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THE STRUCTURE OF MELANINS AND MELANOGENESIS—III

THE STRUCTURE OF SEPIOMELANIN[1,2]

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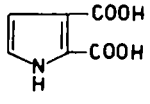
Abstract—New degradation products of sepiomelanin have been obtained. Alkali fusion yields, in addition to other compounds, 5,6-dihydroxyindole, 5,6-dihydroxyindole-2-carboxylic acid, 4-methylcatechol and a compound, which is probably 5,6-dihydroxyindole-4,7-dicarboxylic acid. These products constitute the first proof of the indole structure of a natural melanin. The carboxyl group of 5,6-dihydroxyindole-2-carboxylic acid is not formed during alkali fusion, but pre-exists in the macromolecule. Cysteic acid, taurine, glycine and aspartic acid were obtained by oxidation of sepiomelanin with hydrogen peroxide in acetic acid. The formation of cysteic acid indicates that sepiomelanin is bound to the protein by means of cysteine. Taurine is clearly an artifact generated by decarboxylation of cysteine. Glycine and aspartic acid probably are derived from the pyrrole moiety of the indole units: they also result from the oxidation of 5,6-dihydroxyindole-2-carboxylic acid.

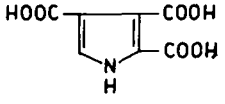
Oxidation of methylated sepiomelanin yields 3-carbomethoxypyrrole-2,5-dicarboxylic acid and 5-carbomethoxypyrrole-2,3-dicarboxylic acid; isolation of the former further proves the presence of pyrrole units in sepiomelanin, whereas formation of the latter is further evidence that some indole (probably dopachrome) units of the macromolecule have a carboxyl group in position 2.

PYRROLE-2,3-DICARBOXYLIC (I), pyrrole-2,3,4-tricarboxylic (II), pyrrole-2,3,5-tricarboxylic (III) and pyrrole-2,3,4,5-tetracarboxylic acids (IV) have been obtained by oxidative degradation of melanin from *Sepia officinalis*.[3] Although they supply valuable information about the melanin structure, they do not constitute rigorous proof as to the existence of indole units in the macromolecule.[4]

We have shown that free carboxyl groups make up ca. 9% by weight of melanin; at least a part of these carboxyl groups are linked to pyrrole rings, whereas the remainder are probably bound to indole rings in position 2. Further, the presence of sulphur



I



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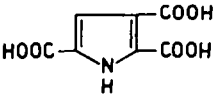

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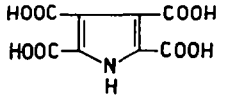


IV

[1] This investigation was supported by the National Cancer Institute, research grant C-5220, C2, Public Health Service, U.S.A.

[2] Part II. M. Piattelli, E. Fattorusso, S. Magno and R. A. Nicolaus, *Tetrahedron* **18**, 941 (1962).

[3] R. A. Nicolaus, *Biogenesis of Melanins* Suppl. no. 1. Rassegna di Medicina Sperimentale (1962).

[4] *In vitro* studies on the biogenesis of tyrosine and dopa melanins, based on the assumption that biosynthetic pigments are polymers of 5,6-indolequinone, also failed to offer convincing proof of the presence of the indole rings in the polymer. For a more detailed discussion of the melanin problem see: R. H. Thomson in M. Florkin and H. S. Mason, *Comparative Biochemistry* Vol. III. Academic Press, New York (1962).

in even highly purified samples of sepiomelanin, is not understood. Consequently, the object of this investigation was the following:

(a) to furnish rigorous proof of the presence of indole units* in the macromolecule;

(b) to obtain new experimental data confirming the presence of pyrrole rings;

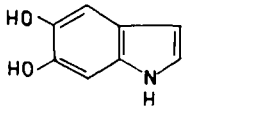
(c) to explain the presence of sulphur in even purified pigment.

For this purpose sepiomelanin was extracted and purified as described in a previous paper,[5] and the weight relation between the protein and the melanin (ca. 1:9) was determined by analysis of the amino acids derived from the hydrolysis of the pigment. This analysis will be further discussed in the present paper.

In order to determine whether the indole skeleton exists in the melanin molecule, the oxidation products were isolated in the hope that some compound would prove beyond doubt the presence of indole units. Unfortunately, the only new product isolated was malonic acid, which, at present, does not supply any useful information on the structure of melanin.

However, fragments proving the presence of the dihydroxyindole units in the polymer were obtained by alkaline fusion.[6]

Treatment of the pigment with sodium hydroxide at ca. 300° in the presence of sodium dithionite yielded a number of degradation products among which, by



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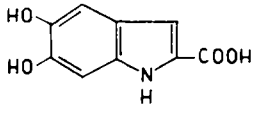

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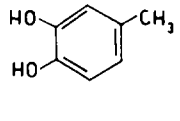
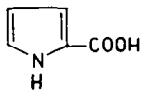


VII



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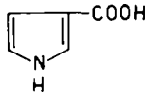

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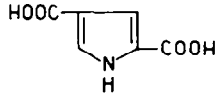

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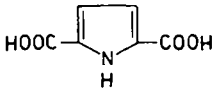
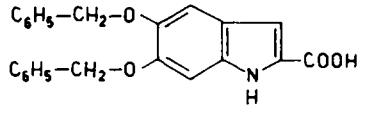


XI



XII

* The hypothesis that some natural melanins are constituted of indoleun its derives from the well known Raper's works on the formation of tyrosin melanine *in vitro*. On the other hand the formation *in vivo* of compounds containing the indole skeleton starting from tyrosine does occur, e.g. in lycorine (A. R. Battersby, R. Binks and W. C. Wildman, *Proc. Chem. Soc.* 410 1960).

