Studies related to the Chemistry of Melanins. Part X.[1] Quantitative Assessment of Different Types of Units present in Dopa-melanin

By G. A. Swan* and A. Waggott, Department of Organic Chemistry, The University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU

(±)-3,4-Dihydroxyphenyl[*carboxy*-^{14}C]alanine was converted into melanin, which was oxidised to give pyrrole-2,3,5-tricarboxylic acid, and also decarboxylated. The specific activities of the precursor, the melanin, the acid, the decarboxylated melanin, and the carbon dioxide evolved during the decarboxylation were compared. From the results it was possible to assess approximately the fractions of the polymer units which are (*a*) uncyclised amino-acid units, (*b*) carboxylated indole and indoline units, and (*c*) carboxylated pyrrole units. From the results of methylation experiments the relative proportions of units in the quinonoid and phenolic states were deduced.

EARLIER work [2] in which melanins were prepared from (±)-[*carboxy*-^{14}C]tryosine and (±)-3,4-dihydroxyphenyl[*carboxy*-^{14}C]alanine suggested that approximately one sixth of the polymer units retain a carboxy-group originating from the carboxy-group of the amino-acid. Nicolaus and his collaborators [3] found no significant difference in the yield of either pyrrole-2,3-dicarboxylic acid or pyrrole-2,3,5-tricarboxylic acid formed by the oxidation of dopa-melanin before or after decarboxylation by heat. They concluded that dopa-melanin could not have a carboxy-group in the 2-position of the indole nucleus. The detection of trimethylamine among the oxidation products of methylated dopa-melanin was put forward as evidence for the presence in the polymer of uncyclised amino-acid units.

In the earlier work with radioactive precursors,[2] an enzyme preparation of poor quality was used, and the radioactivity measurements were done on thick layers of barium carbonate. We therefore decided to carry out more extensive research, comparing autoxidative and enzymic experiments, the latter with a large proportion of enzyme of high purity. Moreover, oxygen was used as oxidant in the earlier experiments; but later [4] it was found that, at any rate in the case of 3,4-dihydroxyphenethylamine, the results obtained by the use of air differed from those obtained with oxygen. In the present work air was used. Most methods for the determination of the ^{14}C radioactivity of a melanin require that the sample be burnt. In the first experiments of our new series, the resulting carbon dioxide was absorbed in 'Hyamine-10X' hydroxide, prior to scintillation counting. As, however, we obtained high background counting rates, probably owing to the occurrence of chemiluminescence, we replaced the Hyamine by piperidine–water (1 : 1), in conjunction with a scintillator solution consisting of PPO, POPOP, and naphthalene in dioxan.[5] When this method was used, there sometimes appeared to be inconsistencies in our results (possibly because of carbon dioxide absorption being incomplete), so we used the accurate and sensitive gas-counting method on $^{14}CO_2$ in all the work described in this paper.

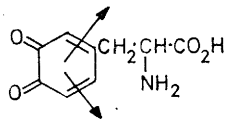
Preliminary experiments, in which quantities of dopa-melanin (*ca.* 1 g.) had been oxidised with hydrogen peroxide once, or smaller amounts (0·3—0·5 g.) had been subjected to three successive oxidations, showed that it was possible to isolate a few mg. of pyrrole-2,3-dicarboxylic acid and of pyrrole-2,3,5-tricarboxylic acid from the degradation products by preparative paper chromatography. Dry-column chromatography [6] on silica did not give satisfactory separation of these acids.

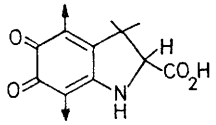
When a sample of (±)-3,4-dihydroxyphenyl[*carboxy*-^{14}C]alanine was burnt, and the resulting carbon dioxide was counted in a particular gas-counter, it gave a counting rate of 729 counts/min./cm. pressure of the carbon dioxide at 20°. For convenience, this will be referred to as a specific activity of 729. When this

[1] Part IX, F. Binns, J. A. G. King, A. Percival, N. C. Robson, and G. A. Swan, *J. Chem. Soc.* (*C*), 1970, 1134.
[2] G. R. Clemo, F. K. Duxbury, and G. A. Swan, *J. Chem. Soc.*, 1952, 3464.
[3] M. Piattelli, E. Fattorusso, S. Magno, and R. A. Nicolaus, *Tetrahedron*, 1962, **18**, 941.
[4] G. A. Swan, *Ann. New York Acad. Sci.*, 1963, **100**, 1005.
[5] N. C. Robson and G. A. Swan, in 'Symposium on Structure and Control of the Melanocyte,' Springer, Berlin, 1966, p. 155.
[6] B. Loev and K. M. Snader, *Chem. and Ind.*, 1965, 15.

particular sample was converted into melanin by autoxidation, the melanin had a specific activity of 160.

The polymer might contain radioactive carboxy-groups in uncyclised amino-acid units (I), 2-carboxyindoline-5,6-quinone units (II), or 2-carboxyindole-5,6-quinone units (III). Variations in the state of oxidation of the units is possible (*e.g.* instead of being quinonoid as shown, they could be diphenolic), but this is of no consequence so far as the discussion in the earlier part of this paper is concerned. There could also be some units derived from types (II) and (III) by oxidative


(I)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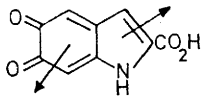
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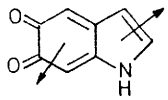

(IV)

fission of the benzene ring; but for the moment these will be disregarded, as it will be shown later that allowing for them does not greatly alter the result of the calculation.

Suppose that a fraction x of the polymer units are of the C_9 type, *i.e.* (I), (II), and (III) together, and that the remainder, *i.e.* $(1 - x)$ are of the C_8 type *e.g.* (IV). Then

$$9x/[9x + 8(1 - x)] = 160/729, \quad i.e.\ x = 0{\cdot}2$$

This would mean that one out of every five polymer units would contain a carboxy-group derived from that of the original amino-acid. Table 1 shows the results of a number of such experiments. The weight percentages of carboxy-group (derived from the amino-acid carboxy-group) in the melanins are based on an average molecular weight of 163 for a polymer unit.

