# STRUCTURE AND BIOSYNTHESIS OF MELANIN

a Thesis submitted by

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# A B S T R A C T

The problems of melanin biogenesis and structure are reviewed up to date.

Preparative procedures are outlined for the labelling of phenols with deuterium and tritium by exchange in alkaline and in acidic solutions. The use of phenols, labelled with tritium at the ortho and para positions, in biosynthetic investigations is discussed. The methods developed are illustrated by the synthesis of (±)-3,4-dihydroxyphenylalanine (dopa) labelled specifically with tritium at each of its nuclear positions; and also at its side chain positions.

Tracer studies with variously labelled dopas have shown that the melanin pigment is quite irregular in structure and that regular structures hitherto postulated cannot be correct.

A radio dilution study has shown that pyrrole-2,3-dicarboxylic acid is produced in very small amount from the oxidative degradation of melanin.

## ACKNOWLEDGEMENTS

I wish to record my sincere gratitude to Dr. G.W. Kirby for a most helpful and inspiring guidance at all stages of this work. It has indeed been a privilege and a pleasure to work under his supervision.

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CONTENTS

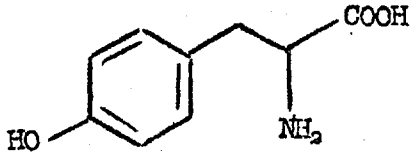
# INTRODUCTION

Melanin is a black, amorphous and insoluble pigment found in animals and in many plants; and it is probably responsible for most of the dark pigmentation observed in them. Melanins formed by chordates, (for example, sea squirts, fish, salamandas, toads, snakes, crocodiles, birds and mammals) seem to have a mainly protective function, for example, in shielding underlying tissues from shortwave irradiation, in heat control and in adaptive colouration. Association of melanins in human skin and hair with certain malignant tumours has attracted the interest and the attention of geneticists and cytologists. It is therefore not surprising that the pigment is of considerable biochemical interest.

Structural work on melanin has been retarded largely because of its insolubility and amorphous nature. In fact, no melanin, whether natural or synthetic, has yet been isolated and characterised in the conventional manner as a single chemical compound of definite composition. Consequently, no precise definition of it has and can be given yet; and the name melanin has been applied indiscriminately to many animal and plant pigments though these may be quite different structurally.

## Definition of Melanin

The name melanin has been loosely applied to pigments of high molecular weight formed by the enzymatic oxidation of phenols. Fürth and Schneider[1] defined melanin as the black precipitate obtained by the action *in vitro* of insect tyrosinase on tyrosine (1). Thompson[2] however broadened this definition to include the product of tyrosinase action on tyrosine and closely related compounds like 3,4-dihydroxyphenylalanine (dopa) (2), tyramine (3) and dopamine (4). Angeli (1915) had defined the pigment as the last product of enzymatic oxidation and polymerisation of pyrrole derivativesproduced from protein or from phenolic amino acids such as tyrosine and congeners possessing the characteristic group (5)

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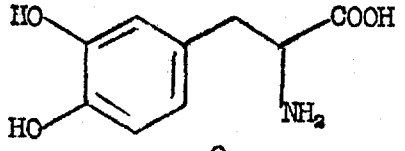


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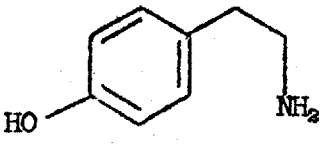


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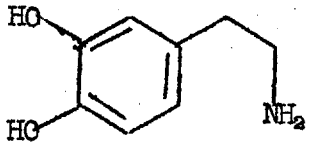


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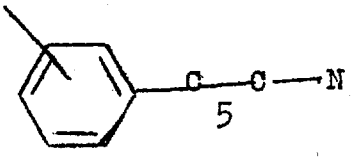


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Thomas[3] applied the name melanin to black nitrogeneous pigments which are indolic derivatives and Swan[4] accepted this definition. Mason[5] however described the natural pigments as compounds of high molecular weight formed by the enzymatic oxidation of phenols and usually occurring as granules in which the melanin may be combined with protein. Nicolaus[6], has on the basis of certain typical degradation products, postulated two main classes of melanins: the indole melanins which are mainly of animal origin and the catechol melanins which are of plant origin. Elementary analyses and general properties like infusibility, insolubility and typical degradation products do suggest structural similarity between the natural pigments and those formed in vitro from tyrosine and dopa.

The present account will be confined to the first and most important class of pigments, that is melanins derived by oxidation of tyrosine and dopa.

## BIOGENETIC STUDIES

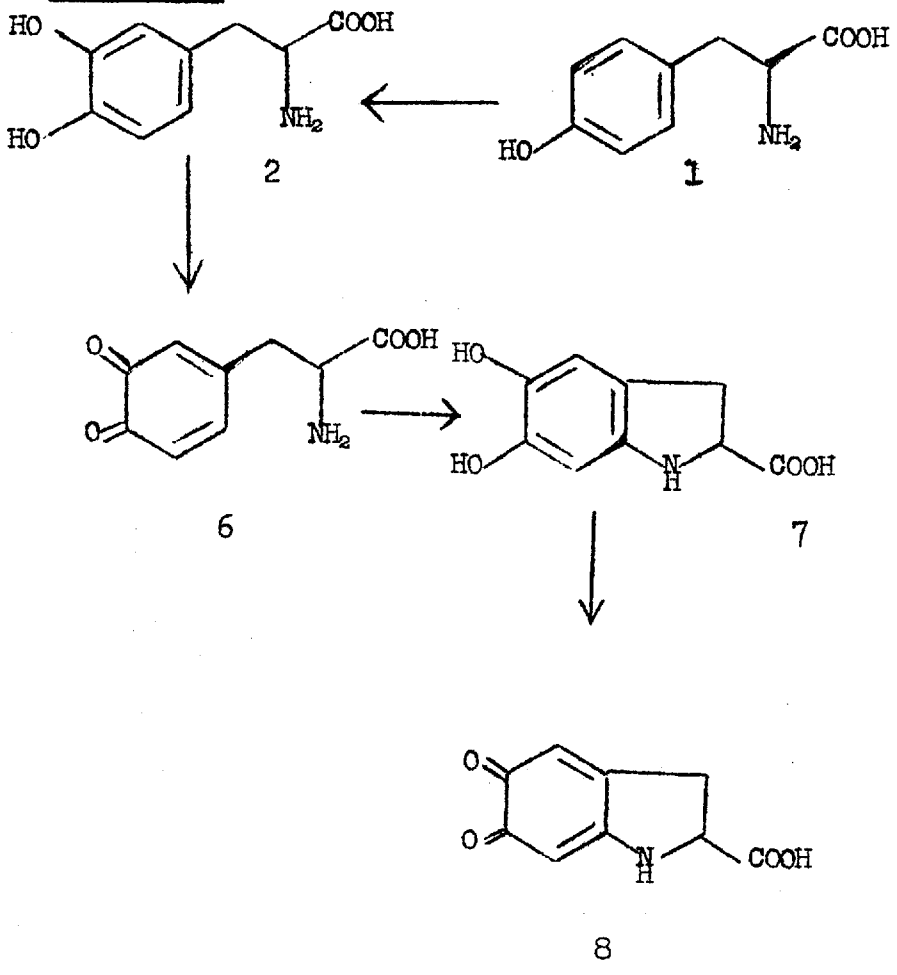
In natural product chemistry, it is usual to first determine the structure of a compound and then to find out how that structure could be derived biogenetically. Tyrosine or dopa melanin, on the other hand falls into the small group of natural products of which more is known about their biogenesis than their structures. The first indication of the origin and biogenesis of melanin coincided with the discovery of tyrosinase[7] in certain higher plants and fungi which are prone to blacken when exposed to air. Bertrand[8] isolated tyrosine from the fungus Russula nigricans and noticed that when this amino acid came in contact with an enzyme from this plant, it gave a red solution which finally deposited a black precipitate. Similar results were obtained with extracts from other plants and animals[1]. Onslow[11] and Pugh[9], having demonstrated the occurrence of tyrosinase and tyrosine in mammals and invertebrates, concluded that tyrosinase oxidation of tyrosine could be responsible for much of the dark pigmentation observed in animals and plants. Bloch[10] also demonstrated the presence of the enzyme in the pigment-forming cells of the skin which produces melanin from dopa. More recent work[47] has shown the conversion of [$^{14}$C] tyrosine into the melanin of the

skin and hair. It is now firmly established that the enzyme tyrosinase is in fact a phenol oxidase which hydroxylates monohydric phenols to catechols and catalyses the oxidation of the latter to o-quinones. Tyrosinase is a copper-protein complex of wide distribution. Enzymes from different sources show qualitative differences. Mammalian tyrosinase is relatively specific for L-tyrosine and L-dopa whereas enzymes from lower animals and especially from plants are active towards the D- and L- amino acids and a wide range of other monohydric and dihydric phenols[12].

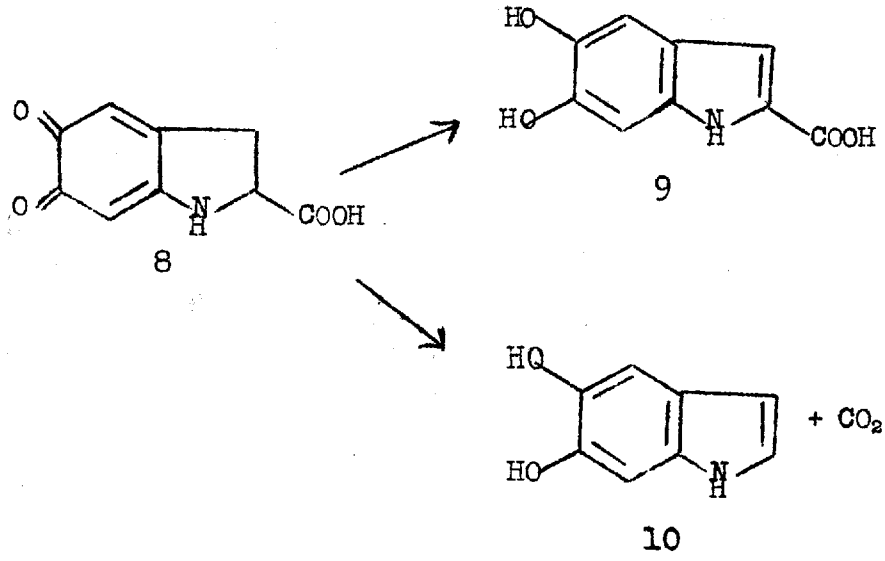
The first comprehensive scheme of melanin biogenesis was put forward by Raper[13]. With tyrosine as substrate, and by modifying the pH of the reaction, he showed three separate stages of oxidation (effected with molecular oxygen) and hence postulated the sequence of transformations outlined in Scheme 1.

Scheme 1

1st stage




2nd stage



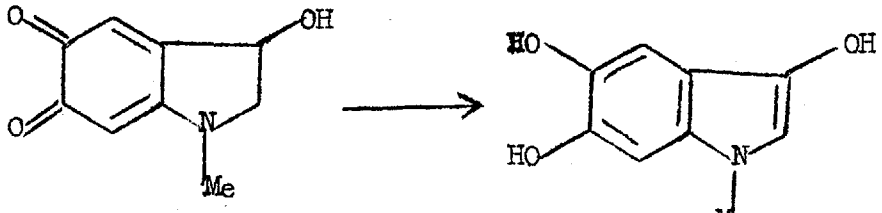
3rd stage: 9 and 10 ——→ Melanin

According to this scheme, the first observable intermediate was dopachrome (8) recognised by its characteristic red colour. Raper[14] observed that only the formation of dopaquinone (6) in this scheme was necessarily enzymatic, the other reactions being spontaneous although accelerated by the enzyme. In the second stage, dopachrome then underwent further changes to form either or both of the colourless dihydroxyindoles (9) and (10) depending upon the experimental conditions. The third stage consisted of the oxidative polymerisation of (9) and (10) to yield the black pigment melanin. In support of this scheme, the intermediate, dopa (2) was isolated[15] in ca. 3% yield as its lead salt which was decomposed with hydrogen sulphide to give dopa. Furthermore, when the red pigment first formed was allowed to decolourise in the presence of sulphorous acid and then methylated with dimethyl sulphate in an atmosphere of hydrogen, 5,6-dimethoxyindole and 5,6-dimethoxy-2-carboxyl indole were isolated[16] as crystalline solids and characterised. Dopa was also shown to be readily oxidisable by the same enzyme and by silver oxide to the same end-products. More recently[17] 5,6-dihydroxyindole has been isolated and

identified by paper chromatography by Nicolaus and his coworkers during the enzymatic oxidation of dopa at pH 6.8. This compound together with 5,6-dihydroxy-2-carboxylindole has also been isolated[56], by paper chromatography from the alkaline fusion products of sepiomelanin. They were identified by comparison of their ultraviolet spectra with those of authentic samples.

Formation of dopachrome from dopaquinone is a normal quinone-amine addition reaction followed by rapid oxidation of the resulting quinol. Dopachrome then rearranges to the dihydroxyindoles. The rearrangement of dopachrome and structurally related compounds have been examined extensively. Mason[18] found that this rearrangement was catalysed by acid to give mainly 5,6-dihydroxy-2-carboxylindole (9) and also by alkali to give 5,6-dihydroxyindole. At pH 5.6-6.8, the rearrangement was accompanied by decarboxylation, unlike at pH 1.3-2.0. Fischer and his coworkers[19] also found that zinc ions caused a rapid rearrangement of adrenochrome (11) into 3,5,6-trihydroxy-1-methylindole (12) - an internal reduction corresponding to the decarboxylative rearrangement of dopachrome. This rearrangement has been shown by Harley-Mason, Bu'Lock[20] and Mason to be

general for dihydroindole-5,6-quinones and has been used by these authors as a convenient synthetic route to dihydroxyindoles. For example when dopa was treated with alkaline ferricyanide, it gave a red solution, (believed to be dopachrome) which on treatment with zinc acetate gave an excellent yield of 5,6-dihydroxyindole. Similarly N-methyldopa gave 5,6-dihydroxy-1-methylindole. The reaction was extended to halogen-substituted compounds.

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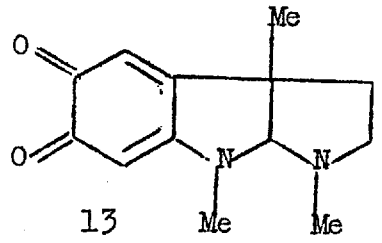
Colour changes during melanin formation

During the enzymatic oxidation of dopa at pH 6.8, the solution first became red ($\lambda_{max}$ 305, 475 mμ)[4,18], then slowly turned purple ($\lambda_{max}$ 300, 535 mμ) and later showed a general absorption corresponding to the black appearance of melanin. The red colour is generally believed to be due to the formation of dopachrome (of Raper's scheme) the

chromophore of which was deduced[18] by spectroscopic comparison with the known 2,3-dihydroindole-5,6-quinones adrenochrome (11) ($\lambda_{max}$ 305, 480 mμ in water) and rubreserine (13) ($\lambda_{max}$ 300, 480 mμ in water).