[5] M. Piattelli and R. A. Nicolaus, *Tetrahedron* **15**, 66 (1961).

[6] As is known, oxidation and reduction reactions take place at the same time during alkaline fusion. A typical example of alkaline fusion with reduction reactions is that of papaverine, which yields 3,4-dimethoxytoluene and 6,7-dimethoxyisoquinoline. On the other hand, several natural substances, having an alkylated benzene ring with two hydroxyl or alkoxyl groups in position 3,4, when fused with alkali, undergo oxidative degradation with formation of protocatechuic acid.

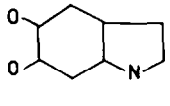
We performed alkali fusion on a compound having two directly linked benzene rings (2,2'-dihydroxydiphenyl; see Experimental), which yielded phenol, *o*-cresol and salicylic acid, as degradation products. It is clear that in this case both reduction and oxidation reactions take place.

means of paper chromatography, 5,6-dihydroxyindole (V), 5,6-dihydroxyindole-2-carboxylic acid (VI), 4-methylcatechol (VII) and the following pyrrolic acids: pyrrole-2-carboxylic (VIII), pyrrole-3-carboxylic (IX), pyrrole-2,4-dicarboxylic (X) and pyrrole-2,5-dicarboxylic (XI), were identified. In addition, among the breakdown products, a dihydroxyindolecarboxylic acid which is probably 5,6-dihydroxyindole-4,7-dicarboxylic acid was isolated.

Only small quantities of these products were obtained; but only a small portion of sepiomelanin is degraded during alkaline fusion. In fact, if the recovered pigment is re-subjected to alkaline fusion, the same degradation products are obtained even if the operation is repeated several times. Furthermore, it should be noted that the experimental conditions used are favourable for the further degradation of the dihydroxyindole products formed.

5,6-Dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid were also isolated by paper chromatography and identified by comparison of their UV spectra with those of authentic samples. 5,6-Dihydroxyindole-2-carboxylic acid, earlier described by Beer *et al.*,[7] was prepared more simply by catalytic reduction of 5,6-dibenzyloxyindole-2-carboxylic acid (XII).

Whereas no structural significance may be attached to the fact that pyrrolic acids VIII–XI are obtained, as they can arise from both pyrrole and indole units, the formation of 5,6-dihydroxyindole indicates that units of the type XIII exist in the macromolecule.



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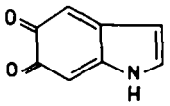
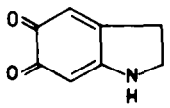


XIV



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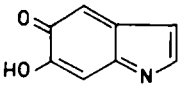
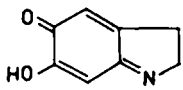
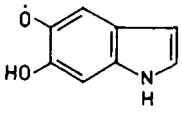


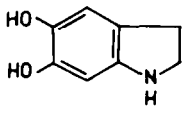
XVI



XVII



XVIII

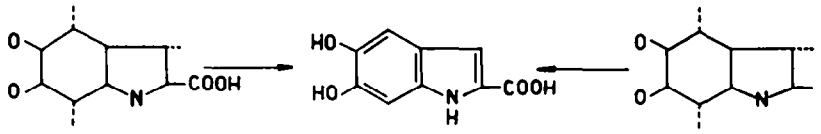


XIX

For instance, units like V and XIV–XIX may be present, all of which are capable of yielding 5,6-dihydroxyindole and pyrrolic acids by alkaline fusion. On the other hand, these indole fragments may be of immediate practical value in classifying natural black pigments particularly if there is the possibility of distinguishing between melanins of the indole type and those of different structure.

The origin of the carboxyl group in 5,6-dihydroxyindole-2-carboxylic acid may be illustrated by the two following hypotheses.

[7] R. J. S. Beer, L. McGrath, A. Robertson and A. B. Woodier, *J. Chem. Soc.* 2061 (1949).



Either indole units with a carboxyl group in position 2 exist in the macromolecule or indole units linked to this position generate the carboxyl groups during degradation.

In order to clarify this question, synthetic melanin, prepared by autoxidation of 5,6-dihydroxyindole at pH 8, was subjected to alkaline fusion under the same conditions as those adopted for sepiomelanin. It is evident that this pigment cannot have indole units with a carboxyl group in position 2; however, indole units linked through this position do undoubtedly exist in the polymer.[8] Since, among the products of alkaline fusion of melanin, 5,6-dihydroxyindole and catechol and not 5,6-dihydroxyindole-2-carboxylic acid were isolated, it must be concluded that indole units bearing a carboxyl group at position 2 are present in sepiomelanin.

