185. *Melanin Formation.*

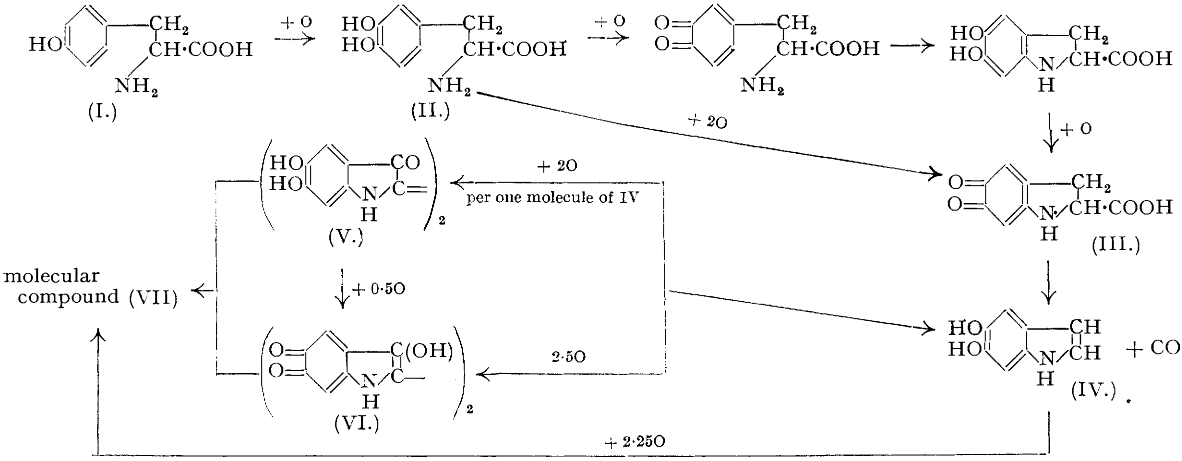
By G. R. Clemo and J. Weiss.

Raper and his co-workers have shown that 5 : 6-dihydroxyindole is an intermediate product in the formation of melanin *in vivo* and *in vitro,* and that it is formed from this compound by a non-enzymatic oxidation process. This suggests that 5 : 6 : 5′ : 6′-tetrahydroxyindigo, possibly as the corresponding quinone or semiquinone, is an important constituent of the melanin pigments. This conclusion is based primarily on the mechanism of oxidation. In particular, 2 : 3-dimethyl-5 : 6-dihydroxyindole does not oxidise in the same fashion, whereas apparently 5 : 6-dimethoxyindole does.

The amount of oxygen required for the oxidation process and some other properties of melanin (*e.g.*, reversible reduction in alkaline solution) are discussed, and attempts to prepare the indigo compound are reported.

Melanin is the dark pigment which is produced by the oxidation of tyrosine or dihydroxyphenylalanine in the presence of certain enzyme catalysts. Although melanin prepared from animal or plant sources is often not identical with the above mentioned melanin the difference is possibly due to the difficulty of purification from adsorbed and admixed substances (cf. Schaaf, *Biochem. Z.*, 1929, **209**, 79). Bloch (*Z. physiol. Chem.*, 1917, **98**, 226) has also shown that the melanin in the melanoblasts of the epidermis is produced from **3** : 4-dihydroxyphenylalanine by the enzyme, dopa oxidase.

Raper (*Biochem. J.*, 1927, **21**, 89) has studied the formation of melanin by the oxidation of tyrosine (I) and 3 : 4-dihydroxyphenylalanine (II) in the presence of tyrosinase and he has shown that one of the main intermediate products is the 5 : 6-dihydroxyindole (IV) which is also fomerd *in vivo* according to Linnell and Raper (*Biochem. J.*, 1935, **29**, 76). The latter substance readily oxidises to give melanin in a non-enzymatic process. This can be represented by the following scheme which up to (IV) follows that given by Raper (*loc. cit.*, also Evans and Raper, *Biochem. J.*, 1937, **31**, 2162).



Raper's scheme of reactions seems to be essential for the formation of melanin although it is clear from the work of Bloch and Schaaf (*Biochem. Z.*, 1926, **162**, 181) that, under changed conditions of pH and temperature, side reactions appear to a greater or smaller extent which lead to other products contaminating the melanin.