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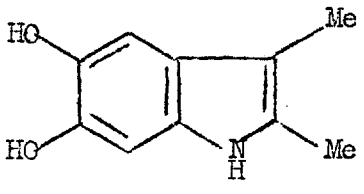
On both physical and chemical grounds, the previously[21] drawn analogy between dopachrome and hallochrome (a red pigment extracted from Halla parthanopaea) ($\lambda_{max}$ 539) has been dismissed[22]. Dopachrome is slowly converted to 5,6-dihydroxyindole and then to indole-5,6-quinone and finally to melanin. Mason[18] regarded the purple melanochrome which he observed during the enzymatic oxidation of 5,6-dihydroxyindole as indole-5,6-quinone (16). Beer and coworkers[23] however suggested that the true indole-5,6-quinones (16) are yellow and that the intensely coloured melanochromes are simple short polymers of quinonoid indole molecules. Harley-Mason and Cromartie[24] found that 2,3-dimethyl-5,6-dihydroxyindole (14) formed a comparatively stable red substance ($\lambda_{max}$ 470 mμ) whose light absorption is similar to dopachrome. This same product was formed

when the catechol was oxidised with silver oxide in methanol or with aqueous ferricyanide. This product therefore appeared to these authors to be the corresponding quinone. Since 2,3-dimethyl-4,7-quinone (15) shows a peak at 470 mμ [25], the purple pigment can hardly be monomeric and it is therefore probably a low molecular weight polymer. Bu'Lock, more recently, has proposed, on spectroscopic grounds, that the first purple pigment ($\lambda_{max}$ 530 mμ) formed from the oxidation of 5,6-dihydroxyindole is a 5,6-dihydroxyindolyl-5,6-indolequinone (17). Later oxidation products have flatter maxima at 540 mμ and are probably oligomers, intermediates between the dimers and the melanin polymer. Bu'Lock was able to measure the concentration of dihydroxyindoles in solution by their fluorescent intensities. All the above ideas are summarised in Scheme 2. Autoxidation of dopa and dopamine produced no band at 305 mμ, only a slight peak at 480 mμ and no purple stage. Enzymatic oxidation of dopamine showed colour changes similar to those of dopa.

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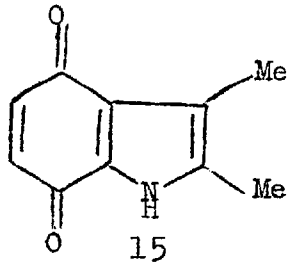


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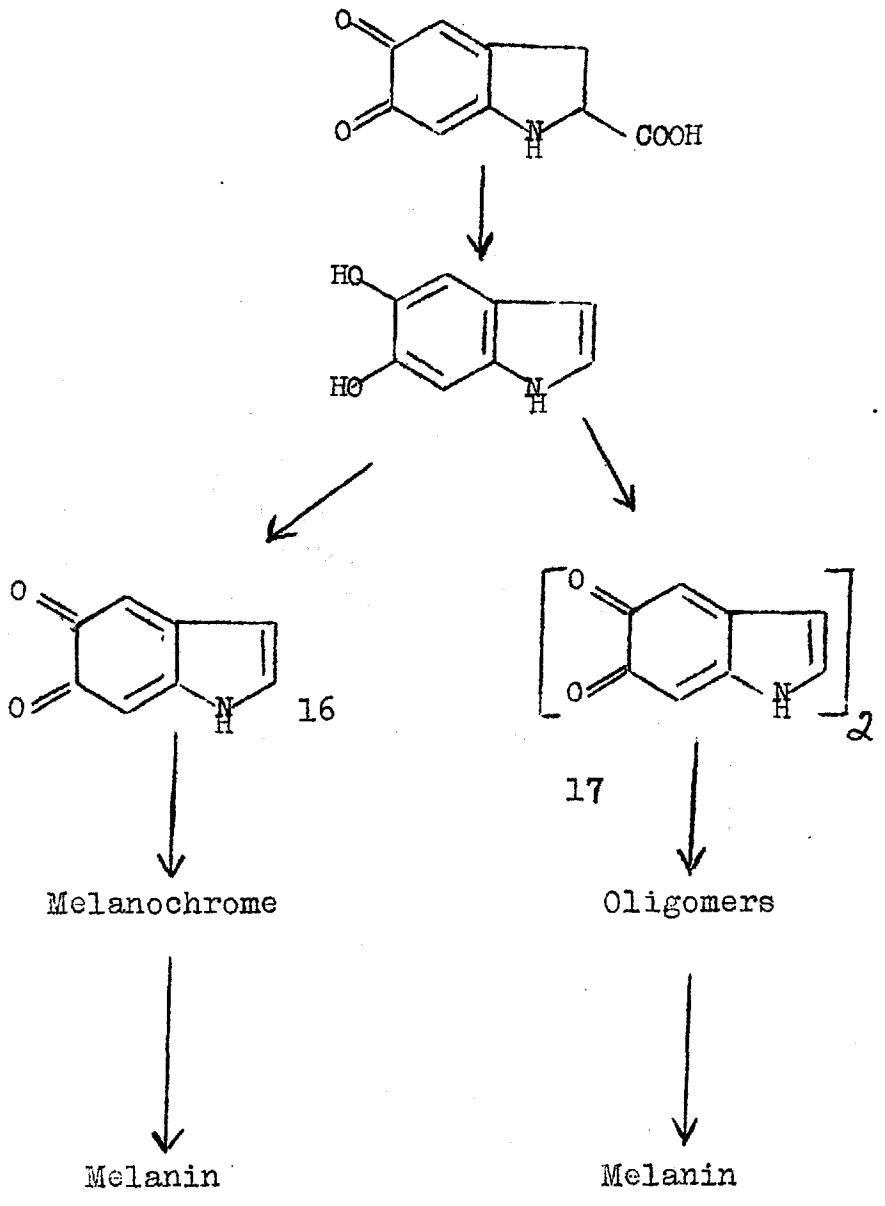
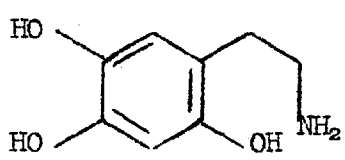
Scheme 2




The possibility that further hydroxylation of the aromatic nucleus might occur during melanin formation has also been investigated. In their autoxidation

experiments with dopamine, Witkop and his coworkers[26,27] found that nuclear hydroxylation of this compound occurred very readily to form 2,3,5-trihydrophenethylamine (18). This would suggest that the latter might be an intermediate in the oxidation of the former to melanin. The idea of a trihydroxylated intermediate is supported by the more recent observations of Rolland and Lissitzky[28] who observed the presence of an intermediate (during the enzymatic oxidation of tyrosine) whose behaviour suggest the structure of a trihydroxylated derivative of phenylalanine. The probability that a trihydroxylated derivative might be a significant intermediate has been refuted by Swan and Percival[29] on various grounds. They found that the yield of melanin from (18) was less than a quarter that obtained under comparable conditions from the autoxidation of dopamine, and the yield from 2-[2,4,5-trihydroxyphenyl] alanine (19) was less than half that obtained from dopa. The melanins from the amines (18) and (19) generally had higher carbon contents. Benzoylation experiments with [$^{14}$C] benzoyl chloride showed that only a fraction of one benzoyl group per 8-carbon unit was present in the polymer. On the other hand each unit of benzoylated dopamine melanin contained one benzoyl group. That these trihydroxy compounds are unlikely to be inter-

mediates was further confirmed by spectroscopic studies by the same authors. During the oxidation of (18) and (19) at pH 8 in the absence or in the presence of tyrosinase (30 units) only a slight maxima at 490 mμ together with strong maxima at ca 270 and 215 mμ were observed[29]. For the hydroxyquinone (20) at pH 7.5, Senoh and Witkop reported $\lambda_{max}$ 270, 495 m (ε 10,800; 2,250 respectively).

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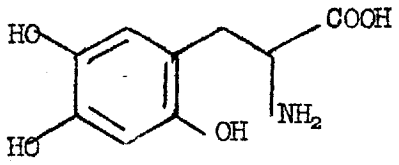


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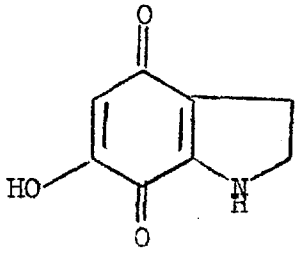


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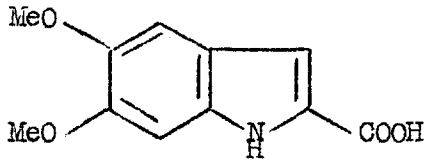


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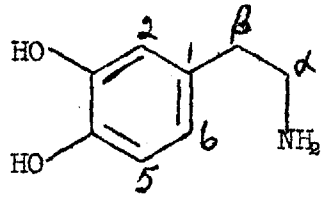
Carbon dioxide Evolution During Melanin Formation

Decarboxylation of dopachrome is implied in Raper's scheme of melanogenesis. Raper prevented this

decarboxylation by using sulphorous acid and hence was able to isolate 5,6-dimethoxy-2-carboxylindole (21). Further evidence for this decarboxylation was obtained by Mason and Wright[30] who investigated quantitatively the amount of carbon dioxide evolved during the oxidation of dopa. Clemo and his coworkers[31] However, by using (±) [$^{14}CO_2H$] tyrosine and (±) [$^{14}CO_2H$] dopa found that the amount of carbon dioxide evolved, during the enzymatic oxidation of the former at pH 6.8 and the autoxidation of the latter at pH 8, generally exceeded one mole. The yield of melanin was only about 60% by weight of the amino acid used. Furthermore only about 50% of the evolved carbon dioxide arose from the carboxyl group of the amino acid. Also it was found that dopamine and 5,6-dihydroxyindole, both of which have no carboxyl groups, also evolved carbon dioxide during their autoxidations to melanin which suggested degradation of one or several of the intermediates during melanin formation. Swan and Wright[32] found that at least 96% of carbon dioxide evolved during the autoxidation of [$\alpha$-$^{14}C$] - and [$\beta$-$^{14}C$] dopamines arose from the breakdown of the benzene nucleus and only 1.9% and 2.3% respectively from the labelled positions (22). These authors, using

[$^{14}$C] nuclear labelled dopamines, later showed that 29.8, 27.2 and 3.5% of the carbon dioxide evolved during the autoxidation of this precursor arose from positions 3,4 and 5 respectively, and by inference 30% from position 2 (22). These results are consistent with the oxidative splitting of catechols under these conditions. By comparing the activities of the melanins with those of precursors, the authors suggested that approximately 1 unit in every 5 in the melanin polymer has undergone oxidative fission with the loss of probably $C_4$, $C_5$ and $C_6$ of indole nucleus (22), (23).

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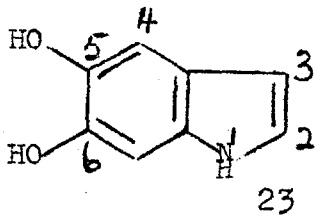


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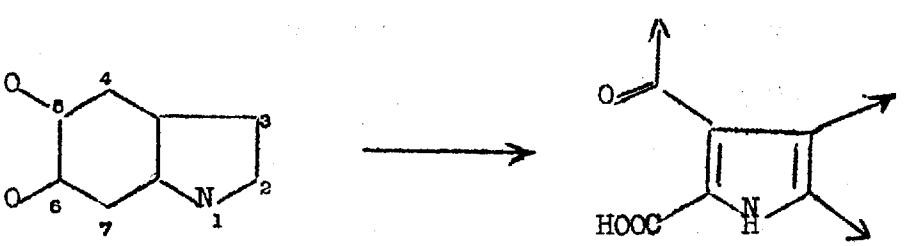
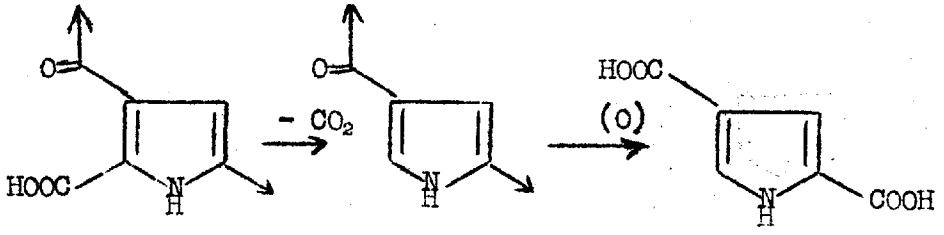
It is now well established[33,34] that autoxidation of diphenols to quinones leads to the formation of hydrogen peroxide which degrades the aromatic nucleus in melanin or any of its quinonoid precursors with the consequent evolution of carbon dioxide. Catalase which destroys hydrogen peroxide inhibits the evolution of carbon dioxide during melanin formation from dopamine

and 5,6-dihydroxyindole[33]. This has been confirmed by Beer[23] and Swan[32] and their coworkers who found that during the autoxidation of these compounds, the addition of catalase halves the amount of carbon dioxide evolved. Nicolaus and his coworkers[35] observed that the melanins prepared from dopa and 5,6-dihydroxyindole yielded, on heating at 190-200°, about half the amount of carbon dioxide evolved during melanogenesis, and also that the pigments prepared in the presence of catalase yield, when heated, about half the quantity of carbon dioxide obtained from the pigments prepared in the absence of catalase. These observations were interpreted as agreeing with Swan's results[32] on the hydrogen peroxide induced degradation of melanin. For example, during melanogenesis, from the rupture of one indole unit, two moles of carbon dioxide (from $C_5$ and $C_6$) and one carboxyl group should be formed. Hence the amount of carbon dioxide evolved during the heating of melanin should be half that obtained during melanogenesis.



This postulate however does not seem consistent with Swan's results which in fact suggested that positions 4, 5 and 6 of the indole nucleus contributed approximately equally to the carbon dioxide evolved. Nicolaus and his coworkers[35] also reported the presence of pyrrole-2,3-dicarboxylic acid (24) and pyrrole-2,5-dicarboxylic acid (25) among the oxidation products of decarboxylated melanins whereas these were absent among the oxidation products of the undecarboxylated melanins. They submitted these observations as a further proof of the oxidative cleavage of the benzenoid portion of some indole units during melanogenesis.