It is noteworthy that, besides the indole derivatives, catechol is obtained from 5,6-dihydroxyindole during alkaline fusion of melanin, whereas 4-methylcatechol is obtained from sepiomelanin. At present it is difficult to explain the diverse behaviour of the two pigments; considering the nature of the degradation[6] it may be that 4-methylcatechol is formed from dopachrome units of sepiomelanin, obviously not present in the synthetic pigment.

After having thus obtained rigorous proof of the existence of indole units in sepiomelanin, it was necessary to demonstrate the presence of pyrrole units based on the fact that pyrrole-2,4-dicarboxylic and pyrrole-2,5-dicarboxylic acids were obtained by oxidation of decarboxylated sepiomelanin whereas they are absent among the oxidation products of undecarboxylated sepiomelanin.[9] This result indicates the presences of units of the type XX–XXII in the polymer. These units may be derived by hydrogen peroxide degradation of the benzenoid portion of dihydroxyindole or indolequinone nuclei during melanogenesis.

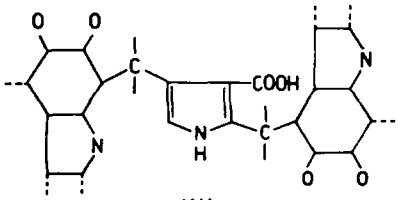
Sepiomelanin previously methylated with diazomethane was oxidized and if the foregoing hypothesis is valid, then esters of pyrrolic acids such as XXIII–XXV would be among the oxidation products.

Fractionation of the oxidation products by countercurrent distribution and by preparative chromatography, hitherto only yielded acid XXV, which is evidently derived from the unit XXII. The identity of this acid was proved by mixed melting point and comparison of its IR spectrum with that of an authentic sample, synthetized by mild alkaline hydrolysis of 2,3,5-tricarbomethoxypyrrole.

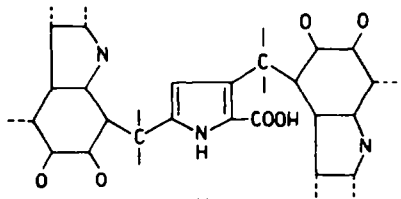
Now, in addition to acid XXV another monoester of pyrrole-2,3,5-tricarboxylic acid was isolated and shown to be identical with acid XXVI (mixed m.p. and IR spectrum) prepared by oxidation of 2-carbomethoxy-5,6-dihydroxyindole (XXVII). In this synthesis the acid XXVI was obtained in low yields, indicating that the dihydroxyindole nucleus tends to be degraded completely by the oxidizing agent—an interesting result

[8] This is demonstrated by the formation of pyrrole-2,3,5-tricarboxylic acid among the oxidation products of 5,6-dihydroxyindole melanin. See Part II of this series.

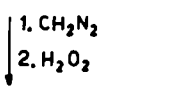
[9] M. Piattelli, E. Fattorusso, S. Magno and R. A. Nicolaus, *Rend. Ac. Sci. fis. e mat. Napoli* serie IV, Vol. XXVIII, 165 (1961).



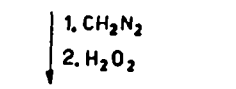
XX



XXI



1. CH_2N_2
2. H_2O_2



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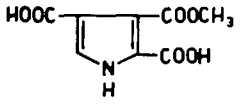
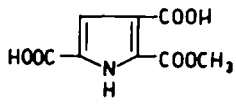
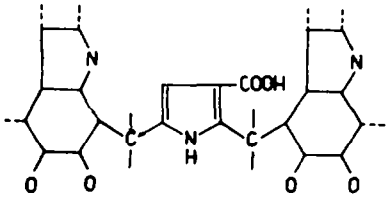
2. H_2O_2



XXIII

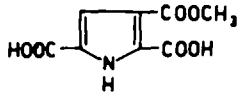


XXIV



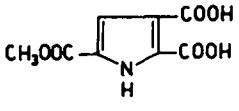
XXII

1. CH_2N_2
2. H_2O_2

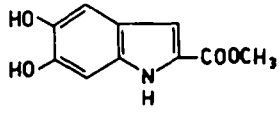


XXV

which may explain why melanin gives on oxidation pyrrolic acids in very low yields. The isolation of acid XXVI further proves that indole units with a carboxyl group in position 2 are present in the macromolecule.



XXVI



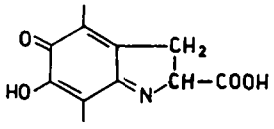
XXVII

The presence of these units shows that a carboxylated intermediate of Raper's scheme, probably dopachrome, partakes in the formation of the polymer. Whether these units retain an aminochrome structure in the polymer or rearrange to units of dihydroxyindole type was determined by the preparation of a melanin by enzymic oxidation of 5,6-dihydroxyindole 2-carboxylic acid.[10] This polymer, which on titration showed a slightly higher carboxyl group content than anticipated, upon heating

[10] Surprising is the statement in literature (see, e.g., R. H. Thomson *loc. cit.*) that 5, 6-dihydroxyindole-2-carboxylic acid does not yield melanin.

evolved carbon dioxide corresponding to only 5·9% by weight of the pigment, i.e. considerably less than the amount corresponding to total decarboxylation.