The enzymic experiments were carried out in the presence of a large excess of mushroom polyphenol oxidase (10,000 units/100 mg. of substrate) of high purity (1030 units/mg. of protein). The melanin thus obtained by precipitation with hydrochloric acid contained protein derived from the enzyme, and long boiling with 2N-hydrochloric acid was needed to remove this protein. When such a melanin, which had been boiled for 48 hr., was then boiled with fresh 2N-hydrochloric acid for a further 12 hr., it was not possible to detect the presence of amino-acids in the remaining acid by using ninhydrin and paper chromatography. Unfortunately it is not known what, if any, structural change in the melanin itself is effected by this acid treatment. It does, however, seem reasonable to compare the results of such an experiment with those obtained by similar acid treatment of an autoxidative melanin. In fact, the values obtained after 48 hr. boiling are very close, suggesting similar retentions of carboxy-groups.

In some experiments catalase was also present, but in this case the amount of protein was much smaller

TABLE 1

Preparation of melanin from (±)-3,4-dihydroxyphenyl-[*carboxy*-^{14}C]alanine

Melanin preparation	Specific radioactivity: Pre-cursor	Specific radioactivity: Melanin	Fractional retention of amino-acid carboxy-group in melanin	Amino-acid carboxy-group in melanin (wt. %)
1 Autoxidation	729	160	0·20	5·5
2 Autoxidation	670	152	0·21	5·8
4 Autoxidation	817	174	0·19	5·3
5 Autoxidation, then 48 hr. with 2N-HCl (reflux)	817	135	0·15	4·1
6 Autoxidation, then 16 days with 2N-HCl (reflux)	817	81	0·09	2·5
7 Autoxidation in presence of catalase	817	234	0·26	7·1
8 Enzymic, then 48 hr. with 2N-HCl (reflux)	817	138	0·15	4·2
9 Enzymic, in presence of catalase, then 48 hr. with 2N-HCl (reflux)	817	155	0·17	4·7

in relation to the weight of the melanin than that introduced as polyphenoloxidase, and it could have little effect on the calculations.

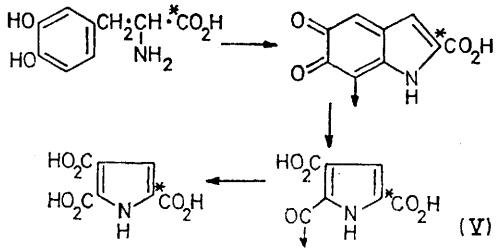
When the melanins were boiled with hydrochloric acid for long periods their radioactivity slowly diminished. Such treatment has been shown to result in the formation of small amounts of pyrrole-2,3,5-tricarboxylic acid,[7] and if this was derived from end groups containing ^{14}C in the polymer, their loss (V) would result in a lowering of the specific activity of the melanin. This explanation is also attractive, in that it could account for certain other findings (mentioned later in this paper); but unfortunately it seems, as will be shown subsequently, that the proportion of the units (V) is too small to account entirely for the observed decrease in radioactivity. It could be that this acid treatment might bring about cyclisation of some hitherto uncyclised units (I). As oxidative cyclisation of dopaquinone under neutral conditions is known to give dopachrome, which then undergoes rearrangement and (to a large extent) accompanied decarboxylation, and as the boiling of our melanins with acid was carried out in the presence of air, this latter process might result in the decarboxylation of units such as (I) and (II), with accompanying lowering of the specific activity of the melanin. Also, if peroxide groups are present in the melanin, these might undergo changes during acid treatment; without evidence of the nature of

[7] F. Binns, R. F. Chapman, N. C. Robson, G. A. Swan, and A. Waggott, *J. Chem. Soc.* (*C*), 1970, 1128.

such peroxides, it is impossible to predict what effect such changes would have on the radioactivity of the melanins.

The presence of catalase during the melanogenesis increased the retention of amino-acid carboxy-group in the melanin, more especially in the autoxidative experiments, where this increase amounted to approximately one quarter. It is probable that hydrogen peroxide, produced during the melanogenesis, could attack α-amino-acid groups present in the polymer, *i.e.* units (I) and (II); and that the presence of catalase could reduce the extent of this attack by decomposition of hydrogen peroxide. It is true that attack on the benzene rings of indole units by hydrogen peroxide should result in loss of non-radioactive carbon from the polymer, and the effect of catalase here should work in the opposite direction.[8] However, this second effect might well be smaller than the first.

Pyrrole-2,3,5-tricarboxylic acid was isolated from the products of oxidation by hydrogen peroxide of autoxidative melanin prepared from (±)-3,4-dihydroxyphenyl[*carboxy*-^{14}C]alanine of specific activity 670, and had specific activity 376. If all the carboxy-groups in the 5-position of the acid arose from the radioactive carboxy-groups of the 3,4-dihydroxyphenyl[*carboxy*-^{14}C]alanine, as shown in Scheme 1, then the specific

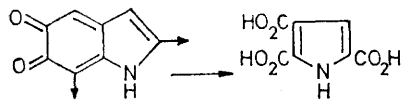


SCHEME 1

activity (y) expected of the pyrrole-2,3,5-tricarboxylic acid should be given by the equation:

$$9/7 = y/670, \quad i.e.\ y = 861$$

So the acid had, in fact, only 0·435 of the expected activity. In other words, for every mole of radioactive acid arising in this way, 1·3 mole of inactive acid must arise in some other way, presumably largely from the oxidative fission of the benzene rings of indole units which are linked through their 2-position to other polymer units; see Scheme 2. Unfortunately no



SCHEME 2

quantitative conclusions regarding the relative proportions in the polymer of these two types of units can be drawn, because the degradation with hydrogen peroxide is not quantitative. The results of a number of such experiments are shown in Table 2. From these it is seen that autoxidative melanin which has been boiled with acid yields pyrrole-2,3,5-tricarboxylic acid in which the radioactivity has been more diluted than in that derived from melanin not treated with acid. This seems reasonable on the assumption that the acid treatment hydrolyses off pyrrole-2,3,5-tricarboxylic acid from units of type (V). Again, it should be noted that there is little difference between the results obtained on autoxidative and enzymic melanins which have been boiled with acid.