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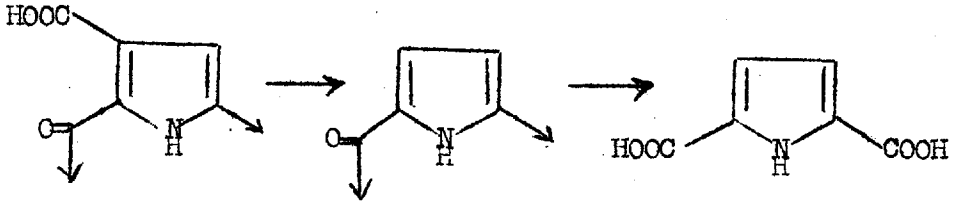


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Generally higher yields of melanins were obtained when oxidations were carried out in the presence of catalase.[33,35]

## STRUCTURAL STUDIES

Synthetic melanin is readily obtained in the laboratory by the tyrosinase catalysed oxidation, at pH 6.8, of tyrosine or dopa with molecular oxygen. Alternatively, dopa may be converted into melanin by autoxidation at pH 8. Sepia (cuttle fish) melanin is generally obtained by extraction into strong alkali followed by recovery with acid. Extraneous matter is removed by solvent treatment, and protein is separated by prolonged strong acid hydrolysis[36].

### Difficulties encountered in structural work

The physical nature of this pigment has made structural work particularly difficult. Spectroscopic techniques do not seem of great value for characterising melanins, since all the pigments examined so far absorb both visible and ultraviolet light without revealing any well defined maxima; even infrared spectra show only a few broad bands which are useless for characterisation[6]. Its insolubility in common organic solvents has made other physical techniques, notably n.m.r. of no use at all. It has so far not been possible to obtain a crystalline specimen or a crystalline derivative of melanin, hence

x-ray crystallographic analysis has not been possible. There is in fact no rigid proof, up to date, that the pigment isolated in various preparations is one single chemical compound. Nor has it been possible to even compare melanins produced under different conditions, (see below). Melanin is very hygroscopic and rather difficult to burn; hence elementary analyses on different samples prepared under apparently identical conditions, and even repeated analyses on the same sample have been inconsistent and consequently unreliable. Thus it is not possible to give the pigment any precise molecular formula. Nicolaus[35] has ascribed this difficulty to the high complexity and irregularity of the polymer. Strong acid treatment of samples before analysis is open to obvious criticisms.

Melanin prepared by autoxidation with its accompanying production of hydrogen peroxide and its attendant complications may be quite structurally dissimilar from that formed enzymatically in the presence of tyrosinase with or without catalase. Autoxidatively prepared melanins and the natural melanins (the latter are undoubtedly formed enzymatically) have been reported generally to have better solubility properties and lower carbon content than those prepared enzymatically in the laboratory. Mason

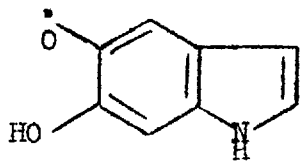
and Wright[30], from their investigations of oxygen-uptake and carbon dioxide evolution during the oxidation of dopa, concluded that variations in the experimental conditions, like pH, buffer concentration, enzyme concentration and duration of oxidation may affect the course of the reaction and hence exercise a significant effect on the final structure of the pigment. In fact, an increase in all these factors caused an increase to a certain limit of overall oxygen consumption and carbon dioxide evolution. These authors therefore stressed the necessity of stating the precise experimental conditions of the preparation of melanin samples in order to permit reproducibility of results.

Tyrosinase has two well defined functions in melanin formation. First, it catalyses the hydroxylation of tyrosine to give dopa, and second, it catalyses at least the first step in the oxidation of dopa. It is not clear however if tyrosinase has other minor functions in melanin biosynthesis. There is some evidence to suggest that the course of melanin formation might depend on the purity and quantity of the enzyme used. Swan's latest tracer experiments present a case in point. This author and Percival[29] found that when dopa labelled with deuterium at the $\beta$-carbon was

oxidised to melanin, there was an increasing loss of deuterium with increasing amount of enzyme used. Swan and Binns[37] had claimed earlier on that they obtained different melanins by the autoxidation of dopamine at pH 8 in the presence of molecular oxygen or air. In view of all these variations, it is not surprising that workers in different laboratories cannot reproduce each other's works.

Free Radical Properties of Melanins

Commoner and his coworkers[38] observed paramagnetic properties of various natural and synthetic melanins which proved that they contain free radicals. These observations have been confirmed by other workers.[39,40,41] Mason[41] established that the free radical property of tissues containing melanin is a property of the contained melanin and established a correlation between the degree of pigmentation in the tissues and free radical content in Calliphora puparia, human hair and sepia ink. Invariably, increased pigmentation was associated with increase in free radical content. This property was ascribed to the presence of a semi-quinone in melanin structure itself. This free radical (26) would be stabilised by resonance with the rest of the conjugated system.



26

Electron spin resonance studies[42] on melanins have suggested that the polymer is highly irregular both in the nature of its component units and the way they are linked together.

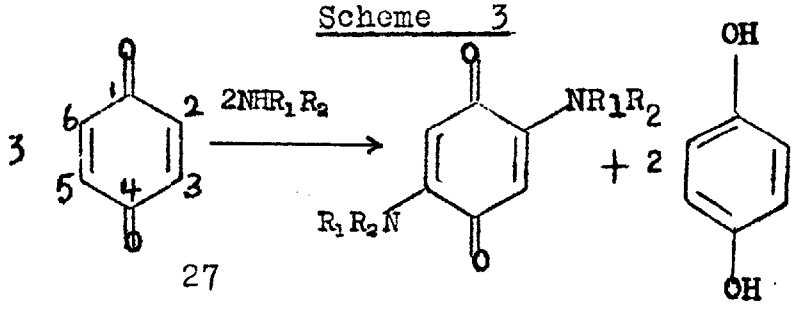
Studies with variously substituted precursors

Even if Raper's scheme for the early stages of melanin biosynthesis is accepted, it still remains to be decided how the repeating units of the melanin polymer are joined together. Harley-Mason and Bu'Lock[43] considered that melanin might be produced by the self-condensation of indole-5,6-quinone (16). These authors confirmed[43] the feasibility of such a condensation by model experiments with simple quinones and indoles.

Amines react with simple quinones to form substitution products, nucleophilic attack by the amine taking place preferentially at positions 2 and 5 of a p-quinone (27) and at 4 and 5 of an o-quinone (28) if these are free. If they are not free however,

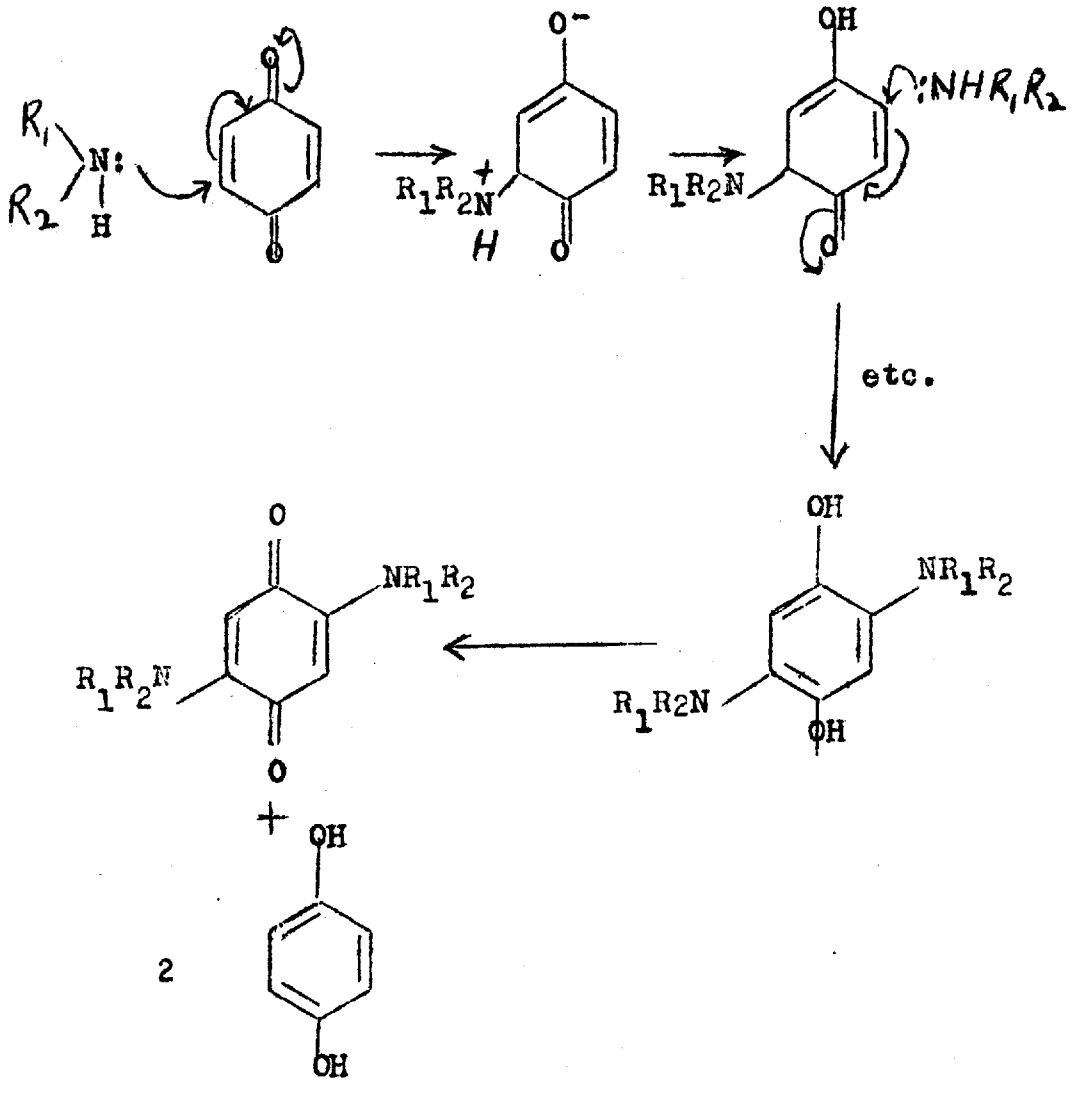
quinonimines (29) are formed, or side-chain substitution takes place[44]; (see Scheme 3.).

Scheme 3




27

Mechanism



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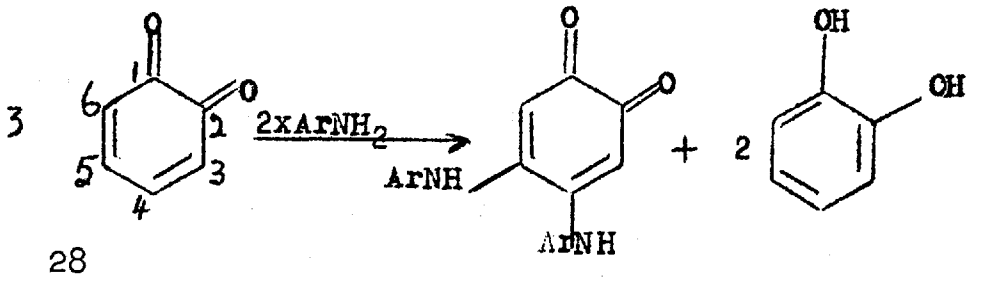


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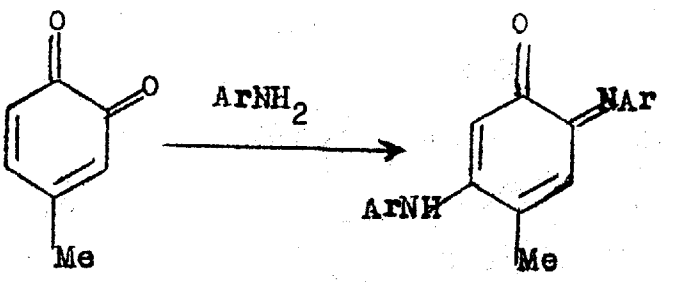
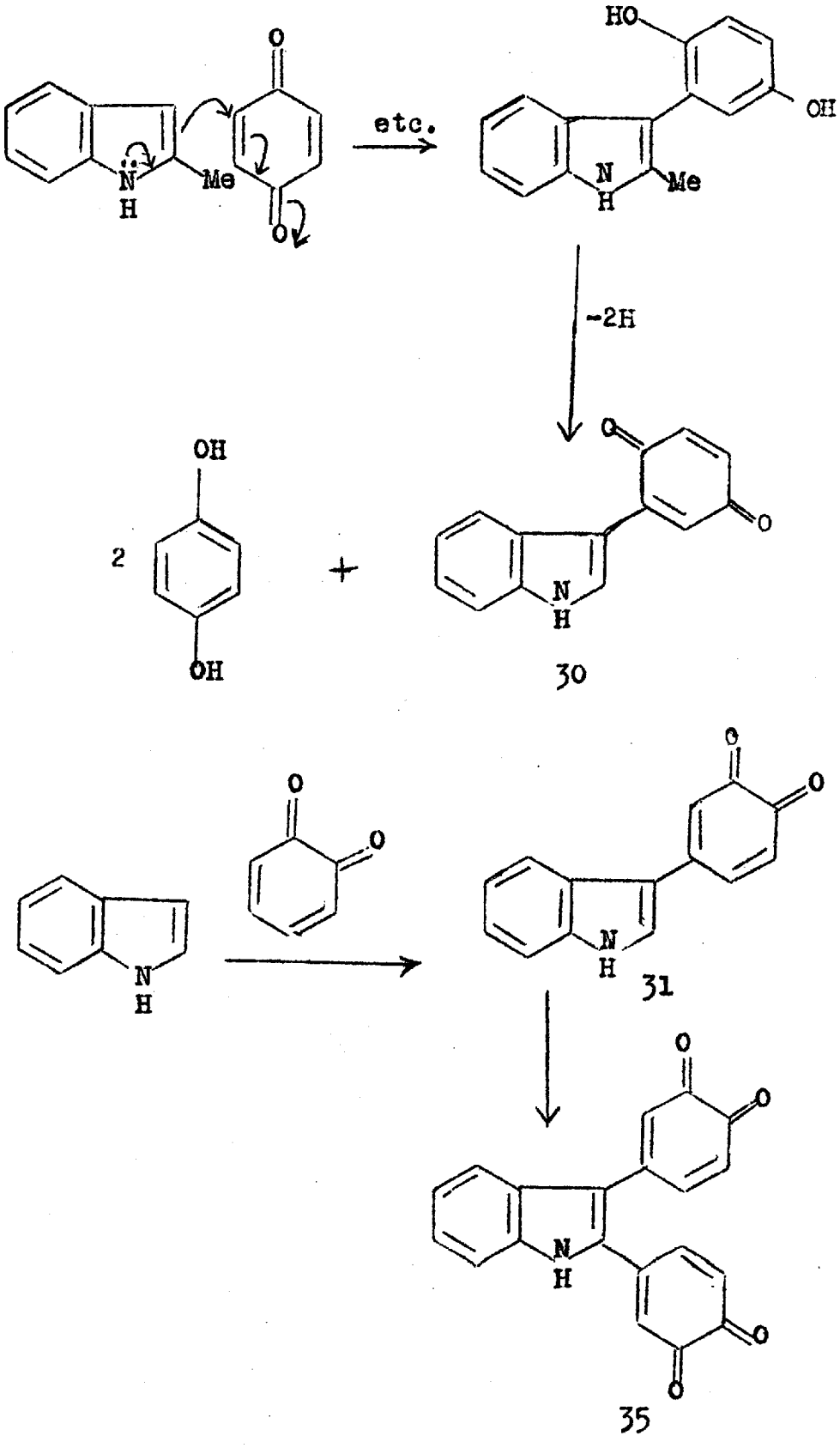
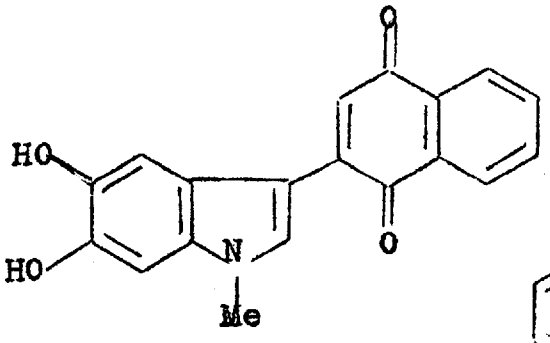



