664. *Formation of Tyrosine Melanin. Part III.* The Use of Carboxyl-labelled Tyrosine and Dihydroxyphenylalanine in Melanin Formation.*

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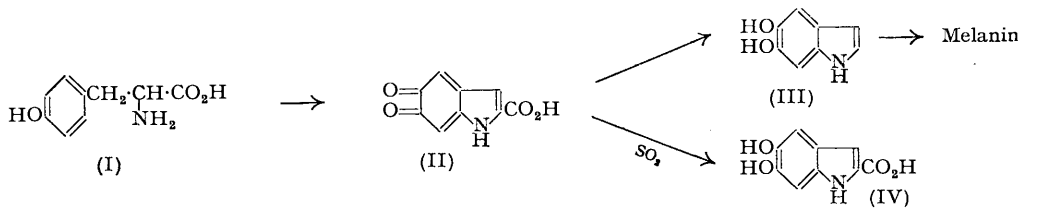
DL-[*carboxy*-^{14}C]-Tyrosine and 3 : 4-dihydroxyphenyl-DL-[*carboxy*-^{14}C]-alanine have been synthesised and used to study melanin formation. It was found that nearly half of the carbon dioxide evolved during the reaction arises from carbon atoms of the amino-acid molecule other than the carboxyl group. Moreover, 2-(3 : 4-dihydroxyphenyl)ethylamine and 5 : 6-dihydroxyindole evolved carbon dioxide during their ærobic conversion into melanin. It was also found that the melanin obtained from the amino-acids retains some of the original carboxyl group.

Current theories concerning the structure of melanin involve polymerisation of indole-5 : 6-quinone. Beer, Clarke, Khorana, and Robertson (*J.*, 1948, 2223) synthesised 5 : 6-dihydroxyindole (III) and 5 : 6-dihydroxyindole-2-carboxylic acid (IV) and found the

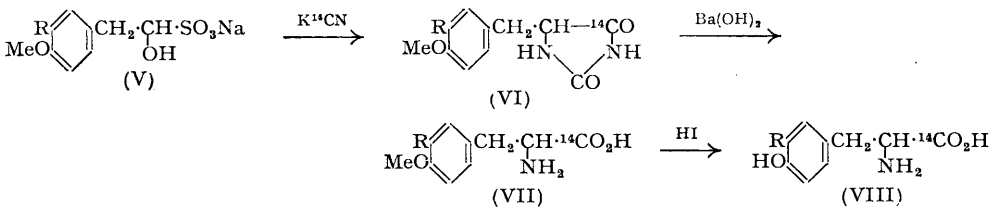


latter, unlike the former, to be stable in alkaline solution. These results imply that tyrosine (I) cyclises and is oxidised to the *o*-quinone (II) which is decarboxylated and

* Part II, *J.*, 1950, 1795.

rearranged to form the indole (III). Raper (*Biochem. J.*, 1927, **21**, 89) prevented decarboxylation by the use of sulphurous acid and so isolated 5 : 6-dimethoxyindole-2-carboxylic acid (cf. IV). Further evidence for this decarboxylation was obtained by Mason (*J. Biol. Chem.*, 1949, **180**, 235) who investigated quantitatively the amount of carbon dioxide evolved during oxidation of 3 : 4-dihydroxyphenylalanine.

We have found that the amount of carbon dioxide evolved during ærobic oxidation of a solution of 3 : 4-dihydroxyphenyl-DL-alanine at pH 8 is equal to, or even greater than, that which could theoretically result from the complete decarboxylation of the amino-acid, but that the yield of melanin is only 60% of the weight of the amino-acid employed.



(V) (VI) (VII) (VIII)

We have, therefore, prepared DL-[*carboxy*-^{14}C]tyrosine and 3 : 4-dihydroxyphenyl-DL-[*carboxy*-^{14}C]alanine and used these amino-acids to investigate the oxidation. Trial experiments showed that the behaviour of DL-amino-acids in melanin formation was little different from that of their L-forms. In our hands, Loftfield's tyrosine synthesis (*J. Amer. Chem. Soc.*, 1950, **72**, 2499) afforded low yields of an impure product, which could not be purified without considerable loss, and we found it desirable to hydrolyse the intermediate hydantoin (VI; R = H) with barium hydroxide and demethylate the resulting *p*-methoxyphenylalanine (VII; R = H) with concentrated hydrochloric acid. The labelled dihydroxyphenylalanine (VIII; R = OH) was similarly prepared from 3 : 4-dimethoxyphenylacetaldehyde bisulphite compound (V; R = MeO) but the final demethylation was brought about by hydriodic acid. Very pure amino-acids were thus obtained.