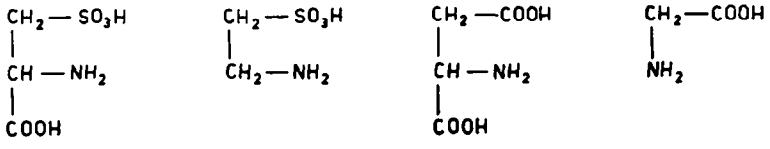
This behaviour suggested that the carbon dioxide evolved comes, not from the carboxyl groups in position 2 of the indole nuclei, but from the carboxyl groups formed by degradation of the benzenoid portion of a few units. In accordance with this view, the decarboxylated pigment gave pyrrole-2,3,5-tricarboxylic and pyrrole-2,3,4,5-tetracarboxylic acids by oxidation, and not pyrrole-2,3-dicarboxylic and pyrrole-2,3,4-tricarboxylic acids, as should result if there were a loss of the carboxyl group in position 2 of the dihydroxyindole nuclei. Since it has been shown that the carboxyl groups bound in position 2 and those derived from partial degradation of some indole nuclei during melanogenesis are eliminated by heating the sepiomelanin,[5] it must be assumed that in the natural pigment the carboxylated indole units have a dopochrome structure (XXVIII).



XXVIII

Although not confirmed experimentally, there is only one plausible hypothesis for the presence of sulphur namely, that the sulphur is part of the amino acids responsible for binding the sepiomelanin to the protein.

This supposition was verified by subjecting the pigment, obtained by centrifugation of *Sepia* ink after adding acetone, to hydrolysis with 6N hydrochloric acid. During hydrolysis the hydrolyzate was removed after 20, 40, 90, 180, 270 and 360 hr and replaced by fresh hydrochloric acid. Amino acids were not found in the last hydrolyzate. The amount of amino acids present in the liquid after 180 and 270 hr was negligible but as a pigment entirely free of amino acids linked to it by means of peptide linkages was required, hydrolysis was continued up for a total 360 hr. The pigment thus obtained was oxidized with hydrogen peroxide in acetic acid inorder to free, as a sulphonic derivative, any sulphurated amino acid bound to the macromolecule through a thioether linkage.



XXIX XXX XXXI XXXII

The resulting amino acids were identified by means of ion exchange chromatography. Besides two sulphurated products, *viz.* cysteic acid (XXIX) and taurine (XXX), comparable amounts of aspartic acid (XXXI) and glycine (XXII) were found among the oxidation products.

The presence of cysteic acid shows that the bond between the prosthetic part and the protein in sepiomelanoprotein is effected by the intervention of the —SH group

of cysteine molecules. Taurine is likely an artifact originating as a result of decarboxylation of the cysteine residues.

It is highly probable that aspartic acid and glycine are derived from the non protein moiety of the pigment. This hypothesis is substantiated by examination of the oxidation products obtained from 5,6-dihydroxyindole-2-carboxylic acid with hydrogen peroxide in acetic acid; besides pyrrole-2,3,5-tricarboxylic acid, aspartic acid and glycine were identified by paper chromatography. This result is significant since in sepiomelanin dopachrome units may be present and in very close structural relationship to 5,6-dihydroxyindole-2-carboxylic acid.

EXPERIMENTAL

All m.p. are uncorrected. IR spectra were taken on a Perkin-Elmer 13 C double beam spectrophotometer in KBr pellets. UV spectra were recorded on a Beckman DB spectrophotometer. Chromatograms were carried out on Whatman no. 1 paper (descending technique). Whatman no. 1 and 3 MM papers, washed with 2% HCl, were used for preparative chromatograms. The solvent systems used, prepared on a vol/vol basis, with their abbreviations are as follows: EAW, ethanol-33% ammonia-water (80 : 4 : 16); PAW, n-propanol-33% ammonia-water (60 : 30 : 10); BAW, n-butanol-acetic acid-water (60 : 15 : 25); HCl, 0·005N HCl; PBW, pyridine-n-butanol-water (33 : 33 : 34); CMFW, chloroform-methanol-formic acid-water (100 : 10 : 0·4 : 9·6; organic phase). Electrophoresis on Whatman no. 1 paper was carried out for 1 hr at 16 V/cm in the following electrolytes: PF, 0·05 M pyridine formate; KH_2PO_4, 0·03 M KH_2PO_4. As spraying reagents the following were used: A, diazotized sulphanilic acid and N NaOH; B, 3% ethanolic $FeCl_3$; C, ammoniacal $AgNO_3$; D, ninhydrine (0·2% solution in acetone). Tentative identification of degradation products was always substantiated by co-chromatography with authentic samples. R_f values and colours produced by the action of different reagents on the degradation products are presented in Table 1.

