Studies related to the Chemistry of Melanins. Part XI.[1] The Distribution of the Polymeric Linkages in Dopa-melanin

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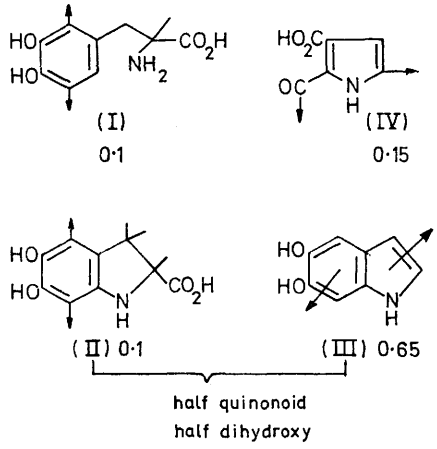
Samples of (±)-3,4-dihydroxyphenylalanine deuteriated at the α-, β-, 2-, 5-, and 6-positions were each converted into melanin, both by autoxidation and enzymically, and the incorporation of deuterium into these melanins was measured. The results were interpreted in terms of an outline structure suggested for dopa-melanin on the basis of other experiments (Part X); and the relative numbers of polymeric linkages at different positions of the polymeric units were estimated. No evidence was found that enzymic dopa-melanin was fundamentally different from the autoxidative melanin. Dopa-melanin, prepared *in vitro*, appears to be an irregular polymer, containing a number of different types of unit, linked in various ways.

FROM the results of experiments on (±)-3,4-dihydroxyphenyl[*carboxy*-^{14}C]alanine, we suggested that the main units in the polymer dopa-melanin might be uncyclised units (I), indolinecarboxylic acid type (II), indole type (III), and pyrrole-carboxylic acid units (IV) in the ratio 0·1 : 0·1 : 0·65 : 0·15, although perhaps we gave no very clear reason for writing the type (II) units in the indoline, rather than the indole form.[1]



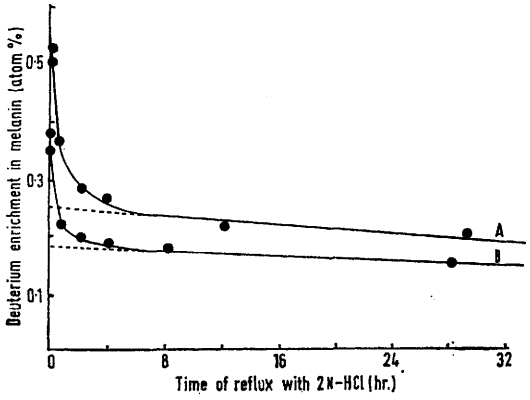
With a view to determining the proportion of indoline-type units in the melanin, and the relative numbers of linkages at different positions of the polymeric units, we have converted samples[2] of (±)-3,4-dihydroxyphenylalanine, specifically deuteriated at each of the α- and β-positions of the side chain, and the 2-, 5-, and 6-positions of the benzene ring, into melanins, and compared the enrichment of deuterium in the precursor and melanins. As an isotope effect could obscure the interpretation of the results, we carried out two series of experiments; in one series the precursor contained only a tracer concentration of deuterium, while in the other the relevant position of the molecule was deuteriated to as near 100% as possible.

For isotopic assay the samples were burnt,[3] and in experiments in which only tracer concentrations of deuterium were used, the resulting water was reduced with zinc,[4] and the isotopic abundance of deuterium in the hydrogen formed was determined mass spectrometrically. 'Memory' effects in the combustion and reduction necessitated several successive runs on the same sample before consistent results could be obtained. In the case of fully β-deuteriated 3,4-dihydroxyphenylalanine, containing over 16 atom % of deuterium, the difficulties were too great, so we measured the deuterium content of the water by the thermal conductivity method.[5] The latter method suffers from two disadvantages: the fragility of the wire used in the conductivity cell, and (more serious) the difficulty of maintaining a steady temperature (−21°) of the thermostatic block for more than 2 hr. Haldeman[6] described

[1] Part X, G. A. Swan and A. Waggott, preceding paper.
[2] F. Binns, J. A. G. King, A. Percival, N. C. Robson, and G. A. Swan, *J. Chem. Soc.* (C), 1970, 1134.
[3] R. C. Anderson, Y. Delabarre, and A. A. Bothner-By, *Analyt. Chem.*, 1952, **24**, 1298.
[4] J. Graff and D. Rittenberg, *Analyt. Chem.*, 1952, **24**, 878.
[5] G. R. Clemo and G. A. Swan, *J. Chem. Soc.*, 1942, 370.
[6] R. G. Haldeman, *Analyt. Chem.*, 1953, **25**, 787.

a modification of this method, claimed to overcome these disadvantages. We modified our apparatus accordingly, and although this then gave satisfactory results when comparatively large samples (>10 mg.) of water were available, the values obtained on small samples (*ca.* 1 mg.) were irreproducible, so the method proved useless for the present problem. Haldeman's measurements were made while water-vapour flowed continuously through the cell. The precursors other than the β-labelled one (which contains two deuterium atoms in the molecule) contain *ca.* 9 atom % of deuterium, and it proved feasible to measure these mass spectrometrically, although in a few experiments the thermal conductivity method was used.