Experimental

Combustion of Samples for Isotope Analyses.—Samples (in duplicate) were converted into carbon dioxide by conventional combustion methods. The carbon dioxide was absorbed in barium hydroxide solution, and the barium carbonate separated and washed by centrifugation under ether.

Radioactivity Determinations.—These were carried out on samples of "infinite thickness," approximately 0·2 cm.^{2} in area, with an end-window Geiger counter, the background of which was 9—10 counts/minute. A sample containing 10^{-2} μc of ^{14}C per mg. of substance gave approx. 1300 counts/minute. Sufficient counts to give a standard error of less than 2% were taken; but slight variation in disc size introduced an additional error of ±5%. All values quoted are corrected for background.

Tyrosinase was prepared by the procedure of Mallette, Lewis, Ames, Nelson, and Dawson (*Arch. Biochem.*, 1948, **16**, 283).

5 : 6-Dihydroxyindole was prepared by the method of Beer, Clarke, Khorana, and Robertson (*loc. cit.*).

DL-[carboxy-^{14}C]*Tyrosine.*—A mixture of *p*-methoxyphenylacetaldehyde sodium bisulphite (600 mg.), ammonium carbonate (600 mg.), potassium [^{14}C]cyanide (3·2 mg.; 50 μc), potassium [^{12}C]cyanide (130 mg.), and aqueous ethanol (3 ml.; 50% v/v) was heated in a sealed tube for 4 hours at 100°. The mixture was diluted with water (3 ml.), heated at 105—110° for ½ hour, and cooled overnight. 5-*p*-Methoxybenzylhydantoin was filtered and washed with a little water. This hydantoin (247 mg.), m. p. 175—176°, recrystallised barium hydroxide (5 g.), and water (10 ml.), were refluxed for 8 hours. The warm solution was diluted with water (5 ml.), neutralised to methyl-red with sulphuric acid (6N), cooled, and filtered, the barium sulphate washed with hot water (4 × 10 ml.), and the solution evaporated to 2 ml. under reduced pressure. After 12 hours at 0° the *p*-methoxyphenylalanine was filtered off, washed with alcohol, and dried. This product (190 mg.), m. p. 245—250°, DL-tyrosine (60 mg.), and concentrated hydrochloric acid (2 ml.) were heated together in a sealed tube for 1 hour at 120°. The contents were then diluted with water (7 ml.). The boiling solution was adjusted to pH 6 with ammonium hydroxide

(*d* 0·88), treated with charcoal, filtered, and cooled. The product (140 mg.) was filtered off and recrystallised with carrier DL-tyrosine (40 mg.) from water, yielding DL-[*carboxy*-^{14}C]tyrosine (140 mg.), m. p. 312—315°, specific radioactivity 83 μc/g. (radiochemical yield, 23%) (Found : C, 59·4; H, 6·3. Calc. for $C_9H_{11}O_3N$: C, 59·7; H, 6·1%). A sample was decarboxylated by treatment with ninhydrin (Van Slyke, MacFadyen, and Hamilton, *J. Biol. Chem.*, 1941, **141**, 671) as a check on the distribution of ^{14}C in the molecule and the reliability of the combustion procedure (see Table 1). We were, however, unable to apply this method to 3 : 4-dihydroxyphenylalanine.

