

The Chemistry of the Melanins. Part V. The Autoxidation of
5 : 6-Dihydroxyindoles.*

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The autoxidation of 5 : 6-dihydroxyindoles, which results in the formation of melanin-like pigments and hydrogen peroxide, has been examined by measurements of oxygen uptake, by elementary analysis of the pigments produced, and by spectroscopic studies of intermediates obtained by enzymic and silver oxide oxidation. With silver oxide yellow indole-5 : 6-quinones of varying degrees of stability were obtained. The results support the quinone polymerisation theory of melanin formation but there is evidence that some of the component indole units are more extensively oxidised.

EARLIER papers in this series have dealt with the synthesis of 5 : 6-dihydroxyindole and its derivatives, which were prepared with the object of comparing their behaviour on autoxidation and thus obtaining information about the mode of formation of the melanin-like product derived from 5 : 6-dihydroxyindole. As the work progressed (see, *e.g.*, Part IV *) it became clear that a qualitative and rather superficial comparison of the autoxidations was inadequate, and the experiments described in the present communication were developed in an effort to discover more objective methods. The autoxidations have been studied by measurements of oxygen uptake and of the absorption spectra of intermediates obtained by enzymic and silver oxide oxidations. Solubility and analytical data are recorded for the "melanins" produced in the autoxidations.

Oxygen Uptake and Hydrogen Peroxide Formation.—In preliminary measurements of oxygen uptake it was discovered that the buffer solution in which the dihydroxyindole had been oxidised contained appreciable amounts of hydrogen peroxide. In all subsequent experiments, therefore, the amount of oxygen absorbed by each indole and the amount of hydrogen peroxide produced at pH 7 and 8 were measured as described in the Experimental section. The results obtained are summarised in Table 1. Oxygen uptakes are calculated as moles of oxygen per mole of indole, and the peroxide formed is similarly expressed. The values in the column "Oxygen to polymer" represent uptakes corrected by subtracting the amount of oxygen consumed in peroxide formation. The variation of oxygen uptake with time at pH 8 is represented by sigmoid curves which indicate that the reactions are autocatalytic. The end of the induction period corresponds with the first appearance of colour in the solution.

The "oxygen to polymer" values of Table 1 are in reasonable agreement with values for the oxygen consumption involved in the conversion of 5 : 6-dihydroxyindole into melanin calculated from data presented by Duliere and Raper (*Biochem. J.*, 1930, **24**, 247) and by Mason (*J. Biol. Chem.*, 1947, **168**, 433) for enzyme-catalysed oxidations of tyrosine and

* Part IV, *J.*, 1951, 2426.

similar substrates. Any hydrogen peroxide formed in the enzymic oxidations would presumably be decomposed by the catalase present in the tyrosinase preparations.

The maximum rate of oxygen absorption by 5 : 6-dihydroxyindole at pH 8 was not materially affected by the addition of mushroom tyrosinase but the (apparent) induction period was reduced and the total amounts of oxygen absorbed and hydrogen peroxide

TABLE 1. *Oxygen consumption by 5 : 6-dihydroxyindoles.*

5 : 6-Dihydroxyindole	O ₂ uptake (moles)	H ₂ O ₂ (moles)	Oxygen to polymer (moles)	Maximum rate (mole/min.)	O ₂ uptake (moles)	H ₂ O ₂ (moles)	Oxygen to polymer (moles)	Maximum rate (mole/min.)
	At pH 7.				At pH 8.			
Unsubstituted	1.31	0.46	1.08	0.020	1.38	0.52	1.12	0.110
1-Methyl	1.13 *	—	—	0.0056	1.41	0.36	1.23	0.021
2-Methyl	1.24	0.69	0.90	0.051	1.45	0.62	1.14	0.19
3-Methyl	1.22	0.79	0.83	0.093	1.27	0.58	0.98	0.45
2 : 3-Dimethyl	0.99	0.66	0.66	0.064	1.44	0.70	1.09	0.19
7- <i>n</i> -Propyl	1.12	0.67	0.79	0.021	1.42	0.60	1.12	0.091
3-Methyl-7- <i>n</i> -propyl	0.73	0.60	0.43	0.017	1.09	0.65	0.76	0.173
2 : 4 : 7-Trimethyl	1.43	0.48	1.19	0.075	1.67	0.46	1.44	0.382

* Oxygen was still being absorbed when experiment terminated.

