Tetrahedron, 1961, Vol. 15, pp. 66 to 75. Pergamon Press, Ltd. Printed in Northern Ireland

THE STRUCTURE OF MELANINS AND MELANOGENESIS—I

THE STRUCTURE OF MELANIN IN *SEPIA*[1]

M. PIATTELLI and R. A. NICOLAUS
Institute of Organic Chemistry, University of Naples, Italy

(*Received* 6 *March* 1961)

Abstract -Melanin from *Sepia officinalis* is a natural polymer containing many carboxylic groups. It is present in the ink sac of the animal as the Mg and Ca salt. Purified sepiomelanin is able to combine with cations and, to a less extent with anions; by heating CO_2 is evolved. A formula closely related to . . . [—$C_8H_3O_2N$—$C_8H_4O_2N$·COOH·]$_x$. . . is suggested for sepiomelanin and its derivatives.

Owing to the presence of a tyrosinase in the ink sack it is possible that 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) plays an important part in the biological process of polymerization.

THE chemistry of melanins is still obscure in spite of the work accomplished.[2] For example, the composition of a natural melanin is yet unknown and consequently, the relations between the various natural, biosynthetic and synthetic pigments are unknown.[3] Chemically, the natural melanin of *Sepia* has been most studied.[4] This pigment is found in the so-called "ink sac" of *Sepia* (Fig. 1). One or two grams of melanin can be obtained from an average-sized animal—a large amount in relation to the body weight (300–400 g). The ink is easily collected by cutting open and lightly squeezing the ink sac. The melanin in the ink occurs in the form of small granules suspended in a colourless plasma.

The biological function of melanin appears to be defensive but the mechanism is uncertain.[5]

It is known that the ink gland contains an enzyme capable of converting tyrosine into melanin,[6] and it may be assumed that the mechanism described by Raper *et al.*[7]

[1] This investigation was supported by the National Cancer Institute, Research Grant C. 5220, Public Health Service, U.S.A.

[2] The literature of melanin chemistry is excellently reviewed by: A. Quilico, *I pigmenti neri animali e vegetali* Tip. Fusi, Pavia (1937); H. S. Mason in M. Gordon, *Pigment Cell Growth* AP. New York; H. S. Mason in F. F. Nord, *Advances in Enzymology* Vol. XVI. IP. New York (1955); H. S. Masonin M. Gordon, *Pigment Cell Biology* AP. New York (1959).

[3] Notably tyrosine melanin, catecol melanin and pyrrole black.

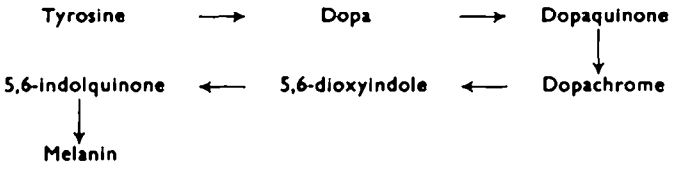
[4] L. Panizzi and R. A. Nicolaus, *Gazz. Chim. Ital.* **82**, 435 (1952); R. A. Nicolaus, *Ibid.* **83**, 239 (1953); **85**, 659 (1955); R. A. Nicolaus and L. Mangoni, *Ibid.* **85**, 1397 (1955); R. A. Nicolaus and L. Caglioti, *Ric. Sci.* **27**, 113 (1957); R. A. Nicolaus, A. Vitale and M. Piattelli, *Rend. Acc. Sci. Fis. Mat. Napoli* Vol. XXV (1958); R. Scarpati and R. A. Nicolaus, *Ibid.* Vol. XXVI (1959); R. A. Nicolaus, M. Piattelli and G. Narni, *Tetrahedron Letters* No. 21 (1959); R. A. Nicolaus, M. Piattelli and G. Narni, *Rend. Acc. Sci. Fis. Mat. Napoli* Vol. XXVII (1960).

[5] D. Tompsett, *Memoirs on Typical British Marine Plants and Animals* Vol. XXXII. University Press, Liverpool (1939).

[6] H. Pribram, *Hofm. Beitr.* **1**, 229 (1901); C. Gessard, *Compt. Rend. Soc. Biol.* **54**, 1304 (1902); P. Rondoni, *Sperimentale* **75**, 33 (1921); L. Califano, *Pubbl. Staz. Zool. Napoli* **13**, 289 (1933); L. Califano and D. Kertész, *Enzymology* **6**, 233 (1939).

