Pergamon

Tetrahedron, Vol. 53, No. 24, pp. 8281-8286, 1997

0040-4020/97 $17.00 + 0.00

PII: S0040-4020(97)00494-8

An Integrated Approach to the Structure of Sepia Melanin. Evidence for a High Proportion of Degraded 5,6-Dihydroxyindole-2-carboxylic Acid Units in the Pigment Backbone

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Abstract:The relative proportion of 5,6-dihydroxyindole (DHI) versus 5,6-dihydroxyindole-2-carboxylic acid (DHICA) units in Sepia melanin and their degree of structural integrity were assessed by an integrated approach involving quantitative determination of pyrrole-2,3-dicarboxylic acid (PDCA) and pyrrole-2,3,5-tricarboxylic acid (PTCA), specific degradation products of DHI- and DHICA-derived units; carboxyl content; rate of ferricyanide consumption; and absorption spectrum. Analyses of both intact and surfactant-solubilised pigment samples, in comparison with synthetic DHI and DHICA melanins, revealed that Sepia melanin consists of a mixture of oligomeric structures incorporating over 75% of DHICA-derived units and only 20% of DHI-derived units, occurring for the most part in an irreversibly degraded form, possibly as pyrrolecarboxylic acids.

In spite of their unique academic, biomedical and industrial interest, spurring vigorous and continuous research efforts since the turn of the century, the melanins still represent the most enigmatic and elusive pigments found in nature.[1] The challenges posed to the organic chemists are of many kinds and relate to the virtual insolubility of these pigments in all solvents, their marked heterogeneity, and the lack of well defined spectral and physicochemical features.[2] Additional difficulties, that have thwarted direct investigation of skin, hair and eye melanins, stem from their occurrence in exceedingly small amounts intimately associated with proteins and other tissue components that can hardly be removed without damaging the pigment backbone.

Most of what is currently known about natural melanins has been earned from studies of the black ink of the cuttlefish *Sepia officinalis* which has been widely accepted as a standard for natural melanins.[3-5] Extensive chemical degradations,[6,7] aided by titration data,[8,9] elemental analyses,[7,9,10] and mass spectrometric determinations,[11] have led to the notion that Sepia melanin consists, besides some protein and other impurities, of a mixture of highly cross-linked, irregular polymers made up of different structural units derived biogenetically from tyrosine. These include 5,6-dihydroxyindole (DHI), 5,6-dihydroxyindole-2-carboxylic acid

(DHICA), pyrrolecarboxylic acids, as well as leucodopachrome and uncyclised dopa units, at various states of oxidation and linked fairly randomly.

In fact, the extreme flexibility of this model, permitting virtually any combination of monomer units with no constraints put upon their nature, redox state and integrity, has considerably demotivated further insights into the structural features of Sepia melanin, favouring unwarranted extrapolations and generalisations that have eventually blurred the distinction from its mammalian congeners or, even worse, from synthetic pigments. Taking advantage of the conceptual and technological advances made in the last few years which have highlighted *inter alia* the unexpected instability of melanins to oxidation[2], we have re-examined the basic structural features of Sepia melanin, with a view to addressing two fundamental pending issues concerning the relative proportion of DHI vs. DHICA units, and their degree of structural integrity.

Preliminarily, the isolation and storage protocol was optimised, to minimise potentially damaging interactions with acids, alkali or oxygen. To this end, freshly collected ink was rapidly centrifuged in 0.01 M HCl in the cold, and then stored wet at 4°C in the absence of oxygen, under which conditions the pigment remained virtually unchanged for at least 5 days.

Structural investigation of the pigment was carried out by acid decarboxylation with hot 6 M HCl, to selectively determine carboxyl-groups linked to aromatic rings,[7,9,12] and mild oxidation with alkaline H_2O_2, leading to the formation of pyrrole-2,3,5-tricarboxylic acid (PTCA) and pyrrole-2,3-dicarboxylic acid (PDCA).[13] While the former fragment originates mainly by disruption of DHICA units, and only to a minor extent from 2-substituted DHI units, the latter derives exclusively from 2-unsubstituted DHI units, and is introduced in the present study as a reliable marker of DHI-derived units (Figure 1).