H. S. Mason [7] has stated that there is good reason to think that the structures of autoxidative melanins



Deuterium enrichment in melanin plotted against time of boiling with acid: A, from (±)-3,4-dihydroxyphenyl[α-^{2}H]alanine; B, from (±)-3,4-dihydroxyphenyl[β-^{2}H]alanine

might be different from those of the enzymic ones, only the latter perhaps corresponding to natural melanins. We agree that there may be small quantitative differences (*e.g.* in the proportions of different polymeric units); but during our work we have never found clear evidence of fundamental differences. Nevertheless, in the present investigation we studied the incorporation of deuterium into the melanins formed both by autoxidation and enzymically, although we were unable to make our series of measurements comprehensive (Table 1).

Melanins were prepared by autoxidation of (±)-3,4-dihydroxyphenyl-[α-^{2}H]alanine and -[β^2-H]alanine containing tracer concentration of deuterium, and their deuterium enrichments were measured. The ratio of deuterium enrichment in the melanin to that in the precursor is represented as *R*. Portions of each melanin were then boiled under reflux with 2N-hydrochloric acid for periods up to 29 hr., after which the deuterium enrichments were measured again. The Figure shows deuterium enrichment in the melanin plotted against time of boiling with acid. This graph suggests that two distinct exchange processes occur; during the first 2 hr. of boiling, a very rapid loss of deuterium occurs, and this is followed by an extremely slow loss, which has never been pursued to completion.

The initial, very rapid loss of deuterium is presumably due to exchange of deuterium present in the 2- or 3-position of an indole type unit such as (III), or in a pyrrole unit like (IV). Exchange of hydrogen in the 3-position of indole itself is known to be rapid under acidic conditions, whereas exchange in the 2-position is much slower.[8] However, in the melanin, 5,6-dihydroxyindole or indole-5,6-quinone units are presumably present, and the exchange rates of these could be very different from that of indole itself; thus the 6-hydroxy-group might activate the 2-position to electrophilic substitution,[9] and the presence of polymeric linkages in the 2- or 3-position might have a profound effect on the rate of exchange in the adjacent position. Indeed, the close parallel between the *R* values of the α- and β-labelled melanins, which have been boiled with acid for the same time, indicates that the rates of exchange of the 2- and 3-indole positions cannot be greatly different, if the initial phase involves only such exchange. This might also suggest comparable rates of coupling at the 2- and 3-positions during the melanogenesis process. Incidentally, the possibility exists that loss of deuterium could occur from the 3-position of 5,6-dihydroxyindole before the latter undergoes oxidation and polymerisation; but we have no reason to think that this loss is appreciable at pH 6·8 or 8.[10]

At the end of this initial very rapid exchange, the melanin is left with deuterium enrichment only in units of types (I) and (II). It is possible that the subsequent extremely slow exchange is the result of cyclisation and/or dehydrogenation of such units. To find the deuterium enrichment after indole- and pyrrole-deuterium had been lost, and to correct for loss owing to the simultaneous slow process, we extrapolated a straight line representing the latter back to zero time, and measured the intercept on the axis representing deuterium enrichment. The *R* value corresponding to this should be representative of units of types (I) and (II) taken together, and should be the same whether obtained from the α- or β-labelled precursor; the observed values were 0·38 and 0·37, respectively.

We also boiled the melanins derived from (±)-dihydroxy-[2-^{2}H]phenylalanine, -[5-^{2}H]phenylalanine, and -[6-^{2}H]phenylalanine with acid for varying periods, when loss of part of the deuterium again occurred.

In the case of enzymic experiments, the melanin initially precipitated contains protein derived from the enzyme, and therefore the found value of *R* must be expected to be too low. After long boiling with acid, the protein is lost;[1] deuterium retentions were then not greatly different from the corresponding values

[7] H. S. Mason, in 'Pigment Cell Biology,' ed. M. Gordon, Academic Press, New York, 1959, p. 582.

