Melanin and its Precursors

8. THE OXIDATION OF METHYLATED 5:6-DIHYDROXYINDOLES*

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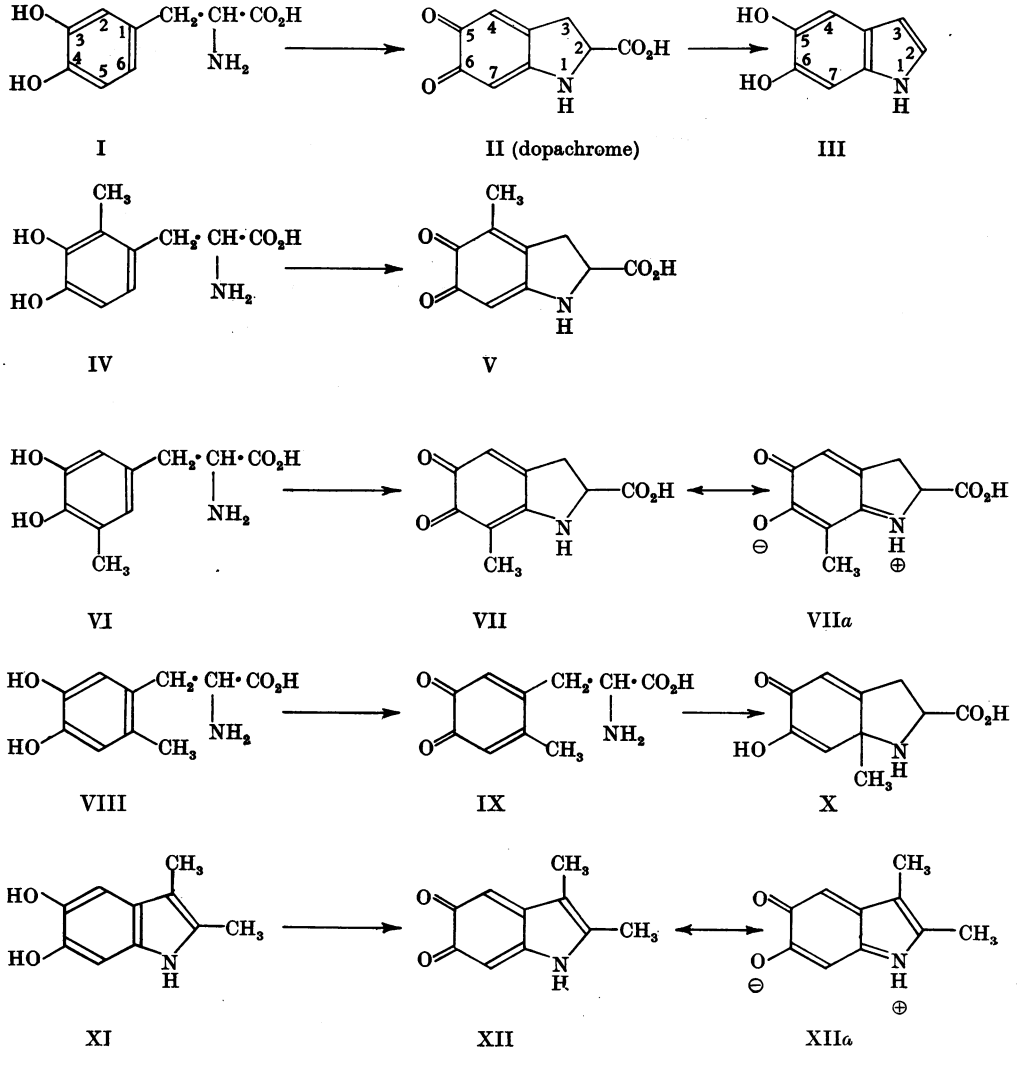
The conversion of tyrosine into melanin by oxygen in the presence of tyrosinase has long been known to proceed by way of 3:4-dihydroxyphenylalanine (dopa) (I) (Raper, 1926) and 5:6-dihydroxyindole (III) (Raper, 1927), which is the last intermediate that has been definitely established in this series of reactions. 5:6-Dihydroxyindole was synthesized by Beer, Clarke, Khorana & Robertson (1948), and shown to undergo rapid autoxidation to melanin in aqueous solution. In part 2 of this series (Bu'Lock & Harley-Mason, 1951) it was suggested that melanin might be derived from the corresponding *o*-quinone,