3 : 4-*Dihydroxyphenyl*-DL-[carboxy-^{14}C]*alanine*.—A solution of ethyl chloroacetate (37 g.), veratraldehyde (50 g.), and anhydrous ether (70 ml.) was added, during 1 hour with stirring, to a solution of sodium (7 g.) in ethanol (50 ml.) and methanol (50 ml.) cooled in ice–salt. The mixture was then allowed to warm to room temperature, poured into water (650 ml.), and extracted with ether, and the ethereal extract was washed successively with water, sodium hydrogen carbonate solution (3%), and water. The solution was then diluted with a further 400 ml. of ether, cooled to 5°, and poured into an ice-cold solution of sodium (6·9 g.) in methanol (100 ml.) and water (6 ml.). After 10 minutes the sodium salt was filtered off, made into a paste with ether (30 ml.) and methanol (6 ml.) and again filtered off. The salt was then mixed with ethanol (20 ml.) and water (15 ml.) and gradually added to stirred boiling aqueous sodium hydrogen sulphite (250 ml.; *d* 1·29). The solution was kept overnight at 0° and the 3 : 4-dimethoxyphenylacetaldehyde bisulphite compound (40 g.) then filtered off. A mixture of the above (600 mg.), ammonium carbonate (600 mg.), potassium [^{14}C]cyanide (2·6 mg.; 41 μc), potassium [^{12}C]cyanide (130 mg.), and aqueous ethanol (3 ml.; 50%) was heated in a sealed tube for 4 hours at 100°. The hydantoin (VI; R = OMe) (250 mg.), m. p. 156—160°, isolated as above, was refluxed for 8 hours with recrystallised barium hydroxide (8 g.) and water (10 ml.). The warm solution was diluted with water (5 ml.), neutralised to methyl-red with sulphuric acid (6N), cooled, and filtered, the precipitate washed with hot water (4 × 10 ml.), and the solution evaporated to 2 ml. Ethanol (5 ml.) was then added and the solution kept for 12 hours at 0°. 3 : 4-Dimethoxyphenyl[*carboxy*-^{14}C]alanine was filtered off, washed with ethanol, and dried. The product (160 mg.), m. p. 240—245°, 3 : 4-dihydroxy-DL-phenylalanine (60 mg.), redistilled hydriodic acid (2·5 ml.), and red phosphorus (10 mg.) were heated in an evacuated Pyrex tube for 1 hour at 110°. The contents were filtered, the phosphorus washed with water, and the resulting solution evaporated to dryness under reduced pressure (nitrogen). The residue was dissolved in water (3 ml.), and the solution adjusted to pH 6 with ammonia (*d* 0·88) and evaporated to dryness. The residue was then dissolved in water (3 ml.) and diluted with ethanol (20 ml.), and the solution was distilled under reduced pressure in an atmosphere of nitrogen until precipitation occurred, whereupon it was cooled at 0° for 1 hour, and filtered. This procedure was repeated until no more 3 : 4-dihydroxyphenylalanine was obtained. This amino-acid (124 mg.), together with carrier 3 : 4-dihydroxyphenyl-DL-alanine (80 mg.), was recrystallised from water (3 ml.) saturated with sulphur dioxide (charcoal), to give the product (103 mg.), m. p. 286—288°, specific radioactivity 33 μc/g. (radiochemical yield, 8·5%) (Found : C, 54·3; H, 6·0. Calc. for $C_9H_{11}O_4N$: C, 54·8; H, 5·8%).

2-(3 : 4-*Dihydroxyphenyl*)*ethylamine Hydrochloride*.—3 : 4-Dimethoxyphenylethylamine (1 g.) (Bide and Wilkinson, *Chem. and Ind.*, 1945, **64**, 89) and concentrated hydrochloric acid (8 ml.) were heated for 2 hours at 140—160°. The whole was taken to dryness, the residue dissolved in a minimum amount of warm ethanol, and ether was added until the hydrochloride was precipitated. The solution was cooled and filtered and the 2-(3 : 4-dihydroxyphenyl)ethylamine hydrochloride recrystallised from hydrochloric acid (1 : 1) (yield, 0·5 g.; m. p. 247°) (Found : C, 50·5; H, 6·2. Calc. for $C_8H_{12}O_2NCl$: C, 50·7; H, 6·3%).

Oxidation Procedure.—A slow stream of carbon dioxide-free oxygen was passed first through a test bottle containing barium hydroxide and then into a two-necked flask, immersed in a thermostat at 20° and containing freshly boiled distilled water (10 ml.) and phosphate buffer solution of pH 8 [in the tyrosine experiment dihydroxyphenylalanine (5 ml.), dihydroxyindole (15 ml.) and dihydroxyphenylethylamine hydrochloride (30 ml.) were used]. A solution (1 ml.) of tyrosinase was also added in the tyrosine experiment. The flask was equipped with dropping funnel and inlet and outlet tubes. The oxygen was then passed through a series of four bubblers, the first three empty and the fourth containing 2N-sodium hydroxide.

The apparatus was swept out for 4 hours. Carbon dioxide-free sodium hydroxide solution (1·5 and 0·5 ml. respectively) was then placed in the second and third bubblers, and a solution of the compound under test in carbon dioxide-free distilled water (for tyrosine, 90 ml.; for dihydroxyphenylalanine, dihydroxyindole, and dihydroxyphenylethylamine hydrochloride,

50 ml.) added from the dropping funnel. The experiment was continued for 3 days with an oxygen flow of approx. 25 bubbles (1·2 c.c.) per minute. The melanin was then completely precipitated by 2N-hydrochloric acid (4 ml.), and the apparatus swept out with oxygen for a further 4 hours. The absorption tubes were washed out with carbon dioxide-free water, and the alkaline solution protected from the atmosphere with a layer of ether and treated with 4N-ammonium chloride (0·25 ml.), excess of barium hydroxide added, and the precipitated barium carbonate centrifuged off and, after one washing with saturated barium chloride solution and two with carbon dioxide-free water, dried at 140° for 1½ hours and weighed (radioactive experiments showed that not more than about 1% of the carbon dioxide escaped absorption). The melanin was centrifuged, washed three times with water, and dried to constant weight over concentrated sulphuric acid *in vacuo*, and the melanin (except the sample analysed) was then refluxed for 24 hours with hydrochloric acid (2N), and then water, as described by Raper and Wormall (*Biochem. J.*, 1925, **19**, 90), then centrifuged and washed, and a further sample analysed. The procedure was once more repeated. The weights of melanin quoted in Tables 1 and 2 are corrected for any inorganic

TABLE 1.