29

Although the nitrogen atom in heterocyclic substances is relatively inert towards quinones, other positions in the molecule may be very reactive. For example, indoles were shown[43] to condense with quinones to form 3-indolyl-quinones (30), (31), (32), (33), (34), (see Scheme 4)

Scheme 4





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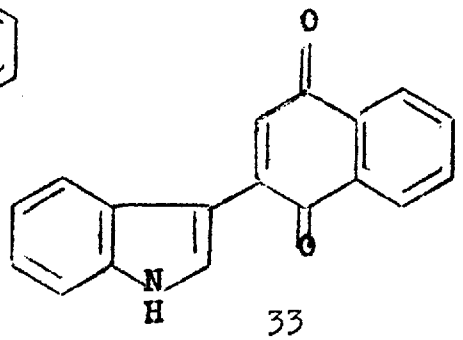

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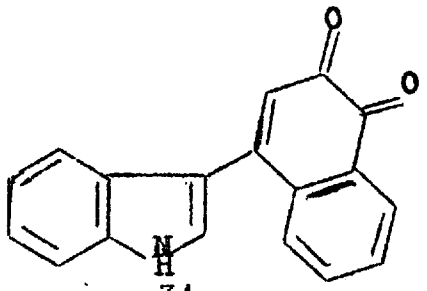

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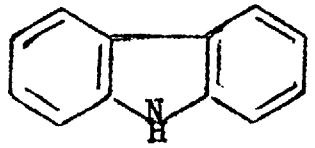


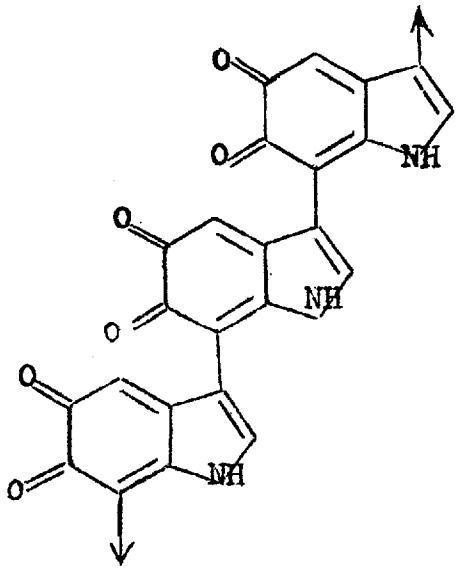
36

If the 2-position of indole is free, then a 2,3-disubstituted product (35) may be formed. However steric effects are important. When overlapping of substituents would cause large deviations from co-planarity in the product, reactions proceeded with difficulty if at all. Reactions with o-quinones were found to be generally faster than with p-quinones. However when 3-substituted and 2,3-disubstituted indoles or carbazole (36) were employed with p-benzoquinone, very slow reaction occurred forming colourless products. Similarly no reaction occurred between indole and 4,5-dimethyl-o-quinone while the 4-methyl-o-quinone reacted. These 3-indolylquinones were obtained by Harley-Mason and Bu'Lock as lustrous crystals varying in colour from blue-violet to almost black; they have

similar properties and generally behave as acids.

On the basis of the above results, Harley-Mason and Bu'Lock[43] postulated the polymerisation of indole-5,6-quinone by repeated oxidative condensation involving the anionic 3-position of one molecule with the cationic 4 or 7 position of another to give structures of the types (37) and (38). Structure (38) derived by condensation in 4-position cannot be coplanar, hence a product of such a structure is less likely. They therefore postulated a 3-7 linkage as the major back-bone structure for melanin. They considered also that minor contributions from cross-linkages involving all the other positions to give a highly irregular three-dimensional polymer would account for the extreme insolubility of the polymer. The extensive conjugation in the mesomeric structure (37a) could account for the general light absorption of the polymer.

To test the above postulate further, Harley-Mason and Cromartie[24] studied the oxidation of various methyl substituted dopas and 5,6-dihydroxyindoles. If the 3-7 linked structure were valid, then 5-substituted dopa, and 3-, and 7-substituted 5,6-dihydroxyindoles should not give melanins. (See (39) and (40) for the numbering systems).



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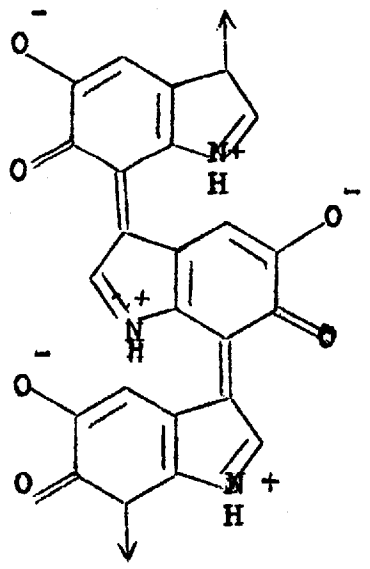
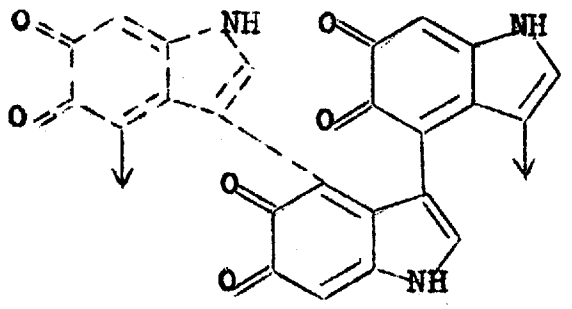


37(a)



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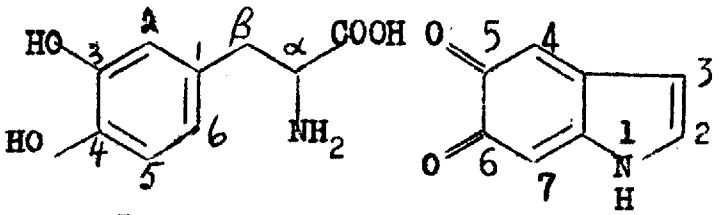


39 40

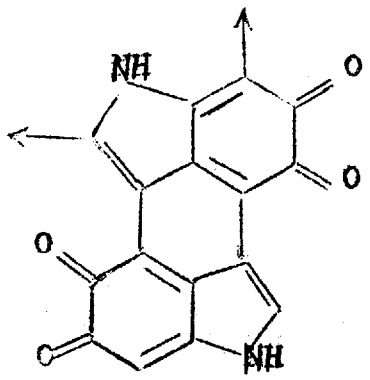
These authors[24] reported that 2-methyldopa and 5-methyldopa both gave melanins, while N-methyl-, 2-methyl-, and N, 7-dimethyl-5,6-dihydroxyindoles all gave insoluble 'melanin-like' pigments. On the other hand, 3-methyl-, and 4,7-dimethyl-5,6-dihydroxyindoles gave deep blue-violet pigments which were however, unlike "true" melanins, soluble in pyridine. 2,3-Dimethyl-5,6-dihydroxyindole gave an orange red solution which was stable to further oxidation. Similarly 6-methyldopa gave an orange-yellow solution. On the basis of the above results, they concluded that a free position 3 in the indole is essential for the formation of a "true" melanin which, according to them, is a black pigment of high molecular weight. These results are hardly clear-cut, but are in general agreement with the work of Robertson and his coworkers.[45,46] The latter found that 3-methyl-, 4-isopropyl-, 7-isopropyl-, 3-methyl-4-isopropyl-, and 3-methyl-7-isopropyl-5,6-dihydroxyindoles in faintly alkaline solution rapidly form blue-violet precipitates which in the case of the 4-isopropyl-, and 7-isopropyl derivatives went virtually black within a few hours. There was a discrepancy between the two sets of results; for while Harley-Mason and Cromartie obtained a stable red solution by oxidation of 2,3-dimethyl-5,6-dihydroxyindole

at pH 6.85 , Robertson[23] and his coworkers obtained, at pH 8, a black polymer of high molecular weight and hence concluded that a 4-7 linkage was possible. In support of their 3-7 linked structure, Bu'Lock and Harley-Mason[43] cited the reported[45] failure of 2-carboxy-5,6-dihydroxyindole (in contrast to 5,6-dihydroxyindole itself and its 2-methyl derivative) in alkaline solution to give a melanin. They attributed this failure to a deactivation of the 3-position by the adjacent carboxyl group. These authors also reported that the enzymatic effect on the rate of oxidation of dihydroxyindoles was negligible. More recently however, Nicolaus and his coworkers[56] reported the enzymatic oxidation of this compound to melanin.

These discrepancies further confirm the necessity for stating the precise experimental conditions in these studies (cf. ref. 30) It does not, of course, follow that the autoxidation of model compounds can be used to deduce the structure of melanin formed enzymatically from unsubstituted dopa.

The above results of Harley-Mason and Robertson and their coworkers are difficult to interpret and do not exclude the earlier structures (41), (42) and (43) proposed by Mason and Lada[48]. These authors

reported an average consumption of 4.6 atoms of oxygen per molecule of dopa during enzymatic oxidation. This is more than the 4 atoms per molecule required for a simple polymer of indole-quinone. This high oxygen consumption they suggested could be due to autoxidative degradation taking place even at pH 6.6-7.4 or to oxidative cross-linking in the polymer. On the basis of the latter reason, they postulated the structures (41), (42) and (43) as being consistent with an average of 3 inter-unit linkages with overall caoplanarity and with the known chemistry of indolylquinones.

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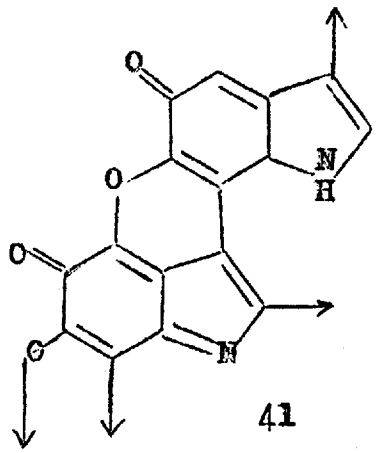


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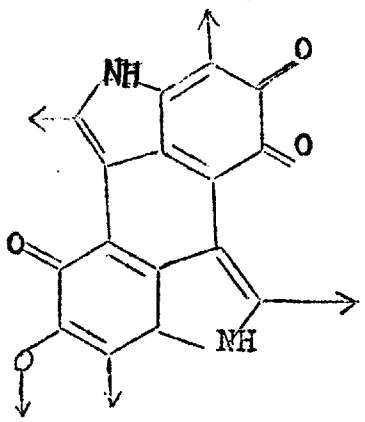
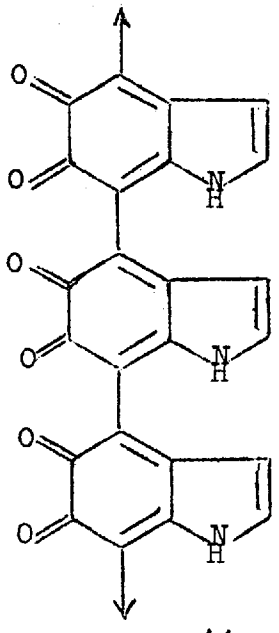



43

The obvious criticism of this postulate is that since these oxidations were conducted in the absence of catalase, the high oxygen consumption might be due to degradation by hydrogen peroxide.

Robertson's[23] suggestion of a 4-7 linkage of indole-5,6-quinone units has been supported by the results of Nicolaus and his coworkers[35] on the oxidative degradation of melanin (see below). Mason[5] also argued that position 3 may in fact not be essential for melanin formation. This author pointed out that 3-methyl-5,6-dihydroxyindole is more susceptible to oxidation than 5,6-dihydroxyindole itself and, as the products of autoxidation are rapidly removed from solution, extensive polymerisation and consequent melanin formation is prevented. Thus a 4-7-back-bone structure was postulated[5,49], and assumed to include some 2-4 and 2-7 linkages to give species like (44), (45) and (46).