TABLE 2

Pyrrole-2,3,5-tricarboxylic acid formed by oxidation of melanin prepared from (±)-3,4-dihydroxyphenyl-[*carboxy*-^{14}C]alanine

Melanin preparation	Wt. of melanin oxidised (g.)	Specific activity of dopa	Pyrrole-2,3-dicarboxylic acid		Pyrrole-2,3,5-tricarboyxlic acid		
			Wt. (mg.)	Specific activity	Wt. (mg.)	Specific activity	Dilution
2 Autoxidation	1·865	670	6·4	0	4·4	376	1 : 1·3
5 Autoxidation, then 48 hr. with 2N-HCl (reflux)	1·024	817	3·5	0	4·2	375	1 : 1·8
7 Autoxidation in presence of catalase	0·583 †	817	2·4	0	2·9	501	1 : 1·1
8 Enzymic, then 48 hr. with 2N-HCl (reflux)	0·61 †	817	2·1	0	3·1	389	1 : 1·7

† Three successive oxidations.

As a check on the purity of the radioactive pyrrole-2,3,5-tricarboxylic acid isolated, a sample prepared from (±)-3,4-dihydroxyphenyl[*carboxy*-^{14}C]alanine of specific activity 729 was treated with excess of ethereal diazomethane, and the resulting trimethyl 1-methylpyrrole-2,3,5-tricarboxylate [9] was sublimed, after which its specific activity was found to be 265, corresponding to a dilution of 1 : 1·25 (*cf.* value of 1 : 1·3 referred to before).

These results are contrary to the conclusions drawn by Naples school, already mentioned. They also show that Cromartie and Harley-Mason's [10] suggestion that amino-acid carboxy-groups retained in the melanin in the form of 5,6-dihydroxyindole-2-carboxylic acid end-groups account for the pyrrole-2,3,5-tricarboxylic acid formed by oxidation of the melanin is only partly true.

[8] G. A. Swan and D. Wright, *J. Chem. Soc.*, 1954, 381, and 1956, 1549.

[9] G. A. Swan and A. Waggott, *J. Chem. Soc.* (*C*), 1970, 285.

[10] R. I. T. Cromartie and J. Harley-Mason, *Biochem. J.*, 1957, **66**, 713.

The pyrrole-2,3-dicarboxylic acid derived from (±)-3,4-dihydroxyphenyl[*carboxy*-^{14}C]alanine was inactive, as expected.

Piattelli and Nicolaus [11] decarboxylated sepiomelanin by heating a suspension in Vaseline oil for 10 hr. at 140—150° in a current of nitrogen, the evolved carbon dioxide being collected in barium hydroxide solution. From the weight of the resulting barium carbonate it was concluded that the melanin contained 9·1% of carboxy-group. We decarboxylated dopa-melanin by heating the dry, finely ground solid in a vacuum system and trapped the evolved carbon dioxide at —195°. The amount and activity of this carbon dioxide were subsequently measured.

Indole-2-carboxylic acid undergoes decarboxylation at its m.p. 202—204°,[12] whereas indoline-2-carboxylic acid decomposes between 120 and 150°.[13] Tyrosine, on the other hand, is more stable, not undergoing loss of weight until 260°.[14] We therefore thought that there might be the possibility of selective decarboxylation of different types of units in the melanin at suitable temperatures.

Preliminary experiments showed that neither (±)-3,4-dihydroxyphenylalanine nor its hydrochloride underwent decarboxylation at 200°, although the latter evolved hydrogen chloride, which was trapped and identified mass spectrometrically, and its yield was shown, by titration, to be quantitative. A similar experiment on 3,4-dihydroxyphenethylamine hydrochloride showed that this also evolved 1 mol. of hydrogen chloride. As dopa-melanin was usually precipitated from solution by acidification with hydrochloric acid, and as we had found the resulting precipitate to contain small quantities of chlorine (presumably in the form of the hydrochloride of uncyclised units), it was thought that hydrogen chloride released during its decarboxylation might interfere with the determination of quantity and activity of carbon dioxide. Fortunately, however, it was found that this hydrogen chloride could be trapped at —78°, along with water evolved by the melanin, and the carbon dioxide could subsequently be trapped at —195° and was then found to be pure by mass spectrometric analysis.

In attempts towards selective decarboxylation a radioactive melanin sample was heated at 150°, and the amounts of evolved water and carbon dioxide as well as the specific activity of the evolved carbon dioxide were measured at intervals of time up to 27 hr. Evolution of water and carbon dioxide was rapid at first, then gradually became slow. The specific activity of the carbon dioxide fell gradually from *ca.* 3000 to 2000. The temperature of the sample was then raised to 200° and the rate of evolution of carbon dioxide became fairly rapid again, after which it gradually fell during 15 hr. to almost zero. The rate of loss of water also increased at 200°, and fell almost to zero after *ca.* 20 hr. The specific activity of the carbon dioxide evolved initially at 200° was again *ca.* 3000 and fell to 2000 after 15 hr. The amount of carbon dioxide evolved during this second period of heating amounted to 20% of the total. As it thus appeared that no selectivity had been achieved between 150 and 200°, all subsequent decarboxylations were carried out at 200°.

The following calculations are based on the assumption that at 200° all units of the melanin containing carboxy-groups undergo decarboxylation with the exception of those containing uncyclised amino-acid side chains. Radioactivity measurements on the decarboxylated melanin should therefore give an indication of the number of the latter type of units present (Table 3). Titration of evolved hydrogen chloride might seem to provide an alternative way of determining the number of these units. However, although samples of dopamine-melanin which have been washed with hydrochloric acid contain considerable amounts of chlorine (presumably as the hydrochloride of uncyclised side-chains),[15] samples of dopa-melanin which had been precipitated with hydrochloric acid lost most of their chlorine when kept for 2 or 3 days over phosphoric oxide and sodium hydroxide in a vacuum desiccator.