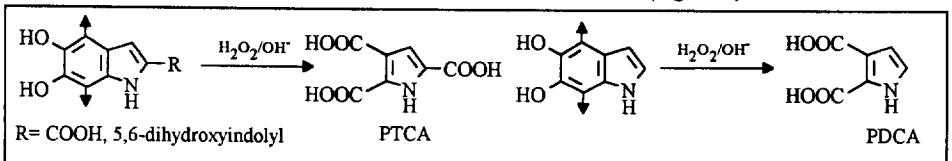

Figure 1. Main pyrrolecarboxylic acids obtained by hydrogen peroxide degradation of melanins.

Table 1 shows carboxyl content, PTCA and PDCA yields for Sepia melanin and different synthetic melanins prepared by tyrosinase oxidation of dopa, DHI and DHICA. In line with previous observations,[13] the data indicate that pyrrolic acids are formed in relatively higher yields from DHI and DHICA melanins, and only in small amounts from dopa melanin, due possibly to the occurrence in the latter of uncyclised, non indolic units. If the yields of PDCA and PTCA are corrected for the amino acid and ash contents of Sepia melanin, determined as 5.7±0.4 % and 14.0±0.8 % w/w, respectively, and it is assumed that no more than 15% of the total yield of PTCA derives from DHI units, which is consistent with the values obtained from pure DHI and DHICA melanins, it could then be argued that the Sepia pigment contains 75 % of DHICA-derived units, and 20 % of DHI units, the remainder being accounted for by other units.

Table 1. Degradative Analyses of Sepia Melanin and Reference Synthetic Melanins.

Melanin	PTCA[a] (nmol/mg)	PDCA[a] (nmol/mg)	CO_2[a] % (w/w)
Sepia melanin	97.7	4.3	8.4
DHICA-melanin	127.3	-	18.2
DHI-melanin	15.8	22.4	5.8
DHICA/DHI melanin[b]	93.2	4.1	11.2
dopa-melanin	11.5	11.6	4.8

[a] Average of three experiments, S.D. ≤ 5%.
[b] DHICA/DHI molar ratio 3:1

Such a conclusion was supported by analysis of a melanin prepared *ad hoc* by copolymerisation of DHICA and DHI in the mole ratio of 3:1, which gave PTCA and PDCA values closely matching those of Sepia melanin.[14]

That DHICA is involved in the biosynthesis of Sepia melanin has already been pointed out in previous studies[8,15,16] and has recently been corroborated by the isolation of a pattern of pyrrolecarboxylic acids closely similar to that obtained from DHICA melanins.[17] However, the present estimate is worthy of note because, even though rather conservative, it far exceeds previous ones.[7,18] Several factors may account for this discrepancy, including differences in sample preparation and analytical procedures. Yet, the degradative approach described in this paper combines mild oxidation conditions with reproducible and improved product yields, and is the first to rely on analysis of two specific markers for DHI and DHICA.

Less straightforward is the interpretation of the carboxyl content data. Although the carboxyl groups might in principle be taken as an indicator of DHICA-related units, in fact pure DHI melanins give carboxyl content values that are far from negligible, denoting evidently the occurrence of significant peroxidative breakdown of the indole units during pigment synthesis.[19,20] This limits the structural significance of carboxyl content for natural melanins, unless supplementary information on the origin of carboxyl groups is available.

Confirmatory evidence for the presence of a high proportion of DHICA units in Sepia melanin was sought through direct analysis of fresh pigment samples by spectrophotometric techniques. To circumvent problems caused by extensive light scattering, the effect of Soluene® 350, a solubilising agent recently exploited for the spectrophotometric determination of melanins in pigmented tissues,[16] was initially investigated. Unfortunately, the method proved inapplicable for the intense absorption of Soluene® 350 in the UV region and its strong alkaline character. However, use of surfactants like polyethylene glycol (PEG), sodium dodecyl sulphate (SDS) or myristyltrimethylammonium bromide (MTMAB) in dilute ammonia under oxygen-depleted atmosphere resulted in a limited, yet significant solubilisation of Sepia melanin.[21] In 0.1 M ammonia, pH 10.0, 0.2 M MTMAB solubilised some 16 % of a 9 mg/ml pigment suspension, due probably to favourable electrostatic interactions with the negatively charged carboxyl groups, whereas PEG 5000 and SDS were somewhat less effective. Figure 2 shows the UV spectrum of the MTMAB-solubilised fraction in aqueous phosphate buffer under nitrogen. Remarkably, the spectrum exhibited a distinct maximum at around 315 nm, virtually superimposable to that of freshly prepared MTMAB-solubilised DHICA melanins. Partial or complete loss of this feature was observed with time or with aged pigment samples, on account of a marked instability of the chromophore to autoxidation.