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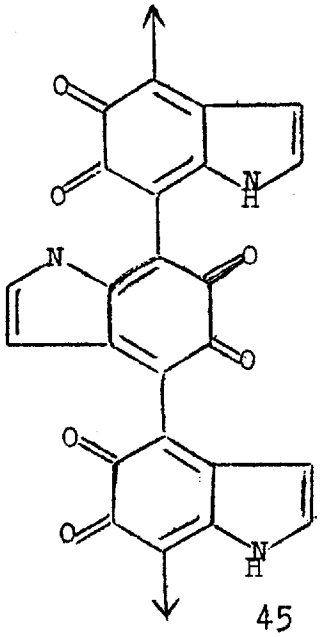

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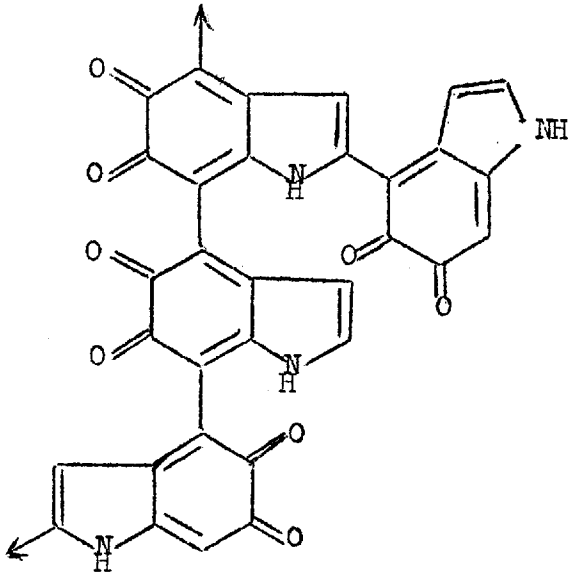
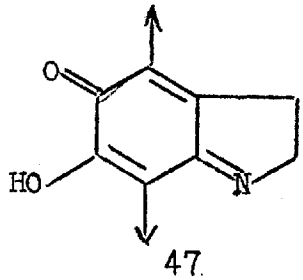


46

The predominance of 4-7 linkage has been strongly supported by the more recent work of Swan and his collaborators[4]. These authors found that when dopamine and dopa, labelled with deuterium at the $\beta$-carbon, were autoxidised to melanin, most of the deuterium was retained. They also found that melanin, prepared by autoxidation of dopamine in the presence of air, gave, on benzoylation, a chloroform-soluble derivative which contained 1 to 1.25 benzoyl groups per 8-carbon unit according to whether or not the melanin was reduced with alkaline dithionite before benzoylation. This was confirmed by using 4-chlorobenzoyl chloride (the chlorine content of the derived melanin was determined) and [$^{14}$C]-benzoyl chloride for benzoylation. Benzoylation of melanin formed by autoxidation of dopa yielded a chloroform-insoluble product that contained 1/22 to 1/6 benzoyl group per 8-carbon unit with or without reduction respectively. The infrared spectrum of the chloroform-soluble benzoylation product of the melanin formed by the autoxidation of dopamine showed bands at 1746 $cm^{-1}$. This latter value indicated that benzoylation had occurred predominantly at a phenolic hydroxyl group rather than at the nitrogen atom. These spectroscopic results together with the

deuterium retention obtained above suggested to Swan that in the melanin, formed by the autoxidation of dopamine, the methylene groups in the majority of the polymer units had not undergone dehydrogenation. On the basis of these results, Swan suggested that melanin prepared by autoxidation of dopamine might be mainly a 4-7 linked polymer of the unit (47). The unit is the enolised form of dopachrome (48) lacking the carboxyl group. However, the melanin obtained by the enzymatic oxidation of this $\beta$-deuterodopamine had a lower deuterium content than that obtained by autoxidation, although it still contained substantial amount of deuterium. Again, he favoured a 4-7 linked structure but now included an appropriate number of indole-quinone units to explain the loss of deuterium.

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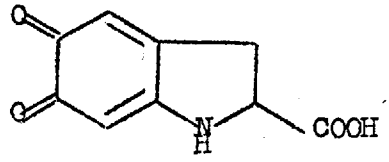



48

With melanin prepared from dopas labelled with deuterium at the $\beta$-carbon, they reported increasing loss of deuterium with increasing enzyme concentration. They therefore do not regard these results as final but leave the structure of this pigment undecided. These latest postulates of Swan

and his coworkers do raise the very serious question as to whether or not 5,6-dihydroxyindole is in fact an intermediate in the formation of melanins from dopamine autoxidation.

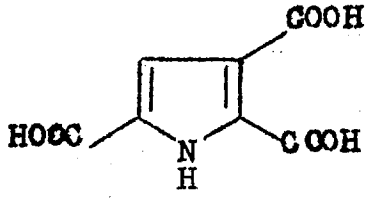
Chemical Degradation of Melanin

Melanin is resistant to strongly acidic and alkaline hydrolyses and to mild oxidation and reduction. Consequently, chemical degradation of the pigment has been unusually difficult. However, a measure of success has been achieved by Nicolaus and his coll a borators on the oxidative degradation of this pigment as a result of continuous work from 1952. Work in this field was also held back because the chemistry of the major products of oxidative degradation, namely pyrrole carboxylic acids, was not well known. This deficiency was first put right by the same group of Italian workers who have now made an extensive study of the pyrrole carboxylic acids. On account of the very small yields of degradation products invariably obtained, it is still rather difficult to make a quantitative assessment of the significance of these results in relation to melanin structure. Nonetheless they provide some useful

information on the probable nature of the nuclei present in the melanin pigment.

When sepia melanin (the natural melanin from cuttlefish) was oxidised with alkaline hydrogen peroxide, pyrrole-2,3,5-tricarboxylic acid (49) and pyrrole-2,3-dicarboxylic acid (50) were identified among the degradation products. The former was isolated as a crystalline compound[50] and the latter was identified[51] by paper chromatographic comparison with an authentic sample. Swan and Binns[52] partially oxidised, with alkaline hydrogen peroxide, melanins synthesised in vitro from dopa, tyrosine, dopamine and 5,6-dihydroxyindole. From the oxidation products they identified, by paper chromatographic comparison with authentic samples, the same pyrrole carboxylic acids (49) and (50). Since Chemo and coworkers[31] had shown that some of the original carboxyl groups of dopa and tyrosine were retained in the melanins prepared from them, Swan and Binns argued that pyrrole-2,3,5-tricarboxylic acid (since it was formed in very small quantities) might arise from indole units which retained the amino acid carboxyl group. However Nicolaus and his coworkers[35] have some evidence to show that such 2-carboxylindole units do not exist in the synthetic pigments (see below).

Nicolaus and his collaborators[53] later identified pyrrole-2,3,4,5-tetracarboxylic acid (51) among the products of oxidation of sepia melanin with hydrogen peroxide in acetic acid.

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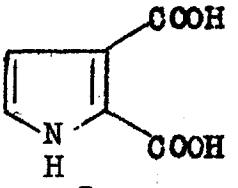


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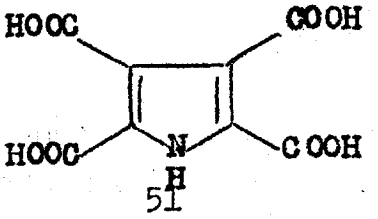


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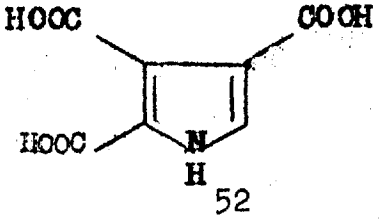



52

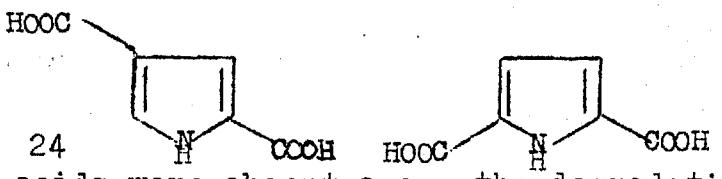
The most significant was the failure to detect pyrrole-2,3,4-tricarboxylic acid (52). This acid should be the main product if the Harley-Mason 3-7 backbone structure is correct. Since this acid was detected among the degradation products when violacein was oxidised under similar conditions[54], the failure to detect it among the oxidation products of melanin could not be entirely due to its destruction under the experimental conditions. These results therefore implied little coupling at position 3 of the indole-quinone unit, and hence led Nicolaus and his collaborators to give further support to the idea

of a 4-7 backbone structure which has already been discussed (see above). However, Piattelli and coworkers[55] have been able to isolate, by counter-current distribution followed by successive paper chromatographic purification, pyrrole-2,3,4-tricarboxylic acid (52) and pyrrole-2,3,4,5-tetracarboxylic acid (51) from the product of oxidation of sepia melanin with hydrogen peroxide in acetic acid. The former was obtained in extremely low yield. These acids were identified by comparison of their infrared spectra and chromatographic and electrophoretic behaviours with those of the authentic samples; neither of them was isolated as a crystalline solid.

More recently, Nicolaus and his coworkers have demonstrated the presence of the pyrrole-carboxylic acids (49), (50) and (51), in that order of decreasing quantity, among the oxidation (with alkaline permanganate) of sepia melanin, of melanins synthesised from dopa in the presence of tyrosinase (with or without catalase) and from 5,6-dihydroxyindole in the presence and absence of tyrosinase. These acids, which were obtained in very small yields as usual, were not isolated as crystalline solids. They were identified by paper

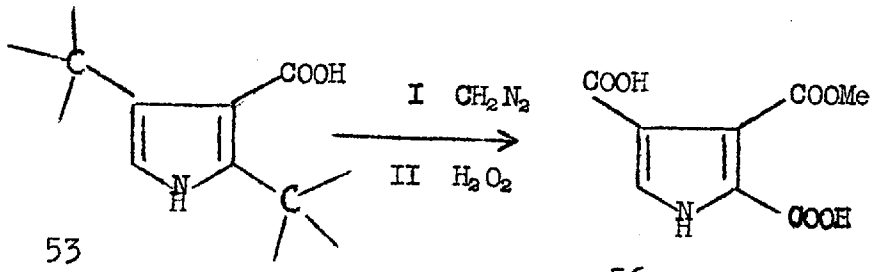
chromatographic comparison with the authentic samples. A significant observation from these oxidative degradations of melanins is that the same pyrrole carboxylic acids were obtained whether the melanin was natural or synthetic.

Further information has been obtained by Nicolaus and his collaborators[56] from studies on decarboxylation of melanins. When sepia melanin, which had been decarboxylated by heating at 190-200°, was oxidised with alkaline permanganate, pyrrole-2,4-dicarboxylic acid (24) and pyrrole-2,5-dicarboxylic acid (25) were found among the degradation products.




24 25

These acids were absent among the degradation products of the undecarboxylated sepia melanin. These observations were interpreted to indicate the presence of pyrrole units of type (53),(54) and (55) in the melanin polymer. These units may be derived from hydrogen peroxide degradation of the aromatic nuclei of 5,6-dihydroxyindole or indole-5,6-quinone units during melanogenesis (see the discussion of the evolution of carbon dioxide during melanin formation given above). This interpretation was supported in

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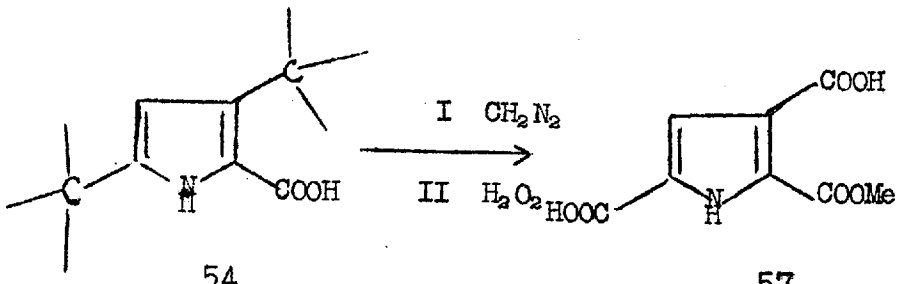


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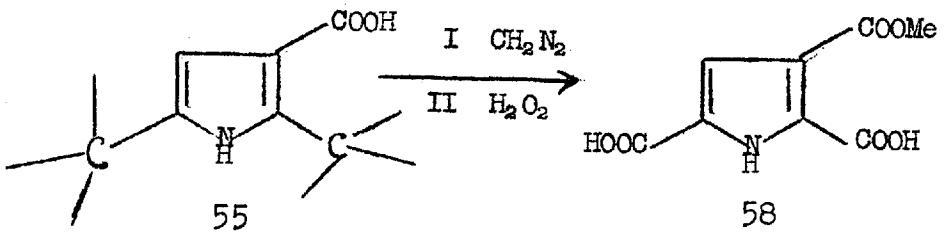


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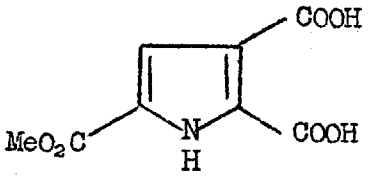


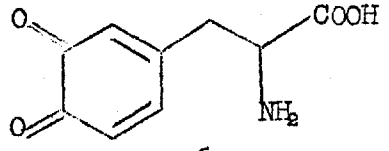

59

the following way. Sepia melanin was methylated with diazomethane and oxidised to give a small amount of 3-methoxycarbonylpyrrole-2,5-dicarboxylic acid (58). This compound was believed to be derived from the unit (55). Unfortunately, the other expected products (56) and (57) of this oxidation were not detected. This hypothesis of participation of pyrrole units in the building of the melanin polymer was further supported by the observation[35] that pyrolysis of melanin from the autoxidation of 5,6-dihydroxyindole gave large amounts of carbon dioxide arising presumably from carboxyl groups produced by partial breakdown of some indole units (cf. ref. 57)

From the oxidation products of methylated sepia melanin was isolated and characterised[56] 5-ethoxycarbonypyrrole-2,3-dicarboxylic acid (59). This was taken to imply the presence, in sepia melanin, of indole units with carboxyl groups at the 2-positions. This interpretation is strengthened by the earlier observations by the same authors[35] that higher yields of pyrrole-2,3-dicarboxylic acid and lower yields of pyrrole-2,3,5-tricarboxylic acid were produced by the oxidation of sepia melanin after decarboxylation than by the oxidation of the undecarboxylated pigment. All these results together

suggested that a carboxylated intermediate in Raper's scheme of biogenesis, probably dopachrome is built into the polymer framework. This is in broad agreement with Swan's results described above.

The hypothesis that uncyclised intermediates like dopaquinone might participate in building up the melanin polymer has also been considered. The retention of carboxyl groups of some dopa molecules in dopa melanin was proved by Clemo and his coworkers[31] by the use of [$^{14}$C] carboxyl labelled dopa and tyrosine. They showed that, by enzymatic oxidation of tyrosine and autoxidation of dopa, 1/6 of the carboxyl groups were retained in the polymer. This was explained by assuming the incorporation of carboxylated intermediates which might be cyclised like dopachrome (8) or uncyclised like dopaquinone (6). Nicolaus and his

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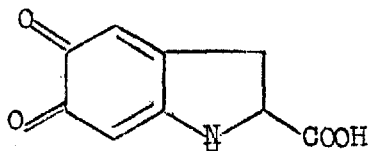


8

coworkers favoured the idea of uncyclised amino acid units on the basis of his pyrrole acids work. He found no significant variation in the yields of the pyrrole-2,3-dicarboxylic acid and pyrrole-2,3,5-tricarboxylic acid in the oxidation products of melanins before and

after decarboxylation. Furthermore, since pyrrole-2,3,5-tricarboxylic acid was not found among the hydrolysed oxidation products of methylated dopa melanin, this pigment, unlike sepia melanin (see above), is unlikely to have carboxyl groups in position 2 of the indole nuclei. This would imply that all the dopaquinone molecules which cyclise decarboxylate. This hypothesis was further supported by the detection of trimethylamine and ammonia, identified by gas-liquid chromatography, among the oxidation products of methylated dopa melanin[35] and methylated sepia melanin[56].

