A Study of the Evolution of Carbon Dioxide during Melanin Formation, including the Use of 2-(3 : 4-*Dihydroxyphenyl*)[1-^{14}C]- *and* 2-(3 : 4-*Dihydroxyphenyl*)[2-^{14}C]-*ethylamine.*

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[Reprint Order No. 4723.]

2-(3 : 4-Dihydroxyphenyl)[1-^{14}C]- and 2-(3 : 4-dihydroxyphenyl)[2-^{14}C]-ethylamine hydrochloride have been synthesised and used to study melanin formation. It was found that about 96% of the carbon dioxide evolved during the reaction arises from the breakdown of the benzene nucleus. A study of the effects of catalase and of hydrogen peroxide on the formation of melanin from 2-(3 : 4-dihydroxyphenyl)ethylamine and from 5 : 6-dihydroxyindole suggested that at least half of the evolved carbon dioxide arises as a result of a secondary reaction due to attack by hydrogen peroxide produced during the primary oxidation.

It has been shown (Clemo, Duxbury, and Swan, *J.*, 1952, 3464) that carbon dioxide is evolved during the conversion of 2-(3 : 4-dihydroxyphenyl)ethylamine and of 5 : 6-dihydroxyindole into melanin by the action of oxygen at pH 8. We now describe further experiments attempting to discover the significance (if any) of this carbon dioxide in the structure and formation of melanin.

We have prepared 2-(3 : 4-dihydroxyphenyl)ethylamine hydrochloride labelled with ^{14}C in the α- and the β-position of the side chain, severally. 3 : 4-Dimethoxybenzaldehyde was condensed with nitromethane, the resulting nitrostyrene derivative reduced with lithium aluminium hydride, and the product demethylated to give 2-(3 : 4-dihydroxyphenyl)ethylamine. The use of nitro[^{14}C]methane gave the α-labelled compound, 2-(3 : 4-dihydroxyphenyl)[1-^{14}C]ethylamine hydrochloride; whereas the β-labelled derivative, 2-(3 : 4-dihydroxyphenyl)[2-^{14}C]ethylamine hydrochloride was obtained by using 3 : 4-dimethoxybenz[^{14}C]aldehyde. The results obtained when these materials were converted into melanin in duplicate experiments (Table 1) show that the evolved carbon dioxide was of very low specific activity in both cases, indicating that only about 1·9 and 2·3% of it was derived from the α- or β-carbon atom respectively. (If it were all derived from the labelled atom, the specific activity of the evolved carbon dioxide should be eight times as great as that of the carbon dioxide produced by combustion of the starting material.) This means, therefore, that the evolved carbon dixoide has its main origin (96%) in the breakdown of the benzene nucleus, although this does not, of course, prove that the residues so oxidised are necessarily incorporated in or into the melanin. If such residues are in fact incorporated, then the specific activity of the carbon dioxide formed by combustion of the melanin should be slightly higher than that by combustion of the original dihydroxyphenylethylamine, but the expected difference is only of the order of the possible experimental error in the radioactivity measurements. We therefore defer further discussion of this aspect until we have available 2-(3 : 4-dihydroxyphenyl)ethylamine labelled in specific positions of the benzene nucleus.

TABLE 1.

	Weight (mg.)		Radioactivity (as $BaCO_3$) (counts/min.)	
	(1)	(2)	(1)	(2)
2-(3 : 4-Dihydroxyphenyl)[1-^{14}C]ethylamine hydrochloride	70·6	79·6	375	378
$BaCO_3$ resulting from melanin formation	20·4	21·2	59	63
Melanin	27·0	28·5	379	380
Melanin (refluxed for 24 hr. with 2N-HCl)	—	—	365	380
2-(3 : 4-Dihydroxyphenyl[2-^{14}C]ethylamine hydrochloride	72·4	74·5	667	667
$BaCO_3$ resulting from melanin formation	24·3	22·6	122	128
Melanin	27·3	26·9	695	684
Melanin (refluxed for 24 hr. with 2N-HCl)	—	—	694	717

The possibility that hydrogen peroxide is formed during the oxidation of catechol to *o*-benzoquinone has been discussed by various authors (Onslow and Robinson, *Biochem. J.*, 1926, **20**, 1138; Platt and Wormall, *ibid.*, 1927, **21**, 29; Wagreich and Nelson, *J. Amer. Chem. Soc.*, 1938, **60**, 1545; Dawson and Ludwig, *ibid.*, p. 1617; cf. also Wieland and Fischer, *Ber.*, 1926, **59**, 1180; Raper, *Biochem. J.*, 1937, **31**, 2155). It seemed to us possible that hydrogen peroxide might be similarly formed during the formation of melanin from tyrosine, 3 : 4-dihydroxyphenylalanine, 2-(3 : 4-dihydroxyphenyl)ethylamine, and 5 : 6-dihydroxyindole; and, if so, it seemed conceivable that this hydrogen peroxide might be concerned in the carbon dioxide evolution. We now present evidence in support of this, the results being summarised in Table 2, which shows the weights of carbon dioxide (as barium carbonate) and melanin formed from 1 mmole of dihydroxyphenylethylamine or dihydroxyindole with or without the addition of catalase or hydrogen peroxide. When 5 : 6-dihydroxyindole was converted into melanin, the addition of catalase to the solution caused a reduction in the amount of carbon dioxide evolved to approximately one-half of the normal quantity. The addition of hydrogen peroxide (10 mmoles per mmole of dihydroxyindole), on the other hand, caused an increase of carbon dioxide evolution to about twice the normal value; and the melanin was then formed in reduced amount, and it was brown, gelatinous, and readily soluble in alkali (it was necessary to wash it with water containing a little hydrochloric acid to lower its solubility). Analytical data on melanin (Table 3) showed a very slight increase in carbon content in the case of catalase addition, and a considerable decrease when hydrogen peroxide was added. The melanin samples formed from dihydroxyindole in absence of catalase were sometimes "electric" in properties; and great difficulty was experienced in obtaining consistent analytical results on these specimens. The yields of melanin were high, except in the hydrogen peroxide experiments.