Compound	Weight (mg.)	Radioactivity (as $Ba^{14}CO_3$) (counts/min.)
Tyrosine	59·8	353
$BaCO_3$ resulting from melanin formation	41·0	1730
$BaCO_3$ formed by action of ninhydrin on tyrosine	—	3004
Melanin	21·0	57
Melanin (refluxed for 24 hrs. with 2N-HCl)	—	23
Melanin (refluxed for 48 hrs. with 2N-HCl)	—	19
Water-soluble residues (containing 20% of C)	92·0	395

$$\frac{Ba^{14}CO_3 \text{ from melanin formation}}{Ba^{14}CO_3 \text{ from combustion of tyrosine}} = 4{\cdot}9$$

TABLE 2.

Compound	Weight (mg.)	Radioactivity (as $Ba^{14}CO_3$) (counts/min.)
Dihydroxyphenylalanine	62·5	475
$BaCO_3$ resulting from melanin formation	68·0	2410
Melanin	37·4	83
Melanin (refluxed 24 hrs. with 2N-HCl	—	61
Melanin (refluxed 48 hrs. with 2N-HCl)	—	15
Water soluble residues (containing 4% of C)	100	260

$$\frac{Ba^{14}CO_3 \text{ from melanin formation}}{Ba^{14}CO_3 \text{ from combustion of dihydroxyphenylalanine}} = 5{\cdot}1.$$

residue (less than 3%), and the weights of barium carbonate evolved in melanin formation corrected for any extraneous barium carbonate (less than 2%).

The liquors obtained from centrifuging the melanin were evaporated to dryness, dried to constant weight *in vacuo* over concentrated sulphuric acid, and analysed for carbon, and 20 mg. were burnt for isotope analysis.

If the evolved carbon dioxide arose from the carboxyl group of the amino-acid, then the ratios given in the Tables should both be 9. It is therefore clear that nearly half of this carbon dioxide (subsequently referred to as " non-carboxyl carbon dioxide ") arises from one or more of the other eight carbon atoms of the amino-acid molecule. This was further substantiated by the ærobic oxidation of the carboxyl-free 2-(3 : 4-dihydroxyphenyl)ethylamine and 5 : 6-dihydroxyindole at pH 8, both of which led to the production of melanin with the evolution of carbon dioxide (Table 3).

TABLE 3.

	2-(3 : 4-Dihydroxyphenyl)-ethylamine	5 : 6-Dihydroxy-indole
Compound (mg.)	80·0	47·0
$BaCO_3$ resulting from melanin formation (mg.)	21·3	30·5
Melanin (mg.)	31·2	43·0

At present we have no proof that the amino-acid, dihydroxyphenylethylamine, or dihydroxyindole residues which evolve this non-carboxyl carbon dioxide are in fact incorporated into the melanin molecule. It may, however, be significant that the amounts of carbon dioxide evolved by dihydroxyindole, and the amounts of non-carboxyl carbon dioxide evolved by the two amino-

acids, appear to bear some relation to the weight of melanin produced. If it should emerge that this non-carboxyl carbon dioxide is really evolved from residues which are incorporated into the melanin, then a ring contraction or a ring fission would be implied. The oxidative fission theory (*loc. cit.*) was rejected on the basis that hydroxylamine did not prevet pigment formation. Later work has shown that a higher concentration of hydroxylamine does inhibit pigment formation. Perhaps, however, our results mean simply that a small proportion of the amino-acid is being oxidised completely to carbon dioxide. We are therefore now attempting to settle this point by synthesis of the amino-acids or (preferably) the dihydroxyphenylethylamine labelled at specific positions in the benzene nucleus and at the α- or β-position of the side chain, for use in a further study of melanin formation. One can picture the ring contraction occuring by a benzilic acid type of rearrangement (*e.g.*, on the quinone derived from 5 : 6-dihydroxyindole) or by oxidative fission (Clemo and Duxbury, *J.*, 1950, 1795).

It is also interesting that some of the carboxyl groups are apparently retained in the melanin (either combined in the molecule or adsorbed as amino-acid). This should be borne in mind when considering analytical data on melanins prepared from amino-acids.

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