The total amount of carbon dioxide evolved during decarboxylation should indicate the number of carboxy-groups capable of undergoing decarboxylation, *i.e.* the number of units of types (II) and (III), together with pyrrolecarboxylic acid units such as (VI), (VII), and (VIII), formed by attack of hydrogen peroxide on indole-5,6-quinone units. Radioactive carbon dioxide arising from (II) and (III) would be diluted with inactive carbon dioxide from the pyrrolecarboxylic acid units, so the specific activity of the evolved gas should provide information as to the relative proportions of the two different groups of units. Thus from Table 3 it is seen that in the case of preparation 1 (autoxidation) the carbon dioxide evolved during decarboxylation has a specific activity of only 2596; whereas if it had all arisen from units of types (II) and (III) it should have been 6561, *i.e.* the active carbon dioxide from the latter type of units has been diluted with inactive gas in the ratio 1 : 1·53.

The amount of pyrrole-2,3,4,5-tetracarboxylic acid formed on oxidation of sepiomelanin [16] and dopa-melanin [7] seems to be extremely small. If, therefore, units of type (VII) are disregarded for the moment, *i.e.* if it is assumed that each pyrrole unit has only one carboxy-group, then for each unit of type (II) and/or (III) there must be 1·53 pyrrole units. It should be noted that the presence of pyrrole units containing a radioactive carboxy-group in the 2-position (V) has also been neglected as the number of such units present is probably very small.

[11] M. Piattelli and R. A. Nicolaus, *Tetrahedron*, 1961, **15**, 66.
[12] A. Reissert, *Ber.*, 1897, **30**, 1030.
[13] C. B. Hudson and A. V. Robertson, *Austral. J. Chem.*, 1967, **20**, 1935.
[14] W. W. Wendlant, *Texas J. Sci.*, 1960, **12**, 138.
[15] G. A. Swan, *Rend. Accad. Sci. fis. mat.* (*Napoli*), 1964, [4] **31**, 1.
[16] M. Piattelli, E. Fattorusso, and S. Magno, *Tetrahedron Letters*, 1961, 718.

From the activity of the melanin (an average of results from preparations 1 and 3 in Table 3), it is calculated that 10% of the amino-acid carboxy-group is incorporated into the decarboxylated melanin, *i.e.* that the melanin contains one such carboxy-group in ten polymer units. This is calculated on the assumption that the number of pyrrole units in the melanin is small, *i.e.* that these 10% of carboxylated units are C_9 and that the remaining units are all C_8.

Combined with the result already obtained, *i.e.* that the original melanin contained 20% of carboxy-group derived from the original amino-acid, this gives a value of 10% for units of type (II) and/or (III) (Table 4). If it is then accepted, as already deduced, that for each unit of type (II) and/or (III) there are 1·53 pyrrole units, the fraction of pyrrole units in the melanin comes to 0·15. The wt. percentages of carboxy-group given in Table 4 are based on an assumed average molecular weight for a polymer unit of 163. The values for the total decarboxylatable carboxy-group in the last column of Table 4 are obtained by adding the values in the two previous columns (*i.e.* values deduced entirely from radioactivity measurements). These values are given again in the last column of Table 5, along with values calculated from the amount of carbon dioxide evolved during the decarboxylation of melanin derived from inactive (±)-3,4-dihydroxyphenylalanine.

In these calculations, the effect of the pyrrole units

TABLE 3

Decarboxylation of melanin prepared from (±)-3,4,dihydroxyphenyl[*carboxy*-^{14}C]alanine

Melanin preparation	Specific radioactivity: Precursor	Specific radioactivity: CO_2	Specific radioactivity: Decarboxylated melanin	Fractional dilution of CO_2	Fractional retention of amino-acid carboxy-group in decarboxylated melanin
1 Autoxidation	729	2596	90	1 : 1·53	0·11
3 Autoxidation	729	2550	73	1 : 1·57	0·09
4 Autoxidation	817	2770		1 : 1·65	
5 Autoxidation, then 48 hr. with 2N-HCl (reflux)	817	2190	59·7	1 : 2·36	0·07
7 Autoxidation in presence of catalase	817	4596	109	1 : 0·6	0·12
8 Enzymic, then 48 hr. with 2N-HCl (reflux)	817	2284	52	1 : 2·22	0·06
9 Enzymic in presence of catalase, then 48 hr. with 2N-HCl (reflux)	817	3569	52	1 : 1·06	0·06

TABLE 4

Distribution of various types of carboxylated units in dopa-melanin

Melanin preparation	Fraction per polymer unit: Uncyclised units (I)	Fraction per polymer unit: Carboxylated indole and indoline type units (II) and/or (III)	Fraction per polymer unit: Carboxylated pyrrole units (VI) and (VIII)	Carboxy-group (wt. %): In uncyclised units (I)	Carboxy-group (wt. %): In carboxylated indole and indoline type units (II) and/or (III)	Carboxy-group (wt. %): In carboxylated pyrrole units (VI) and (VIII)	Carboxy-group (wt. %): Total decarboxylatable *i.e.* sum of two previous columns
1 Autoxidation	0·11	0·09	0·14	3·0	2·5	3·9	6·4
3 Autoxidation	0·09	0·11	0·17	2·5	3·0	4·7	7·7
5 Autoxidation, then 48 hr. with 2N-HCl (reflux)	0·07	0·08	0·19	1·9	2·2	5·5	7·7
7 Autoxidation in presence of catalase	0·12	0·14	0·09	3·3	3·9	2·5	6·4
8 Enzymic, then 48 hr. with 2N-HCl (reflux)	0·06	0·09	0·20	1·7	2·5	5·5	8·0
9 Enzymic in presence of catalase, then 48 hr. with 2N-HCl (reflux)	0·06	0·11	0·12	1·7	3·0	3·2	6.2

TABLE 5

Decarboxylation of melanin prepared from (±)-3,4-dihydroxyphenylalanine

Melanin preparation	Wt. of melanin decarboxylated (mg.)	Time at 200° (hr.)	Pressure of CO_2 (cm.)	Decarboxylatable carboxy-group (wt. %): From amount of CO_2	Decarboxylatable carboxy-group (wt. %): From Table 4
1 Autoxidation	25·4	72	2·8	7·8	6·4
2 Autoxidation	36·5	45	3·75	7·3	
3 Autoxidation	75·2	40	8·0	7·9	7·7
5 Autoxidation, then 48 hr. with 2N-HCl (reflux)	103·2	128	11·9	8·0	7·7
	21·2	9	2·0	6·6	
7 Autoxidation in presence of catalase	71·3	22	7·2	6·4	6·4
8 Enzymic, then 48 hr. with 2N-HCl (reflux)	35·2	48	4·3	8·7	8·0
9 Enzymic in presence of catalase, then 48 hr. with 2N-HCl (reflux)	21·5	20	1·5	4·9	6·2

on the number of carbon atoms in the average polymer unit was disregarded. If one now accepts that 15% of such units are present, and supposes that these have only five carbon atoms [in fact, if units of types (VII) and (VIII) are present, the average will be greater than five], *i.e.* one takes the average unit as being $C_{7\cdot75}$, and if one then introduces the latter value into the calculations, one obtains a value of x of 0·19 instead of 0·2. It would be surprising if the experimental errors involved did not exceed such a difference.