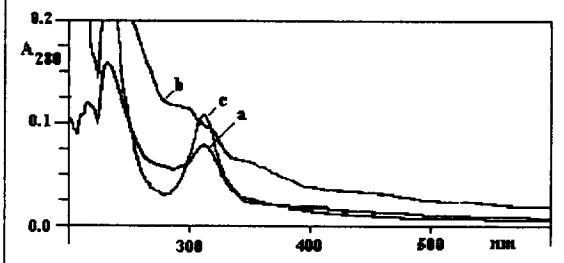

Figure 2. UV spectra of MTMAB-solubilised melanins, (a) fresh Sepia melanin, (b) aged Sepia melanin and (c) DHICA melanin.

Table 2 Chemical Data for MTAMB-Soluble and Insoluble Melanins

Melanin	PTCA[a] (nmol/mg)	PDCA[a] (nmol/mg)	CO_2[a] % (w/w)
Sepia (soluble)	74.2	4.0	8.2
Sepia (insoluble)	78.7	3.7	8.3
DHICA (soluble)	113.7	-	17.0
DHICA (insoluble)	143.9	-	16.8
DHI (soluble)	15.9	20.5	5.2
DHI (insoluble)	14.0	20.9	5.8

[a] Average of three experiments, S.D. ≤ 5%.

Table 2 reports degradative analyses of the MTMAB-soluble fractions of Sepia melanin, DHI and DHICA melanins, compared to the insoluble portions. In all cases the solubilised fractions were found to closely reflect the whole pigment, with no apparent modification due to structural degradation. No meaningful difference in the protein content was observed between the soluble (5.0± 0.4 % w/w) and unsoluble (5.5± 0.4 % w/w) fractions of Sepia melanin. To gain information about the average redox state and structural integrity of the pigment backbone, fresh intact and MTMAB-solubilised melanin samples were analysed for their reducing behaviour toward potassium ferricyanide.

Inspection of the relative rates of ferricyanide consumption, as determined by potentiometric titration and spectrophotometric analysis (Table 3), revealed that, however fresh, Sepia melanin is far less oxidisable than synthetic melanins. Although the values for intact pigments are affected by differences in the size and general properties of the particles,[5,22] the results obtained on solubilised fractions are expected to reflect more closely the actual redox properties of the pigments. If possible spurious effects caused by non-pigment components of Sepia melanin are disregarded, which seems reasonable considering the relatively low impurity content and the high surfactant concentration, these data would suggest that Sepia melanin contains a surprisingly low proportion of oxidisable catechol-type units.

Table 3. Ferricyanide Consumption of Sepia Melanin and Reference Synthetic Melanins

Melanin	$K_3Fe(CN)_6$ consumption[a] (μmol/mg min)
Sepia	0.16[b](0.16)[c]
DHICA	10.0[b]
DHI	8.8[b]
Sepia (soluble)	0.30[d](0.28)[c]
DHICA (soluble)	12.4[d]
DHI (soluble)	9.8[d]

[a] Average of three separate experiments, S.D. ≤ 7%.
[b]Determined potentiometrically [c]After $NaBH_4$ reduction.
[d]Determined spectrophotometrically

Pre-treatment of solubilised Sepia melanin samples with $NaBH_4$, to reduce possible quinone units, did not affect significantly the rate of ferricyanide reduction, indicating that most of the pigment units are present in a redox inactive, irreversibly degraded form, possibly as pyrrolecarboxylic acids. From a quantitative UV spectrum of the MTMAB-solubilised fraction (1.4 mg/ml, Figure 2), assuming an average molar extinction coefficient for DHICA units of 16,850 M^{-1} cm^{-1},[23] it could roughly be estimated that intact DHICA units account for less than 5% of Sepia melanin, which is in agreement with

relative ferricyanide reduction rates. A high degree of degradation of the pigment backbone provides also a plausible explanation for the relatively low carboxyl content of Sepia melanin, since in separate experiments (data not shown) it was found that mild oxidation of DHICA melanins with hydrogen peroxide affords to degraded pigments with a lower carboxyl content compared to the parent melanin. Notably, a preliminary investigation of fresh intact Sepia melanin, using matrix-assisted laser desorption (MALDI) mass spectrometry,[24] provided evidence for clusters of pseudomolecular ion peaks that were fully consistent with the prevalent occurrence of partially degraded DHICA oligomers.