Compound (III) was subsequently found in the marine worm *Halla parthenopea* (Mazza and Stolfi, *Arch. Sci. biol.*, 1931, **61**, 183) and is capable of giving a reversible oxidation reduction system (Friedheim, *Biochem. Z.*, 1933, **259**, 254).

According to this scheme 3 atoms of oxygen are required to convert one molecule of (I) and 2 atoms of oxygen to convert one molecule of (II) to (IV). Dulière and Raper (*Biochem. J.*, 1930, **24**, 259) found that starting from (I) 5·23 atoms of oxygen and starting from (II) 4·12 atoms of oxygen were required to convert these substances into melanin. This leaves for the conversion of dihydroxyindole into melanin 2·23 and 2·12 atoms of oxygen respectively.

The question now arises as to the nature of the oxidation process by which (IV) is converted into melanin and there are two obvious possibilities : (1) oxidation with the formation of an *o*-quinone (cf. Wagreich and Nelson, *J. Amer. Chem. Soc.*, 1938, **60**, 1545) and not involving the 5-membered N ring, and (2) the inclusion of the latter in the oxidative reaction with the formation of an indigoid system.

The fact that 5 : 6-dimethoxyindole is very sensitive to oxidising agents and rapidly darkens even in contact with the air (Oxford and Raper, *J.*, 1927, 417) would appear to favour the second alternative above.

Further, it is well known that indoles with free hydrogen atoms in the α- and β-positions yield indigoid compounds on oxidation at room temperature (cf. Nencki, *Ber.*, 1875, **8**, 727; G.PP. 130,629 and 132,405). Porcher (*Bull. Soc. Chim.*, 1909, [4] **5**, 526) who has studied in detail the oxidation of indole by hydrogen peroxide (at 37°) found that the reaction goes through indoxyl which is subsequently oxidised to give indigo. (A certain amount of indirubin is also formed, particularly at higher temperatures.) It is, therefore, to be expected that dihydroxyindole will react in a similar way, its rate of reaction being favoured by the presence of the hydroxy groups which also render it much more soluble in water. This course for the reaction is schematically represented above. This could lead to either tetrahydroxyindigo (V) needing 5 atoms of oxygen and thence to the quinone (VI) needing 5·5 atoms of oxygen. The fact that the melanin obtained from tyrosine is practically insoluble in sodium hydroxide is against (V) and some of the properties of melanin do not fit either with the simple quinone structure (VI). It is well known, however, that *o*-quinones tend to dimerise or polymerise (cf. Willstätter and Müller, *Ber.*, 1911, **43**, 271; Wagreich and Nelson, *loc. cit.*) and also that the quinone (VI) and the catechol (V) might well form a molecular compound or a semiquinone (cf. Michaelis, *Chem. Rev.*, 1935, **16**, 274) which would more nearly agree with the physical properties of melanin. A further possibility is that (V) and (VI) might condense through one hydroxyl and one keto group either from the benzene rings or the 5-membered rings whereby a chain structure could be obtained which would appear in harmony with the physical properties of melanin. For either of these latter possibilities 5·25 atoms of oxygen would be needed from (I), in good agreement with the 5·23 atoms of oxygen found by Dulière and Raper (*loc. cit.*). However, any small deviations could be easily accounted for by a certain amount of non-oxidative side reactions and it is also possible that the state of oxidation varies to some extent in different preparations of melanin.

The mechanism suggested above is further supported by some observations on the 2 : 3-dimethyl-5 : 6-dihydroxyindole in which the reactive α- and β-positions are methylated. This substance (the preparation of which is described below) is actually stable towards oxygen in acid solutions but oxidises in alkaline solution (in conformity with other *o*-dihydroxy compounds) to give a violet-coloured product which dissolves in alcohol and acetone with a purplish colour and gives a deep red solution in conc. sulphuric acid.

Indigoid substances and some of their precursors are well known to occur in biological systems. All the facts given above suggest that 5 : 6 : 5′ : 6′-tetrahydroxyindigo, presumably as a quinone or semiquinone, is an important constituent of the natural melanins.