[7] H. S. Raper, *Biochem. J.* **21**, 89 (1927); W. L. Dulière and H. S. Raper, *Ibid.* **24**, 239 (1930); R. D. H. Heard and H. S. Raper, *Ibid.* **27**, 36 (1933); see also H. S. Mason, *J. Biol. Chem.* **172**, 83 (1948).

may be applied to the formation of the pigment *in vivo*. It should, therefore, be considered as a polymer of 5,6-indolquinone:



The problem of purifying melanin from *Sepia* was never seriously faced, and only

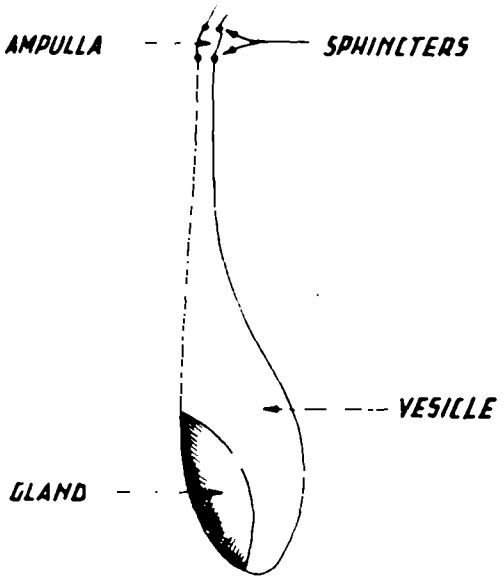


FIG. 1.

recently have two simple methods been put forward which give encouraging results. The first consists, briefly, of the following processes:

(a) washing the pigment with 1 per cent HCl.
(b) hot extraction with acetone.
(c) prolonged boiling with concentrated HCl.

The second method involves:

(a) dialysis of the contents of the ink sac against distilled water.
(b) washing with 1 per cent HCl.
(c) digestion for 370 hours with concentrated HCl.[8]

During purification it was observed that melanin is combined with calcium and magnesium. Melanin may also combine with small quantities of acid (in this case HCl) and may lose CO_2 on heating.[9]

The amorphous pigment obtained was hygroscopic, insoluble in all solvents, did not crystallize and contained traces of sulphur (0·2–0·4 per cent). The substance

[8] These methods of purification remove proteins and inorganic material. The nature of this material will be reported soon. It is notable that on oxidation it yields no pyrrolic acids.
[9] These properties explain the disagreement of analytical data in the literature.

obtained according to the first method of purification was entirely free from ashes and on analysis corresponded with a polymer of Type I or better, Type II.[10]:

$$\cdots\cdots[\ (C_9H_9O_2N)_4\ \ C_9H_7O_2N{\cdot}COOH\ ]_x\cdots\cdots \quad \text{I}$$

$$\cdots\cdots[\ (C_9H_9O_2N)_4\ \ C_9H_6O_2N{\cdot}COOH\ ]_x\cdots\cdots \quad \text{II}$$

$$\cdots\cdots[\ C_9H_9O_2N\ \ C_9H_7O_2N{\cdot}COOH\ ]_x\cdots\cdots \quad \text{III}$$

$$\cdots\cdots[\ C_9H_6O_2N\ \ C_9H_6O_2N{\cdot}COOH\ ]_x\cdots\cdots \quad \text{IV}$$

The second method of purification performed at room temperature, gave a less pure substance but the danger of decarboxylation was avoided. Analysis of this pigment agreed with that of a polymer of Type III, or better Type IV.

The presence of carboxyl groups in sepiomelanin has been proved by the following observations:

(a) the carboxyl groups could be titrated with alkali.

(b) CO_2 was evolved on heating at 140°-150°.

(c) a methyl ester was obtained on treatment with methyl alcohol in the presence of hydrogen chloride.

Moreover, quantitatively, the number of the —COOH groups deducible from the analytical results shown in Table 1 agree satisfactorily with the formulae given, provided that the special physical properties of the substance are taken into account and because of these properties it is not possible to expect reactions to proceed to completion.

