Tetrahedron, 1964, Vol. 20, pp. 1163 to 1172. Pergamon Press Ltd. Printed in Northern Ireland

THE STRUCTURE OF MELANINS AND MELANOGENESIS—IV

ON SOME NATURAL MELANINS[1,2]

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Abstract—Melanins have been isolated from the following sources: rat-tumor, human hair, dog-hair, horse-hair, ox-hair, ox-choroid, squid-ink, octopus-ink, chicken-feathers, pigeon-feathers, *Amphiuma*-liver, and axolotl-liver; on the basis of certain typical degradation products these melanins can be classified as "indole" melanin.

A few melanins from the plant kingdom have also been taken into consideration. Like the recently studied ustilago melanin, sunflower seed and water-melon seed melanins have proved similar to cathechol melanin on the basis of the available chemical evidence; it may be that "catechol" melanin is a pigment widespread in nature.

The black from the parasite *Capnodium nerii* belongs to an as yet unknown type of polymer.

As is known, many animal and plant pigments are indicated by the name *melanin*.[3] This name is at present so broadly applied that it can be attributed without distinction to compounds of considerably different structure. On the other hand, since there is a great dearth of information on the structure of most of these pigments, no attempt of chemical classification has yet been possible.

The only melanins hitherto studied fairly extensively are *Sepia*-ink pigment and *Ustilago maydis*-spore pigment. The early studies on sepiomelanin had shown it to be quite similar to the black obtained *in vitro* by enzymic oxidation of tyrosine. Biogenetic studies had lead to assign a polymeric structure made up of 5,6-indolequinone units to tyrosine black. Later it was demonstrated that the structure of sepiomelanin, whose precursor is actually tyrosine, is a great deal more complex than what would appear from the Raper-Mason biogenetic scheme.[4] In fact, it has been shown that, besides the 5,6-dihydroxyindole and 5,6-indolequinone units, pyrrole units bearing a carboxyl group and dopachrome units partake in the building up of the sepiomelanin molecule as well. It is interesting to note that dopachrome, 5,6-dihydroxyindole and 5,6-indolequinone are intermediates in the Raper-Mason biogenetic scheme.[3] The presence of pyrrole units in the melanin molecule can be explained by assuming that the benzenoid part of some "indole" units undergoes an oxidative breakdown during or just after polymerization.[5] Furthermore, the polymer is an irregular one, not only as far as the units which make it up are concerned, but also on account of the way in which these units are linked among themselves. These

[1] This investigation was supported by the National Cancer Institute, research grant CA-05220-04, Public Health Service, U.S.A.

[2] Part III. M. Piattelli, E. Fattorusso, S. Magno and R. A. Nicolaus, *Tetrahedron* **19**, 2061 (1963).

[3] R. H. Thomson in M. Florkin–H. S. Mason, *Comparative Biochemistry*, Vol. III, Academic Press, New York (1962).

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

[5] Our supposition (*Tetrahedron Letters* No 21, 14 (1959)) that sepiomelanin is a copolymer of 5,6-indolequinone-2-carboxylic acid and 5,6-indolequinone in the ratio of 4:1 becomes implausible in the light of these results.

conclusions have been confirmed by the ESR studies lately carried out on melanins.[6] As for the black from *Ustilago maydis* spores, the chemical study of which is being carried out at this laboratory, it has been ascertained that this black is not an indole derivative but is similar to the polymer obtained *in vitro* by enzymic oxidation of catechol.[7]

Much less is known about the other natural black pigments; moreover, specific tests are lacking which would reveal which of them have identical or similar structure. The properties which biologists hold to be characteristic of melanins, e.g. insolubility in ordinary solvents, bleaching by oxidizing agents and ability to reduce ammoniacal silver nitrate, are most likely reactions common to any substance that derives from oxidative polymerization of phenols.

At the same time, spectroscopic techniques do not seem of great value for characterization purposes, since all the pigments examined so far absorb both visible and UV light without revealing any distinguishing features. Even IR spectra show only a few broad bands, which, in our opinion,[8] are useless as a means of characterization.