TABLE 2. *Visible changes in autoxidations at pH 8.*

5 : 6-Dihydroxyindole	Intermediate colour	Final appearance
Unsubstituted	Purple	Black; black ppt.
1-Methyl	Yellow → pink → violet	Black; slight ppt.
2-Methyl	Brown	Black; black ppt.
3-Methyl	Blue	Intense dark blue; dark ppt.
2 : 3-Dimethyl	Brownish-purple	Almost black; no visible ppt.
7-Propyl	Purple	Black; no visible ppt.
3-Methyl-7-propyl	Blue	Dark blue; slight ppt.
2 : 4 : 7-Trimethyl	Red-purple	Intense red-brown; no visible ppt.

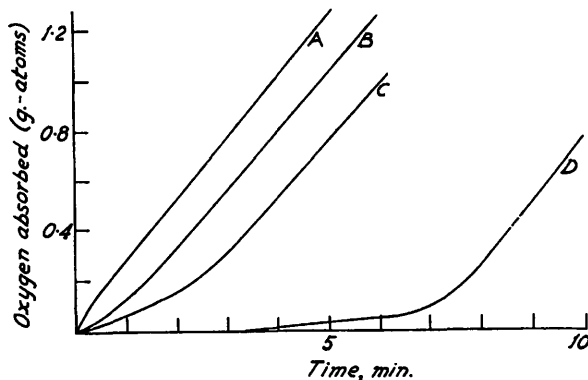


FIG. 1.

Curve	Enzyme units
A	5.0
B	2.5
C	1.0
D	nil

produced were also decreased, whilst the "oxygen to polymer" value remained virtually the same. When the reaction was carried out at various enzyme concentrations expressed as arbitrary enzyme units, the results shown in Fig. 1 were obtained. It appears that the enzyme has an effect on the *initial* rate of oxygen uptake which is roughly proportional to enzyme concentration but thereafter has little effect on the course of the reaction.

Visible changes in the autoxidising solutions at pH 8 (without enzyme) are summarised in Table 2. In every case except that of the 2 : 4 : 7-trimethylindole, filtration of the oxidised solution through a fine-mesh sintered funnel led to almost complete removal of the coloured products. The pigment from 5 : 6-dihydroxy-2 : 4 : 7-trimethylindole was obtained by acidification of the oxidised solution with dilute sulphuric acid.

Polymer yields and analytical data are collected in Table 3.

Spectrophotometric Experiments.—The formation of melanin from 3 : 4-dihydroxy-

phenylalanine (dopa) has been studied spectroscopically by Mason (*J. Biol. Chem.*, 1948, **172**, 83) who detected a purple intermediate characterised by a flat absorption maximum at 540 $m\mu$. This result has now been confirmed with authentic 5 : 6-dihydroxyindole. When a solution of mushroom tyrosinase in phosphate buffer (pH 5.6) was added to a solution of the indole (*ca.* $5 \times 10^{-5}M$) in the same solvent, a purple colour developed which reached maximum intensity in approximately 20 minutes. The solution then showed an absorption maximum at 540 $m\mu$ (curve A in Fig. 2). It is interesting that an almost identical curve is obtained at pH 8, at which conversion into melanin is normally rapid. The

TABLE 3. *Polymer yields and analytical data.*^a

5 : 6-Dihydroxyindole	Yield (% by wt.)	Composition (%)			Atomic proportions			
		C	H	N	C	H	O	N
Unsubstituted (A)	109	{ 56.3	3.0	8.2	8.00	5.20	3.42	1.00
		{ 56.6	3.1	8.2				
		{ 55.0	3.2	8.45				
		{ 55.2	3.3	8.45				
(B)	—	{ 55.5	3.2	7.85	8.00	5.43	3.60	0.97
(C)	—	{ 55.8	3.1	7.8				
(D)	—	{ 54.7	3.4	7.6	8.00	5.92	3.77	0.98
		{ 54.5	3.3	8.0				
2-Methyl	93	56.1	3.8	7.3	9.00	7.36	3.95	1.00
3-Methyl	103	57.4	4.2	7.0	9.00	7.90	3.70	0.95
1-Methyl	102	55.4	4.0	—	9.00	7.78	4.09	1.00
2 : 3-Dimethyl	96	62.0	4.1	—	10.00	8.00	3.55	1.00
7-Propyl	—	62.0	5.1	—	11.00	10.85	3.55	1.00
3-Methyl-7-propyl	87	64.4	6.1	—	12.00	13.70	3.25	1.00

^a The polymer samples had appreciable ash-contents (1–3%). The results have been calculated on an ash-free basis. ^b Expected C : N ratio assumed in calculation.

stability of the intermediate is probably a consequence of the very low concentrations used.