Clearly this degradation work suffers from the serious disadvantage that all the recognisable products are produced in very poor yields. It is quite impossible to decide the relative importance of the numerous structural units which have been suggested as being present in melanin. Nor is it even clear if the various melanins used in the work have closely similar structures. For these reasons, it was decided to develop a method that might give quantitative information concerning the manner in which the units are linked in melanin and which could be used for comparing the structure of enzymatic, autoxidative and natural melanins.

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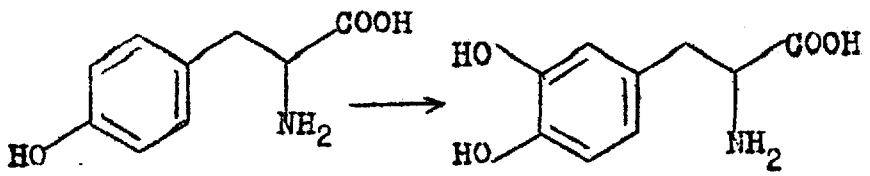
## Purpose of Present Work

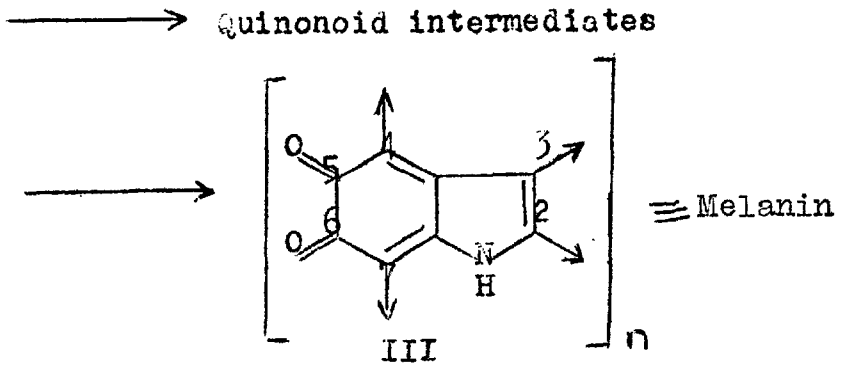
In the discussion of this work, the name melanin will be restricted to the insoluble black pigment obtained by the tyrosinase-catalysed oxidation of tyrosine (I) and 3,4-dihydroxyphenylalanine (dopa) (II) with molecular oxygen (in the presence of catalase) at pH 6.8 or by the autoxidation of dopa at pH 8. The earlier stages of the biosynthesis of this pigment as postulated by Raper[40] and confirmed spectroscopically by Mason[41] have been generally accepted. Nicolaus and his coworkers[42] have recently produced further evidence in support of this biogenetic scheme. It is now generally believed that this pigment is largely a polymer of indole-5,6-quinone units (III) with possible minor contributions from other entities like pyrrole units and dopaquinone units[43]. For the sake of convenience, the polymer shall be regarded as consisting essentially of indole-5,6-quinone units (see Scheme 1).

Scheme 1




I II

Scheme 1 (Contd.)



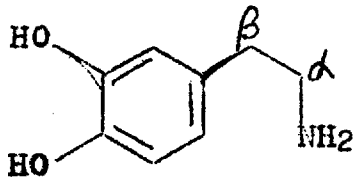

III

The problem of melanin structure therefore has been to find out which positions of the indolequinone unit are involved in coupling.

As described in the review section, there have been three main approaches to this problem. Harley-Mason's approach[44] was the study of melanin formation using dopa variously substituted with methyl group in it. aromatic nucleus. This approach suffers from the obvious drawback that these methyl dopas may not necessarily behave like ordinary dopa under the oxidation conditions. Another approach is illustrated by the degradative studies carried out by Nicolaus and his collaborators[43] and by Binns and Swan[45]. The most important products of degradation (pyrrole carboxylic acids) have never been isolated as crystalline solids, but always identified by paper chromatography of the degradation products. These pyrrole carboxylic acids

were invariably detected in very low yields (for which no precise figures are available in the literature), and hence it has not been possible to rationalise these results quantitatively in relation to melanin structure.

A more recent approach by Swan and his collaborators[46] has involved the oxidation of dopamine (IV), labelled with deuterium at its $\alpha$ and $\beta$ positions, and of dopa, labelled in its $\beta$-position, to give melanin.




IV

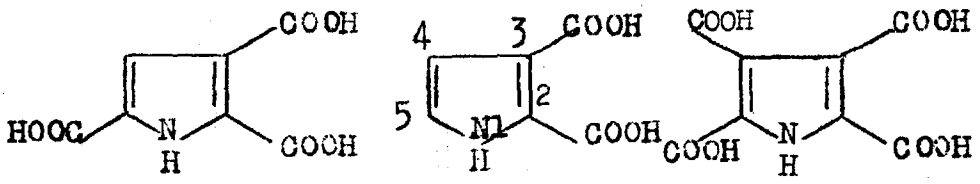
The results from the oxidation of dopamine were discussed earlier but do not bear directly on the dopa melanin problem. These authors were not decided on the interpretation of their results with $\beta$-deuterodopa.

It appeared to us however that information about the positions of linkage could be obtained by using tritium labelled dopa. Such an approach would (subject to certain assumptions which are discussed in details below) provide a quantitative measure of how far each position of the indole-quinone unit is involved in coupling. This approach could also be developed to provide the first quantitative method

for comparing the structures of melanins produced under different conditions. This forms the main theme of the present work. However it was thought desirable to see if useful information could be obtained from the oxidative degradation of melanin using radio dilution techniques. This preliminary work will be discussed first and will be followed by a discussion of the nuclear exchange reactions of phenols. Adaptations of such exchange reactions for the synthesis of specifically labelled dopa will be described next. Finally the use of such precursors in the study of melanin structure will be discussed.

## Isotope Dilution Study of Melanin Degradation

The very poor yields of the pyrrole carboxylic acids (V,VI,VII)

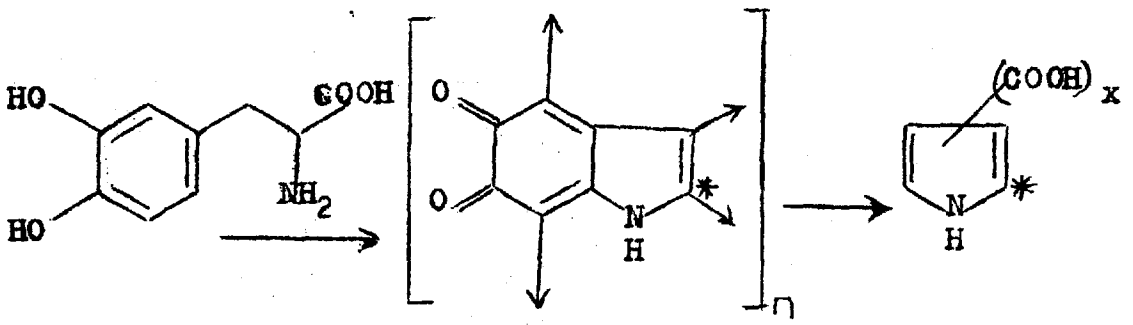


V VI VII

obtained from the oxidative degradation of melanin could be due to, among other reasons, the difficulty of isolation and the instability of these compounds under the conditions of oxidation. These difficulties appeared to us to be surmountable by a radio-dilution study of the oxidative degradation. This technique would provide the first quantitative information on the yields of these acids from melanin degradation.

The principle behind this technique is briefly as follows[47]. [$\alpha$-$^{14}$C] Dopa is oxidised to give melanin as in Scheme II.

Scheme II



≡ Melanin

A sample of this material, of known total activity, is degraded in the usual way to give the pyrrole carboxylic acids which must be labelled as indicated (Scheme II). A known weight of an inactive sample of one of the acids is then added to the oxidation product. After re-isolation and rigorous purification the specific activity of the acid can be used to calculate the total activity of this species present in the oxidation mixture. Thus it is possible to obtain an accurate value for the yield of various acids produced during the oxidation. If the diluting, inactive acid is added at the beginning of the oxidation then it will "protect" from over-oxidation the small amounts of material produced by break-down of the melanin. In this way, one can obtain an estimate of the true yield of oxidation product free from losses caused by further oxidation or isolation methods.

Pyrrole-2,3-dicarboxylic acid (VI) was chosen for the first study. This acid was obtained from the hydrolysis[29] of the 3-monoethyl ester. The latter was obtained by the condensation[28] of aminoacetaldehyde (prepared[27] by the sodium amalgam reduction of glycine ester hydrochloride in water) with ethyl oxaloacetate.

An intensive study of melanin oxidation with potassium permanganate (oxidation with hydrogen peroxide gave the same products but was found to be rather slow) was made in order to settle the following points.

(a) The rate and duration of oxidation in the absence and presence of added pyrrole-2,3-dicarboxylic acid (referred to henceforth as the 'diacid'),

(b) the consumption of the oxidant by melanin, and

(c) the stability of the diacid under the conditions of oxidation.

Melanin was treated with alkaline permanganate and the rate of consumption of the oxidant was followed by titrating aliquot portions, withdrawn at intervals from the oxidation mixture, against standard ferrous ammonium sulphate. Oxidation was very rapid and practically complete within the first hour (see Table 6 and graph B). When oxidations were repeated in the presence of added diacid, similar results were obtained but much more oxidant was consumed (see Table 7 and graph C). The stability of alkaline permanganate under the conditions of oxidation was confirmed by titrating aliquot portions of it, from time to time, as above (see Table 5 and graph A). The

completeness of oxidation which is so important in the present work was ascertained by treating the oxidation mixture with sulphur dioxide, the latter dissolved manganese dioxide leaving a clear solution if all the melanin was oxidised. From Table 7, it was obvious that the diacid consumed a significant quantity of the oxidant, a fact which was kept in mind in later investigations. The precise quantities of the oxidant consumed by melanin and the diacid were determined by running oxidations on them separately and on a mixture of them in a known small excess of oxidant. The unused oxidant was determined by back-titrations as above.

Since the diacid is susceptible to oxidation under these conditions, the crucial problem was to regulate the amount of oxidant present such that all the melanin would be oxidised, while some diacid was left undestroyed. This problem was solved by further oxidation experiments which revealed the following facts (see Table 9). (a) Melanin and the diacid were oxidised simultaneously; (b) when oxidation was conducted in the presence of a certain optimum quantity of oxidant (surprisingly, approximately equal to the total consumption by the melanin and diacid present

Table 5

Stability of Alkaline Potassium Permanganate

(10 ml. of N-permanganate plus 0.6 ml. 2N-potassium carbonate).

| Time (hr). | 0 | 5 | 10 | 24 | 48 | 72 |
|---|---|---|---|---|---|---|
| *N/40 $Fe^{++}$ (ml.) | 18.10 | 17.90 | 17.80 | 17.50 | 17.30 | 13.50 |

Table 6

Oxidation of Melanin in Alkaline Potassium Permanganate

(Melanin, 22.2 mg.; N-permanganate, 10 ml.; 2N-potassium carbonate, 0.6 ml.).

| Time (hr.) | 0 | 0.25 | 0.5 | 0.75 | 1 | 2 | 4 |
|---|---|---|---|---|---|---|---|
| *N/40 $Fe^{++}$ (ml.) | 18.20 | 13.03 | 12.50 | 12.10 | 11.95 | 11.70 | 11.20 |
| Time (hr.) | 8.5 | 24 | | | | | |
| *N/40 $Fe^{++}$ (ml.) | 10.65 | 10.10 | | | | | |

Table 7

Oxidation of Melanin in the Presence of added Pyrrole-2,3-dicarboxylic acid in Alkaline Potassium Permanganate

(melanin, 18.2 mg.; diacid, 20.8 mg.; N-permanganate, 10 ml.; 2N-potassium carbonate, 0.6 ml.)

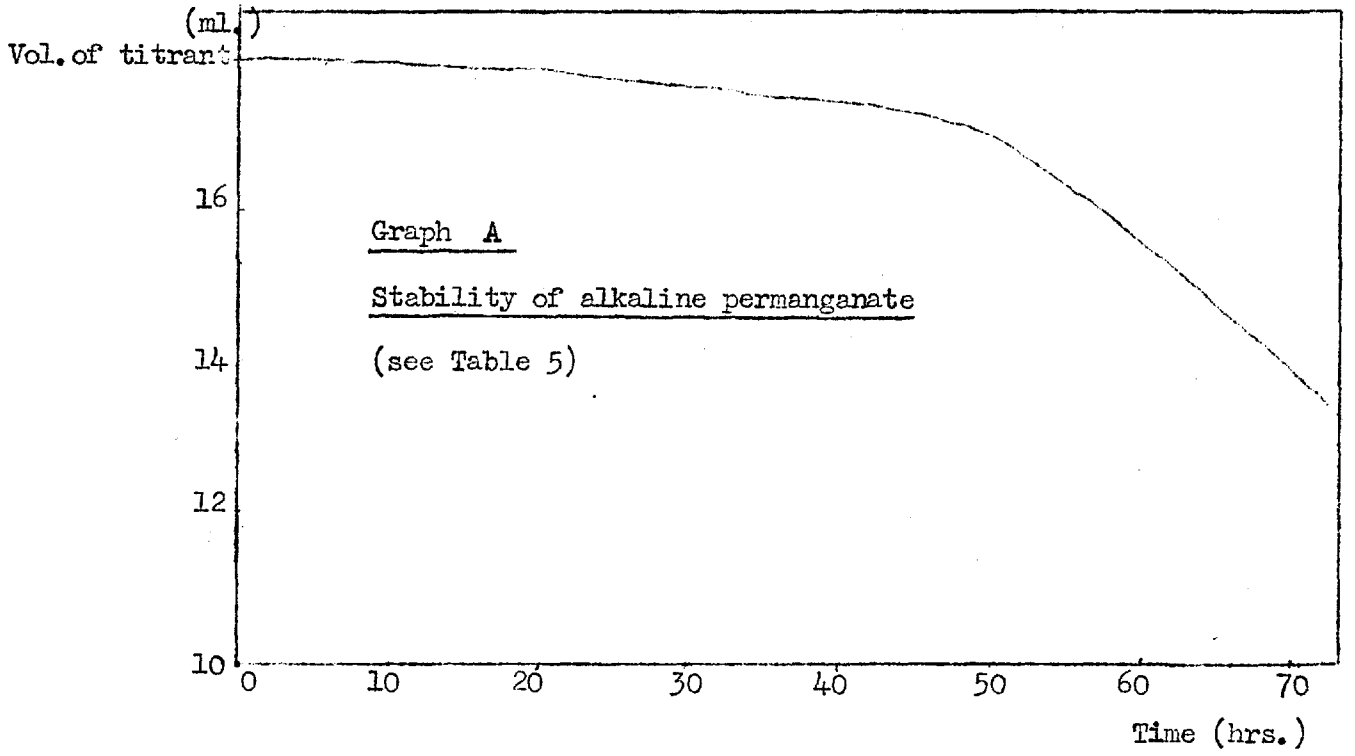
| Time (hr.) | 0 | 0.25 | 0.5 | 0.75 | 1 | 2 | 4 |
|---|---|---|---|---|---|---|---|
| *N/40 $Fe^{++}$ (ml.) | 18.10 | 11.05 | 10.35 | 10.15 | 9.00 | 8.65 | 8.30 |

over/....