TABLE 1

	Found	Calculated
Neutralization equivalent of melanin:	306	338·3
Percentage CO_2 evolved at 140°-150°:	9·1	13·0
Percentage OCH_3 of the methyl ester:	6·0	8·8

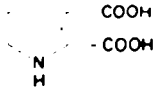
The behaviour of the pigment with diazomethane is interesting. A compound was obtained which on analysis gave values near the theoretical for a polymer .... $[-C_9H_7O_2N-C_9H_6O_2N{\cdot}COOCH_3-]_x$..... This indicates the presence of numerous hydroxyl groups or desmotropic functions. The percentage value of methoxyl found (18·8) was much lower than that calculated for polymer IV (24·4). Probably, by reason of its complete insolubility, the pigment is never entirely methylated (on heating small quantities of carbon dioxide are still evolved) and some units of the polymer can also react as orthoquinones with diazomethane. This can be demonstrated qualitatively by the positive colour test for $-O-CH_2-O-$ group.[11] We have also been able to show that diazomethane reacts slightly with nitrogen; in fact,

[10] These formulae and other like them show only the statistical distribution of the two types of units in the polymer.

[11] Indirectly this is the only proof of the existence of orthoquinone groups in the melanin of *Sepia*.

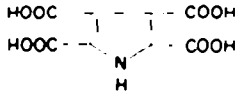
on oxidation of the methylated pigment with $KMnO_4$, monomethylamine is obtained. This is not formed from melanin under similar conditions.

The most important compounds isolated or identified from the products of oxidation with H_2O_2 or $KMnO_4$ are, as is known, pyrrolic acids V, VI and VII:



V

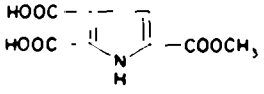
VI



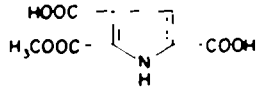
VII

Pyrrolic acids V and VI have been isolated, but pyrrolic acid VII has only been identified by paper chromatography. The yields of these acids are low: 1·2 per cent for pyrrolic acid VI and less than 0·05 per cent for the other two. However, as we shall see, these acids have a certain significance for structural investigation.

The behaviour of methylated melanin on oxidation is different because the pyrrolic acids V, VI and VII disappear almost completely from the degradation products. This is easily shown by chromatography. When chromatograms are sprayed with DZA the pyrrolic acids are recognizable as intense red spots. In the case of methylated melanin, however, a yellow spot is visible, with an R_f near that of pyrrolic acid VI. But if the degradation products are hydrolysed with NaOH and chromatographed, this yellow spot disappears and a spot identifiable with pyrrolic acid VI appears instead. Considering the R_f value of the yellow spot and the colour which is characteristic of esterified pyrrolic acids in the α-position, one must presume that such a spot is caused by an ester of Type VIII or IX:



VIII

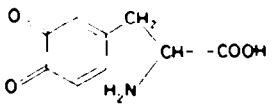


IX

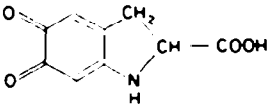
Since it is very probable that the carboxyl groups in position 4 and 5 are derived from the destroyed benzenoid portion of the pigment, one has to conclude that the ester in question is 2-carbomethoxy-4,5-dicarboxypyrrole (VIII). This indicates that pyrrolic acid VI is derivated essentially from the carboxylated units of the pigment. This was confirmed by comparing chromatographically the oxidation products of sepiomelanin before and after heating at 140°-150°. Melanin purified at low temperature yielded traces of 2,3-pyrroledicarboxylic acid V and 2,3,4,5-pyrroletetracarboxylic acid VII, and large quantities of 2,3,5-pyrroletricarboxylic acid VI. With melanin heated at 140°–150° the amount of pyrrolic acid VI clearly decreased, whereas pyrrolic acid V was found in relatively large quantities. This also makes it clear that pyrrolic acid VI derives from carboxylated units of the pigment and pyrrolic acid V from decarboxylated units.

These results are easily interpreted if one partially accepts Raper's mechanism of melanin formation. However, the higher hydrogen content than that calculated for a polymer of formula $(C_8H_3O_2N)_x$ and the presence of a large number of carboxylic groups in sepiomelanin lead one to think that polymerization occurs with the participation of a compound carrying a —COOH group. The possible monomers are

dopaquinone (X) and dopachrome (XI):



X



XI

With X a polymer should contain α-aminoacid chains.[12] In order to show the possible presence of groups of this type, sepiomelanin was methylated with diazomethane and oxidized with $KMnO_4$. The volatile basic products were analysed by gas chromatography (see Figs. 2 and 3).