Since classical methods cannot be applied to these pigments, degradation and identification of the resultant products seems at present to be the only possible way of characterizing them. Naturally, the conclusions which may be drawn from the application of this method, as to similarities and dissimilarities between various blacks, do not pretend to having an absolute value, for the same degradation products could also be had from melanins which were not perfectly identical. In spite of these limitations, we felt it would be useful to apply this method to the study of a number of natural blacks in order to determine the distribution of "indole" and "catechol" melanins in living organisms. Furthermore, a research of this type would allow us to single out any black pigments having a different structure from that of the two mentioned above. These could thereafter be investigated more amply.

Up to now we have succeeded in isolating twelve pigments of animal origin and three of plant origin. The insolubility of these substances makes their purification an extremely troublesome matter; the only way is to remove the extraneous material by means of a drastic acid hydrolysis and extraction with solvents.[9]

The pigments thus isolated were examined by chromatographic and electrophoretic analyses of the products which had been obtained by two different degradation methods: permanganate oxidation and alkali fusion.

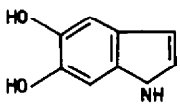
Among the degradation methods which have already been successfully applied to the study of sepiomelanin and ustilago melanin, we chose the above two because of the speed and ease with which they reveal whether a melanin is indolic, catecholic or some other type.

[6] M. S. Blois, A. B. Zahlan and J. E. Maling, *Electron Spin Resonance Studies on Melanin*, to be published.

[7] M. Piattelli, E. Fattorusso, S. Magno and R. A. Nicolaus, *Tetrahedron Letters* No. 15, 997 (1963).

[8] In spite of what maintained by T. G. Bonner, A. Duncan, *Nature, Lond.* **194**, 1078 (1962).

[9] The advantage of such a procedure is that a product obtains which is free from protein material. It is to be observed that, although the action of HCl produces a loss of CO_2 and NH_3, the pigments are not basically changed, as it can be observed from their degradation products. As for the possible formation of melanoidins due to the action of hydrochloric acid which we adopted as a hydrolizing agent, care was always taken to remove the acid frequently, replacing it with fresh acid, thus avoiding the occasional precipitation of melanoidins. In any case, we have ascertained experimentally that melanoidins do not yield any of the typical degradation products of melanins.



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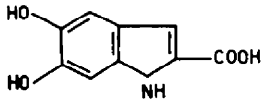

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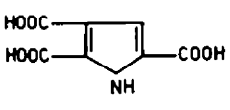

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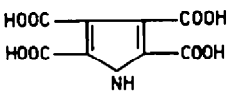

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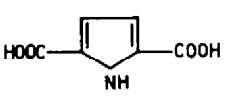

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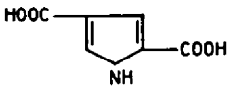

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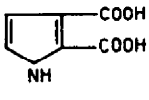

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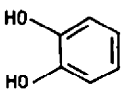

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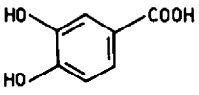

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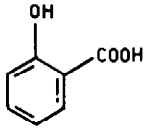


X

The results are shown in Table A. As can be seen all the melanins of animal origin yield the same degradation products; acids V and VI are the only ones which are not always present. This difference may be imputed to a partial decarboxylation of the pigments taking place during the hot acid hydrolysis used in the course of the purification. In fact, these two acids, which are not obtained by oxidation of cold purified sepiomelanin, turn up among the oxidation products of thermically decarboxylated sepiomelanin.

Another fact to be noted from observation of the data in Table A is that all the melanins of animal origin examined so far are of the "indole" type.[14]

As far as the black pigments of plant origin are concerned, due to the small number hitherto investigated no overall conclusions can be drawn. Still, it is to be noticed that two out of the three blacks examined are similar to ustilago melanin; this would lead one to suppose that "catechol" melanin may be fairly widespread in the plant kingdom. This supposition is supported by the fact that pigments of the

[10] Both *Amphiuma* and *Ambystoma* are cold-blooded, tailed amphibians, whose livers contain varying numbers of pigment cells which are believed to contribute fundamentally to their normal metabolism.

[11] Vegetable melanins are so little known that R. H. Thompson (*loc. cit.*) states . . . "it is more difficult to say, with certainty, which plant pigments are melanins since no presumed melanin has yet been isolated and subjected to adequate chemical examination."

[12] The parasite which causes incalculable damage to maize crops.

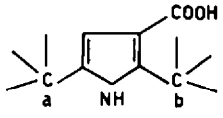
[13] *Capnodium nerii* is an oleander parasite commonly found in low damp places. The leaves of the host become coated with a black crust made up of a dense mycelial stratum of thickly tangled hyphae. Species of the same genus attack economically valuable plants such as grape-vines and olive trees.