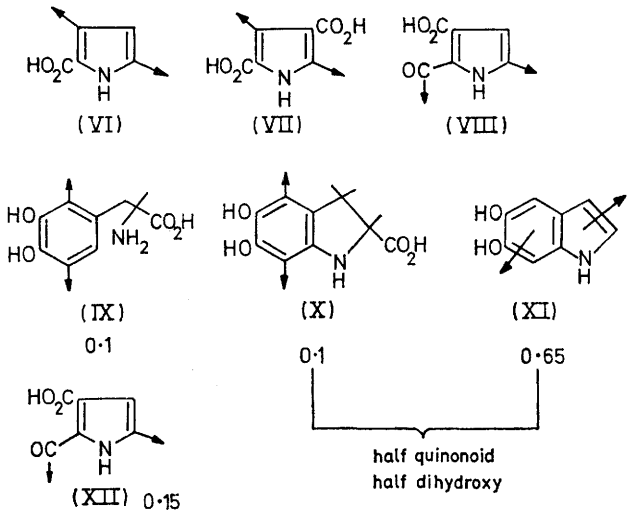
Table 4 shows that boiling a melanin with hydrochloric acid appears to increase slightly the fraction of carboxylated pyrrole units present. This could be a real effect. On the other hand, the presence of pyrrole units containing a radioactive carboxy-group (V) has been ignored in our calculations. If the effect of boiling with acid was simply to hydrolyse off radioactive pyrrole-2,3,5-tricarboxylic acid from such groups, then the apparent greater dilution of the radioactivity in the carbon dioxide formed by decarboxylation of the acid-treated melanin, as compared with that not treated with acid, might not be the result of the presence of a larger amount of inactive carbon dioxide, but rather of a smaller amount of active carbon dioxide. In fact, if one reconsiders the results in Tables 3 and 4, and allows for the presence of units of type (V), one has to conclude that the fraction of the latter units must be small, and that the loss of these units by hydrolysis could not account for the apparent increase in the fraction of carboxylated pyrrole units present, which one therefore has to regard as being a genuine change.

The low yield of pyrrole-2,3,5-tricarboxylic acid obtained by boiling the melanin with hydrochloric acid [7] is not proof that the fraction of units (V) in the melanin is very low, as this hydrolytic reaction is not quantitative. However, suppose that for every 0·1 unit of type (II) and/or (III), there are a units of type (VI) and/or (VIII), and b units of type (V). Then, considering the decarboxylation results,

$$(0\cdot1 + b)/a = 1/1\cdot53, \quad i.e.\ b = (a - 0\cdot153)/1\cdot53$$

Unless $a \geqslant 0\cdot153$, b is negative. The value already deduced for the fraction of the pyrrole units is, in fact, 0·15. If type (V) units are introduced, the *total* number of pyrrole units, *i.e.* (V), (VI), and (VIII), increases steeply; *e.g.* if $b = 0\cdot05$, $a = 0\cdot23$, and the total number of pyrrole units becomes 0·21; or if $b = 0\cdot1$, a 0·31, and the total 0·41.

As already deduced, the fraction of uncyclised amino-acid units (I) and that of units of types (II) and/or (III) in the melanin are each 0·1. If one introduced type (V) units, one would have to correspondingly reduce the number of type (II) and/or (III), to keep the proportion of the amino-acid carboxy-group right; and this would conflict with the results mentioned in the previous sentence. As will be seen from Part XI,[17] there is independent evidence from deuteriation experiments for the presence of type (II) rather than (III), and that the combined fraction of (I) and (II) must be 0·2. One way out of this conflict would be to suggest that the melanin contains the following units, with the fractions shown: (I) (0·1), 5,6-dihydroxyindoline (or the corresponding quinone) (0·1), (VI) + (VIII) (0·05), and (V) (0·1). However, although this would fit the results of the β-deuteriation experiments, it would not fit the α-deuteriation.[17] It appears to be impossible to explain our decarboxylation results satisfactorily, taking into consideration probable limits of error, unless the fraction of type (V) units in the melanin is considerably less than 0·05.



It may also be noted that boiling a melanin with acid causes a decrease in the fraction of uncyclised units (I) present (Table 4). It is also clear that both autoxidative and enzymic melanins prepared in the presence of catalase contain a considerably lower fraction of carboxylated pyrrole units than similar melanins prepared in the absence of catalase. Again one may note the absence of any significant difference between autoxidative and enzymic melanins.

The losses of carbon dioxide on decarboxylation of enzymic dopa-melanin, with and without catalase, and which has been kept with concentrated hydrochloric acid for 7 days before decarboxylation, found by the Naples school,[3] were 5·2 and 7·5% respectively. Our corresponding figures on melanin which had been boiled with 2N-hydrochloric acid for 48 hr. were 4·9 and 8·7%, respectively.

We had earlier deduced the presence of units of type (I) and/or (II) in dopa-melanin through experiments with deuteriated precursors.[4,5,15] A more extensive series of experiments on these lines, which supports our present conclusions, will be reported later.[17] Kirby and Ogunkoya [18] also carried out experiments with tritiated precursors; and their results could also be explained in terms of the presence of units of type (I).

[17] J. A. G. King, A. Percival, N. C. Robson, and G. A. Swan, following paper.
[18] G. W. Kirby and L. Ogunkoya, *Chem. Comm.*, 1965, 576.

As their results were based on a comparison of ^{3}H and ^{14}C radioactivity, it was not necessary that the enzymic melanin should be boiled with acid.