It has only been possible to show the presence of ammonia and methylamine. If

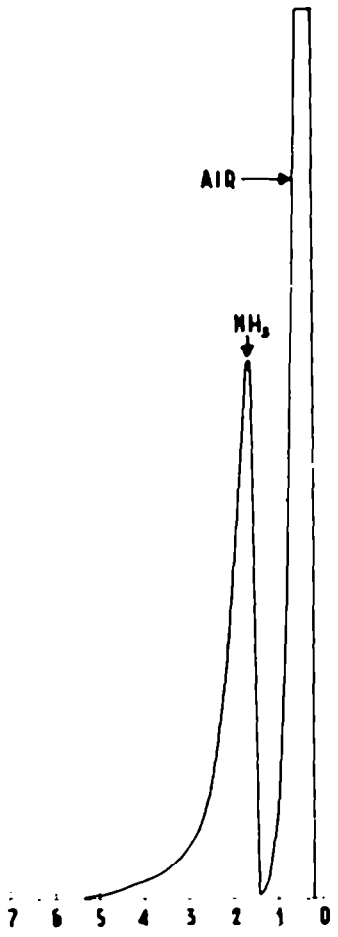


FIG. 2.

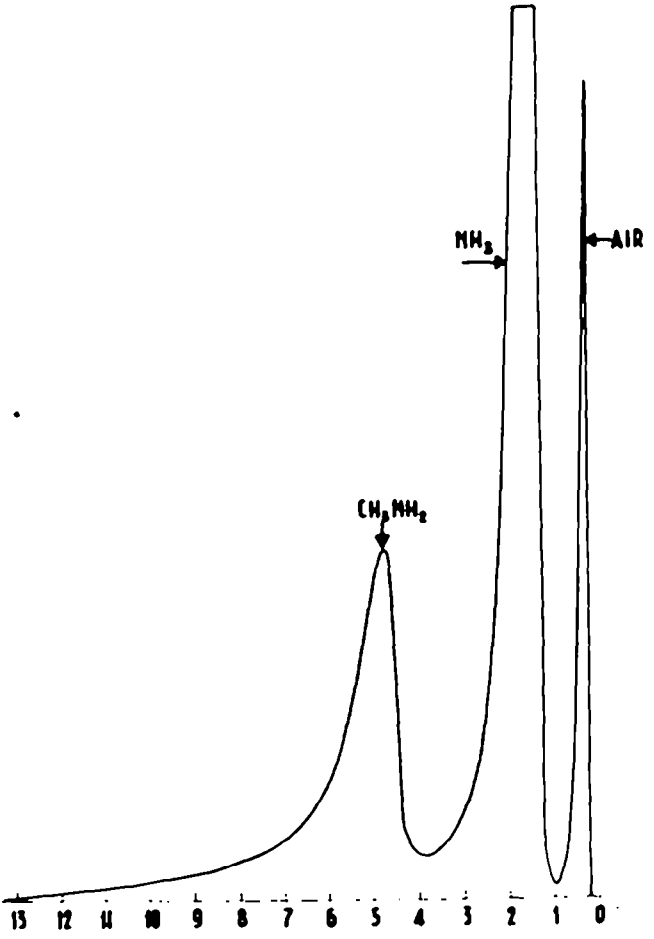


FIG. 3.

[12] This was considered unlikely since 6-methyldopa, which cannot be transformed into indolequinone, does not form melanin (R. I. T. Cromartie and J. Harley-Mason, *Biochem. J.* 66, 713 (1957). The degradation described here was performed as a further control.