* Part 7: Cromartie & Harley-Mason (1953*b*).



indole-5:6-quinone, by repeated condensation between the anionoid centres in the pyrrole ring of one molecule and the cationoid centres in the quinonoid rings of adjacent molecules, giving rise to a polymeric material of high molecular weight. In order to test this theory and to assess the participation of the various centres in the 5:6-dihydroxyindole molecule in its oxidative polymerization, a series of homologues have been prepared in which different positions are blocked by methyl groups, and their oxidation by molecular oxygen in aqueous solution has been followed spectroscopically under identical conditions, as has already been briefly reported (Cromartie & Harley-Mason, 1953*a*). The various current theories of the formation and constitution of melanin have recently been discussed in a comprehensive review by Mason (1955).

EXPERIMENTAL

Materials

Silver oxide was prepared by the method of Helferich & Klein (1926). The buffer solutions were prepared from 0·2M-Na_2HPO_4 and 0·1M-citric acid (McIlvain, 1921). Tyrosinase was obtained by extracting an acetone-dried powder from *Psalliota campestris* with water as required and filtering.

Dihydroxyindoles. All the dihydroxyindoles were freshly resublimed in a high vacuum. They were prepared by methods described in the literature as follows: 5:6-dihydroxyindole and 5:6-dihydroxy-2-methyl- and -2:3-dimethylindole (Harley-Mason, 1953); 5:6-dihydroxy-1-methylindole (Harley-Mason, 1950); 5:6-dihydroxy-4-methyl-, -7-methyl- and -4:7-dimethyl-indoles (Cromartie & Harley-Mason, 1953*b*); 5:6-dihydroxy-3-methylindole (Beer, McGrath, Robertson & Woodier, 1949).

Dihydroxyphenylalanines. 3:4-Dihydroxy-2-methyl- and -5-methyl-phenylalanine were prepared by the method of Cromartie & Harley-Mason (1952), and 3:4-dihydroxy-6-methylphenylalanine by that of Cromartie & Harley-Mason (1953*b*).

4:5-*Dimethyl*-o-*benzoquinone.* This was prepared by the method of Bu'Lock & Harley-Mason (1951). Light-absorption maxima in water: 2610 and 4120Å (ϵ 3300 and 1000 respectively).

Oxidation of dihydroxyphenylalanines

With silver oxide. 3:4-Dihydroxy-2-methylphenylalanine, 3:4-dihydroxy-5-methylphenylalanine and 3:4-dihydroxy-6-methylphenylalanine were oxidized with Ag_2O by the method of Mason (1948). Solutions (10 ml.; 0·137 mM) of the amino acids in a buffer solution of pH 5·6 were shaken with Ag_2O (0·05 g.) for various periods of time, and filtered through Whatman no. 42 filter paper. The absorption spectrum was then measured for the region 2400–6000Å. The spectra of the original solutions were also measured. The position and extinction coefficients of the maxima for the original solution and after the optimum time of shaking are given in Table 1 (see also Fig. 4).

With tyrosinase. Pairs of tubes containing 3:4-dihydroxyphenylalanine and its 2-methyl, 5-methyl, and 6-methyl derivatives dissolved in a buffer solution of pH 6·85 (0·01 g. of amino acid in 12·5 ml.) were set up side by side. Mushroom tyrosinase (0·1 ml.) was added to one tube of each pair and a stream of oxygen was bubbled through all 8 tubes simultaneously. Observations of the visible changes

Table 1. *Spectra of methylated dopas and of the products of their oxidation with silver oxide in aqueous buffer* (pH 5·6)

The results for dopa itself are quoted from Mason (1948) for comparison

Position of methyl substituents	Original spectrum		Spectrum of oxidized solution					Time of shaking (min.)
	$\lambda_{max.}$ (Å)	$\epsilon_{max.}$	$\lambda_{max.}$ (Å)	$\epsilon_{max.}$	$\lambda_{max.}$ (Å)	$\epsilon_{max.}$	Colour	
2	—	—	3070	11 200	4850	3400	Red	45
5	2780	2200	3070	7100	5100	4200	Violet	45
6	2850	3400	3170	5500	4500	1000	Yellow	90
None	2800	2700	3050	9300	4750	3500	Red	10

Table 2. *Autoxidation of dopa and its methyl derivatives in aqueous buffer* (pH 6·85)

Addition of tyrosinase made no difference in the first two cases and only a slight difference in the third case.