[14] Biologically it was foreseeable that "indole" melanins would turn out to be considerably widespread in the animal kingdom.

TABLE A. SOME TYPICAL MELANIN DEGRADATION PRODUCTS

Source of melanin	Products obtained by	
	Alkali fusion	Permanganate oxidation
Rat-melanoma	I, II	III, IV, V,* VI,* VII
Human hair	I, II	III, IV, V, VI, VII
Dog-hair	I, II	III, IV, V, VI, VII
Horse-hair	I, II	III, IV, V, VI, VII
Ox-hair	I, II	III, IV, V, VI, VII
Ox-choroid	I, II	III, IV, VII
Sepia-ink	I, II	III, IV, VII
Squid-ink	I, II	III, IV, VII
Octopus-ink	I, II	III, IV, VII
Chicken-feathers	I, II	III, IV, V, VI, VII
Pigeon-feathers	I, II	III, IV, V, VI, VII
Amphiuma-liver[10]	– –†	III, IV
Axolotl-liver	– –†	III, IV
Sunflower seeds[11]	VIII, IX, X	— — —
Water-melon seeds	VIII, IX, X	— — —
Ustilago maydis-spores[12]	VIII, IX, X	— — —
Capnodium nerii[13]	unidentified products	

* Acids V and VI arise from pyrrole units of the polymer. In Part III of this series we have obtained evidence of the structure A, in which carbon atom *a* may belong or not to an "indole" unit, whereas carbon atom *b* results from the breakdown of the benzenoid part of an "indole" unit.



A

† On account of the small amounts available of these two melanins alkali fusion could not be performed. Nevertheless the presence of III and IV among the permanganate oxidation products lead one to conclude that also melanins from livers of *Amphiuma* and axolotl are of the "indole" type.

same type are to be found in vegetable which are phyletically quite unlike (Ustilaginales, Campanulales and Cucurbitales).

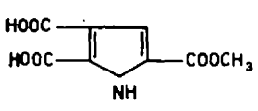
Two of the melanins which we studied, the one from human hair and the one from rat-melanoma, are of greater biological interest and were consequently investigated at greater length. Besides being oxidized with permanganate and fused with alkali, they were subjected to all the chemical operations which had previously been performed on sepiomelanin.

In order to ascertain whether free carboxyl groups were present and estimate their quantity, the two pigments were subjected to thermic decarboxylation at 200°. The amount of carbon dioxide evolved proved equal to both cases (4·1 % by weight of the melanin). This amount is quite less than what was obtained by decarboxylation of sepiomelanin (9·1 % by weight of the melanin), performed under identical experimental conditions.

Permanganate oxidation of the decarboxylated pigments and analysis of the degradation products lead to the identification, in both melanins, of the same compounds already identified among the oxidation products of the pigments which had not been decarboxylated. However, a change was noted in the relative quantities of these acids: oxidation of the decarboxylated pigments yielded a greater quantity of pyrrole-2,3-dicarboxylic acid whereas there was a decrease in the yields of pyrrole-2,3,5-tricarboxylic acid. These results coincide with the behaviour of sepiomelanin. It ought to be mentioned, though, that in the case of sepiomelanin, pyrrole-2,4-dicarboxylic acid and pyrrole-2,5-dicarboxylic acid are to be found among the oxidation products of the pigment only after it has been decarboxylated; not so for the melanins from rat-tumor and human hair.[15]

The melanins from melanoma and human hair were methylated by suspending them in an ethereal solution of diazomethane and the percentage of methoxyl groups was determined (melanin from melanoma, 15%; melanin from human hair, 14%). The test for methylenedioxy groups was positive in both cases. Comparison of these data against what was obtained from sepiomelanin reveals that in sepiomelanin the $-OCH_3$ content is appreciably higher (18%).

The methylated melanins were oxidized with $KMnO_4$ and analysis of the degradation products revealed the presence of 5-carbomethoxypyrrole-2,3-dicarboxylic acid (XI) as in the case of sepiomelanin. The presence of XI leads one to conclude that at least a part of the carboxyl groups must be in position 2 of some indole nuclei.