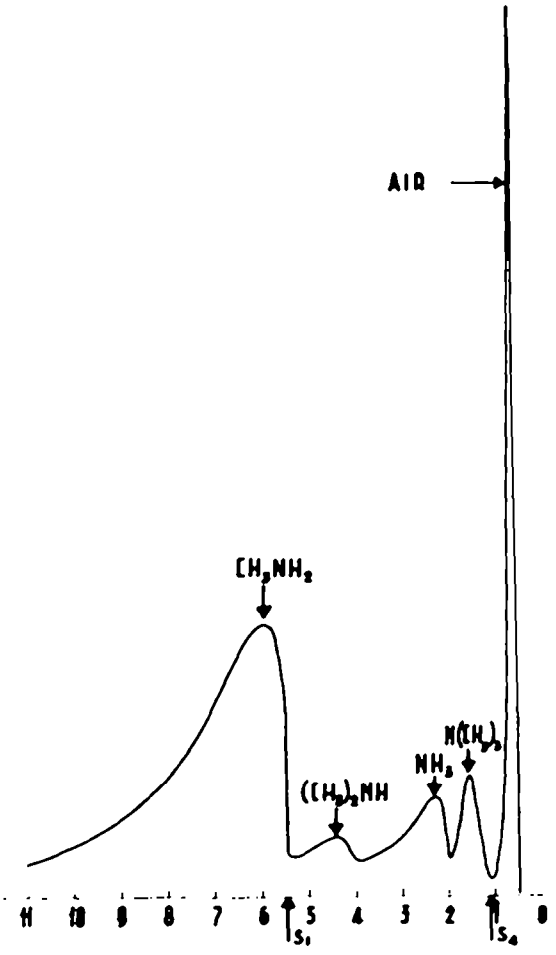
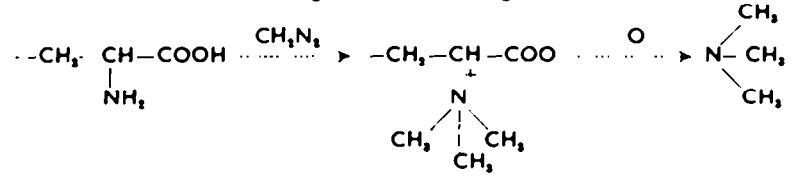


FIG. 4.

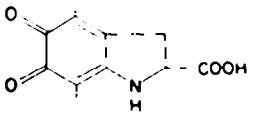
chains of the type $-CH_2-\underset{\displaystyle NH_2}{\underset{|}{CH}}-COOH$ had been present, trimethylamine would have been found, formed according to the following scheme:

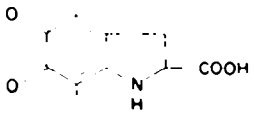


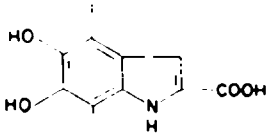
As a control, a sample of tyrosine was methylated with diazomethane and oxidized with $KMnO_4$: the presence of trimethylamine among the volatile degradation products is clear from the graph (Fig. 4).

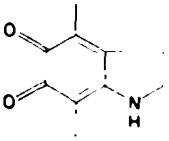
The presence of dopaquinone units in the polymer being, therefore excluded, one must conclude that dopachrome plays an important part in the biological formation of sepiomelanin.

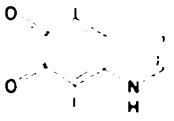
All the results so far obtained can be explained by the presence of units of the following types:

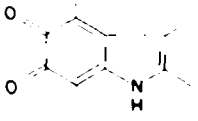

XII

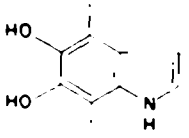

XIII

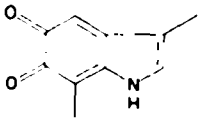

XIV

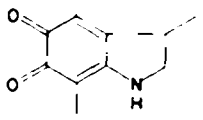

XV


XVI


XVII


XVIII


XIX


XX

Units such as XII, XIII, XIV, may account for the presence of 2,3,5-pyrroletricarboxylic acid VI and 2-carbomethoxy-4,5-dicarboxypyrrole (VIII) among the oxidation products of sepiomelanin and sepiomelanin methyl ether respectively. 2,3-Pyrroledicarboxylic acid V may arise from units of the Type XV, XVI and XVIII.

The possible existence of cross-linking between different chains is depicted in XVII. This structure is supported by the presence of the 2,3,4,5-pyrroletetracarboxylic acid VII in the oxidation mixture.[13]

Finally units XIX and XX, although there is no proof, may not be excluded *a priori* in sepiomelanin, considering the high reactivity of the position 3 in 5,6-dioxyindole derivatives.[14] Although these results seem significant, we think further work is necessary and expect further indications on the structure of sepiomelanin from work now in progress.