The nature of this intermediate is clearly of considerable importance and, in order to facilitate further discussion, the general trivial name *melanochrome* is suggested for the coloured compounds obtained by enzymic oxidation of dihydroxyindoles in very dilute solutions. Absorption curves for melanochromes derived from several dihydroxyindoles (at pH 5.6) are reproduced in Fig. 2.

The purple melanochrome obtained from 5 : 6-dihydroxyindole was regarded by Mason (*loc. cit.*) as being indole-5 : 6-quinone, but the position of its absorption is comparable with the maxima shown by the indolylbenzoquinones reported by Bu'Lock and Harley-Mason (*J.*, 1951, 703). In an attempt to detect monomeric quinones as intermediates in the formation of melanochromes, a study of the oxidation of dihydroxyindoles with silver oxide in a variety of solvents was made. In acetone solution all the indoles studied, except 5 : 6-dihydroxy-2-methylindole, gave evidence of a yellow intermediate, the stability of which varied considerably. In most cases the initially yellow solution rapidly became green and then blue or purple, changes which were catalysed by acids or bases, but with 5 : 6-dihydroxy-2 : 3-dimethylindole the yellow solution was stable for 20–30 minutes and the intermediate had a sharp absorption maximum at 362 $m\mu$ without appreciable absorption in the 500–600 $m\mu$ region (see Fig. 3, which also shows the very similar absorption curve of a solution obtained by silver oxide oxidation of 1 : 2 : 3 : 4-tetrahydro-6 : 7-dihydroxycarbazole).

It seems likely that the yellow intermediates in the silver oxide oxidations are the true indole-5 : 6-quinones and that the more intensely coloured melanochromes are formed from them and are composed of a number, possibly a small number in some cases, of linked quinonoid indole molecules.

With 5 : 6-dihydroxy-2-methylindole a well-defined colour was not observed either in the enzymic or the silver oxide oxidations. In this case the oxidation may proceed so rapidly that the product is precipitated or perhaps absorbed by the oxidant. The behaviour of 5 : 6-dihydroxy-1-methylindole resembled that of 5 : 6-dihydroxyindole whilst the enzymic oxidation of 5 : 6-dihydroxy-2 : 3-dimethylindole gave a solution with an absorption maximum at *ca.* 445 $m\mu$.

On this view, the melanochromes observed in the enzymic oxidations are polyindole-quinones of the same type as, but less complex than, the insoluble melanins, the formation of which probably involves cross-linking of melanochrome chains.

The formation of considerable quantities of hydrogen peroxide in the autoxidation of each indole studied is presumably associated with the first reaction, *i.e.*, oxidation of indole to quinone. It is known, for example, that the autoxidation of duroquinol to duroquinone produces one mol. of hydrogen peroxide (James and Weissberger, *J. Amer. Chem. Soc.*, 1938, **60**, 98). The formation of the quinone almost certainly involves a free-radical reaction and, in this connection, it is interesting that the only significant effect of tyrosinase observed in this work is to eliminate the normal induction period. The possibility that hydrogen peroxide is derived in some way from an intermediate indoleninyl hydroperoxide analogous to the tetrahydrocarbazolyl peroxides (see Beer, Broadhurst, and Robertson, *J.*, 1952, 4946) has been rejected on the grounds that hydrogen peroxide is produced even in the autoxidation of 5 : 6-dihydroxy-1-methylindole, since *N*-substituted tetrahydrocarbazoles do not form hydroperoxides (*J.*, 1950, 2118).

By analogy with the autoxidation of duroquinol, one mole of peroxide should be produced per mole of indole.* The observed values (Table 1) vary between 0.36 and 0.79 mole and the difference between these values and the theoretical minimum of one mole must represent peroxide which has either decomposed to give oxygen or been utilised in some oxidation process. Recent work by Dr. Duxbury in these laboratories (*Chem. and Ind.*, 1953, 1364) suggests that hydrogen peroxide oxidation, possibly of the quinone but more probably of the polymerised material, is responsible for the formation of carbon dioxide observed by Clemo, Duxbury, and Swan (*J.*, 1952, 3464) in autoxidation experiments with 2-(3 : 4-dihydroxyphenyl)ethylamine and with 5 : 6-dihydroxyindole. It seems likely that carbon dioxide formation is *incidental* to the process of melanin formation (see Cromartie and Harley-Mason, *Chem. and Ind.*, 1953, 972) and cannot be regarded as strong evidence for the ring-fission hypothesis of melanogenesis (cf. Clemo *et al.*, *loc. cit.*; Jolles, *Chem. and Ind.*, 1953, 845) which therefore rests largely on analogy.