The following observations are in support of this suggestion. The nitrogen content of (VI) is in good agreement with the value (8·65%) given by Raper and Wormall (*Biochem. J.*, 1925, **19**, 84) and with the value (8·3%) given by Bloch and Schaaf (*loc. cit.*) for preparations of dopa melanin. Neither of the above workers give a full analysis for their preparations, but Piettre (*Compt. rend.*, 1911, **153**, 782, 1037) found the following values for several purified specimens of melanin : horse melanin (C, 56·0; H, 3·5; N, 9·6%, small amounts of Fe and S were not determined), melanin from *sepia officinalis* (C, 58·0; H, 3·4; N, 11·3; S, 0·4%) and melanin produced enzymatically from tyrosine [C, 58·5; H, 3·55; N, 8·1%. Calc. for $C_{16}H_{10}O_6N_2$ (V) : C, 58·9; H, 3·1; N, 8·6%. Calc. for $C_{16}H_8O_6N_2$ (VI) : C, 59·2; H, 2·5; N, 8·6%. Calc. for $C_{16}H_9O_6N_2$ (VII) : C, 59·0; H, 2·8; N, 8·6%].

Melanin is relatively unstable towards strong oxidising agents, as would be expected from its structure, and can be easily reduced in the presence of sodium hydroxide whereby it is (partially) decolorised. Some melanins are soluble in pyridine and in alkali hydroxides (cf. Bloch and Schaaf, *loc. cit.*).

It was found (Figge, *Proc. Soc. Expt. Biol. Med.*, 1939, **41**, 127) that melanin is actually capable of giving a reversible oxidation reduction system and can be (reversibly) reduced by hydrosulphite.

Hayduck (*Ber.*, 1903, **36**, 2930) tried to prepare 5 : 6 : 5′ : 6′-tetrahydroxyindigo from *o*-nitroprotocatechuic aldehyde with acetone and alkali but did not succeed. He started with the corresponding tetramethoxyindigo, which he prepared from dimethoxy-*o*-nitroprotocatechuic aldehyde, but he was unable to demethylate this compound without considerable decomposition.

The authors started with piperonalindigo which was prepared from *o*-nitropiperonal and acetone with alkali (Haber, *Ber.*, 1890, **23**, 1566), but so far it has not been found possible to remove the methylene groups smoothly. Various methods were tried (hydrochloric and sulphuric acid at different concentrations and temperatures with and without the addition of phloroglucinol; cf. Clowes, *Ber.*, 1899, **32**, 2841) and although most of the products obtained were very similar to melanin in their appearance and also behaved similarly towards various solvents they did not give a satisfactory analysis. According to the analysis the best products contained approximately 90 per cent. of the required substance.

The preparation of 2 : 3-methylenedioxyaniline is described from the corresponding nitro compound (Hesse, *Annalen*, 1879, **199**, 341) and from the nitro carboxylic acid (Mameli, *Atti R. Accad. Lincei*, 1905, (5), **14**, II, 514) and from the amide by the action of sodium hydroxide and bromine (Rupe and von Majewski, *Ber.*, 1900, **33**, 3403; van Linge, *Rec. Trav. chim.*, 1897, **16**, 50). The yields of amine claimed are either poor or bad, but it has now been found that it can be conveniently obtained by the action of freshly prepared sodium hypochlorite solution on piperonylamide.

The amine is easily converted to the hydrazine by the reduction of its diazonium chloride with stannous chloride and the hydrazine readily gives the hitherto undescribed 2 : 3-*dimethyl*-5 : 6-*methylenedioxyindole* by treatment with methyl ethyl ketone under standard Fischer indole conditions (cf. *Annalen*, 1886, **236**, 129) and also is converted into 6 : 7-*methylenedioxy*-1 : 2 : 3 : 4-*tetrahydrocarbazole*.

When the indole is boiled gently with dilute sulphuric acid formaldehyde distils off with the steam and can be isolated in a 75% yield in the form of its crystalline dimedon derivative. If the residue is made alkaline it oxidises to a purple substance.

EXPERIMENTAL.

Action of Sulphuric Acid on 5 : 6-*Methylenedioxyindigo*.—In acetic acid : the compound 0·1 g.) in acetic acid (20 c.c.) and sulphuric acid (7 c.c.) was heated at 120—130° for 5 hours with frequent shaking. The solution became dark brown and the starting material was converted into a black substance. After washing, extraction with dilute alkali, washing with dilute HCl and hot water the substance was dried in vacuum [Found : C, 57·7; H, 3·5; N, 7·6. $C_{16}H_{10}O_6N_2$ (V) requires C, 58·8; H, 3·1; N, 8·6%].