Position of methyl substituents	Coloration	Time of maximum colour (hr.)	Final appearance
2	Red	2·5	Fine black ppt.
5	Violet	2·5	Fine black ppt.
6	Yellow	12	Yellow; no ppt.
None (with enzyme)	Red	0·25	Flocculent black ppt.
None (without enzyme)	None	None	Slight black ppt.

that occurred are recorded in Table 2. The presence of the enzyme made no noticeable difference in the cases of the 2-methyl and 5-methyl compounds.

Oxidation of dihydroxyindoles

Autoxidation. The freshly resublimed dihydroxyindole (15 μmoles) was brought into solution as rapidly as possible in a buffer (100 ml.) of pH 6·85. A stream of oxygen was then passed through the solution from a capillary tube. Portions were withdrawn at intervals, the first before starting the oxygen stream; their optical densities at a series of wavelengths from 2400 to 3500 Å were rapidly measured with a Unicam spectrophotometer. The results of some typical cases are shown graphically (Figs. 1–3), and further observations of each individual case are recorded in Table 3. The precipitate, if any, was filtered off and examined. In a separate series of experiments it was shown that adding mushroom tyrosinase had no effect beyond shortening the induction period in the cases when it was longest, i.e. in those of 5:6-dihydroxyindole and its 1-methyl derivative.

Oxidation with silver oxide. A solution of 5:6-dihydroxy-2:3-dimethylindole (0·025 g.) in methanol (2 ml.) was shaken with Ag_2O (0·15 g.) for 30 min. and filtered. The resulting red solution was stable for some hours; but removal of the solvent at room temperature caused decomposition, and no solid separated on cooling in an acetone–CO_2 bath. The absorption spectrum was superposable on that of the autoxidation product (Fig. 3). The addition of a trace of HCl caused a slow irreversible change of colour to blue (absorption maxima at 3050 and 6080 Å with inflexions at 2600, 3400 and 4000 Å). Hydrogenation of the red solution over palladium–charcoal rapidly decolorized it, and 5:6-dihydroxy-2:3-dimethylindole could then be recovered after removing the solvent.

RESULTS AND DISCUSSION

Oxidation of methylated dopas

The aerial oxidation of aqueous solutions of 2-methyl-dopa (IV) and 5-methyl-dopa (VI) was not accelerated by the presence of tyrosinase, but some

Table 3. *Autoxidation of methylated 5:6-dihydroxyindoles in aqueous buffer* (pH 6·85)

The dihydroxyindole (15 μmoles) was dissolved in 100 ml. of buffer (pH 6·85) and oxygen was bubbled through the solution.

Position of methyl substituents	Times of appearance (min.)			Appearance of ppt.	Solubility of ppt.
	Colour	Opalescence	Precipitate		
None	20	30	90	Black, flocculent	Insol. in pyridine
1	None	90	240	Black, fine	Dispersed in hot pyridine
2	10	30	360	Black, fine	Insol. in pyridine
3	5	None	30	Blue, flocculent	Sol. in ethanol and alkali
2:3	10	None	None	None	None
4	15	40	360	Black, fine	Dispersed in pyridine
7	10	20	180	Black, fine	Readily dispersed in pyridine
4:7	3	None	25	Purple, fine	Extracted by ethyl acetate