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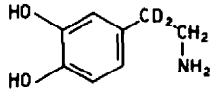

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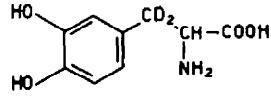

XIII

In addition, trimethylamine and ammonia were found among the oxidation products of the methylated pigments. The former had not been produced by oxidation of methylated sepiomelanin.[16] Since trimethylamine forms during oxidation of compounds having α-amino acid chains previously methylated with diazomethane, it is to be supposed that, different from sepiomelanin, $-CH(NH_2)-COOH$ groups are present in melanins from rat-tumor and human hair.

This would appear to be borne out in Swan's work[17] on the autoxidation of 3,4-dihydroxyphenethylamine (XII) and dopa (XIII) which have been deuterated in positions β. Melanins formed from these precursors retain most of the deuterium, which might signify that open-chained units are present in the polymer.[18]

It should especially be noted the high sulphur content found in melanin from human hair. As we are sure that the drastic purification methods eliminated the proteins, the sulphur probably comes from an amino-acid–cystein, likely enough—

[15] The question whether such a decarboxylation process occurs in the living cells or it is due to the purification procedure must be further investigated.

[16] Part I of this series.

[17] G. A. Swan, *Ann. New York Ac. of Sciences* **100**, 1005 (1963).

[18] This hypothesis had already been made in Part II of this series on the basis of experimental evidence.

linked to the melanin and that originally serves to bind it to the protein as we recently demonstrated in the case of sepiomelanin. However, one notes a great difference between the sulphur content in sepiomelanin (0·35%) compared with that found in human hair (3·7%). This may be on account of a higher content of protein-pigment bonds in the melanin from human hair.

From the results obtained it may be concluded that melanin from melanoma and melanin from human hair are similar in every aspect taken into consideration, whereas there are differences between them and sepiomelanin. Nevertheless, it is clear that both pigments may be classified as "indole" melanins.

EXPERIMENTAL

The systems (prepared on a vol/vol basis):ethanol:33% ammonia:water; 80:4:16 (EAW), n-propanol:33% ammonia:water; 60:30:10 (PAW), n-butanol:acetic acid:water; 60:15:25 (BAW), chloroform:methanol:formic acid:water; 100:10:0·4:9·6 (organic phase; CMFW), 0·005N HCl, were used for paper chromatography on Whatman no. 1 paper by the descending technique.

Thin layer chromatographies were performed on Kieselgel G Merck by using the following solvent systems: chloroform:formic acid; 80:20 (CF), benzene:acetic acid; 50:50 (BA).

Whatman no. 1 paper was used for paper electrophoresis, a potential of about 16 V/cm being applied for 40 min. The electrolytes used were: 0·03M KH_2PO_4, 0·1M formic acid, 0·05M pyridine formate. Papergrams and thin layer chromatograms were sprayed with the following reagents: diazotized sulphanilic acid followed by N NaOH (DZA), 3% $FeCl_3$ in ethanol, ammoniacal $AgNO_3$, 2% phosphomolybdic acid.

Gas-liquid chromatography was performed at 35° with a 100 × 0·6 cm column of Celite (60–80 mesh) coated with triethanolamine (7·5%) and vaseline oil (7·5%). The carrier gas was hydrogen at a flow rate of 8 l. per hr.

Tentative identification of degradation products was always substantiated by co-chromatography and co-electrophoresis with authentic samples.

For elemental analyses, samples of the pigments were dried *in vacuo* over P_2O_5 at 80° for 48 hr. Unless otherwise stated pigments were ash-free. Analyses were carried out by E. Thommen, Dept. Org. Chem., University of Basel, Switzerland.

In the purification of the pigments hydrolysis, extractions and washings were usually carried out until appreciable amounts of soluble products were no longer removed.

Melanin from rat-melanoma

Purification and chemical behaviour. To the homogenate from 110 g of rat melanomas (strain of Istituto Regina Elena per lo Studio e la Cura dei Tumori, Roma, Italy) was added 300 ml conc. HCl; the mixture was kept at room temp for 3 days. After addition of water (800 ml), the precipitate was collected by centrifugation and washed with 1% HCl. The suspension of the crude pigment in conc. HCl (300 ml) was kept at room temp for 13 days, water (1000 ml) was added and the mixture was centrifuged. The black precipitate was first washed with water and successively with ethanol, ether, light petroleum and was eventually dried. In order to obtain complete removal of the proteins, the pigment was treated with boiling 6N HCl for 20 hr, collected by centrifugation and washed with water (10 times), acetone (3 times) and ether (twice), thus obtaining 2·3 g of a black, amorphous powder. (Found: C, 64·5; H, 5·5; N, 7·1%; mean values of four determinations).