Hempel [19] attempted to investigate the structure of natural melanin without isolating it. He injected mice bearing Harding–Passey melanoma simultaneously with specifically tritiated (±)-3,4-dihydroxyphenylalanine and (±)-3,4-dihydroxyphenyl[α-^{14}C]alanine. In autoradiographs of the melanomas 2—10 days later, it was found that the silver grains lay over the melanin granules, and it was therefore assumed that all the radioactivity was contained in the melanin. The melanomas were homogenised, acid-soluble and lipid fractions were removed, and the ^{3}H and ^{14}C activities of the residual material were measured by liquid scintillation counting. On the reasonable assumption that the α-carbon atom is incorporated quantitatively into the melanin, the ratio of ^{3}H to ^{14}C activity was taken as a measure of the incorporation of the particular hydrogen atom labelled by ^{3}H in the precursor. Hempel concluded that at least 49% of the polymer units of the melanin must retain the hydrogen atom of position 6 of dopa (*i.e.* these units were presumably uncyclised), and that 71% of the units retain the carboxy-group of the original amino-acid.

Hempel's method is elegant; but its value is based entirely on the assumption that all the measured radioactivity originated from melanin. The validity of this assumption has been questioned,[20,21] and indeed there is indication [22] that dopa can be released by hydrolysis of melanoma homogenates such as those used by Hempel, *i.e.* that there is present dopa which is not part of the melanin polymer proper, but is at the most linked to it as a peptide. Hempel [23] has replied to this criticism, but further investigation of the situation is still required.

Nicolaus and his collaborators [3] treated enzymic dopa-melanin with diazomethane in ether and oxidised the product; but they failed to detect the formation of the pyrrolecarboxylic acids which are usually formed by oxidation of melanins, even when the methylated melanin was hydrolysed before oxidation. We also treated autoxidative dopa-melanin three times successively, each for 12 hr., with excess of diazomethane. In addition, we treated autoxidative dopa-melanin with methanol in the presence of hydrogen chloride.

There is a wide range of variation in the elemental analytical results for melanins in the literature, as well as in values determined on our own samples. Even repeat analyses on the same sample are sometimes widely divergent. Moreover, there is no guarantee that two samples of melanin apparently prepared in exactly the same way will be identical. In addition, the hygroscopic nature of melanins has no doubt resulted in samples having been analysed under varying degrees of hydration. It is therefore impossible to decide on an empirical formula for dopa-melanin from these values.

However, the Naples school [3] has published duplicate sets of values on methylated (diazomethane) enzymic dopa-melanin which show good agreement; and we obtained reasonably reproducible values on methylated (diazomethane) autoxidative dopa-melanin. Moreover, their results and ours show a striking agreement. In other respects also, we have found little difference between autoxidative and enzymic dopa-melanins. However the Italian workers used a relatively low ratio of enzyme to substrate, so it is possible that their preparations should be regarded as intermediate between enzymic and autoxidative. From these values it seems that dopa-melanin which has been methylated exhaustively with diazomethane has the approximate empirical formula $C_{10}H_{9\cdot6-10}NO_3$. If a methoxy-group value of 21% is accepted, this would mean that approximately $C_{1\cdot3}H_{2\cdot6}$ must represent methoxy-group. If all the carbon which arose from diazomethane is split off as methyl iodide during the Zeisel estimation, then the empirical formula of the melanin should be approximately $C_{8\cdot7}H_{7-7\cdot4}NO_3$. Although the latter agrees fairly well with the results which include the highest values for carbon and hydrogen quoted by the Naples school, these values appear to be higher than many of those found by other authors and ourselves.[24] Moreover, the product which we obtained by the action of methanolic hydrogen chloride on autoxidative dopa-melanin gave analytical figures approximately corresponding to $C_8H_6NO_3$, of which perhaps $C_{0\cdot35}H_{0\cdot7}$ represents methoxy-group. The hydrogen and oxygen values may vary according to the degrees of hydration of the melanin, but it is important to establish the C : N ratio. Such a high ratio as 8·7 : 1 must imply either a high retention of carboxy-group (ruled out by our results in Table 1), or incorporation in the polymer of units which have lost their nitrogen. The most likely occurrence of the latter type of unit would involve attack of uncyclised amino-acid side chains by hydrogen peroxide, with loss of the original carboxy-group and the amino-group. The comparatively small effect of catalase on the retention of units of type (I), as seen from Table 4, suggests that nitrogen-free units are unlikely to be present in sufficient abundance to bring the C : N ratio up to 8·7 : 1. We are therefore reluctant to accept the formula $C_{8\cdot7}H_{7-7\cdot4}NO_3$ for dopa-melanin.

We suggest that the results are best interpreted on the assumption that treatment of a melanin with diazomethane results in the methylation of carboxy- and phenolic groups (to give methoxy-groups, determined

[19] K. Hempel, in 'Symposium on Structure and Control of the Melanocyte,' Springer, Berlin, 1966, p. 162; *Z. Naturforsch.*, 1967, **22***b*, 173.

[20] G. A. Swan, Sixth International Pigment Cell Conference, held in Sofia, May 1965, discussion of Dr. K. Hempel's paper, unpublished.

[21] H. S. Mason, in 'Advances in Biology of Skin, vol. VIII, The Pigmentary System,' Pergamon, Oxford, 1967, pp. 315, 316.

[22] H. Takahashi and T. B. Fitzpatrick, *Nature*, 1966, **209**, 888.

[23] K. Hempel, in 'Advances in Biology of Skin, vol. VIII, The Pigmentary System,' Pergamon, Oxford, 1967, p. 315.

[24] A. B. Lerner and T. B. Fitzpatrick, *Physiol. Rev.*, 1950, **30**, 91; H. Burton, *Chem. and Ind.*, 1947, 383.

by the Zeisel method); but that in addition a substantial amount of CH_2 is introduced into the melanin in a form which does not yield methyl iodide under Zeisel conditions. This could be in the form of methylenedioxy-groups, formed from quinonoid units as suggested by Nicolaus,[3] or in the form of *N*-methyl groups; we have already shown,[9] for example, that treatment of pyrrole-2,3,5-tricarboxylic acid with excess of diazomethane results in *N*-methylation, as well as esterification. It might even be in the form of $C{\cdot}CH_2$ groups. To free the methylated melanin completely from ether before Zeisel determination is clearly important.