TABLE 1. MELANINS DEGRADATION PRODUCTS IDENTIFIED BY PAPER CHROMATOGRAPHY

R_f values × 100						Detection			
EAW	PAW	BAW	HCl	CMFW	Compounds	A	B	C	D
—	—	70	35	—	5,6-Dihydroxyindole	red	green	brown	black
—	—	49	05	—	5,6-Dihydroxyindole-2-carboxylic acid	red	blue	brown	—
—	—	87	65	80	4-Methylcatechol	red	green	brown	—
—	—	84	77	58	Catechol	red	green	brown	—
52	64	79	—	—	Pyrrole-2-carboxylic acid	red	—	—	—
45	35	75	—	—	Pyrrole-3-carboxylic acid	red	—	—	—
22	13	72	—	—	Pyrrole-2,4-dicarboxylic acid	red	—	—	—
28	17	70	—	—	Pyrrole-2,5-dicarboxylic acid	red	—	—	—
40	—	78	—	—	3-Carbomethoxypyrrole-2,5-dicarboxylic acid	red	—	—	—
60	52	65	—	—	5-Carbomethoxypyrrole-2,3-dicarboxylic acid	red	—	—	—

5,6-Dihydroxyindole-2-carboxylic acid (VI)

5,6-Dibenzyloxyindole-2-carboxylic acid[11] (3 g), dissolved in ethanol (200 ml), was hydrogenated (10% Pd-C (400 mg) at 95° and 100 atm for 48 hr). After removal of the catalyst, the solution was evaporated (*in vacuo*), thus obtaining 1·6 g of an almost white product, which was recrystallized from dil. acetic acid. The m.p. of this material (230°) was not depressed by admixture with an authentic sample of VI, prepared according to R. J. S. Beer *et al.*[7]

[11] H. G. Schlossberger and H. Kuch, *Chem. Ber.* **93**, 1318 (1960).

2-Carbomethoxy-5,6-dibenzyloxyindole

5,6-Dibenzyloxyindole-2-carboxylic acid (1 g), suspended in ether (15 ml), was treated with an excess of ethereal solution of diazomethane. The unreacted diazomethane was destroyed with acetic acid and the solvent removed *in vacuo*. The crude product gave 2-carbomethoxy-5,6-dibenzyloxyindole (m.p. 149–150°) as colourless needles on recrystallization from benzene (Found: C, 74·1; H, 5·5; N, 3·6; $C_{24}H_{21}O_4N$ requires: C, 74·4; H, 5·5; N, 3·6%).

2-Carbomethoxy-5,6-dihydroxyindole (XXVII)

2-Carbomethoxy-5,6-dibenzyloxyindole (1 g), suspended in ethanol (50 ml), was hydrogenated (10% Pd-C (150 mg) at 4 atm and room temp for 10 hr). The residue (0·53 g) obtained after evaporation of the filtered solution was recrystallized from acetic acid. The product had a m.p. (255–260°) and UV spectrum which conformed to those reported for 2-carbomethoxy-5,6-dihydroxyindole.[13]

5-Carbomethoxypyrrole-2,3-dicarboxylic acid (XXVI)

To a stirred solution of XXVII (550 mg) in 2N K_2CO_3 (10 ml) a 3% $KMnO_4$ solution (60 ml) was added dropwise at 5° until the colour persisted for 15 min. Excess permanganate was destroyed with a conc $NaHSO_3$ aq. The filtered solution, acidified (conc HCl), was extracted with ether (300 ml in 8 portions). The crude material, obtained by evaporation of the solvent, was dissolved in 1% H_2SO_4 (25 ml) and purified using countercurrent distribution between water and ether. After 90 transfers, the acid XXVI (100 mg) was present in tubes 8–15. It was further purified by passing through a strongly acidic resin (Dowex 50). Evaporation of the solvent afforded 70 mg of crude product, which was crystallized from acetic acid, colourless needles (30 mg), m.p. 246–247° (Found: OCH_3, 14·7; $C_8H_7O_6N$ requires: OCH_3, 14·5%).

3-Carbomethoxypyrrole-2,5-dicarboxylic acid (XXV)

2,3,5-Tricarbomethoxypyrrole[13] (100 mg) was suspended in 0·1N NaOH (9 ml). After 14 hr, the clear solution on acidification (conc HCl) yielded a precipitate, which was recrystallized from water, needles (25 mg), m.p. 249–251° (Found: OCH_3, 14·8; $C_8H_7O_6N$ requires: OCH_3, 14·5%). The monoester so obtained gave a red colour with the diazonium salt of sulphanilic acid; this demonstrated that no α-carboxylic group was still esterified[14] and therefore it was formulated as XXV.

Sepiomelanin

The cold purification of sepiomelanin was carried out as previously reported.[5]

5,6-Dihydroxyindole melanin

5,6-Dihydroxyindole melanin was prepared by autoxidation of 5,6-dihydroxyindole in 0·05 M phosphate buffer at pH 8 as previously described[2] (Found: C, 59·4; H, 3·1; N, 9·3; $(C_8H_3O_2N)_x$ requires: C, 66·2; H, 2·0; N, 9·6%).

Melanin from 5,6-dihydroxyindole-2-carboxylic acid

Compound VI (2 g) was dissolved in 1500 ml 0·05 M phosphate buffer at pH 6·8. After adding 25 mg of tyrosinase (1000 U/mg), a rapid stream of O_2 was bubbled into the solution during 6 hr. The solution turned pink in colour with the formation of a brown precipitate. After acidification (conc HCl), the precipitate was collected by centrifugation and washed 3 times with 2% HCl. The melanin obtained was kept in conc HCl (ca. 50 ml) for 15 days to remove the absorbed enzyme. Water (50 ml) was added and the pigment collected by centrifugation and washed with water (7 times), with acetone (once) and finally with ether (once). A black powder (1·3 g), infusible and insoluble in any solvent, was obtained (Found: C, 58·1; H, 3·1; N, 7·6; $(C_9H_5O_4N)_x$ requires: C, 57·1; H, 1·6; N, 7·4%).