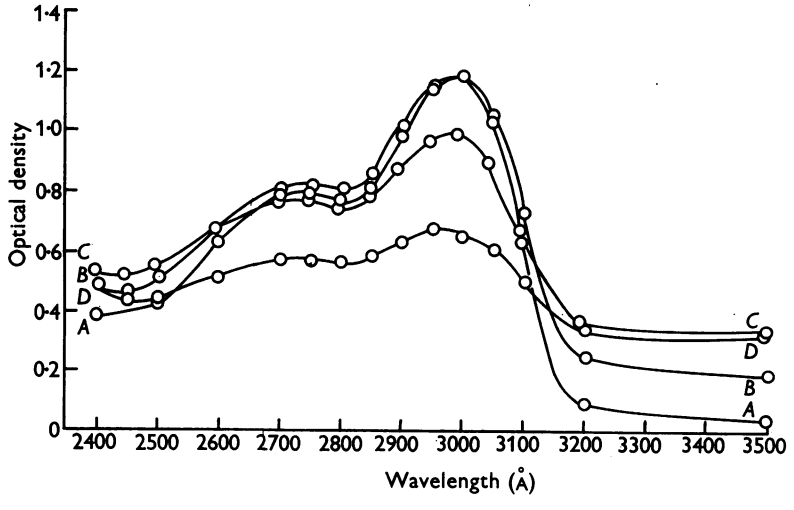


Fig. 1. Spectrum of 0·15 mM-5:6-dihydroxyindole in buffer (pH 6·85): *A*, before oxidation; *B*, *C*, *D*, after passing O_2 for 23, 52, and 100 min. respectively.

acceleration was observed in the case of 6-methyl-dopa (VII) (Table 2). Since tyrosinase acts by combination of the copper atom of the prosthetic group with the *o*-dihydroxy grouping of the substrate (Mason, 1956), its inactivity in the first two cases may be ascribed to steric inhibition of the approach of the enzyme molecule by the adjacent methyl group. These observations are in harmony with those of Schmalfuss & Peschke (1929), who showed that insect tyrosinase could not convert 2-methyl-, 3-methyl- or 2:5-dimethyl-tyrosines into melanin. Similarly, Roth, Miller & Dawson (1944) found that 3-methylcatechol was much more slowly oxidized by tyrosinase than was catechol or 4-methylcatechol.

The similarity between the spectra of the solutions of 2-methyl- and 5-methyl-dopa after oxidation with silver oxide and those obtained by Mason (1948) with dopa showed that cyclization to methyldopachromes (V and VII) had taken place (Table 1). The bathochromic shift of the visible maximum with 7-methyldopachrome (VII) as compared with dopachrome is due to the attachment of the methyl group directly on the chromophore of the zwitterionic form (VII*a*). On the other hand, the oxidation of 6-methyl-dopa (VIII) with

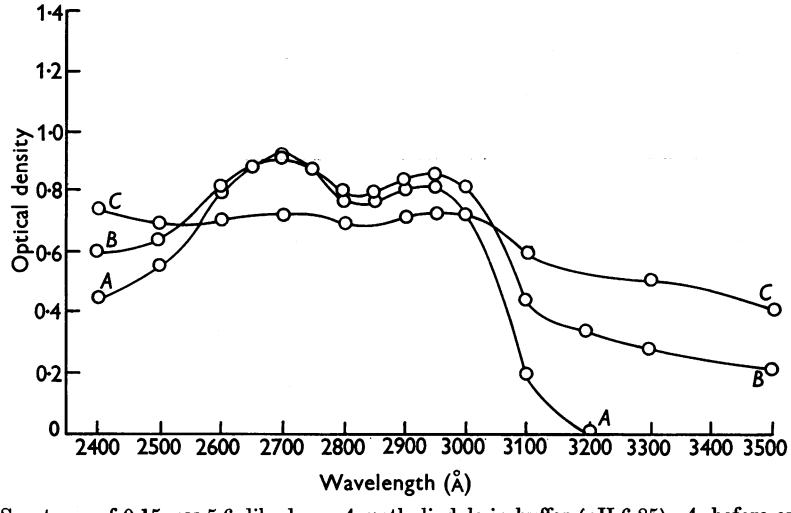


Fig. 2. Spectrum of 0·15 mM-5:6-dihydroxy-4-methylindole in buffer (pH 6·85): *A*, before oxidation; *B*, *C* after passing O_2 for 12 and 40 min. respectively.