[8] R. L. Hinman and C. P. Bauman, *J. Org. Chem.*, 1964, **29**, 2437; M. Koizumi and T. Titani, *Bull. Chem. Soc. Japan*, 1938, **13**, 307.

[9] N. C. Robson and G. A. Swan, in 'Symposium on Structure and Control of the Melanocyte,' Springer, Berlin, 1966, p. 155.

[10] S. N. Mishra and G. A. Swan, *J. Chem. Soc.* (*C*), 1967, 1431.

on autoxidative melanins which had been boiled with acid for similar periods. We see nothing in our results to suggest a fundamental difference in structure between autoxidative and enzymic dopa-melanin.

Table 1 shows that whilst there is some divergence between the values of R found for melanins which were similarly prepared and treated, but which differed in the extent of deuteriation nevertheless on the whole values found with nearly 100% deuteriation tend to be slightly higher than those with tracer concentration. Although values obtained with tracer concentrations are slightly higher in a few experiments, particularly those with the 6-labelled precursor, in the latter case the retention of deuterium is low, so the measured values are low (0·07—0·22%); the error involved in such measurements is greater than in cases where a higher retention occurs. If an isotope effect occurred in the dehydrogenation of dopachrome to give indole-5,6-quinone, this would be expected to favour the retention in the melanin of deuterium rather than hydrogen in experiments involving tracer concentration, and so the value of R found by 100% labelling should be lower than that with tracer concentration. Our results suggest that an isotope effect cannot have a very great influence on the results. However, in general, the results from the nearly 100% labelling experiments will be used in the following calculations. It is also assumed that decarboxylation and dehydrogenation of

TABLE 1

Conversion of specifically deuteriated (±)-3,4-dihydroxyphenylalanine into melanins

Sample no.	Melanin preparation	Method of measurement *	Position of label	Enrichment of ^2H (atom %) Precursor	Melanin	R	Precursor	Melanin	R
1	Autoxidative, wash H_2O †	m.s.	α	0·69	0·52	0·75			
2	Autoxidative, wash H_2O	t.c.	α				7·2	7·0	0·97
1	Autoxidative, wash 0·01N-HCl †	m.s.	α	0·69	0·52	0·75			
1	Autoxidative, shake 2N-HCl, 0·5 hr.†	m.s.	α	0·69	0·51	0·74			
1	Autoxidative, reflux 2N-HCl, 8 hr.†	m.s.	α	0·69	0·24	0·35			
3	Autoxidative, reflux 2N-HCl, 8 hr.	m.s.	α	3·35	1·25	0·37			
2	Autoxidative, reflux 2N-HCl, 8 hr.	t.c.	α				7·2	3·5	0·49
4	Enzymic, wash H_2O	t.c.	α				7·2	4·9	0·68
5	Enzymic, reflux 2N-HCl, 8 hr.	m.s.	α	3·35	1·1	0·33			
4	Enzymic, reflux 2N-HCl, 8 hr.	t.c.	α				7·2	3·1	0·43
6	Autoxidative, wash 0·01N-HCl †	m.s.	β	0·48	0·36	0·75			
7	Autoxidative, wash H_2O	t.c.	β				16·2	13·3	0·82
6	Autoxidative, shake 2N-HCl, 0·5 hr.†	m.s.	β	0·48	0·34	0·71			
6	Autoxidative, reflux 2N-HCl, 8 hr.†	m.s.	β	0·48	0·17	0·35			
7	Autoxidative, reflux 2N-HCl, 8 hr.	t.c.	β				16·2	3·6	0·22
6	Autoxidative, reflux 2N-HCl, 24 hr.†	m.s.	β	0·48	0·15	0·30			
8	Autoxidative (catalase), wash 0·1N-HCl	m.s.	β	0·70	0·54	0·77			
9	Autoxidative (catalase), wash 0·1N-HCl	m.s.	β	1·13	0·83	0·73			
10	Enzymic, wash H_2O	t.c.	β				16·2	11·4	0·7
11	Enzymic, reflux 2N-HCl, 8 hr.	m.s.	β	3·13	0·92	0·29			
10	Enzymic, reflux 2N-HCl, 8 hr.	t.c.	β				16·2	4·8	0·3
12	Enzymic (catalase), reflux 2N-HCl, 24 hr.‡	m.s.	β	1·13	0·22	0·20			
13	Autoxidative, wash 0·01N-HCl †	m.s.	2	1·22	0·82	0·67			
14	Autoxidative, wash 0·01N-HCl †	m.s.	2				7·6	4·7	0·62
14	Autoxidative, shake 2N-HCl †	m.s.	2				7·6	4·4	0·58
14	Autoxidative, reflux 2N-HCl, 2 hr.†	m.s.	2				7·6	2·2	0·29
13	Autoxidative, reflux 2N-HCl, 4 hr.†	m.s.	2	1·22	0·28	0·23			
13	Autoxidative, reflux 2N-HCl, 48 hr.†	m.s.	2	1·22	0·14	0·11			
14	Autoxidative, reflux 2N-HCl, 48 hr.†	m.s.	2				7·6	0·95	0·12
15	Enzymic, wash 0·01N-HCl †‡	m.s.	2				7·8	4·1	0·53
15	Enzymic, reflux 2N-HCl, 24 hr.†‡	m.s.	2				7·8	1·45	0·49
16	Autoxidative, wash 0·01N-HCl †	m.s.	5	1·42	1·07	0·75			
17	Autoxidative, wash 0·01N-HCl †	m.s.	5				8·0	5·8	0·73
16	Autoxidative, reflux 2N-HCl, 2 hr.†	m.s.	5	1·42	0·53	0·37			
16	Autoxidative, reflux 2N-HCl, 8 hr.†	m.s.	5	1·42	0·33	0·23			
16	Autoxidative, reflux 2N-HCl, 48 hr.†	m.s.	5	1·42	0·17	0·12			
17	Autoxidative, reflux 2N-HCl, 48 hr.†	m.s.	5				8·0	1·3	0·16
18	Enzymic, wash 0·01N-HCl †‡	m.s.	5				8·1	5·2	0·64
18	Enzymic, reflux 2N-HCl, 24 hr.†‡	m.s.	5				8·1	1·5	0·19
19	Autoxidative, wash 0·01N-HCl †	m.s.	6	1·00	0·22	0·22			
20	Autoxidative, wash 0·01N-HCl †	m.s.	6				7·0	1·4	0·20
19	Autoxidative, shake 2N-HCl, 5 hr.†	m.s.	6	1·00	0·20	0·20			
20	Autoxidative, shake 2N-HCl, 5 hr.†	m.s.	6				7·0	1·17	0·17
19	Autoxidative, reflux 2N-HCl, 2 hr.†	m.s.	6	1·00	0·14	0·14			
20	Autoxidative, reflux 2N-HCl, 2 hr.†	m.s.	6				7·0	0·79	0·15
19	Autoxidative, reflux 2N-HCl, 48 hr.†	m.s.	6	1·00	0·07	0·07			
20	Autoxidative, reflux 2N-HCl, 48 hr.†	m.s.	6				7·0	0·35	0·05
21	Enzymic, wash 0·01N-HCl †‡	m.s.	6				6·8	1·15	0·17
21	Enzymic, reflux 2N-HCl, 24 hr.†‡	m.s.	6				6·8	0·51	0·08