[13] H. Wyler and A. S. Dreiding, *Helv. Chim. Acta* **42**, 1699 (1959).
[13] R. Nicolaus, *Gazz. Chim. Ital.* **83**, 239 (1953).
[14] R. A. Nicolaus, *Rassegna di Medicina Sperimentale* no. 2, anno VII (1960).

Oxidation of melanin from 5,6-dihydroxyindole-2-carboxylic acid

Melanin from 5,6-dihydroxyindole-2-carboxylic acid (50 mg) was suspended in a mixture of acetic acid (1·5 ml) and 36% H_2O_2 (1·5 ml). After stirring for 10 days at room temp, excess oxidant was destroyed by hydrogenation at room temp and barometric pressure for 10 hr in the presence of 10% Pd-C (10 mg). After removal of the catalyst, the solution was evaporated to dryness and the residue dissolved in water (0·2 ml) and analyzed by paper chromatography. By using solvents EAW, PAW and BAW and spraying reagent A, pyrrole-2,3,5-tricarboxylic and pyrrole-2,3,4,5-tetracarboxylic acids were identified; with spray reagent E, glycine and aspartic acid were detected. The identity of the pyrrolic acids was confirmed by electrophoresis in electrolytes PF and KH_2PO_4.

Thermic decarboxylation of melanin from 5,6-dihydroxyindole-2-carboxylic acid

Melanin from 5,6-dihydroxyindole-2-carboxylic acid (240 mg), dried at 80° over P_2O_5 *in vacuo* for 8 hr, was decarboxylated as previously described.[5] 64 mg of $BaCO_3$, equivalent to 14·3 mg CO_2 (5·9% by weight of the melanin used), was obtained.

Oxidation of decarboxylated melanin from 5,6-dihydroxyindole-2-carboxylic acid

Decarboxylated melanin from 5,6-dihydroxyindole-2-carboxylic acid (50 mg) was oxidized in the usual way[2,5] with 3% $KMnO_4$. The products obtained were analyzed by paper chromatography using EAW, PAW and BAW as solvents. Pyrrole-2,3,5-tricarboxylic acid and trace amounts of pyrrole-2,3,4,5-tetracarboxylic acid were detected by using spray A.

Titration of carboxyl groups in melanin from 5,6-dihydroxyindole-2-carboxylic acid

The titration of carboxyl groups of melanin from 5,6-dihydroxyindole-2-carboxylic acid was performed as previously described for sepiomelanin.[5] A neutralization equivalent of 180 was determined (theoretical neutralization equivalent for $(C_9H_5O_4N)_x$ = 189).

Alkaline fusion of sepiomelanin

Sepiomelanin (5 g), NaOH (15 g), $Na_2S_2O_4$ (1 g) and water (3 ml) were gradually heated in a platinum crucible to 280–300° and kept at this temp for 20 min. After cooling, the fused mass was dissolved in an ice-cooled 5% $Na_2S_2O_4$ solution (200 ml). After acidification with acetic acid (30 ml), the mixture was centrifuged to remove the brown precipitate. The clear solution was extracted with peroxide-free ether (200 ml in 5 portions). The combined ether solutions were extracted with a saturated solution of $NaHCO_3$ containing a small amount of $Na_2S_2O_4$ and then washed twice with a little water. After drying over Na_2SO_4 and $Na_2S_2O_4$, the solvent was evaporated and the residue taken up with water (0·2 ml; fraction 1). Acidic degradation products were isolated by acidifying the $NaHCO_3$ solution and extracting with ether. After washing with water and drying over Na_2SO_4 and $Na_2S_2O_4$, the ether extract was evaporated to dryness and the residue dissolved in water (0·2 ml; fraction 2). Chromatographic analysis of the fraction 1 (BAW, HCl and CMFW as the irrigants and A, B, C and D as spray reagents) showed the presence of 5,6-dihydroxyindole and 4-methylcatechol. 5,6-Dihydroxyindole-2-carboxylic acid was identified in fraction 2 by chromatography on paper using the solvent systems BAW and HCl and the spray reagents A, B and C. Pyrrole-2-carboxylic acid, pyrrole-3-carboxylic acid, pyrrole-2,4-dicarboxylic acid and pyrrole-2,5-dicarboxylic acid were identified by using EAW, PAW and BAW as irrigants and A as spray. The remainder of fraction 1 was used for the isolation of 5,6-dihydroxyindole by preparative chromatography on Whatman no. 1 paper (HCl as the irrigant); the UV spectrum of the substance isolated by elution of the band R_f 0·35 was determined and found to agree closely to the spectrum of authentic sample of 5,6-dihydroxyindole (max = 275 mμ, 298 mμ). The remainder of fraction 2 was similarly chromatographed in HCl; elution of the band R_f 0·05 yielded a product the UV spectrum of which in water showed close agreement with the spectrum of 5,6-dihydroxyindole-2-carboxylic acid (max = 313 mμ). Another compound was obtained by elution of the band R_f 0·30; from preliminary investigation, it appeared to be 5,6-dihydroxyindole-4,7-dicarboxylic acid.