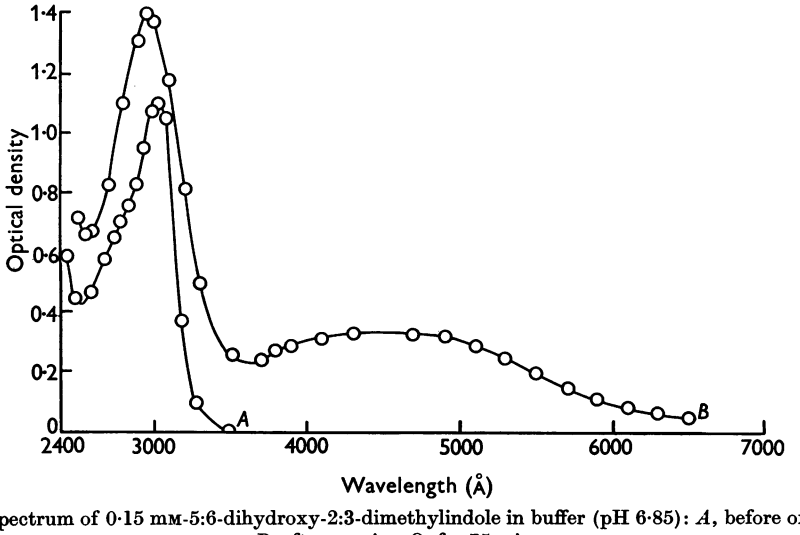


Fig. 3. Spectrum of 0·15 mM-5:6-dihydroxy-2:3-dimethylindole in buffer (pH 6·85): *A*, before oxidation; *B*, after passing O_2 for 75 min.

silver oxide or with tyrosinase furnished a stable yellow solution with a quite different spectrum (see Fig. 4). Since, however, the spectrum bears little resemblance to that of 4:5-dimethyl-*o*-benzoquinone (absorption maxima at 2610 and 4120 Å in water) it can hardly be due to the monocyclic *o*-quinone (IX). It is possible that cyclization to (X) took place; but attempts to isolate any solid product or bring about a rearrangement to an indole were unsuccessful.

2:3-*Dimethylindole*-5:6-*quinone*

Indole-5:6-quinone, which was first proposed as a transient intermediate in melanogenesis by Raper (1938), has never been isolated on account of its instability; but it was hoped that its 2:3-dimethyl derivative would be more stable. Although it could not be isolated in the solid state, the red solution obtained by oxidizing 5:6-dihydroxy-2:3-dimethylindole (XI) with silver oxide was stable for some hours and was considered to contain the quinone (XII). It had a well-defined absorption spectrum with maxima at 2970 and 4700 Å, identical with that obtained by aerial oxidation in aqueous solution (Fig. 3); dopachrome, which has a very similar chromophore, shows maxima at 3050 and 4750 Å. The oxidation of (XI) with potassium ferricyanide in aqueous solution, which produces a similar red colour, has been shown to be reversible (below pH 5) by electrometric titration (N. S. Hush & J. L. Morgan, personal communication). The normal redox potential, measured by Hush & Morgan by the method of discontinuous titration, was +0·562v; this value lies between those of dopaquinone, +0·800v (Friedheim, 1933) and dopachrome +0·443v (Ball & Chen, 1933). Evidently the resonance between the *o*-quinonoid form (XII) and the zwitterionic form (XII*a*), which is responsible for the resemblance of its absorption spectrum to that of dopachrome, more than counterbalances the loss of the aromatic resonance of the pyrrole ring.

The yellow compound obtained by Beer, Broadhurst & Robertson (1954) by oxidation of (XI) with silver oxide in acetone was probably a dimer of (XII), formed by a self-condensation of the Diels–Alder type, since oxidation of catechol with silver oxide *in acetone* yields an analogous dimer of *o*-benzoquinone (J. Harley-Mason & A. H. Laird, unpublished results; cf. Horner & Sturm, 1955). This type of Diels–Alder dimer is not obtained in aqueous solution.

Oxidation of methylated 5:6-*dihydroxyindoles*

In order to appraise the contribution of the various free centres of 5:6-dihydroxyindole to the formation of melanin it was useful to compare the behaviour of this compound and its various methyl and dimethyl derivatives under carefully controlled, identical conditions. To this end freshly sublimed dihydroxyindoles were used, and no tyrosinase was added, since preliminary experiments had shown that its effect on the rate and on visible phenomena of the reaction was negligible. The course of the early phases of the autoxidation in which evanescent intermediates play a predominant role is much