EXPERIMENTAL

Hot purification of sepiomelanin

The samples of melanin used were obtained by a method described in a previous paper. The pigment was in the form of a black powder which was infusible, hygroscopic, insoluble in all solvents and contained small quantities of chlorine and traces of sulphur. It was dried in a vacuum over P_2O_5 at 110° for 17 hr. (Found: C, 63·3; H, 2·4; O, 23·8; N, 8·8; Cl, 2·2; S, 0·3–0·4. Subtracting Cl: C, 64·7; H, 2·5; O, 24·3; N, 9·0. $[-(C_9H_5O_2N)_4-C_9H_7O_2N{\cdot}COOH-]_x$ requires: C, 63·9; H, 2·1; O, 25·0; N, 9·1%. $[-(C_9H_5O_2N)_4-C_9H_6O_2N{\cdot}COOH-]_x$ requires: C, 63·2; H, 3·2; O, 24·7; N, 9·0%). No ashes were left on calcination. Melanin samples are very difficult to burn. Differences of 1–2% were observed in various analyses of the same sample.

[13] According to M. M. Rangier, *C. R. Acad. Sci., Paris* **249**, 1954 (1959) the fundamental unit of natural melanins is adrenochrome. Unfortunately, the paper is so erroneus that it cannot be considered.

[14] J. Bu'Lock and J. Harley-Mason, *J. Chem. Soc.* 703, 2248 (1951); R. I. T. Cromartie and J. Harley-Mason, *Ibid.* 3525 (1953); *Idem., Biochem. J.* **66**, 713 (1957).

Cold purification of sepiomelanin

An ink sac was taken from an urethan-anesthetized cuttlefish, cut open and emptied. The liquid obtained, which was slightly alkaline, was dialysed against distilled water until the reaction for Cl^- was negative. The dialysed fluid, after evaporation in a vacuum, left 71 mg of residue.

The content of the dialysis tube, plus 50 cc 1% HCl, was centrifuged. The black precipitate was washed by centrifuging 5 times with 1% HCl (20 cc each time).

The combined supernatants gave 10·2 mg residue after evaporation and calcination. Analysis of the residue by flame spectrophotometry showed it to consist of magnesium and calcium chlorides in a molecular ratio of 1:1·76.

The pigment was then suspended in 15 cc conc HCl and left at room temp for 15 days. It was then centrifuged, washed 10 times with 1% HCl (20 cc each time) and twice with distilled water; 24 mg of residue were obtained from the washings.

After drying over P_2O_5 in a vacuum at room temp for 48 hr, the purified pigment weighed 140 mg. Analysis showed a chlorine content of about 3%, only partially removable by dialysis, traces of sulphur and negligible quantities of ashes, but the pigment was considered sufficiently pure for investigation. Boiling for 24 hr with conc HCl removed small quantities (2–4%) of colourless material mostly consisting of aminoacid hydrochlorides and traces of inorganic substances. (Found: C, 59·9; H, 3·4; O, 26·3; N, 8·2; Cl, 3·2; S, 0·3 0·4. Subtracting Cl: C, 60·9; H, 3·4; O, 27·1; N, 8·5. [$C_9H_9O_2N$ $C_9H_9O_2N{\cdot}COOH-]_x$ requires: C, 61·1; H, 1·8; O, 28·7; N, 8·4% [$C_9H_9O_2N-$ $C_9H_9O_2N{\cdot}COOH-]_x$ requires: C, 60·9; H, 3·0; O, 28·4; N, 8·3%). No ashes were left on calcination. Drying the samples of melanin in a vacuum at higher temp (80° and 110°) does not alter the pigment.

Methyl ester

100 mg of finely ground melanin were suspended at room temp in 2·5 cc anhydrous methanol, saturated with dry hydrogen chloride. After 24 hr the solid was centrifuged, washed with anhydrous methanol, dried in air, triturated again and re-esterified.

After repeating 3 times, the reaction was considered complete since the amount of methoxyl found did not increase. The samples of esterified melanin were dried to a constant weight in a vacuum at room temp. The ester was in the form of a black powder, insoluble in all solvents and containing traces of sulphur and small quantities of chlorine. (Found: C, 58·9; H, 3·3; O, 26·0; N, 7·9; Cl, 3·9; S, 0·3; OCH_3, 5·7. Subtracting Cl: C, 61·2; H, 3·5; O, 27·1; N, 8·0; OCH_3, 6·0. [$C_9H_9O_2N-C_9H_9O_2N{\cdot}COOCH_3$ $]_x$ requires: C, 62·1; H, 2·3; O, 27·6; N, 8·0; OCH_3, 8·9%. [– $C_9H_9O_2N$ –$C_9H_9O_2N{\cdot}COOCH_3-]_x$ requires: C, 61·4; H, 3·4; O, 27·3; N, 7·9; OCH_3, 8·8%). On oxidation with permanganate the ester behaves in a very similar way to sepiomelanin. Pyrrolic acids V, VI and VII were always obtained, although the tricarboxylic acid VI diminished. On heating under the conditions described later CO_2 was evolved to the extent of 2·8% of the weight of pigment used.