Table 7 (contd.)

| Time (hr.) | 14 | 24 |
|---|---|---|
| *$N/40$ $Fe^{++}$ (ml.) | 7.50 | 7.30 |

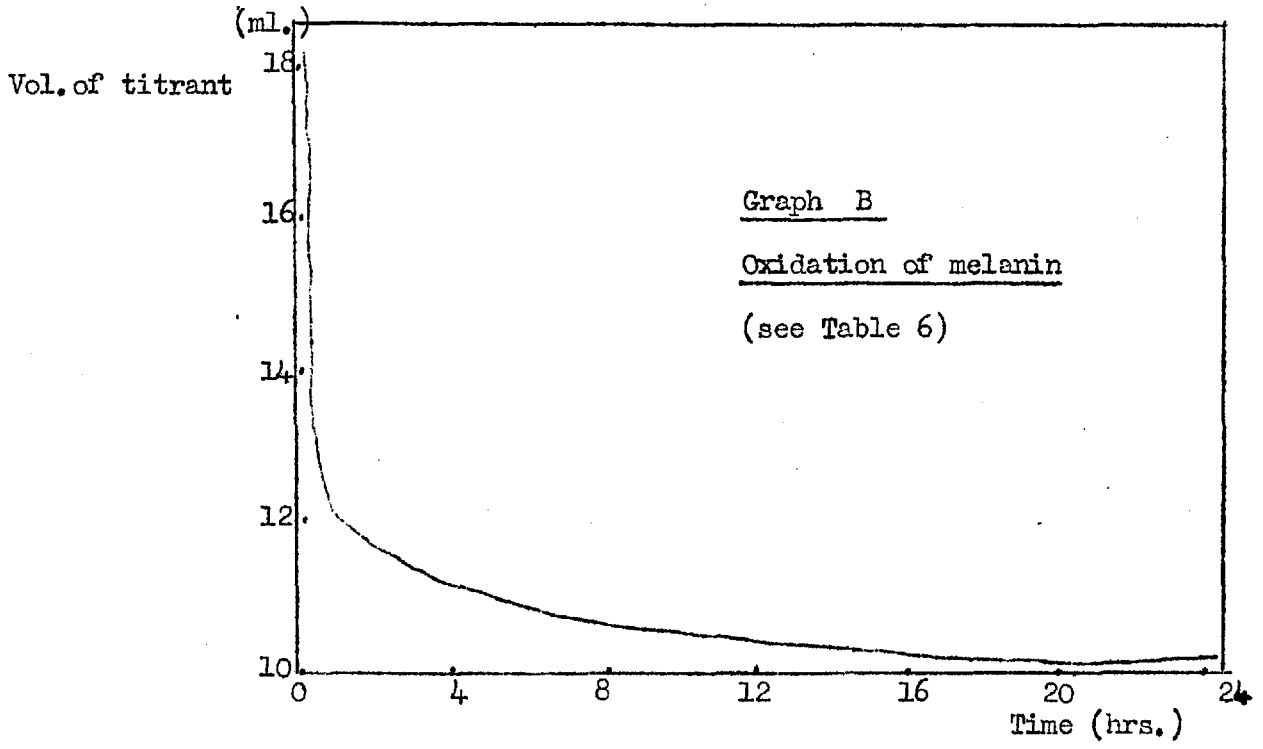
* The figures quoted represent the volume of ferrous ammonium sulphate taken up by 0.5 ml. portions withdrawn at intervals from the reaction mixture.




Graph A

Stability of alkaline permanganate

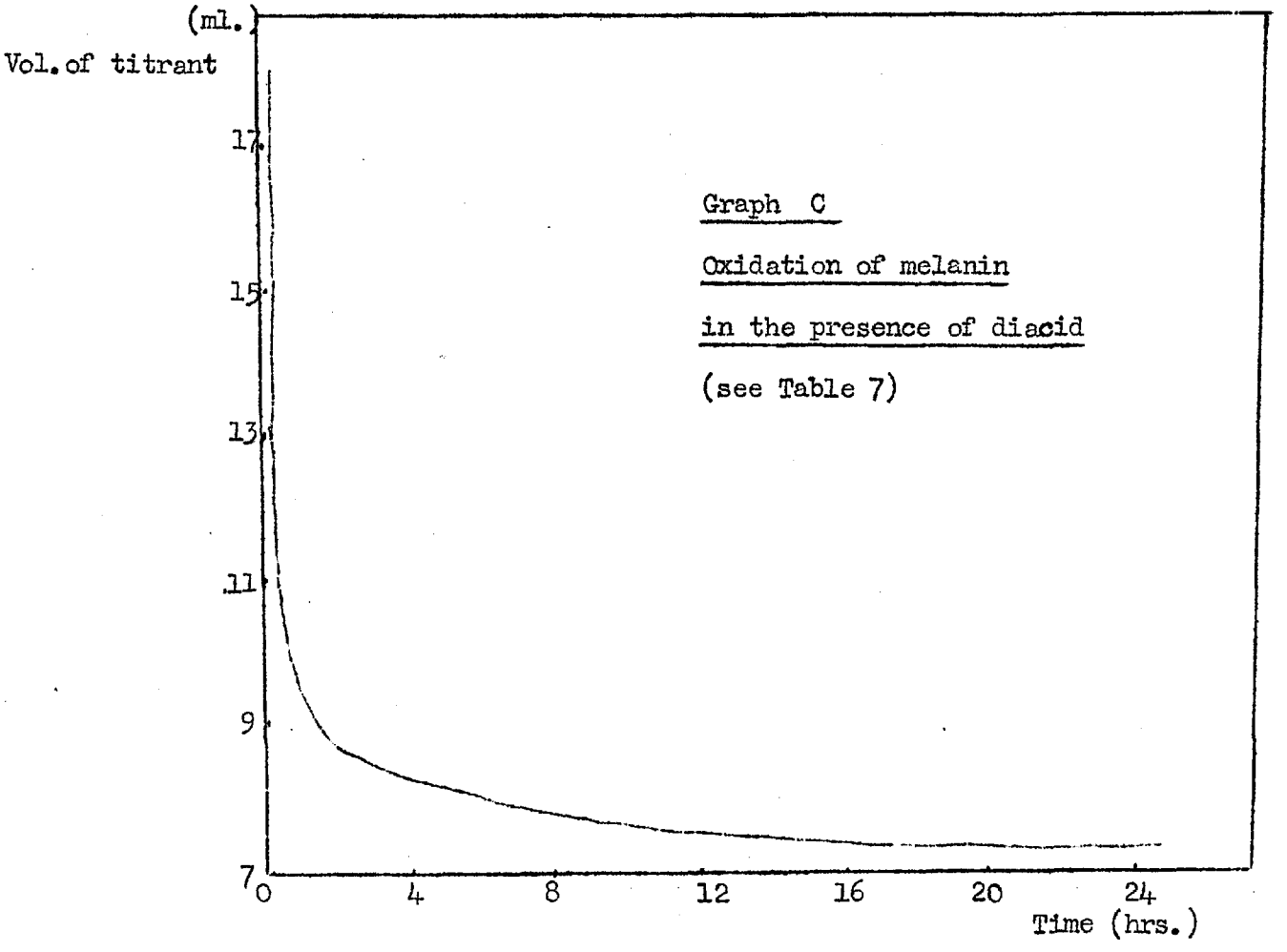
(see Table 5)




Graph B

Oxidation of melanin

(see Table 6)




Graph C

Oxidation of melanin

in the presence of diacid

(see Table 7)

as determined above) all the melanin was oxidised and 20-30% of the acid was recovered pure as its dimethyl ester; (c) two experiments using, in each case, a quantity of oxidant short of the above optimum amount, but greater than the amount normally required for the complete oxidation of the melanin alone, resulted in an incomplete oxidation of the melanin; (d) the use of a quantity of oxidant in excess of this optimum amount destroyed all the added diacid.

Table 9

Recovery of Pyrrole-2,3-dicarboxylic acid from the oxidation of Melanin in the Presence of the diacid.

| Melanin taken (mg.) | Diacid added (mg.) | N-Permanganate added (ml.) +0.6ml $K_2CO_3$ | Melanin oxidised (%) | Diacid recovered (%) |
|---|---|---|---|---|
| - | 46.8 | 5 | - | 30 |
| - | 46.8 | 10 | - | Nil |
| - | 50 | 10 | - | Nil |
| 49 | 52 | 10 | ca 70 | 27 |
| 51 | 50 | 10 | ca 65 | 25 |
| 41 | 52 | 12 | 100 | 25 |
| 44 | 53 | 12.6 | 100 | 20 |

(Each oxidation was run for 1 hr.)

Once the procedures for the oxidation of melanin in the presence of added diacid and for the isolation

of the unreacted diacid had been established, melanins of known activity (prepared from [$\alpha$-$^{14}$C]dopa) was oxidised and the degradation products assayed by a radio dilution method. The total radiochemical content of the pyrrole-2,3-dicarboxylic acid was found to be only 0.2 and 0.3% respectively, in two different runs, of that of the original melanin. Since all the radioactivity will be expected in the pyrrole nucleus, the above values also correspond to the yields of this acid from the oxidation of melanin. However since not less than 20% of the diacid has been shown to survive the conditions of the oxidation, the very low yield cannot be due to the production of this acid and subsequent destruction. Furthermore, some of the added inactive acid must have been destroyed before the production of the active material from melanin degradation. Therefore the measured specific activity would be higher than the true value, and the former must therefore correspond to the maximum yield of the diacid. This yield of diacid, the magnitude of which can be stated for the first time, is obviously too low to be interpreted in relation to melanin structure. Since the other pyrrole carboxylic acids are produced in similar yields, it did not seem to us worthwhile to repeat the above experiments with them.

## Exchange Reactions in Phenols

### General

The main theme of the present work involved the study of the structure of melanin using dopa labelled specifically with tritium in each of its nuclear and side chain positions. Such a precursor would, subject to certain provisions discussed below, give the correspondingly labelled indolequinone (see Scheme I). The main problem was specific labelling of the nucleus.

Ingold and his coworkers[1] had shown that phenols, when heated in alkaline deuterium oxide, exchanged nuclear hydrogens for deuterium. For example, in phenol itself, 3 aryl hydrogens were exchanged under these conditions. These authors and later workers[2] established that exchange occurred predominantly at positions ortho and para to phenolic hydroxyl group. Under more forcing conditions, for example with potassamide in liquid ammonia, meta exchange has also been observed[3]. The possibility[4] that some meta exchange might take place even in aqueous systems must be kept in mind (see below). It was apparent that ortho and para exchange of phenolic precursors in alkaline tritiated water would provide

a convenient and inexpensive procedure for the preparation of these nuclear labelled dopas. With this in mind, these exchange reactions were investigated further on a variety of phenols in order to establish general deuteration and hence tritiation methods which could be applied on a preparative scale. Special attention has been paid to the specificity of the exchange reaction.

In recent years much interest has been shown in the biosynthesis of complex phenolic compounds especially the phenolic alkaloids[5]. Recent experiences in these laboratories[6,7] have shown that monohydric phenols and catechols do not exchange ortho and para tritium in biological systems, and that phenols labelled in this way may indeed be used used successfully in biosynthetic studies. Resorcinol[50] and phloroglucinol[51] derivatives are, however, known to undergo nuclear exchange extremely rapidly. This would seriously limit the use of correspondingly labelled materials in biosynthetic work. These ortho and para exchange reactions of phenols have also been effected under acidic conditions[1,48]. Such acid-catalysed exchange reactions have been investigated on tyrosine[8] and dopa.

Base-catalysed Exchange Reactions in Phenols

The standard base catalyst in the present work was deuteroxide. This was conveniently generated by the addition of dry t-butoxide to deuterium oxide. In order to achieve exchange under milder conditions, several organic amines were tested, triethylamine proved most effective (see below). The exchange reactions were generally effected by heating, in a nitrogen-filled tube at 100°, the phenol in excess deuterium oxide in the presence of half a molar equivalent of deuteroxide or a molar equivalent of amine. These exchange reactions were conveniently followed by n.m.r. spectroscopy.

Tyrosine (I) gave on deuteration (in the presence of an additional mole of deuteroxide to ionise the carboxyl group) the 3,5-dideutero-derivative. The latter on chlorination[26] gave the 3-Cl-5-deutero derivative in which the remaining two aryl protons were well resolved doublets with the expected meta-relationship. [3,5-$^3H_2$]Tyrosine was obtained in a similar manner; chlorination of the latter gave a product (unrecrystallised) whose molar activity was 54% of the starting material.

