also that no detectable amount of the indole 3 is liberated by treatment of native sepiomelanin either with sodium borohydride in organic medium, e.g. methanol, or with 0.1 N NaOH under nitrogen or hydrogen or in the presence of small amounts of sodium dithionite as an antioxidant.

All these evidences, taken together, would suggest that the release of 3 by non-degradative treatment of sepiomelanin with sodium borohydride results from two distinct processes, namely reduction of some specific unit in the polymer coupled with a hydrolytic step. This, however, could hardly be envisaged in terms of partial structures such as 4, tentatively suggested by Nicolaus[1] as a model for sepiomelanin. On the other hand, the low yields of the indole and the unique properties of the pigment render the interpretation of our results open to question and the possibility still remains that 3 arises from some minor component strongly retained into the melanin granule. If so, this could account for most of the degradation products so far obtained, thus further increasing the complexity of the picture and weakening the structural significance of fragments such as 2 and 3 itself, which have represented up to now the basis for most speculations on melanin structure.



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