TABLE 2.

	2-(3 : 4-Dihydroxyphenyl)ethylamine hydrochloride (190 mg., 1 mmole)		5 : 6-dihydroxyindole (149 mg., 1 mmole)	
Solution added	$BaCO_3$ (mg.)	Melanin (mg.)	$BaCO_3$ (mg.)	Melanin (mg.)
Catalase solution (0·3 ml.) ...	29·8	109·4	60·3	156·1
,, (0·03 ml.)	33·7	116·0	—	—
No addition	56·7	70·3	114	153·8
H_2O_2 (1 mmole)	59·2	41·4	152·9	152·1
,, (5 mmoles)	84·5	15·4	—	—
,, (7·5 mmoles)	—	—	204·2	149·9
,, (10 mmoles)	101·8	0	229·8	114·9

From 2-(3 : 4-dihydroxyphenyl)ethylamine, yields of melanin were always much lower (probably owing to side reactions), but the addition of catalase caused a considerable improvement; at the same time, the evolved carbon dioxide was approximately halved in amount. The addition of hydrogen peroxide (1 mmole) reduced the weight of melanin formed by nearly one-half, although the amount of carbon dioxide evolved was not greatly altered and the melanin did not become gelatinous (the percentage of carbon in the melanin was also lowered). When hydrogen peroxide (10 mmoles) was added to a solution of 2-(3 : 4-dihydroxyphenyl)ethylamine at pH 8, the solution remained clear on treatment with oxygen—no melanin was formed, although carbon dioxide was evolved.

TABLE 3.

Addition in melanin formation	Composition * (%) of melanin derived from			
	2-(3 : 4-dihydroxyphenyl)ethylamine		5 : 6-dihydroxyindole	
	C	H	C	H
Catalase	57·2	4·7	57·35	3·05
,, ...	57·15	4·5	57·0	2·8
No addition	56·1	5·0	56·3	3·2
,, ...	56·0	4·9	55·2	3·6
H_2O_2 (1 mmole)	54·85	4·55	53·8	3·65
,, ,, ...	54·25	4·35	54·0	3·35
,, (10 mmoles)	—	—	49·1	3·8
,, ,, ...	—	—	49·05	3·7

* For samples dried to constant weight *in vacuo* over concentrated sulphuric acid.

When melanin which had been formed from dihydroxyphenylethylamine or dihydroxyindole was suspended in a phosphate buffer solution of pH 8 containing hydrogen peroxide (20 moles per molecular unit of melanin, based on the assumption of an arbitrary value of 170 for the molecular weight of the latter) and treated with oxygen, carbon dioxide was evolved and the melanin partly dissolved, forming a brown solution. In one experiment, in which melanin which had been prepared from α-labelled dihydroxyphenylethylamine was similarly treated, the specific activity of the evolved carbon dioxide showed that only about 6% of this carbon dioxide originated from the α-carbon atom (Table 4).

TABLE 4.

Material	Melanin derived from		
	2-(3 : 4-dihydroxyphenyl)[1-^{14}C]ethylamine		5 : 6-dihydroxyindole
	Weight (mg.)	Radioactivity (counts/min. as $BaCO_3$)	Weight (mg.)
Melanin before H_2O_2 treatment ...	21·2	375	19·7
$BaCO_3$ produced	19·8	180	28·0
" Melanin " after treatment	12·8	—	15·0

From the above experiments, we infer that hydrogen peroxide, produced during the aerobic oxidation of 2-(3 : 4-dihydroxyphenyl)ethylamine or 5 : 6-dihydroxyindole brings about an oxidation either of the primarily formed melanin or of one of the intermediate products (probably at the site of the hydroxyl groups of the benzene nucleus) giving rise to carbon dioxide. The striking difference, as regards the weight of melanin formed, between the action of hydrogen peroxide on the two compounds suggests that hydrogen peroxide can decompose one of the intermediates before the 5 : 6-dihydroxyindole stage. At present, we consider this ring fission to be best regarded as a side reaction, rather than directly concerned with the primarv formation of melanin, although its occurrence must be remembered in considering analytical data on melanin. We cannot, therefore, support the recent attempt by Jolles (*Chem. and Ind.*, 1953, 845) to revive Angeli's theory (*Atti R. Accad. Lincei*, 1927, **6**, 87) in which oxidation of 5 : 6-dihydroxyindole to pyrrole-2 : 3-diacetic acid is postulated as an essential feature of the reaction sequence. This is in agreement with the recent work by Cromartie and Harley-Mason (*Chem. and Ind.*, 1953, 972). It should also be noted that, although Panizzi and Nicolaus (*Gazzetta*, 1952, **82**, 435) isolated a pyrroletricarboxylic acid (in very low yield) from the oxidation products of sepia melanin, they were unable to obtain it from tyrosine-melanin.