In conclusion, the innovative approaches that have evolved from the present study have permitted for the first time to delineate to a sufficient degree of definition and from multiple anglepoints the core structural features of Sepia melanin. This now appears as an intimate collection of oligomeric species made up for the most part of pyrrolecarboxylic acid units derived from DHICA. Besides definitively subverting the traditional notion of natural melanins as 5,6-indolequinone-derived polymers, these results fill important gaps that have so far hampered a deeper understanding of the origin and functional significance of such intriguing pigments.

EXPERIMENTAL

HPLC was performed on a Gilson instrument equipped with a 305 model pump and a 316 UV detector using a Spherisorb S5 ODS2 (4.6 x 250 mm) column. Detector was set at 280 nm. Potentiometric titrations were carried out using a platinum-iridium electrode. EMF values were measured with a precision of $1 \cdot 10^{-5}$ V using a Hewlett-Packard 3421 A data acquisition unit controlled by computer. Elemental analyses were performed by Microanalytisches Labor Paschen, Remagen, Germany. Myristyltrimethylammonium bromide (MTMAB) was from Sigma, Soluene® 350 from Packard. Potassium ferricyanide was from Aldrich and was of the highest purity available. DHI and DHICA were prepared by a known procedure.[23] PTCA and PDCA were prepared as previously reported.[13] Nitrogen gas was freed from oxygen by passage through an alkaline pyrogallol solution.

Melanins. Sepia melanin was obtained by centrifugation at 4°C and 15 g of freshly collected ink suspended in 0.01 M HCl. Synthetic melanins were prepared by tyrosinase-catalysed oxidation of dopa[20] DHI[13] and DHICA[13] as described previously. Mixed DHI-DHICA melanin was prepared by co-oxidation of 3.1 mM DHI and 9.4 mM DHICA as described above.

Surfactant-induced solubilisation of melanin. The melanin (160 mg), finely suspended in 0.01 M HCl (4 ml), was added via syringe to a solution of 0.2 M MTMAB in 0.1 M NH_3, pH 10 (16 ml) in a rubber capped Corex® tube previously purged with nitrogen and was vigorously stirred for 4 h under rigorously an oxygen-free nitrogen atmosphere. The suspension was centrifuged under nitrogen, the brown supernatant was filtered through a 0.47 μ Millipore membrane and the absorption spectrum was recorded in a rubber capped cuvette under nitrogen. For chemical analysis and quantitation of solubilised melanin, the supernatant was filtered and acidified to pH 2 with HCl. The resultant precipitate was centrifuged, washed five times with 0.01 M HCl and then dried over P_2O_5.

Analytical procedures. The carboxyl[20] and protein contents[10,25] of melanins were determined by reported procedures. Oxidative degradation of melanins was carried out as described previously.[13]

Ferricyanide consumption by native and solubilised melanin samples. Previously reported procedures[26] were adopted with modifications.

Native melanins. To a vigorously stirred solution of 20 mM potassium ferricyanide in 0.1 M potassium phosphate buffer, pH 7.4 (20 ml) placed in a titration vessel thermostatted at 20.00 ± 0.02 °C was added a suspension of the melanin (10 mg) in 1 ml of 0.01 M HCl. Ferricyanide reduction was determined by measuring the EMF change after 1 min. A vigorous stream of nitrogen was passed through the solution during measurements. All experiments were run in triplicate.

Solubilised melanins. To a solution of potassium ferricyanide (0.4 mM) in 0.1 M phosphate buffer, pH 7.4, in a rubber capped cuvette, thoroughly purged with nitrogen, was added a 100-200 µl aliquot of the appropriate melanin solution obtained as described above. Ferricyanide reduction was determined by monitoring the absorbance change at 410 nm after 1 min. All experiments were run in triplicate.
When necessary, melanin samples, both native and MTMAB-solubilised, were treated with an excess of sodium borohydride in methanol. After acidification, to destroy excess reductant, the pigment samples were subjected to analysis as described above. Volume variations were usually negligible.

Acknowledgements. This work was supported by grants from MURST, CNR and L. M. Gelb Research Foundation (Stamford, CT, USA). We thank Prof. L. Ciavatta and Dr. M. Iuliano (Dept. of Chemistry, University of Naples) for potentiometric facilities, and Miss Silvana Corsani for technical assistance.

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(*Received in UK* 15 *April* 1997; *accepted* 1 *May* 1997)