Methyl ether

100 mg of finely ground melanin was treated with an ethereal solution of diazomethane (200 mg diazomethane in 2 cc ether) and allowed to stand at room temp. After 24 hr the melanin was centrifuged, washed with ether, ground up again and treated with diazomethane. The process was repeated 3 times. After washing with ether and anhydrous methanol the pigment was dried for 48 hr at room temp over P_2O_5 in a vacuum. The methyl ether was in the form of an infusible brown powder, insoluble in all solvents. On heating to 140°–150°, CO_2 was evolved to the extent of 1·8% by weight of the pigment. The $-O-CH_3$ O = group test was positive and performed as follows: 50 mg of methylated pigment were suspended in 5 cc conc H_2SO_4 and treated with 0·2 cc of 0·5% alcoholic solution of gallic acid. After leaving overnight and then centrifuging the pigment, the liquid was coloured emerald green. (Found: C, 64·1; H, 5·5; O, 23·8; N, 7·4; OCH_3, 18·8. [$C_9H_9O_2N-$ $C_9H_9O_2N{\cdot}COOCH_3-]_x$ requires: C, 63·8; H, 3·2; O, 25·5; N, 7·5; OCH_3, 24·7%. [$C_9H_9O_2N-$ $C_9H_9O_2N{\cdot}COOCH_3$ $]_x$ requires: C, 63·1; H, 4·2; O, 25·3; N, 7·4; OCH_3, 24·4%).

Determination of carboxyl groups by titration

570 mg of melanin were placed in a glass column of diameter 8 mm, a N/5 solution of Na_2CO_3 was passed through the column and the first 100 cc were collected. By titration with N/5 HCl

(indicator tetrabromophenolphthalein ethyl ester or fluorescein in u.v. light) a neutralization equivalent of 306 was determined (theoretical neutralization equivalent for $[-C_8H_5O_2N-C_9H_4O_2N\cdot COOH]_x$ 338·3). In the calculation the amount of alkali used to neutralize the hydrochloric acid in the pigment was allowed for.

Thermic decarboxylation of sepiomelanin

Melanin, obtained as described above, was finely ground, suspended in 5 cc vaseline oil and heated at 140°–150° (bath temp) for 10 hr in a current of nitrogen. The amount of CO_2 evolved was measured by absorbing the gas in a saturated solution of barium hydroxide and weighing the barium carbonate formed; 0·4292 g $BaCO_3$ was obtained from 1·0817 g melanin, and was equivalent to 0·0957 g CO_2 (8·85% of the weight of melanin used). Subtracting the chlorine present in the sample, a value of 9·1% is obtained. The theoretical carbon dioxide evolved by a polymer $[-C_8H_5O_2N-C_9H_4O_2N\cdot COOH-]_x$ is 13·0%.

The decarboxylated melanin was washed repeatedly with light petroleum (b.p. 40°–70°) and finally with ether; it was then dried for 48 hr over P_2O_5 in a vacuum. (Found: C, 61·1; H, 3·5; O, 22·0; N, 9·2; Cl, 4·0. Subtracting Cl: C, 63·5; H, 3·6; O, 22·9; N, 9·5. $(C_8H_5O_2N)_x$ requires: C, 65·3; H, 3·4; O, 21·7; N, 9·5%). Decarboxylated melanin was in the form of a black powder, infusible, hygroscopic and insoluble in all solvents. No apparent difference could be observed between it and the original melanin.

Determination of the water content of melanin

After desiccation melanin was highly hygroscopic; the rate of water absorption was very rapid at first, but gradually decreased, although it was still noticeable after many days. In an atmosphere saturated with water vapour at 20° the quantity of water absorbed after 15 days was 30% of the original weight of melanin.

By drying over P_2O_5 in a vacuum the water was totally removed and the original weight of melanin was restored. Drying at various temperatures (room temp; 80°; 110°) varied only the time required for water removal.