The pigment (300 mg) was treated with an excess of ethereal solution of diazomethane (500 mg); after 24 hr the pigment was collected by centrifugation, washed with ether, and again treated with diazomethane. After washing with methanol and ether the pigment was dried for 48 hr over P_2O_5 at 80° *in vacuo*. (Found: C, 64·5; H, 6·0; N, 7·4; OCH_3, 15·0%). The —O—CH_2—O— group test was positive.

Rat-melanoma melanin (278 mg) on thermic decarboxylation, performed as described for sepiomelanin (Part II of this series), yielded 51 mg of $BaCO_3$ equivalent to 11·4 mg CO_2 (4·1% of the weight of melanin used).

Oxidation of melanoma melanin, methylated melanoma melanin and decarboxylated melanoma melanin

The pigment (50 mg) was oxidized with 3% $KMnO_4$ (5.8 ml) and the oxidation mixture worked up as already described in the preceding papers of this series. Paper chromatography (EAW, PAW and BAW as irrigants; spray: DZA), thin layer chromatography (BA and CF as solvents; spray: DZA) and paper electrophoresis (electrolytes: 0·03M KH_2PO_4, 0·1M formic acid; spray: DZA) of the degradation products showed the presence of pyrrolic acids III–VII.

The methylated pigment (150 mg) was oxidized with 3% $KMnO_4$ (14 ml); by paper chromatography (solvent systems: EAW, PAW, BAW; spray: DZA) the ester XI was identified. In order to identify the volatile bases formed by oxidation, a sample of methylated pigment (500 mg) was oxidized with 3% $KMnO_4$ (46 ml) and after removal of MnO_2 the liquid was distilled and collected in 1N HCl. By evaporation of the solution the hydrochlorides of the bases were obtained as a crystalline residue. Gas-liquid chromatography of the free bases gave the result shown in Fig. 1.

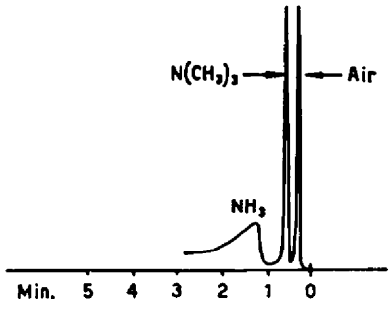

FIG. 1

The decarboxylated melanin was thoroughly washed with light petroleum and finally with ether; it was then dried *in vacuo* to constant weight. 50 mg of this pigment was oxidized with 3% $KMnO_4$ (5·6 ml); paper chromatography (solvent systems: EAW, PAW, BAW; spray: DZA), thin layer chromatography (BA, CF as irrigants; spray: DZA) and paper electrophoresis (electrolytes: 0·03M KH_2PO_4 and formic acid; spray: DZA) of the degradation products showed the presence of pyrrolic acids III–VII.

Alkali fusion of melanoma melanin

A mixture of melanoma melanin (200 mg), NaOH (600 mg), $Na_2S_2O_4$ (100 mg) and a few drops of water were heated at 300° for about 10 min. After cooling, the fused mass was taken up with 10% $Na_2S_2O_4$ (100 ml); the solution was acidified with acetic acid (2 ml), clarified by centrifugation and extracted with peroxide-free ether. The ether fraction, after extraction with a saturated solution of $NaHCO_3$ containing a small amount of $Na_2S_2O_4$ and washing with water, was taken to dryness; the residue was dissolved in water (0·1 ml; fraction 1). The $NaHCO_3$ solution was acidified with conc. HCl and the acid products were recovered by extraction with peroxide-free ether (80 ml in 5 portions); the ether extract was evaporated to dryness and the residue dissolved in water (0·1 ml; fraction 2). Fractions 1 and 2 were analyzed by paper chromatography (solvent systems: 0·005M HCl and BAW) and by paper electrophoresis (electrolyte: 0·03M KH_2PO_4). 5,6-Dihydroxyindole (I) and 5,6-dihydroxyindole-2-carboxylic acid (II) were identified in the fractions 1 and 2 respectively by spraying the papergrams with DZA (I: red spot turning black; II: red spot), $FeCl_3$ (I: green spot; II: blue spot) and $AgNO_3$ (I: black spot; II: black spot).