The analytical data for melanins obtained by autoxidation of 5 : 6-dihydroxyindole, given in Table 3, do not in fact indicate any striking change in the C : N ratio, and we conclude that melanins prepared under the conditions described are built up from C₈-N units derived from indole-5 : 6-quinone molecules. On this basis, the maximum oxygen uptake, corrected for hydrogen peroxide formation, should be two atoms. The "oxygen to polymer" values (see Table 1) for most of the dihydroxyindoles studied exceed the figure. For example, at pH 8, 5 : 6-dihydroxyindole and its 2-methyl derivative take up 2.2 and 2.3 atoms respectively and, if an appreciable amount of carbon dioxide is formed, these values would be smaller than the true uptakes. It appears, therefore, that, although the quinone polymerisation theory probably represents the main course of events, some other process involving consumption of oxygen occurs. This conclusion is supported by the analytical data (Table 3). The analyses of four independent samples of the melanin from 5 : 6-dihydroxyindole are surprisingly consistent if allowance is made for variation in water content (the correction is legitimate because the samples are hygroscopic when exhaustively dried). By subtraction of fractional amounts of water the empirical formulæ can be adjusted for comparison with the formula (C₈H₃O₂N)_n expected on the basis of the polyindolequinone theory, and it then appears that the polymers contain approximately 0.3 atom of oxygen in excess of that required. This additional oxygen could be accommodated on the assumption that the melanins are essentially polyindolequinones in which some of the indole units are in a more highly oxidised state.

The mode of linkage of the quinone units remains to be discussed. Bu'Lock and Harley-Mason (*loc. cit.*) have argued, partly from the results of model experiments with indoles and quinones and partly on theoretical grounds, that the positions involved in the self-condensation of indole-5 : 6-quinone are the 3- and the 7-position. Our earlier observation that 5 : 6-dihydroxy-3-methylindole behaves differently from 5 : 6-dihydroxy-

* It is conceivable that hydrogen peroxide is also formed during the polymerisation stage since this must also involve oxidation of quinols, if the final product is to be largely quinonoid (cf. Bu'Lock and Harley-Mason, *loc. cit.*).

indole on autoxidation (Part II, *J.*, 1949, 2061) suggested that the 3-position was involved and the study of the spectra of melanochromes now reported supports this view. Enzymic oxidations of 5 : 6-dihydroxy-, 5 : 6-dihydroxy-1-methyl-, and 5 : 6-dihydroxy-7-*n*-propylindole gave spectroscopically similar intermediates (λ_{\max} , *ca.* 540 $m\mu$) but compounds with a 3-methyl group and a free 2-position (5 : 6-dihydroxy-3-methyl- and 5 : 6-dihydroxy-3-methyl-7-*n*-propylindole) gave intermediates with maxima at longer wave-lengths (>600 $m\mu$). The inference is clearly that 3-substitution, whilst not preventing the oxidative polymerisation, does deflect its normal course. The formation of a polymer of high molecular weight from 5 : 6-dihydroxyindole by a route involving the 3-position must, for the reasons advanced by Bu'Lock and Harley-Mason (*loc. cit.*), also involve the 7-position.

The 2-position in the indole nucleus shows some reactivity in the model condensations mentioned above and it seems probable that 5 : 6-dihydroxyindoles with a 3-methyl group and a free 2-position (activated by the adjacent alkyl group) will polymerise by linkage at the 2-position. In this case the steric limitations discussed by Bu'Lock and Harley-Mason no longer apply and the other position involved in polymer formation could be either the 4- or the 7-position. Judged by solubility properties (Table 5) the "polymer" obtained from 5 : 6-dihydroxy-7-*n*-propylindole at pH 8 is of lower molecular weight than that from the 3-methyl-7-*n*-propylindole. A possible explanation of this rather surprising result is that the dialkylindole contains an activated 2-position and can readily form a long-chain polymer linked at the 2- and the 4-positions, whereas with the 7-*n*-propylindole continued 3 : 4-linkage is prohibited for steric reasons and 2 : 4-linkage is difficult on account of the low reactivity of the 2-position. The pigment from 5 : 6-dihydroxy-7-*n*-propylindole should therefore be of low molecular weight.