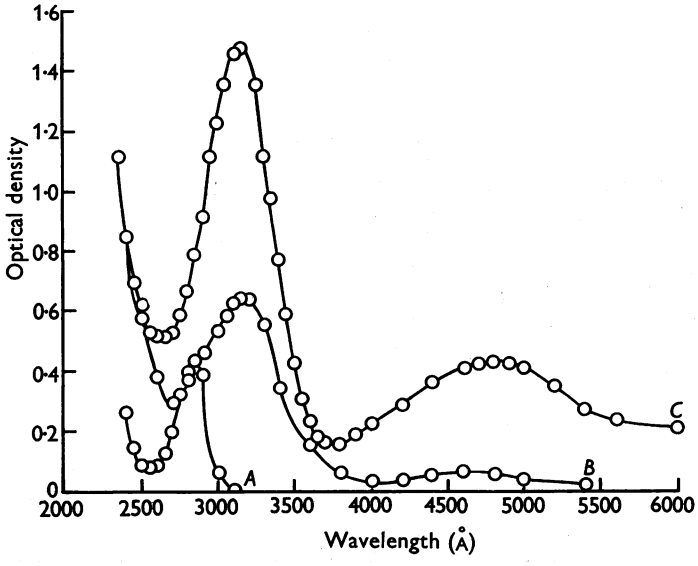


Fig. 4. Spectra of 0·13 mM-solutions of: *A*, 3:4-dihydroxy-6-methylphenylalanine; *B*, the same after oxidation with Ag_2O; *C*, 3:4-dihydroxy-2-methylphenylalanine after oxidation with Ag_2O.

influenced by the conditions of experiment, especially the pH. Reproducible results were obtained under the conditions selected, which possessed the advantage that the pH of 6·85 approximates to that commonly prevailing in living cells. Under these conditions no well-defined maxima were observed in the visible spectrum, except with 5:6-dihydroxy-2:3-dimethylindole, which gave a stable red solution; but the series of absorption curves for the region 2400–3500 Å in the ultraviolet in conjunction with observation of the visible phenomena furnished a semiquantitative picture of the progress of the reaction in each case.

The onset of the reaction was always preceded by a marked induction period, as observed respirometrically by Beer *et al.* (1954), during which the spectrum remained unchanged. In the subsequent reaction two phases could, in general, be distinguished: in phase I the solution became highly coloured; in phase II a precipitate was deposited, which was in some cases black and melanin-like, but in others coloured and distinguishable from melanin. Only with 5:6-dihydroxy-2:3-dimethylindole(XI), from which no precipitate was formed, could phase I be observed in isolation: the orange–red coloration was accompanied by the replacement of the original spectrum by that having maxima at 2970 and 4700 Å (Fig. 3) attributed to the corresponding quinone (XII) (see above). The transient increase in the absorption in the vicinity of 2970 Å that accompanied the colorations of phase I in the case of the 4-methyl (Fig. 2) and 2-methyl compounds can also be attributed to accumulation of the corresponding quinones. With 5:6-dihydroxyindole itself and its 7-methyl derivative phase II evidently supervenes too rapidly for this phenomenon to be observable; but it seems likely that an autoxidation to the corresponding quinone is the essential phenomenon of phase I in these cases also.

The precipitates formed in phase II fell into two quite distinct classes. Those formed from 5:6-dihydroxyindole and its 1-methyl, 2-methyl, 4-methyl, and 7-methyl derivatives were black; they were insoluble in the common organic solvents except pyridine, in which some were very slightly soluble to give brownish solutions; they were always preceded by a characteristic opalescence, which appeared as the colours of phase I faded. On the other hand, the precipitates formed from the 3-methyl and 4:7-dimethyl compounds were highly coloured; they were readily soluble in ethanol to give solutions with well-defined absorption spectra with long-wave maxima near 6000 Å; they were deposited from solutions of the same colour without preliminary opalescence, and they appeared far more rapidly than the melanins. From these differences it was concluded that the black pigments were all polymers of high molecular weight, whereas the coloured ones were oligomeric in nature. The precipitates formed from the 2-methyl, 4-methyl and 7-methyl compounds were more slowly deposited, less flocculent in appearance, and more readily redispersed to a colloidal solution by washing with water than that from the unsubstituted 5:6-dihydroxyindole; this suggests that they were of lower molecular weight on account of inhibition of the normal cross-linking by the methyl groups. This was particularly noticeable in the pigment obtained from 5:6-dihydroxy-7-methylindole, so that the discrepancy between the formation of a melanin from this compound and the reported failure of its 7-*n*-propyl analogue to yield a melanin at pH 8 (Beer *et al.* 1954) is not very significant.

From these results it can be concluded that the 3-position is essential to the formation of a true melanin, and that either the 4-position or the 7-position must be free in addition; but that the 2-, 3-, 4-, and 7-positions all play a part in the building up of a three-dimensional polymer of high molecular weight.

