Structure of Melanin derived from (±)-3,4-Dihydroxy-[^{14}C, ^{3}H]phenylalanine by Oxidation with Tyrosinase

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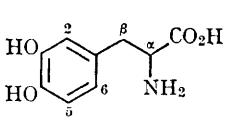
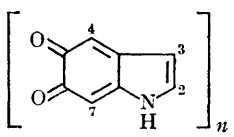
MELANIN is a black, insoluble, polymeric, pigment occurring widely in the animal kingdom.[1] The melanins of hair, skin, melanoma tumours, and *Sepia* ink are all derived biologically by oxidation of tyrosine or 3,4-dihydroxyphenylalanine (dopa) (I). Melanin can also be prepared from dopa *in vitro* by autoxidation or by oxidation in the presence of tyrosinase. Extensive studies on the composition and formation of dopa melanin have led to its formulation as a polyindolequinone (II). This structure, though probably oversimplified, will serve for the present discussion. No firm conclusion has been reached about the way in which the indolic units are linked in the polymer although 3 → 7 [2] and 4 → 7 [3,4] linked structures have at times been favoured.



(I)



(II)

We now describe experiments with tritium-labelled dopa which bear directly on the mode of linkage in the polymer. Specimens of (±)-dopa labelled[5] with tritium singly in the α-, β-, 2-, 5-, and 6-positions and triply in the 2,5,6-positions were each mixed with (±)-[α-^{14}C]dopa. The six [^{14}C, ^{3}H]-labelled samples were then converted into melanin by oxidation at pH 6·8 with oxygen gas in the presence of mushroom tyrosinase[6] and catalase. Catalase was used to prevent accumulation of hydrogen peroxide. The ^{3}H : ^{14}C ratios of the precursors (typically 5 : 1) and of the derived melanins were accurately determined by Dr. H. Simon (Munich) using a combustion method.[7] A comparison of these ratios gave immediately a measure of the tritium loss during melanin formation since it is known[8] that negligible loss of the α-carbon occurs during oxidation. Also, the tritium in all the labelled specimens of dopa was indefinitely stable under the conditions of oxidation. A preliminary experiment with (±)-[α-^{14}C, 2,5,6-$^{3}H_3$]-dopa showed that a fraction, 0·81, of the 3 tritium atoms in the precursor was retained in the melanin. A similar experiment using autoxidation at pH 8 gave a retention of 0·88. Polymerisation to a regular, high-molecular-weight, 4 → 7 linked structure would demand complete loss of tritium and must therefore be discounted. However, linkage through the 3,7-positions would have given a retention (1·0) close to that observed. This second possibility was tested with [5-^{3}H]- and [β-^{3}H]-labelled specimens of (±)-dopa. The melanins from both oxidations were precipitated with sodium chloride rather than with the hydrochloric acid used in the earlier experiments. This was especially desirable for isolating the melanin from [β-^{3}H]dopa since indoles are known[9] to exchange β-hydrogens under acidic conditions. The melanins from [5-^{3}H]- and [β-^{3}H]-dopa each retained 42% of the tritium present in the corresponding precursors. This is incompatible with a regular 3 → 7 linked structure, which would require loss of all the original tritium.

To confirm and extend these observations a complete series of enzymatic oxidations was carried out with a more active batch of mushroom tyrosinase, ammonium nitrate being used as a precipitant. The results (see Table) were in good agreement with those already recorded. As expected, heavy loss of tritium occurred from position-6 in dopa in accord with the general structure (II). However, a significant amount (13%) was retained. The possibility that this might represent unreacted dopa adsorbed on the

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polymer was discounted as follows. A specimen of [^{14}C]melanin, prepared in the usual way from (±)-α-[^{14}C]dopa was heated under reflux in 2N-hydrochloric acid for 3 hr. The acidic extract was then assayed for desorbed dopa by radiodilution. It was found that 1·4% by weight of dopa was removed by this treatment and a similar result was obtained after heating for 24 hr. This amount is less than the accumulated errors of the radioactivity measurements and can be neglected. Nicolaus and his colleagues[10] have suggested that dopa melanin contains some uncyclised amino-acid units; this would account for the finite retention of tritium at position-6. Alternatively, some units might have cyclised on to position-2 rather than -6. Interpretation of the tritium loss (*ca.* 50%) from the β-position of dopa is more difficult since an isotope effect might retard removal of tritium from the methylene group during formation of an indolic intermediate. Experiments are in hand to clarify this point using dopa fully deuterated at the β-carbon.

Racemic specimens of dopa were used throughout since it is known[11] that mushroom tyrosinase oxidises both optical forms at similar rates. However, one experiment was performed with L-[α-^{14}C], [3,5-$^{3}H_2$]tyrosine. Oxidation with tyrosinase in the presence of catalase gave a melanin containing 20% of the original tritium. If half the tritium is lost during hydroxylation to give dopa, the fraction (0·40) retained thereafter is close to that already observed (0·42) in the oxidation of (±)-[α-^{14}C], [5-^{3}H]dopa.

TABLE

Conversion of (±)-α-[^{14}C], [^{3}H]*dopa into melanin at pH* 6·8 *in the presence of tyrosinase and catalase*

Tritium labelling pattern in dopa	2	5	6	2,5,6	α	β
Fraction of tritium retained in melanin	0·37	0·42	0·13	0·86*	0·52	0·54

* This value is expressed as the fraction retained out of 3 tritium atoms, *i.e.* 29% retention of the total activity.

We conclude that melanin produced enzymatically from dopa must have a highly irregular structure. Melanin formed in other ways, for example, by autoxidation or by an intact organism may, of course, differ significantly from our material. The present procedure should however provide the first quantitative method for comparing the structures of dopa melanins produced under different conditions.

Swan and his colleagues have reported[12] experiments with α- and β-deuterated dopa which gave results qualitatively similar to ours. Dr. G. A. Swan (Newcastle) has kindly brought to our attention some recent work[13] by Dr. K. Hempel and his colleagues (Köln) who have studied the formation of melanin, in mouse melanoma, from [^{14}C]- and [^{3}H]-labelled dopa.

(*Received, October 4th*, 1965; *Com.* 623.)

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