We have deduced a combination of units, in terms of which we believe our results can be explained reasonably well. It should be emphasised that we believe our melanins to be irregular polymers, containing a number of different types of units linked in various ways; and therefore our proposal can, at the best, indicate only the approximate proportions of those units which occur in the polymer with greatest abundance, *e.g.* we have not attempted to bring in units which might occur to the extent of 0·05 or less per average polymer unit. We are not denying the possible presence of methylenedioxy-groups in methylated melanin, or of units not containing nitrogen. Moreover the state of oxidation of individual units in the polymer is unknown—only the relative numbers of quinonoid and phenolic units as a whole can be determined (from methoxy-group values).

Let us then assume that autoxidative dopa-melanin is constituted mainly of four types of units: 10% of uncyclised units which are diphenolic (IX), 10% of indolinecarboxylic acid type (X), 65% of indole type (XI), and 15% of pyrrole type (XII), and moreover, that of the units of type (X) and (XI) taken together, one half are quinonoid, and the other half are diphenolic. On this basis the empirical formula of the melanin should be $C_{7\cdot9}H_{4\cdot55}NO_{2\cdot55}$. If this is hydrated to the extent of $0{\cdot}5H_2O$, we obtain the formula $C_{7\cdot9}H_{5\cdot55}NO_{3\cdot05}$. We shall retain this arbitrary $0{\cdot}5H_2O$ throughout the following calculations.

If the treatment with excess of diazomethane involves methylation of all carboxy- and hydroxy-groups, and all indole and pyrrole (but *not* indoline)NH groups, then the fully methylated melanin should have the empirical formula $C_{10}H_{9\cdot75}NO_{3\cdot05}$, which should contain 1·3 methoxy-groups and 0·8 ' extra CH_2 ', corresponding to the following calculated percentages:

	C	H	N	O	OMe
Calc.	62·3	5·06	7·27	25·44	20·92
Found (Newcastle)	62·60	5·60	7·40	24·8	22·1
	62·21	5·62			21·45
					22·12
Found (Naples)	62·8	5·17	7·42		20·6
	62·92	5·14	7·45		

Alongside are shown our found values, and corresponding figures quoted by the Italian workers on methylated enzymic dopa-melanin.

The product of treatment of the melanin with methanol and hydrogen chloride should be $C_{8\cdot25}H_{6\cdot25}NO_{3\cdot05}$, which should contain 0·35 methoxy-group, corresponding to:

	C	H	N	O	OMe
Calc.	58·88	3·72	8·33	29·05	6·46
Found	58·43	3·59	8·34	29·17	6·51
					6·68
					6·75

On decarboxylation, the melanin should lose $0{\cdot}25CO_2$; and unit (X) might well then become indolic, if it was originally of the indoline type. When the product is fully methylated with diazomethane it should give $C_{9\cdot6}H_{9\cdot25}NO_{2\cdot55}$, containing 1·05 methoxy-group, *i.e.* it should contain 18·1% methoxy-group (Found: 16·48%). The discrepancy could be explained if some phenolic units became quinonoid during decarboxylation at 200°.

The fully methylated autoxidative dopa-melanin was treated with [^{14}COCl]benzoyl chloride in pyridine, and the specific radioactivity of the product was found to be 56 when the chloride had been prepared from [$^{14}CO_2H$]benzoic acid of specific activity 1007. The only group likely to react with benzoyl chloride under these conditions is the primary amino-group of units of type (IX), as that of type (X) would probably be too sterically hindered; and, in any case, some of the latter might well be quinonoid. This being so, the benzoylated methylated melanin should have an average unit $C_{10\cdot7}$, containing 0·1 benzoyl group, so that

$$\frac{\text{Sp.act. of benzoylated methylated melanin}}{\text{Sp.act. of benzoic acid}} = \left(\frac{0{\cdot}1}{10{\cdot}7}\right)\Big/\frac{1}{7} = 0{\cdot}065$$

Whereas the found value = 56/1007 = 0·056

It is difficult to assess the probable errors involved in the proportions of different units deduced above, because (*a*) one has no guarantee that reactions (*e.g.* methylations) are complete, (*b*) it is difficult to obtain reproducible analytical results on melanins, (*c*) the presence of some units probably present in small fractional amounts has been ignored, and (*d*) the calculations are based, in any case, on certain fundamental assumptions. However, it may be helpful to summarise the evidence regarding the various units.

The fractions of units (IX) and (X) were deduced from the results of decarboxylation experiments on melanins derived from [*carboxy*-^{14}C]dopa; and both receive confirmation from the results of deuteriation experiments. That of (IX) is to some extent confirmed also by the result of the experiment with [^{14}COCl]-benzoyl chloride. The value of each of these fractions might be given as $0{\cdot}1 \pm 0{\cdot}02$. The fraction of (XII) was also deduced from the results of decarboxylation experiments; but in this case, the accuracy would be influenced by the presence of other pyrrole-type units, *e.g.* (V) and (VI), both of which might well be present in a fraction less than 0·05. However, the methoxy-

value on melanin treated with methanol in the presence of hydrogen chloride provides a check on the total carboxy-group, which might be given as $0{\cdot}35 \pm 0{\cdot}02$. The latter agrees well with the sum of the individual components (0·1, 0·1, 0·15). The difference between the methoxy-values on materials treated with diazomethane and with methanol–hydrogen chloride is used to deduce the proportion of phenolic hydroxy-groups ($0{\cdot}95 \pm 0{\cdot}05$), the number of quinonoid rings being found by difference.

Finally, the elemental analyses provide a check on the overall composition. These values have not been used directly in calculating the proportions of different units, although in certain of these calculations it was necessary to know either the molecular weight of the average polymer unit, or the number of hydrogen atoms in the unit.

Mason [25] concluded from titrations that enzymic dopa-melanin contains six phenolic hydroxy-groups and fewer than one carboxy-group per ten units. It is not possible to make a direct comparison between our results and his, but these values are lower than ours on autoxidative dopa-melanin. Also, even after our enzymic melanin had been boiled for 48 hr. with 2N-hydrochloric acid, it contained more carboxy-groups than Mason's melanin not so treated.