EXPERIMENTAL

The radioactivity determinations and oxidation experiments were carried out as described by Clemo, Duxbury, and Swan (*loc. cit.*); the volumes of solutions stated there for the oxidation experiments were used for approx. 0·3 mmole of the organic compound.

5 : 6-Dihydroxyindole was prepared by the method of Beer, Clarke, Khorana, and Robertson (*J.*, 1948, 2223), as we found the last stage of Harley-Mason's new method (*J.*, 1953, 200) to be unreliable, *i.e.*, the oxidation of 2-(2-amino-4 : 5-dihydroxyphenyl)ethylamine.

The catalase used was a preparation from ox-liver (Sumner and Dounce, *J. Biol. Chem.*, 1937, **121**, 417) kindly supplied by Dr. J. S. Falconer, Physiology Department, King's College, Newcastle-upon-Tyne. Its activity was determined by the method of von Euler and Josephson (*Annalen*, 1927, **452**, 158). The addition of 0·03 ml. of the catalase solution to a solution of hydrogen peroxide (1 mmole) in a volume of 150 ml., at pH 8 and 0°, brought about decomposition at the initial rate of 0·135 mmole of H_2O_2 per min. As shown in Table 2, the addition of this amount of catalase to a solution of 1 mmole of 2-(3 : 4-dihydroxyphenyl)ethylamine produced nearly the same effect as did ten times this quantity of enzyme.

[^{14}C]Methyl iodide was prepared by the method of Cox, Turner, and Warne (*J.*, 1950, 3167) and converted into nitro[^{14}C]methane essentially as described by Arnstein and Bentley (*J.*, 1951, 2385). 3 : 4-Dimethoxybenz[^{14}C]aldehyde was prepared *via* 3 : 4-dimethoxybenz[^{14}C]oic acid in the way described by Calvin *et al.*, " Isotopic Carbon," Chapman and Hall, London, 1949, pp. 183, 199. It was, however, found desirable to distil the acid chloride before reduction. After the reaction, the xylene solution was filtered, washed with sodium hydrogen carbonate solution, and dried (Na_2SO_4), the xylene was removed, and the crude residue was used in the subsequent condensation with nitromethane, which showed that the yield of aldehyde was 58%, based on the acid chloride.

3 : 4-*Dimethoxy-ω-nitrostyrene*.—A solution of methylamine, prepared from methylamine hydrochloride (20 mg.) and sodium carbonate (25 mg.) in methanol (0·25 ml.) was added to one of nitromethane (0·25 g.) and 3 : 4-dimethoxybenzaldehyde (0·8 g., 15% excess) in methanol (2 ml.), and the mixture was kept for 3 days in the dark. The product (0·7 g.) was collected and washed with methanol; it had m. p. 140°, in agreement with Rosenmund (*Ber.*, 1910, **43**, 3412). These conditions (giving a yield of 82% based on nitromethane) were used for the condensation of 3 : 4-dimethoxybenzaldehyde with nitro[^{14}C]methane. When the nitromethane was in excess (15%), the nitrostyrene was obtained in 87% yield based on the aldehyde; under these conditions, 3 : 4-dimethoxybenz[^{14}C]aldehyde was condensed with nitromethane (cf. Gairaud and Lappin, *J. Org. Chem.*, 1953, **18**, 1).

2-(3 : 4-*Dimethoxyphenyl*)*ethylamine*.—The above nitrostyrene (0·5 g.) was introduced into a solution of lithium aluminium hydride (0·8 g.) in ether (40 ml.) during 20 hr. by the Soxhlet technique. Water, followed by sodium hydroxide solution, was added to the cooled solution, the ethereal layer was separated, and the aqueous layer extracted with ether, with the help of the centrifuge. The ether was removed from the dried (Na_2SO_4) extract, and the residue distilled, giving the amine (0·32 g., 74%) as a colourless liquid, b. p. 120° (bath)/1 mm.

This was demethylated as described by Clemo, Duxbury, and Swan (*loc. cit.*), giving 2-(3 : 4-dihydroxyphenyl)ethylamine hydrochloride (0·24 g.; m. p. 238°). Recrystallisation from hydrochloric acid (1 : 1) afforded 0·19 g. of a product, m. p. 238—239°.

Thanks are offered to King's College Research Fund for grants towards the purchase of equipment, to King's College Council for the award of a Postgraduate Studentship (to D. W.), and to Professor G. R. Clemo, F.R.S., for his interest.

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[*Received, October* 15*th*, 1953.]