This result conflicts with the recent report by Cromartie and Harley-Mason (*loc. cit.*) that an insoluble melanin-like pigment is obtained from 5 : 6-dihydroxy-7-methylindole by autoxidation at pH 6.85. These authors also state that autoxidation of 5 : 6-dihydroxy-2 : 3-dimethylindole gives an orange-red solution containing 2 : 3-dimethylindole-5 : 6-quinone which, in contrast to our results obtained at pH 8, is stable to further oxidation. Although the quinone from this indole is more stable than are those derived from other indoles, we find that autoxidation leads to a polymer which appears to be of considerable molecular weight. The polymer was not visible as a precipitate in the oxidised solution but could nevertheless be removed by filtration. The formation of an insoluble pigment from this indole implies that continued 4 : 7-linking of the indolequinone units is possible, and the light absorption of the corresponding melanochrome (λ_{\max} , *ca.* 445 $m\mu$) might be explained on this basis. The colour finally produced in the silver oxide oxidation, however, appears to be quite anomalous.

EXPERIMENTAL

Oxygen Uptake and Hydrogen Peroxide Formation.—In a preliminary study of the autoxidation of 5 : 6-dihydroxyindole by Dr. A. G. White (see Part II, *J.*, 1949, 2062) some difficulty was experienced in obtaining reproducible results in oxygen-uptake experiments. This difficulty was not encountered in the present work for which the various hydroxyindoles, prepared by previously described methods, were rigorously purified, in most cases by vacuum-sublimation. With compounds containing a 2-methyl group, which did not sublime readily, repeated recrystallisation gave satisfactory results. Normal analytical-grade phosphates were used for the preparation of standard *M*/15-solutions (International Critical Tables, Vol. I, p. 81) which were mixed before each run to give buffers of the required pH.

The apparatus consisted of a tubular reaction vessel connected by small-bore pressure tubing to a gas-burette containing mercury. The reaction vessel and burette were kept at 25° by use of a thermostat and standard circulating arrangements. The reaction vessel was shaken vertically by a crank and eccentric at a speed sufficient to maintain the reaction mixture in a froth. The stopper of the vessel was a B19 standard cone carrying a small glass hook on which was suspended a small tube containing the dihydroxyindole. The system could thus be brought to equilibrium before the reaction was started by dislodging the tube.

Runs were normally carried out with 0.2 millimole of indole and 10 ml. of buffer solution. The reaction was followed by direct reading of the volume of the system at suitable intervals.

When oxygen uptake ceased the contents of the vessel were acidified with 2*N*-sulphuric acid (20 ml.), and the mixture was then filtered. Potassium iodide (1.0 g.) was added to the filtrate and after storage for 5 min. in the dark the liberated iodine was titrated with 0.01*M*-sodium thiosulphate. In a separate experiment with 5:6-dihydroxyindole the oxidising agent left in solution after the removal of pigment was shown to be hydrogen peroxide. Vacuum-distillation gave a colourless aqueous solution which reacted positively with acidified potassium iodide solution and with titanous sulphate, and was spectroscopically indistinguishable from distilled water in the range 300—700 μ .

Polymer Yields and Analytical Samples.—The dihydroxyindoles (30 mg.) were introduced into the reaction vessel, the system was swept out with oxygen, the phosphate buffer (pH 8; 10 ml.) then added, and the vessel was shaken until no more oxygen was absorbed. Samples intended for analysis were collected by centrifugation, very thoroughly washed (25 times) in the centrifuge tube with distilled water, and then dried first *in vacuo* at room temperature over phosphoric oxide and finally to constant weight in a vacuum at 60°. In measurements of polymer yields the pigment was collected on a fine-mesh sintered crucible of known weight after acidification of the mixture with 2*N*-sulphuric acid (10 ml.) and was thoroughly washed and dried before weighing.

Silver Oxide Oxidations.—A solution of the dihydroxyindole (3—4 mg.) in pure acetone (5 ml.) was shaken with an excess of alkali-free silver oxide, and the suspension was then clarified by centrifugation. After 5 min., 1 ml. of the supernatant liquid was withdrawn and diluted to 25 ml. with acetone. The absorption spectrum of the resulting solution was measured.

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