Melanin from human hair

Purification and chemical behaviour. Black human hair (280 g), previously degreased by continuous extraction with light petroleum (36 hr), were kept in conc. HCl (400 ml) for 20 days at room temp. After adding water (400 ml) and centrifuging, the collected precipitate was washed with 1% HCl (10 times), water (twice) and acetone (twice). The crude pigment was treated with boiling 6N HCl for 100 hr and collected by centrifugation; it was then washed with 1% HCl (10 times), water (10 times), acetone (3 times) and finally with ether (twice). After drying, the pigment weighed 3·5 g (Found: C, 61·1; H, 4·6; N, 8·4; S, 3·7; Cl, 1·2%; mean values of four determinations).

The methylated pigment was prepared by reaction with diazomethane as described above for melanoma melanin (Found: C, 62·2; H, 4·7; N, 8·4; OCH_3, 14·0%); it gave a positive —O—CH_2—O— group test.

Hair melanin (458 mg) upon thermic decarboxylation yielded 84 mg of $BaCO_3$ equivalent to 18·8 mg CO_2 (4·1% of the weight of melanin used).

Hair melanin, methylated hair melanin and decarboxylated hair melanin were oxidized and the degradation products were analyzed in the usual way: the same pyrrolic acids found in the case of melanoma melanin were identified. By gas-liquid chromatography ammonia and trimethylamine were identified in the basic volatile fraction of the oxidation products of methylated hair melanin.

Hair melanin (500 mg) subjected to alkali fusion (experimental conditions employed for melanoma melanin) gave I and II.

Melanin from dog-hair

Purification and chemical behaviour. Black poodle hair (120 g), previously washed with a hot soap solution, were suspended in conc. HCl (400 ml) and left at room temp for 12 days. After centrifugation the precipitate was washed with 1% HCl and suspended in conc. HCl (400 ml). After 12 days the black precipitate was collected, washed with 1% HCl, kept in boiling 6N HCl for 50 hr and centrifuged.

The supernatant was discarded and the melanin put into boiling 6N HCl again for 50 hr and finally washed with 1% HCl (6 times), water (5 times), ethanol (twice) and ether (twice). The melanin, dried over P_2O_5, weighed 5·5 g and was insoluble in any solvent. (Found: C, 61·2; H, 4·0; N, 5·1; Ashes, 1%; S and Cl, traces). Permanganate oxidation (7 ml of 3% $KMnO_4$) of the pigment (50 mg) afforded III and IV and traces of V–VII.

Among the products obtained by alkali fusion of the pigment (500 mg) I and II were identified.

Melanin from horse-hair

Purification and chemical behaviour. 40 g of black horse hair (tail), washed with a lukewarm solution of soap and then twice with hot water, were treated with conc. HCl (400 ml; 15 days at room temp). The crude pigment was refluxed with 6N HCl for 72 hr (after 36 hr the liquid was removed and replaced by fresh azeotropic HCl). The melanin was washed with 1% HCl (6 times), water (8 times), ethanol (twice) and ether (twice) and dried over P_2O_5 (1·2 g). (Found: C, 64·6; H, 5·1; N, 6·0; S, 1·3; Cl, 0·7; Ashes 2%). 50 mg of the purified pigment was oxidized with 3% $KMnO_4$ (5·5 ml); among the oxidation products III and IV and trace amounts of V, VI, VII were identified. Alkali fusion (300 mg of the pigment) produced I and II.

Melanin from ox-hair

Purification and chemical behaviour. 159 g of black ox hair (tail) were washed first with a hot soap solution and then with hot water and eventually with hot ethanol.

After treatment with conc. HCl (22 days at room temp) the black product was kept in boiling 6N HCl for 70 hr; after 25 and 50 hr the supernatants were removed and replaced with fresh 6N HCl. The melanin, collected by centrifugation, was washed with 1% HCl (9 times), water (8 times), ethanol (twice) and ether (twice). After drying over P_2O_5, the melanin resulted as a brown amorphous powder (1·0 g). (Found: C, 65·5; H, 5·5; N, 5·4; S, 1·6; Cl, 1·4; Ashes, 0·4%). 50 mg of the pigment oxidized with a 3% solution of $KMnO_4$ (4·8 ml) yielded III and IV and traces of V, VI. VII. Alkali fusion of melanin (400 mg) yielded I and II.