* m.s. mass spectrometric; t.c. thermal conductivity. † and ‡ see Experimental section.

dopachrome occur by a concerted mechanism, so that the deuterium of the α-labelled dopa will be retained in the resulting indole-5,6-quinone.

If the structure[1] already discussed is assumed for autoxidative dopa-melanin, the melanin formed from (±)-3,4-dihydroxyphenyl[α-^{2}H]alanine, after boiling with acid, should contain 0·2 atom % of deuterium per unit ($C_{7\cdot9}H_{5\cdot55}NO_{3\cdot05}$ on the assumption that the boiling with acid did not bring about any change in chemical composition), so that

$$R = \left(\frac{0\cdot2}{5\cdot55}\right)\Big/\left(\frac{1}{11}\right) = 0\cdot396 \quad \text{(Found: } R = 0\cdot38\text{)}.$$

Similarly, for the melanin from (±)-3,4-dihydroxyphenyl[β-^{2}H]alanine, after boiling with acid,

$$R = \left(\frac{0\cdot4}{5\cdot55}\right)\Big/\left(\frac{2}{11}\right) = 0\cdot396 \quad \text{(Found: } R = 0\cdot37\text{)}.$$

Suppose that units (I), (II), and (IV) are linked in the way shown, and that a fraction a of units (III) are linked in the 2-position of the indole ring; taking the value of R for the melanin from (±)-3,4-dihydroxyphenyl[α-^{2}H]alanine before acid treatment as 0·97, then

$$11[0\cdot1 + 0\cdot1 + (0\cdot65 - a)]/5\cdot55 = 0\cdot97,$$
$$i.e.\ a = 0\cdot36$$

Suppose that a fraction b of units (III) are linked in the 3-position of the indole ring; taking the value of R for the melanin from (±)-3,4-dihydroxyphenyl-[β-$^2H_2$]alanine before acid treatment as 0·82, then

$$11[0\cdot2 + 0\cdot2 + (0\cdot65 - b) + 0\cdot15]/2 \times 5\cdot55 = 0\cdot82,$$
$$i.e.\ b = 0\cdot37$$

Similarly, supposing that fractions c and d of units (III) are linked in the 4- and 7-positions of the indole ring, respectively; taking the values of R for the melanins from (±)-3,4-dihydroxy-[2-^{2}H]phenylalanine and -[5-^{2}H]phenylalanine as 0·62 and 0·73, respectively, then c and d are 0·34 and 0·28 respectively.