These compounds were obtained in very low yields; but it must be kept in mind that they are readily destroyed under the experimental conditions used. On the other hand, when melanin from one experiment was re-subjected to alkali fusion, the same products were again obtained in comparable yields.

Alkaline fusion of 5,6-dihydroxyindole melanin

Alkaline fusion of 5,6-dihydroxyindole melanin and analysis of degradation products were performed as described for sepiomelanin.

The following products were identified: 5,6-dihydroxyindole, catechol, pyrrole-2-carboxylic, pyrrole-3-carboxylic, pyrrole-2,4-dicarboxylic and pyrrole-2,5-dicarboxylic acids.

Alkaline fusion of 2,2'-dihydroxydiphenyl

Alkaline fusion of 2,2'-dihydroxydiphenyl was carried out under experimental conditions used for sepiomelanin.

Salicylic acid was identified among the degradation products by paper electrophoresis (electrolyte PF); phenol and *o*-cresol were identified by chromatography on Al_2O_3 film using chloroform as the solvent.

Isolation of 5-carbomethoxypyrrole-2,3-dicarboxylic acid from oxidation products of methylated sepiomelanin

Sepiomelanin, methylated with diazomethane as previously reported,[6] (30 g) was oxidized with H_2O_2 in acetic acid, as described for oxidation of melanin from 5,6-dihydroxyindole-2-carboxylic acid. The mixture of oxidation products, dissolved in 1% H_2SO_4 (25 ml), was partitioned by countercurrent distribution between water and ether; after 100 transfers, 214 mg of crude product were obtained from the tubes 15–20. Crystallization from acetic acid afforded colourless needles. The m.p. (246–247°) of this compound was not depressed by admixture with an authentic sample of 5-carbomethoxypyrrole-2,3-dicarboxylic acid. The identification was substantiated by comparison of IR spectra.

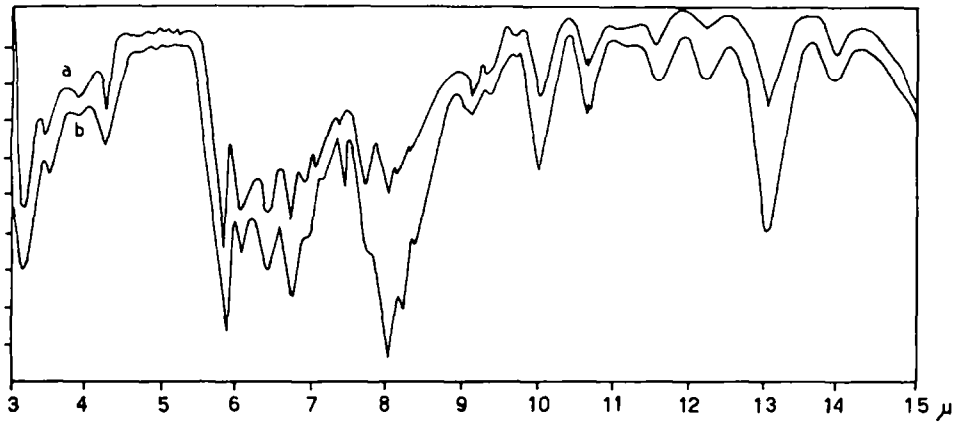


FIG. 1. Infrared spectra of 5-carbomethoxypyrrole-2,3-dicarboxylic acid. a = synthetic b = degradation product of methylated sepiomelanin.

Isolation of 3-carbomethoxypyrrole-2,5-dicarboxylic acid from oxidation products of methylated sepiomelanin

Sepiomelanin, methylated with diazomethane,[5] (15 g) was oxidized as described for the isolation of 5-carbomethoxypyrrole-2,3-dicarboxylic acid. The oxidation products were chromatographed on Whatman 3 MM paper using BAW as solvent. Elution with water of the band R_f 0·8 yielded a partially-crystalline residue (48 mg), which was further purified by re-chromatographying on Whatman 3 MM paper using the same solvent. Upon elution with water, the band R_f 0·8 yielded a white crystalline product, which was recrystallized from water (26 mg); the m.p. of this substance (249–251°) was not depressed by admixture with an authentic sample of 3-carbomethoxypyrrole-2,5 dicarboxylic acid. The identification was confirmed by comparison of IR spectra.

Isolation of malonic acid from oxidation products of methylated sepiomelanin

Sepiomelanin, previously methylated with diazomethane,[5] (50 g) was oxidized with H_2O_2 in acetic acid as described and the products obtained were partitioned by countercurrent distribution