Comparative oxidation of sepiomelanin and decarboxylated sepiomelanin

Quantitative chromatographic analysis of pyrrolic acids. 100 mg of melanin and 100 mg of decarboxylated melanin were each suspended in 2 cc 2N K_2CO_3 and oxidized with 3% $KMnO_4$ until the colour persisted for 15 min.

For oxidation, melanin required 12·5 cc. $KMnO_4$ and decarboxylated melanin required 12·7cc. After removal of MnO_2 and precipitation of oxalic acid with $CaCl_2$ the solutions were extracted with ether (50 cc in 5 portions). After removal of the solvent, melanin yielded 7·6 mg residue and decarboxylated melanin 3·5 mg. These residues were each dissolved in distilled water and the filtered solutions used for quantitative determination of 2,3,5-pyrroletricarboxylic acid VI and 2,3-pyrroledicarboxylic acid V. For this analysis 0·1 cc of each solution and graduated quantities (0·025 cc, 0·10 cc, 0·15 cc) of a standard solution containing 1 mg 2,3,5-pyrroletricarboxylic acid VI per cc were applied to a Whatman No. 1 paper. Descending chromatography, using ethanol–33% ammonia–water (80:4:16) as solvent, was allowed to run to about 25 cm from the starting point. After colour development with DZA the spots were cut out and their colours eluted in 20 cc N/20 Na_2CO_3 solution. The optical densities of these solutions were read with a Lumetron colorimeter (filter λ – 420 mμ) using a blank made from part of the paper sprayed with DZA. From the calibration curve the quantity of 2,3,5-pyrroletricarboxylic acid VI contained in the samples was easily determined.

The 2,3,5-pyrroletricarboxylic acid content of melanin was found to be 1·4%, and that of decarboxylated melanin 0·5%.

The 2,3-pyrroledicarboxylic acid V content was determined by the same method. Decarboxylated melanin gave approximately 0·05% of this acid. Carboxylated melanin, however, gave quantities that they were not measurable by this method.

Oxidation by $KMnO_4$ of the fractions soluble in water and hydrochloric acid

(a) The residues obtained by evaporation of the dialysate and of the washing of raw melanin with 1% HCl resisted oxidation by permanganate under the conditions described and used for melanin

(b) On permanganate oxidation the fraction removable with cold conc HCl yielded small quantities of substances that can be revealed with DZA. Such spots have R_f values differing from those of 2,3-pyrroledicarboxylic acid V, 2,3,5-pyrroletricarboxylic acid VI and 2,3,4,5-pyrroletetracarboxylic acid VII.

Volatile bases from oxidation of melanin and its methyl ether

Examination by gas chromatography. Melanin and its methyl ether were oxidized with $KMnO_4$ as described above. At the end of oxidation the liquid was distilled and collected in N HCl. The hydrochlorides of the volatile bases were obtained by evaporation of the acidic solutions. The yield from melanin was about 10% of the oxidized weight of the pigment and from methylated melanin 13%.

The hydrochlorides were treated with the minimum possible quantity of a saturated solution of KOH and the bases analysed by gas chromatography using C. Erba's Fractovap apparatus. The composition of the column (1 m length) was: celite 85%, triethanolamine 7·5%, vaseline oil 7·5%. The carrier gas was hydrogen, at a flow rate of 4 litres per hour. The experiment was carried out at 20°. Results are shown in (Figs. 2 and 3), melanin yielded only ammonia (Fig. 1), while methylated melanin yielded ammonia and methylamine (Fig. III).

By indirect analysis of the hydrochlorides (Mohr's or Volhard's determination of Cl) ammonium chloride and methylamine hydrochloride were found to be in a 1:1 ratio by weight.

Methylation and oxidation with $KMnO_4$ of tyrosine

Examination of the volatile bases. 100 cc of an ethereal solution containing 2·5 g diazomethane were added to 100 cc of an ethereal suspension of 1 g tyrosine. Reaction was started with some drops of water. After leaving overnight the ether was evaporated to dryness; the residue was dissolved in 13 cc 2N K_2CO_3 and oxidized with a 3% solution of $KMnO_4$ (135 cc). MnO_2 was filtered off and the liquid distilled and collected in N HCl. The solution was evaporated to dryness and hydrochlorides were examined by chromatography as already described. The results are shown in (Fig. 4).