The pattern of melanogenesis

The theory, based on steric considerations, that the main chains of the melanin macromolecule are composed of 3:7- rather than 3:4-linkages has recently received confirmation from tracer studies by Swan & Wright (1956). These authors showed that the hydrogen peroxide formed as a by-product in the autoxidation of 5:6-dihydroxyindole to melanin subsequently attacked the melanin, degrading some of it to carbon dioxide, and that almost all of this carbon dioxide arose from the breakdown of the benzene ring rather than the pyrrole ring. They were, however, able to show that the carbon atom in the 7-position of the original indole nucleus contributed very little to the carbon dioxide formed, which suggests that it is protected while the remaining carbon atoms of the benzene ring are lost. It would be of great interest to convert the various labelled melanins into the melanic acids of Panizzi & Nicolaus (1952) by more drastic treatment with hydrogen peroxide.

Although the occurrence of an induction period and the formation of hydrogen peroxide (Beer *et al.* 1954) show that the preliminary oxidation of 5:6-dihydroxyindole proceeds, like the autoxidation of other phenols, by a free-radical mechanism, the model experiments of Bu'Lock & Harley-Mason (1951) make it likely that the actual coupling steps proceed by an ionic mechanism. Since the completion of phase I for 5:6-dihydroxy-2:3-dimethylindole requires a much longer period than elapses in the other compounds before the onset of phase II, it is evident that polymerization must start while there remains a considerable concentration of the

unchanged 5:6-dihydroxyindole in the solution. This last could act as well as, or probably better than, indole-5:6-quinone as the anionoid component in the coupling reaction; and the intermediate semiquinone could act similarly, leading to an incorporation into the macromolecule of the free-radical centres observed by Commoner, Townsend & Pake (1954), using the method of paramagnetic resonance. These could also arise during the reoxidation that must follow each coupling step, which probably also involves semi-quinonoid intermediates as suggested by Beer *et al.* (1954).

The experiments of Clemo, Duxbury & Swan (1952) with tyrosine and dopa labelled in the carboxyl group have shown that about one-sixth of the carboxyl groups of tyrosine or dopa are incorporated into the melanin formed from them by enzymic oxidation. It had previously been observed by Raper (1927) that rearrangement of dopachrome in neutral solution yielded not only 5:6-dihydroxyindole but also 5:6-dihydroxyindole-2-carboxylic acid. Unlike 5:6-dihydroxy-2-methylindole this acid is not itself converted into a melanin by autoxidation (Beer *et al.* 1949), probably because the nucleophilic reactivity of the 3-position is reduced by the adjacent carboxyl group. The corresponding quinone should, however, be capable of reacting at the 4- or 7-position with the reactive 3-positions of other indolic compounds present to yield a copolymer in which the carboxyl groups would be situated at the ends of chains. Such end-groups could well be the origin of the pyrrole-2:3:5-tricarboxylic acid obtained by Nicolaus and his co-workers (Panizzi & Nicolaus, 1952; Nicolaus, 1953, 1955) by oxidative degradation of natural melanins and of tyrosine–melanin prepared *in vitro*. The higher yields of this acid obtained from natural melanins probably indicate that such end-groups occur more frequently in natural melanins, which are commonly conjugated with proteins to complex melanoproteins. This result is in accord with the suggestion made arlier on the basis of model experiments with peptides (Bu'Lock & Harley-Mason, 1951), that such conjugation might take place by oxidative cyclization of *N*-terminal tyrosine residues in proteins and incorporation of the resulting dihydroxyindole residues into melanin while they remained attached through the 2-position to the protein. Melanoproteins could also arise by the reaction of free thiol and amino groups in proteins with the quinonoid nucleus in melanin itself or with any of its quinonoid precursors (cf. Mason, 1955).

More recently, Nicolaus & Mangoni (1955) have detected pyrrole-2:3-dicarboxylic acid by paper chromatography among the products of the oxidative degradation of tyrosine–melanin and of various natural melanins. Since this acid, too, was formed in very low yields it may well be derived from indole units in which the 2- and 3-positions are unsubstituted. As Nicolaus & Mangoni suggest, these indole units may be linked to others by both the 4- and 7-positions, but the weight of other evidence makes it unlikely that this is the fundamental repeating unit in tyrosine–melanin. The absence of pyrrole-2:3:4-tricarboxylic acid from among the products of oxidative degradation is not altogether surprising, since the pyrrole ring in an indole-5:6-quinone is no longer strictly aromatic, and might be expected to break up on oxidation into small fragments such as oxalic acid and oxamic acid, the main products actually isolated by Panizzi & Nicolaus (1952).