The value of R for the melanin from (±)-3,4-dihydroxy[6-^{2}H]phenylalanine should be 0·20 (Found: 0·20). We have no direct evidence to suggest that units of type (I) must be linked through the 2- and 5-positions of the benzene ring, as shown, rather than through the 6-position. There is also the possibility that dopa-quinone could cyclise through its 2- rather than its 6-position; again we have no direct evidence on this point.

If one then adds up the number of polymeric links to each average polymer unit, accepting the above values, one obtains a result of 2·05, which implies that the majority of units are linked to two other units. In view of the errors involved in estimating the figure of 2·05, this value does not differ significantly from 2·00. It is therefore not possible to make any statement concerning the possible presence of polymer units with three linkages.

Linkages in the main type of unit (III) appear to be fairly evenly shared between the 2-, 3-, 4-, and 7-positions, *i.e.* 0·36, 0·37, 0·34, and 0·28, respectively. Although no high degree of accuracy can be claimed for the proportions of different units and linkages found in the melanin, it seems impossible to escape the conclusion that the autoxidative dopa-melanin, prepared in the way we have described, is an irregular polymer, containing a number of different types of units, linked in various ways. As we have pointed out,[1] it is probable that units other than those already represented are also present, to a lesser extent. It also seems likely that enzymic dopa-melanin, prepared *in vitro* in the way we have described is not greatly different structurally from the autoxidative melanin.

Kirby and Ogunkoya[11] have also investigated the structure of melanin in essentially the same manner, although the details of their work are different, and it is therefore important to compare the methods used. They used tritium instead of deuterium, so a considerable isotope effect might occur. They prepared their melanins by oxidation of a mixture of the specifically tritiated (±)-3,4-dihydroxyphenylalanine with (±)-3,4-dihydroxyphenyl[α-^{14}C]alanine, so that through measurement of ^{3}H and ^{14}C activities separately, the fraction of tritium incorporated into the melanin could be measured. This method has advantages over ours in that the presence of protein in the melanin would not affect the results, the degree of hydration of the melanin would be of no consequence, and it is not necessary to know the composition of the melanin. Kirby and Ogunkoya carried out their experiments in the presence of tyrosinase and catalase in unspecified concentration, and used oxygen (whereas we used air) as oxidising agent, so it is impossible to relate one set of results exactly to the other. We carried out few experiments in the presence of catalase, but in these the catalase did not appear to have any great effect on the retention of deuterium in the melanin. Table 2 shows a comparison of Kirby and Ogunkoya's results with ours on autoxidative melanin, expressed as fractional retention of deuterium in the manner of those authors.

TABLE 2

Comparison of the results obtained by Kirby and Ogunkoya and the present authors

Position of label in dopa	α	β	2	5	6
Fractional retention of ^{3}H (K. and O.)	0·52	0·54	0·37	0·42	0·13
Fractional retention of ^{2}H (this paper)	0·49	0·41	0·31	0·37	0·1

EXPERIMENTAL

Part of the polyphenoloxidase used in this work was extracted from mushrooms as described earlier.[12] However, at one period we were unable to obtain mushrooms which

[11] G. W. Kirby and L. Ogunkoya, *Chem. Comm.*, 1965, 546.
[12] F. Binns, R. F. Chapman, N. C. Robson, G. A. Swan, and A. Waggott, *J. Chem. Soc.* (*C*), 1970, 1128.

gave a satisfactory yield of enzyme. We therefore used a commercial preparation of mushroom tyrosinase, which we purified by use of a DEAE cellulose column,[12] but the enzyme activity of the product was then only 400 units/mg. of protein. This latter enzyme (2700 catecholase units/100 mg. of precursor) was used in experiments designated in Table 1 by a double dagger. In all other enzymic experiments, the enzyme had an activity of *ca.* 1000 units/mg. of protein, and *ca.* 10,000 units of it were used for every 100 mg. of precursor. The melanins were prepared as described earlier,[12] except that in some cases (designated by a dagger in Table 1), they were precipitated by the addition of saturated sodium chloride, then washed with 0·01N-hydrochloric acid (unless stated otherwise).

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