Melanin from ox-choroid

Purification and chemical behaviour. The black pigment mechanically removed from 12 ox eyes was left in conc. HCl (150 ml) at room temp for 28 days; every 7 days the liquid was discarded and fresh conc. HCl added. The pigment was finally washed with 1% HCl (5 times), water (5 times), ethanol (twice) and ether (twice) thus obtaining, after drying over P_2O_5, 500 mg of melanin. (Found: C, 60·4; H, 4·6; N, 8·7; S, 0·9; Cl, 1·6; Ashes, 3·0%). The pigment (50 mg) upon treatment with a 3% $KMnO_4$ solution (6·3 ml) yielded III, IV, VII. 200 mg of the pigment, when fused with alkali, yielded I and II identified in the usual way.

Melanin from squid (Loligo vulgaris) *ink*

Purification and chemical behaviour. To the thick black liquid from the ink sacs of 5 medium-sized squids was added 20% HCl (100 ml) and the mixture was centrifuged. The precipitate was suspended in conc. HCl (200 ml) and left at room temp. for 15 days, centrifuged, washed with 1% HCl (10 times), water (14 times), ethanol (twice) and ether (twice). After drying over P_2O_5 *in vacuo*, the amorphous powder weighed 1·9 g (Found: C, 60·9; H, 3·1; N, 6·0%). Permanganate oxidation (6·6 ml of 3% $KMnO_4$) of the purified pigment (50 mg) yielded III, IV and VII; alkali fusion of the melanin (200 mg) yielded I and II.

Melanin from octopus (Octopus vulgaris) *ink*

Purification and chemical behaviour. Two ink sacs taken from medium-sized octopus were cut open and emptied. To the black liquid 50 ml of 20% HCl was added. The precipitate collected by centrifugation was kept in conc. HCl (90 ml) at room temp for 15 days, centrifuged, washed with 1% HCl (10 times), water (12 times), ethanol (twice) and ether (twice). (Found: C, 59·0; H, 3·3; N, 8·0%). The pigment (50 mg) was oxidized (3% $KMnO_4$; 7 ml) and the degradation products analysed as usual: compounds III, IV, VII were detected. Alkali fusion of octopus melanin (200 mg) yielded I and II.

Melanin from chicken-feathers

Purification and chemical behaviour. 50 mg of feather barbs previously washed with a lukewarm solution of soap and then with hot water were air-dried and kept at room temp in conc. HCl for 20 days. The black product obtained after centrifugation was further purified by treatment with boiling 6N HCl for 48 hr. After centrifugation and washing with 1% HCl the melanin was again left in boiling 6N HCl for 24 hr. The suspension was centrifuged and the precipitate washed with 1% HCl (5 times), water (8 times), ethanol (twice) and ether (twice). After drying over P_2O_5 the pigment weighed 1·6 g. (Found: C, 62·9; H, 4·5; N, 6·8; S, 1·2; Cl, 0·7%). The pigment (50 mg) was oxidized with 3% $KMnO_4$ (5·8 ml) yielding III–VII. Alkali fusion of 200 mg of this melanin yielded I and II.

Melanin from pigeon-feathers

Purification and chemical behaviour. 50 g of pigeon feather barbs, previously washed with a lukewarm solution of soap and with hot water, were air-dried and treated in the same way as described above for chicken-feathers. 1·2 g of a black amorphous powder was obtained thereby. (Found: C, 60·5; H, 5·7; S, 3·2; Cl, 1·2; Ashes, 4·6%). $KMnO_4$ oxidation and alkali fusion of this pigment gave the same products as were obtained in the case of chicken-feather melanin.

Melanin from Amphiuma-*liver*

Purification and chemical behaviour. Amphiuma livers were extracted with chloroform and the dry material (1·5 g) was homogenated: to the homogenate conc. HCl (100 ml) was added and the mixture was left at room temp for 9 days. The black precipitate obtained after centrifugation was washed with 1% HCl and treated with boiling 6N HCl for 36 hr. After washing with 1% HCl (5 times), water (7 times), ethanol (twice) and ether (twice), the dry melanin weighed 45 mg. (Found: C, 60·2; H, 4·0; N, 7·6; Ashes, 3%). 18 mg of this pigment was oxidized with 3% $KMnO_4$ (1·8 ml). Owing to the small quantity of melanin only two paper chromatographies (solvents: EAW, PAW; spray:DZA) were made, thus identifying III and IV.