In conclusion we would emphasize that the type of macromolecular structure proposed for tyrosine–melanin permits of considerable variation, and that the end-groups, side chains and cross-linkages present in any particular sample are certainly dependent on the environment in which it was formed.

SUMMARY

1. The 2-, 5- and 6-methyl derivatives of 3:4-dihydroxyphenylalanine have been subjected to aerial oxidation; only the first two yield melanins. Only the last is a substrate for tyrosinase.

2. A stable red solution prepared by oxidation of 5:6-dihydroxy-2:3-dimethylindole with silver oxide in methanol is considered to contain the corresponding *o*-quinone.

3. The oxidation by molecular oxygen of 5:6-dihydroxyindole and of seven of its derivatives containing methyl groups in various positions has been studied under carefully controlled conditions.

4. The results obtained support the general pattern of melanogenesis proposed by Bu'Lock & Harley-Mason (1951). Other current theories are also discussed.

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REFERENCES

Ball, E. G. & Chen, T.-T. (1933). *J. biol. Chem.* **102**, 691.

Beer, R. J. S., Broadhurst, T. & Robertson, A. (1954). *J. chem. Soc.* p. 1947.

Beer, R. J. S., Clarke, K., Khorana, H. G. & Robertson, A. (1948). *J. chem. Soc.* p. 2223.

Beer, R. J. S., McGrath, L., Robertson, A. & Woodier, A. B. (1949). *J. chem. Soc.* p. 2061.

Bu'Lock, J. D. & Harley-Mason, J. (1951). *J. chem. Soc.* p. 703.

Clemo, G. R., Duxbury, F. K. & Swan, G. A. (1952). *J. chem. Soc.* p. 3464.
Commoner, B., Townsend, J. & Pake, G. E. (1954). *Nature, Lond.*, **174**, 689.
Cromartie, R. I. T. & Harley-Mason, J. (1952). *J. chem. Soc.* p. 1052.
Cromartie, R. I. T. & Harley-Mason, J. (1953*a*). *Chem. & Ind.* p. 972.
Cromartie, R. I. T. & Harley-Mason, J. (1953*b*). *J. chem. Soc.* p. 3525.
Friedheim, K. (1933). *Naturwissenschaften*, **21**, 177.
Harley-Mason, J. (1950). *J. chem. Soc.* p. 1276.
Harley-Mason, J. (1953). *J. chem. Soc.* p. 200.
Helferich, B. & Klein, W. (1926). *Liebigs Ann.* **450**, 219.
Horner, L. & Sturm, K. (1955). *Liebigs Ann.* **597**, 1.
McIlvain, T. C. (1921). *J. biol. Chem.* **49**, 183.
Mason, H. S. (1948). *J. biol. Chem.* **172**, 83.
Mason, H. S. (1955). *Advanc. Enzymol.* **16**, 105.
Mason, H. S. (1956). *Nature, Lond.*, **177**, 79.
Nicolaus, R. A. (1953). *Gazz. chim. ital.* **83**, 239.
Nicolaus, R. A. (1955). *Gazz. chim. ital.* **85**, 659.
Nicolaus, R. A. & Mangoni, L. (1955). *Gazz. chim. ital.* **85**, 1397.
Panizzi, L. & Nicolaus, R. A. (1952). *Gazz. chim. ital.* **82**, 435.
Raper, H. S. (1926). *Biochem. J.* **20**, 735.
Raper, H. S. (1927). *Biochem. J.* **21**, 89.
Raper, H. S. (1938). *J. chem. Soc.* p. 125.
Roth, L. J., Miller, W. H. & Dawson, C. R. (1944). Quoted by Nelson, J. M. & Dawson, C. R. *Advanc. Enzymol.* **4**, 99.
Schmalfuss, H. & Peschke, W. (1929). *Ber. dtsch. chem. Ges.* B, **62**, 2591.
Swan, G. A. & Wright, D. (1956). *J. chem. Soc.* p. 1549.