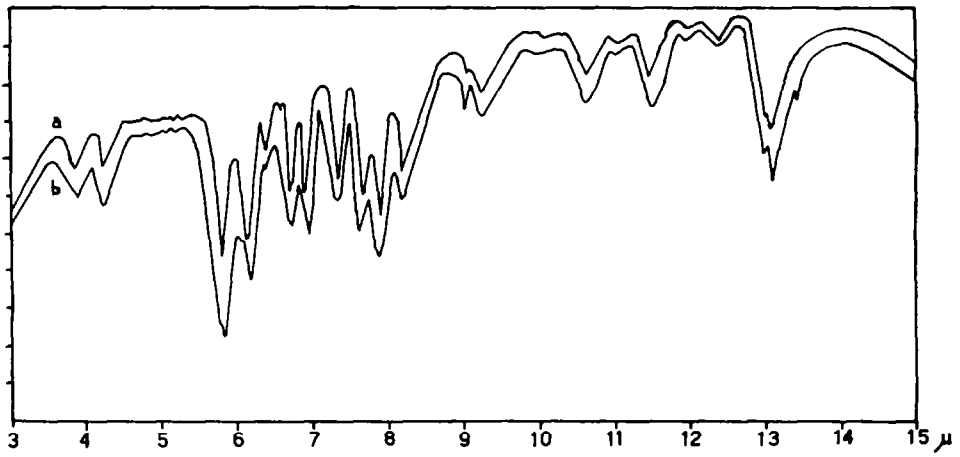


FIG. 2. Infrared spectra of 3-carbomethoxypyrrole-2,5-dicarboxylic acid. a = synthetic b = degradation product of methylated sepiomelanin.

between water and ether (150 stages). The crude product from tubes 18–24 was further purified by chromatography on a column (4 × 30 cm) of cellulose powder using EAW as solvent; 25 ml fractions were collected. Evaporation of fractions 18–30 yielded a crystalline residue (800 mg), which was dissolved in water (50 ml) and passed through a strongly acidic resin (Dowex 50). The eluate was evaporated to dryness, thus giving 550 mg of product which was recrystallized from ethyl acetate, prisms, m.p. 135–136° undepressed in mixture with authentic malonic acid. Comparison of IR spectra confirmed the identity.

Oxidation of 5,6-dihydroxyindole-2-carboxylic acid

To a solution of 5,6-dihydroxyindole-2-carboxylic acid (20 mg) in methanol (4 ml), acetic acid (4 ml) and 36% H_2O_2 (0·2 ml) were added. The mixture was kept at 40° for 40 hr and then heated on a steam bath for 45 min. After evaporation to dryness *in vacuo*, the residue, taken up with water (0·5 ml), was analyzed by chromatography. Using EAW as solvent and A as spray reagent, pyrrole-2,3,5-tricarboxylic acid was identified; by using BAW and PBW as solvents and E as spray, glycine and aspartic acid were identified.

Hydrolysis of sepiomelanoprotein

Amino acids determination. To a suspension of pigment, obtained by squeezing some ink sac of *Sepia officinalis*, acetone was added until precipitation was complete. After centrifugation, the precipitate was washed 10 times with acetone, dried at room temp and weighed (3·71 g). After washing with 1% HCl (10 times with 50 ml portions), the pigment (2 g) was hydrolyzed with boiling 6 N HCl for 20 hr. After centrifugation the supernatant was evaporated to dryness and the residue dissolved in 10% isopropanol (20 ml). An aliquot of 0·5 ml of this solution was used for amino acids determination (C. Erba amino acid analyzer) according to Piez and Morris.[15]

The pigment was again suspended in 6 N HCl and hydrolysis was allowed to proceed for 360 hr; the hydrolyzate was removed after 40, 90, 180, 270 and 360 hr and replaced by fresh 6 N HCl. The results of the amino acids analyses of each hydrolyzate are summarized in Table 2.

Oxidation of sepiomelanin

The pigment (1·8 g), obtained after the hydrolysis described, was oxidized as usual with H_2O_2 in acetic acid. The oxidation product, dissolved in dil. acetic acid, was extracted 5 times with 20 ml portions of ethyl acetate. The aqueous phase was evaporated to dryness and the residue chromatographed on a column of cellulose powder (2 × 20 cm) using solvent EAW in order to remove the

[15] K. A. Piez and L. Morris, *Anal. Biochem.* **1**, 187 (1960).

TABLE 2. AMINO ACIDS ANALYSIS OF THE POLYPEPTIDE PART OF SEPIOMELANIN
(μmoles/g of pigment)

Amino acid	Time of hydrolysis 20 hr	40 hr	90 hr	180 hr	270 hr
Cysteic acid	1·4	1·7	0·3	0·1	—
Aspartic acid	120	0·3	—	—	—
Threonine	78	0·2	—	—	—
Serine	49	0·2	—	—	—
Glutamic acid	93	0·2	—	—	—
Proline	46	—	—	—	—
Glycine	110	0·5	0·4	0·1	—
Alanine	80	0·2	—	—	—
Cystine	57	—	—	—	—
Valine	102	0·6	—	—	—
Methionine	0·6	—	—	—	—
Isoleucine	51	0·4	—	—	—
Leucine	75	0·2	—	—	—
Tyrosine	21	—	0·4	—	—
Phenylalanine	34	0·5	—	—	—
NH_4^+	576	66	56	—	—
Lysine	6	0·2	—	—	—
Hystidine	13	0·2	—	—	—
Arginine	41	—	—	—	—
Taurine	—	—	—	—	—

colouring impurities. The eluate was evaporated to dryness and the residue was taken up in water and analyzed for amino acids. The results were as follows:

Amino acid	mg of amino acid/g of oxidized pigment
Cysteic acid	0·48
Taurine	0·64
Aspartic acid	0·36
Glycine	1·20

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