Under Mason's conditions of melanogenesis, up to 4·6 atoms of oxygen were consumed, and up to 1 molecule of carbon dioxide was evolved per molecule of substrate. He concluded: 'The formation of melanin under these conditions precludes the participation of dopa or 5,6-dihydroxyindole-2-carboxylic acid in the polymerization to any important extent.' However, he oxidised 115 mg. of dopa and obtained only 68 mg. of melanin (perhaps containing protein derived from the enzyme); in view of this low yield (which, of course, is not unique to Mason), it is difficult to accept Mason's conclusion. According to our suggested structure, if all the dopa were converted into melanin, 3·83 atoms of oxygen would be consumed, and 1·1 molecules of carbon dioxide evolved per molecule of substrate, in the case of autoxidation. If 3,4-dihydroxyphenyl[carboxy-^{14}C]-alanine were used, then the ratio of specific activities of evolved carbon dioxide and precursor should be 6·56, whereas a value of 5·28 was found,[5] showing that a higher proportion of the evolved carbon dioxide was derived from carbon atoms other than that of the amino-acid carboxy-group, than on the above assumption.

We included $0{\cdot}5H_2O$ of hydration empirically in our structure. There is a distinct possibility that some melanins might contain peroxide groups. It could be that part or all of the oxygen represented as $0{\cdot}5H_2O$ should really represent peroxidic oxygen. If this were so, the calculated oxygen uptake would be much closer to Mason's value.

[25] H. S. Mason, in 'Advances in Biology of Skin, vol. VIII, The Pigmentary System,' Pergamon, Oxford, 1967, p. 293.
[26] G. A. Swan, *J. Chem. Soc.*, 1955, 1039; R. F. Glascock, 'Isotopic Gas Analysis for Biochemists,' Academic Press, New York, 1954.

EXPERIMENTAL

Combustion of samples and radioactivity measurements were carried out essentially as described earlier,[26] except that the gas-counters were type GA1OM (20th Century Electronics Ltd.). Poly[*methyl*-^{14}C]methacrylate was used as a standard. Polyphenol oxidase and melanins were prepared, and melanins were oxidised, as already described.[7] Beef liver catalase obtained from Mann Research Laboratories, had an enzymic activity of 2235 units/mg., as calculated by the method of v. Euler and Josephson; [27] in those melanin preparations carried out in the presence of catalase, 1 mg. of this sample was used for each 100 mg. of precursor. (±)-3,4-Dihydroxyphenyl[*carboxy*-^{14}C]-alanine was prepared by Clemo, Duxbury, and Swan's method.[2]

Isolation of Pyrrolecarboxylic Acids formed by Oxidation of Melanin derived from (±)-3,4-*Dihydroxyphenyl*[carboxy-^{14}C]*alanine*.—The mixture of pyrrolecarboxylic acids, obtained by oxidation with hydrogen peroxide in alkaline solution of *ca.* 1 g. of the melanin, was dissolved in the minimum volume of ethanol–water (1 : 1), and applied as a band a few cm. from the edge of a sheet of Whatman no. 3 chromatography paper, which had previously been thoroughly washed with 10% aqueous acetic acid. The chromatogram was run (ascending) in butan-1-ol–acetic acid–water (4 : 1 : 5), organic phase (*B*), for 48 hr.; it was then dried, and viewed under a u.v. lamp. The fluorescent bands were cut out. Elution with water was carried out by Reith's method,[28] and the extracts were freeze-dried. Each of the appropriate acids was then further purified by two-dimensional chromatography on pre-washed Whatman no. 3 paper, in the systems propan-1-ol–ammonium hydroxide(*d* 0·88)–water (6 : 3 : 1) (*A*), and *B*. In this way pyrrole-2,3-dicarboxylic acid and pyrrole-2,3,5-tricarboxylic acid were isolated (Table 2), and were identified by m.p. and u.v. and i.r. spectra.

Decarboxylation of Melanin.—The finely-ground sample was placed at the botton of a tube, attached by a ground-glass joint to a high vacuum system. The tube was pumped out for 2 hr., then heated with a bath of a boiling organic liquid of appropriate b.p. The gases released by the melanin were condensed in a trap cooled in liquid nitrogen. Later, the liquid nitrogen was replaced by a bath at −78°, and the carbon dioxide was condensed, by use of liquid nitrogen, into a manometer. By measuring the pressure produced by the carbon dioxide in a space of known volume (29·1 ml. in most cases), the quantity was determined. The carbon dioxide was then transferred to a gas-counter for measurement of radioactivity. The water evolved during the decarboxylation remained in the trap at −78°, and the approximate amount of this was also determined manometrically.

Methylation of Autoxidative Dopa-melanin.—(*a*) An excess of etheral diazomethane (20 ml.) was added to a suspension of the finely ground melanin (100 mg.) in ether (10 ml.), and the mixture was stirred for 12 hr. The ether and excess of diazomethane were distilled off, the residue was dried for 24 hr. (P_2O_5) in a vacuum desiccator,

[27] H. v. Euler and K. Josephson, *Annalen*, 1927, **452**, 158.
[28] E. S. Reith, *Nature*, 1957, **179**, 580.

then reground. The whole process was then repeated twice.

(*b*) A suspension of the finely ground melanin (100 mg.) in methanol (10 ml.) which had been saturated with hydrogen chloride, was shaken for 12 hr. The product was then centrifuged off, washed with methanol (3 × 10 ml.) by centrifugation, and dried for 12 hr. at 40° in a vacuum (P_2O_5). The whole process was then repeated twice.

Benzoylation of Methylated Melanin.—Benzoic [^{14}C]acid (5 g.; 4μCi) was treated for 2 hr. at 80° with thionyl chloride (15 ml.) and the product was distilled twice to give the chloride, b.p. 194—195° (4·57 g.). A mixture of the product from (*a*) (100 mg.), pyridine (20 ml.), and this chloride (5 ml.) was shaken for 20 hr., then filtered on a sintered glass filter; the resulting solid was washed with methanol and dried for 24 hr. at 40° in a vacuum (P_2O_5) (yield 107 mg.).

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