3 : 4-*Methylenedioxyaniline*.—Piperonylamide (8·2 g. crystallised material, m. p. 167°) was ground into a paste with water (100 c.c.), a solution of sodium hydroxide (4 g.) in water (40 c.c.) added, the mixture cooled below 0° and well stirred. Freshly made sodium hypochlorite solution [98 g., made by passing a slow stream of chlorine into a solution of sodium hydroxide (16 g.) in water (184 c.c.) kept below 0° until 14·2 g. had been absorbed during 1½ hours] was then run in so that the temperature was kept below 1°. The cooling bath was then removed, the mixture stirred a further 15 mins. and then warmed in a water bath to 50°, when the colour changed and the reaction was complete after a further 15 minutes' stirring at this temperature. The oil, deposited on cooling, was taken up in ether, the deep yellow aqueous solution extracted four times with ether, and the combined extract dried and fractionated; the almost colourless base (3·8 g., b. p. 108°/1 mm.) passed over and quickly solidified (m. p. 40°). Crystallisation from ligroin (b. p. 40—60°), gave fine colourless needles, m. p. 43—44°. The literature gives the m. p. as 44°.

3 : 4-*Methylenedioxyphenylhydrazine*.—The above distilled base (2·7 g.) was added to cold stirred hydrochloric acid

(5 c.c. of conc. acid diluted with water) (16 c.c.). The solution was cooled to −5° and well stirred whilst a solution of sodium nitrite (1·4 g.) in water (14 c.c.) was dropped in during 10—15 minutes. The solution, at −10°, was efficiently stirred and cooled whilst a solution of stannous chloride (11·2 g.) in conc. hydrochloric acid (9 c.c.) was added during 45 minutes. Although much heat is evolved, by the use of a thin glass vessel and good ice-salt cooling, the temperature was kept at about −10° during the reduction. The hydrazine hydrochloride was collected and crystallised from alcohol giving colourless prisms (2·75 g.). This finely ground hydrochloride (1 g.) was shaken three times with ether in the presence of sodium hydroxide (20 c.c. 8%), the extract dried and fractionated; an oil (0·65 g., b. p. 160°/1 mm.) passed over and immediately set to a yellow crystalline solid which separated from ligroin (b. p. 60—80°) in slightly pink plates, m. p. 75° (Found : C, 55·4; H, 5·3. $C_7H_8O_2N_2$ requires C, 55·3; H, 5·3%). The *m-nitrobenzylidenehydrazone* crystallises from alcohol in beautiful orange plates, m. p. 164—165° (Found : C, 59·2; H, 4·2. $C_{14}H_{11}O_4N_3$ requires C, 59·0; H, 3·9%).

6 : 7-*Methylenedioxy*-1 : 2 : 3 : 4-*tetrahydrocarbazole*.—This separated as a solid mass when the hydrazine and *cyclo*hexanone were warmed a few minutes in acetic acid and crystallised from alcohol in colourless plates, m. p. 191—192° (Found : C, 72·7; H, 6·1. $C_{13}H_{13}O_2N$ requires C, 72·6; H, 6·0%).

2 : 3-*Dimethyl*-5 : 6-*methylenedioxyindole*.—The hydrazine (0·65 g.) was dissolved in methyl ethyl ketone, acetic acid (six drops) added, the solution heated for 1½ hours on the water bath, cooled in ice, and saturated with dry hydrogen chloride when the reaction mixture changed to an intense magenta colour; it was allowed to stand ½ hour in ice and then refluxed gently for 1½ hours on the water bath. The colour changed to a dull brown and a colourless crystalline solid separated. This was left to stand, all solvent removed under reduced pressure, dilute sodium hydroxide added and the whole extracted with ether and fractionated (0·5 g., b. p. *ca.* 180°/1 mm.). This solidified and on crystallisation from ligroin (b. p. 60—80°) colourless prisms (0·3 g., m. p. 132—133°) were obtained (Found : C, 69·7; H, 5·8. $C_{11}H_{11}O_2N$ requires C, 69·8; H, 5·8%).

UNIVERSITY OF DURHAM, KING'S COLLEGE, NEWCASTLE-UPON-TYNE.

[*Received, July 2nd*, 1945.]