Melanin from Ambystoma-*liver*

Purification and chemical behaviour. 750 mg of dry *Ambystoma* livers treated as above gave 20 mg of melanin. On account of the scarcity of material only $KMnO_4$ oxidation was performed: III and IV were identified.

Melanin from sunflower (Helianthus annuus) *seeds*

Purification and chemical behaviour. Sunflower seeds (500 g) were stirred vigorously with water (2 l.) for 3 days. Every 6 hr the reddish-brown suspension was removed and to the seeds fresh water

was added. The combined suspensions were centrifuged and the reddish-brown material was collected and air-dried. The product was continuously extracted with hot ethanol for 100 hr; the residue was kept in conc. HCl (800 ml) for 20 days at room temp, then treated with boiling 6N HCl for 30 hr and successively with hot ethanol for 40 hr. The black product was left in ethanolic HCl for 3 days and after centrifugation was suspended in 6N HCl and heated under reflux for 24 hr. The melanin obtained after centrifugation was washed with 1% HCl (8 times), water (8 times), acetone (twice) and dried over P_2O_5. Yield 5·5 g. (Found: C, 65·0; H, 4·1; N, 0·3; OCH_3, 0·8%). No pyrrolic acids were found by $KMnO_4$ oxidation of this melanin.

To KOH (750 mg) and NaOH (750 mg), fused in a platinum crucible, 500 mg of the melanin was slowly added. The mixture was kept at about 200° for 10 min; after cooling, the fused mass was dissolved in 10% $Na_2S_2O_4$ (130 ml) and the solution acidified with acetic acid (4 ml). After centrifugation the supernatant was extracted with peroxide-free ether (250 ml in five portions); the combined ether extracts were extracted with a saturated solution of $NaHCO_3$ (20 ml in two portions) and then with a little water. The ether layer was taken to dryness and the residue dissolved in 0·5 ml of water (fraction 1). The $NaHCO_3$ solutions were combined, acidified to Congo red with conc. HCl and extracted with ether (200 ml in four portions). The ether extracts were evaporated to dryness and the residue taken up with water (0·5 ml; fraction 2). By paper chromatography (irrigant: 0·005M HCl; spray reagents: DZA, $AgNO_3$, $FeCl_3$, phosphomolybdic acid) and paper electrophoresis (electrolyte: 0·05M pyridine formate) catechol was identified in the fraction 1.

IX and X were found in the fraction 2 by means of paper chromatography (EAW, CMFW and 0·005M HCl as irrigants; spray reagents: DZA, $FeCl_3$, $AgNO_3$ and phosphomolybdic acid) and paper electrophoresis (electrolyte: 0·05M pyridine formate).

Melanin from water-melon (Citrullus vulgaris) *seeds*

Purification and chemical behaviour. 1 kg of water-melon seeds and 3 l. of water were vigorously stirred for 3 days. Every 6 hr the suspension was removed and fresh water added. Further operations followed the same method adopted for sunflower seed melanin, except for the treatment with HCl in ethanol solution, which was not necessary. The dry melanin weighed 10 g. (Found: C, 63·5; H, 4·1; N, 0·7%). It gave no pyrrolic acids by $KMnO_4$ oxidation; VIII–X were found as products of alkali fusion, performed in the same way as for sunflower seed melanin.

Melanin from Capnodium nerii

Purification and chemical behaviour. 20 g of *Capnodium nerii* mycelium obtained by scraping oleander leaves was continuously extracted with hot light petroleum for 36 hr and then with hot ethanol for 40 hr. The air-dried black was left in conc. HCl (400 ml) for 10 days at room temp and then in boiling 6N HCl for 60 hr. The material was washed with water (4 times), dried, continuously extracted with boiling ethanol for 60 hr and finally treated with boiling 6N HCl for 72 hr. The black residue was washed with 1% HCl (3 times), water (6 times), ethanol (twice) ether (twice) and was dried over P_2O_5 for 48 hr, yield 2·0 g. (Found on an ash-free basis: C, 80·0; H, 6·6; Cl, 0·8; OCH_3, 0·8%). No pyrrolic acids were found among the $KMnO_4$ degradation products. The pigment subjected to alkali fusion (performed as described for sunflower seed melanin) yielded products, among which VIII–X were absent.

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