In p-cresol, the replacement of the ortho-

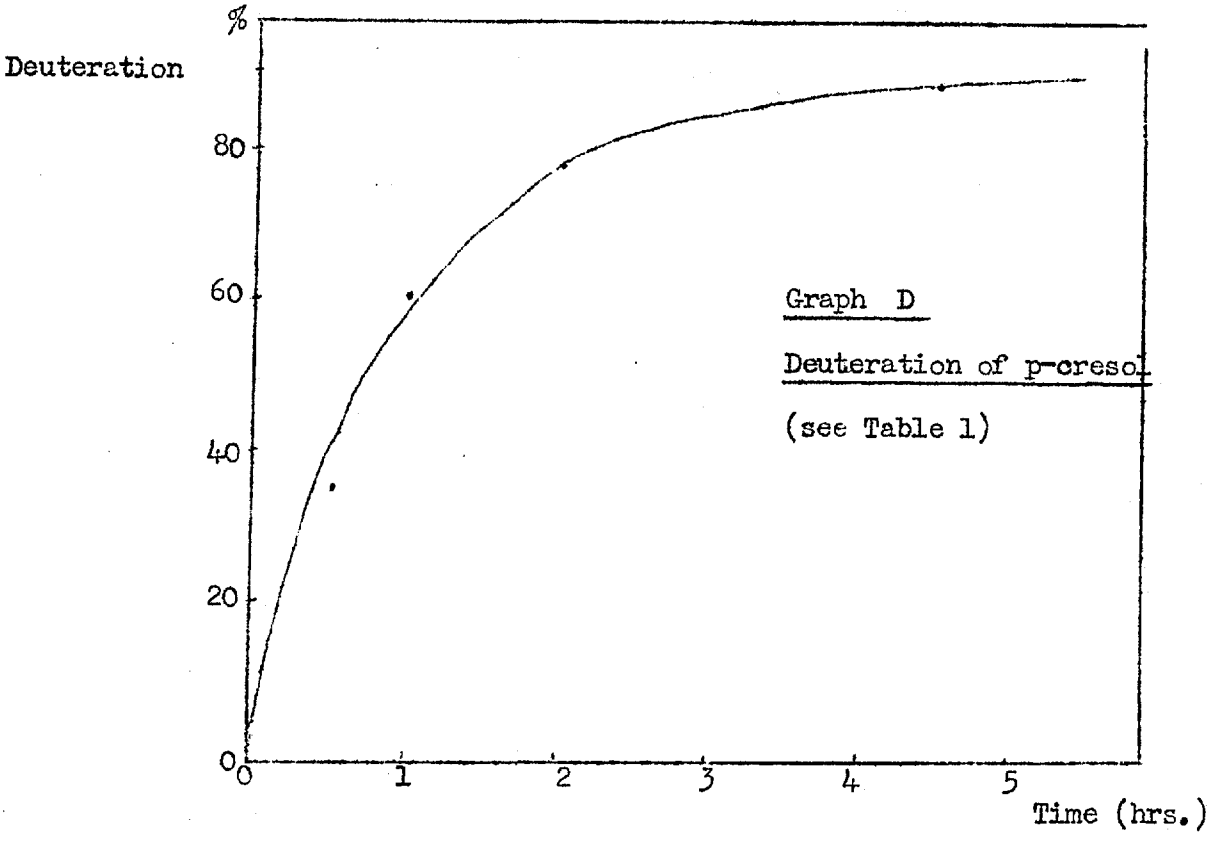
hydrogens by deuterium was observed as the original aryl quartet (4 protons) of the phenol eventually collapsed into a broad singlet (2 protons). The effect of varying amounts of triethylamine and of varying reaction times on the exchange of p-cresol in deuterium oxide is illustrated in Table 1 and Graph D. Even in this two phase system, rapid deuteration was observed, the rate being fastest when equimolar quantities of phenol and amine were present. There was virtually no exchange in this compound and in tyrosine when excess base was present. This agrees with the earlier studies of Ingold and his coworkers who showed[1] that rapid exchange occurred only when appreciable concentrations of phenoxide ion and undissociated phenol (PhOD) were present.

In isoeugenol (VIII), a catechol monoether, the positions ortho and para to the methoxyl group are activated towards electrophilic substitution and might, in principle, be involved in the deuterium exchange reaction. However, with either deuteroxide or triethylamine as catalyst, only the aryl proton ortho to the phenolic hydroxyl group was replaced. Also the olefinic proton vinylogously para to the phenolic hydroxyl group [cf. (IX)] was unaffected.

## Table 1

### Deuteration of p-cresol at 101-103°

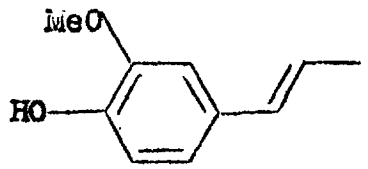
| $Et_3N$ (mole) per mole of phenol | 0.5 | 0.5 | 0.5 | 0.5 | 1.0 | 2.0 | 5.0 |
|---|---|---|---|---|---|---|---|
| Time (hr.) | 0.5 | 1.0 | 2.0 | 4.5 | 0.5 | 0.5 | 0.5 |
| Exchange (%) at ortho positions | 35 | 61 | 78 | 89 | 61 | 58 | 0 |



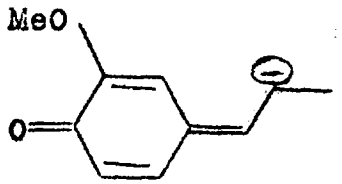

Graph D

Deuteration of p-cresol

(see Table 1)




VIII

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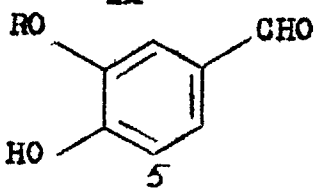
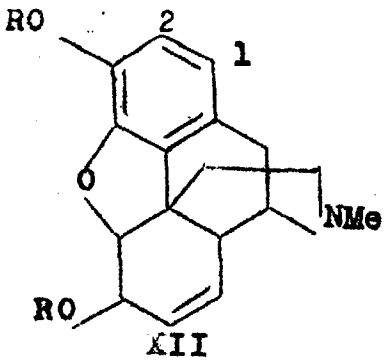
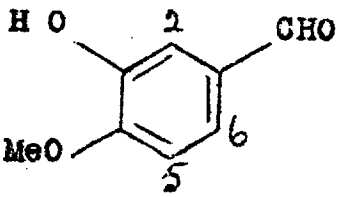


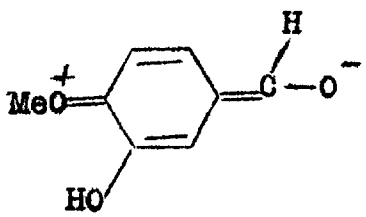

X




XII




XI




XIII

Vanillin (X; R=Me) likewise gave only the 5-deutero-derivative. For preparative work (see below), it was necessary to determine the stability of nuclear deuterium under the normal conditions for demethylation of phenolic ethers and for benzylation and debenzylation of phenolic hydroxyl groups. 5-Deuterovanillin was demethylated[9] with aluminium chloride and pyridine to give 5-deuteroprotocatechuic aldehyde (X; R=H) (n.m.r. control). The monobenzylprotocatechuic aldehyde (X; R=$PhCH_2$) was converted into the

monodeutero-derivative in the usual way. Benzylation, with benzyl chloride and potassium carbonate gave the corresponding benzyl ether without loss of deuterium. Hydrogenation over palladium-carbon in ethanol, containing hydrochloric acid, then yielded 5-deutero-3.4-dihydroxytoluene.

Although n.m.r. spectroscopy provides a convenient qualitative method for following deuteration, it is of limited value in quantitative determinations. Even with well resolved spectra, an error of up to 10% in the integration of proton signals is unexceptional. For this reason, tritiation was used to examine critically the specificity of ortho and para exchange. In isovanillin (XI), the positions (2 and 6) ortho and para to the phenolic hydroxyl group are strongly deactivated by the aldehyde group towards electrophilic substitution. The remaining position 5 which is meta to the phenolic hydroxyl group is less deactivated and is also ortho to the electron-releasing methoxyl. If "meta-exchange" in phenols in aqueous systems is at all important, then it should be observed in isovanillin. Preliminary deuteration studies, under the usual conditions, showed that exchange was generally slow but occurred more rapidly at position 2 than at position 6.

The slower rate of exchange at position 6 might be due to contributions from resonance forms like (XIII) which would diminish the reactivity at position 6 towards electrophilic attack, whereas position 2 is relatively unaffected. However it was not possible to deuterate the latter position exclusively under these conditions. The aryl hydrogen at position 5 appeared not to exchange, but accurate integration was not possible. The Cannizaro reaction did not interfere to any appreciable extent in the exchange reactions of the aromatic aldehydes under these conditions[30]. A sample of tritiated isovanillin, prepared in tritiated water with hydroxide as catalyst was next examined. Benzylation and oxidation afforded 3-benzyloxy-4-methoxy benzoic acid having the same molar activity as the isovanillin. As expected, no exchange of the aldehydic proton had occurred. Demethylation of the labelled isovanillin, under mild conditions[9] with aluminium chloride and pyridine, gave protocatechuic aldehyde (X; R=H) without significant loss of activity. Bromination of the latter gave the known 5-bromo-derivative which again had the same molar activity. Clearly only the 2 and 6 positions were involved in exchange. 6-Bromoisovanillin was deuterated smoothly with triethylamine as catalyst to give 6-bromo-[2-$^{2}$H]isovanillin; similarly 2-bromo-isovanilyl alcohol (in which the

deactivating effect of the aldehyde group no longer exists) gave the 6-deutero-derivative.

Recent studies on the structure[12] and biosynthesis[6] of phenolic alkaloids led us to make a special examination of nuclear exchange in this class of compounds. A phenolic alkaloid contains within its structure both the phenolic hydroxyl group and the amino-function required for the exchange reaction. Indeed specific ortho and para labelling of phenolic alkaloids with deuterium and tritium can be effected very simply. For example, morphine (XII; R=H) when heated alone in dimethylformamide containing deuterium oxide was converted in good yield into the 2-deutero-derivative. The position of deuterium followed from experience with simple phenols, but was supported in an independent way. Acetylation gave the correspondingly deuterated diacetylmorphine (XII; R=Ac). The aryl protons in undeuterated material gave a pair of doublets at 3.25 and 3.47 $\tau$ ($J$=8 cps) in the n.m.r. spectrum. The doublet at 3.47 $\tau$ was assigned to the proton at position 1 since fine coupling with the benzylic methylene had produced broadening and shortening of its component lines in comparison with those of the other doublet. In agreement with this the doublet at 3.25 $\tau$ disappeared on deuteration.

It was possible that traces of dimethylamine in the dimethylformamide had catalysed exchange. However, a similar result was obtained with dimethylsulphoxide as solvent; also morphine hydrochloride was not deuterated in dimethylformamide containing deuterium oxide. This procedure was used successfully for the deuteration and tritiation of sinoacutine[6], a phenolic dienone readily decomposed by aqueous alkali or triethylamine.

Acid-catalysed Exchange in Tyrosine and Dopa

Ingold, Raisin and Wilson[1] had established the electrophilic character of exchange reactions of phenols under acidic conditions. This has been confirmed by later workers[48]. Such exchange reactions were investigated with tyrosine[8] and dopa in order to adapt them for preparative purposes.

At 100° in 4.1 N-deuterium chloride rapid exchange of only the three aryl protons of dopa and the two protons ortho to the phenolic hydroxyl group in tyrosine was observed. No significant exchange had occurred after 7 days at ca 25°. The exchange in dopa was more rapid than that in tyrosine (see Table 4 and Graphs E and F).

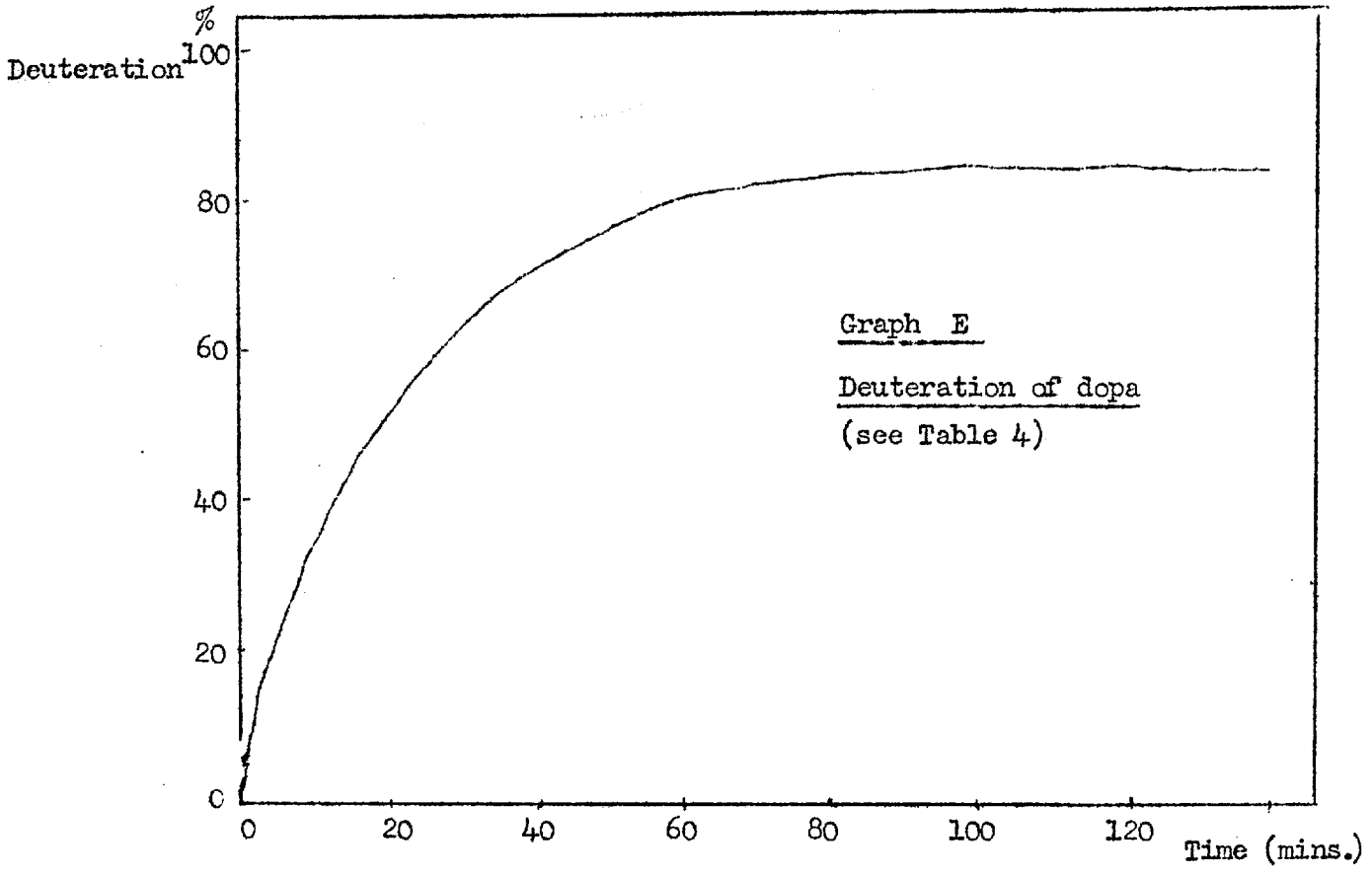
Table 4

Deuteration of L-Tyrosine and (+)-3,4-Dihydroxy phenylalanine (dopa) in 4.1 N DCl at 100°.

| Time (hr.) | | 0.25 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.5 | 4.5 | 5.5 |
|---|---|---|---|---|---|---|---|---|---|---|
| Deuteration (%)* in | Tyrosine | - | 21 | 37 | 52 | - | 66 | 75 | 80 | 83** |
| | Dopa | 44 | 64 | 81 | 82 | 87 | ** | | | |

* 100% deuteration corresponds to the exchange of 2 and 3 aryl protons in tyrosine and in dopa respectively.

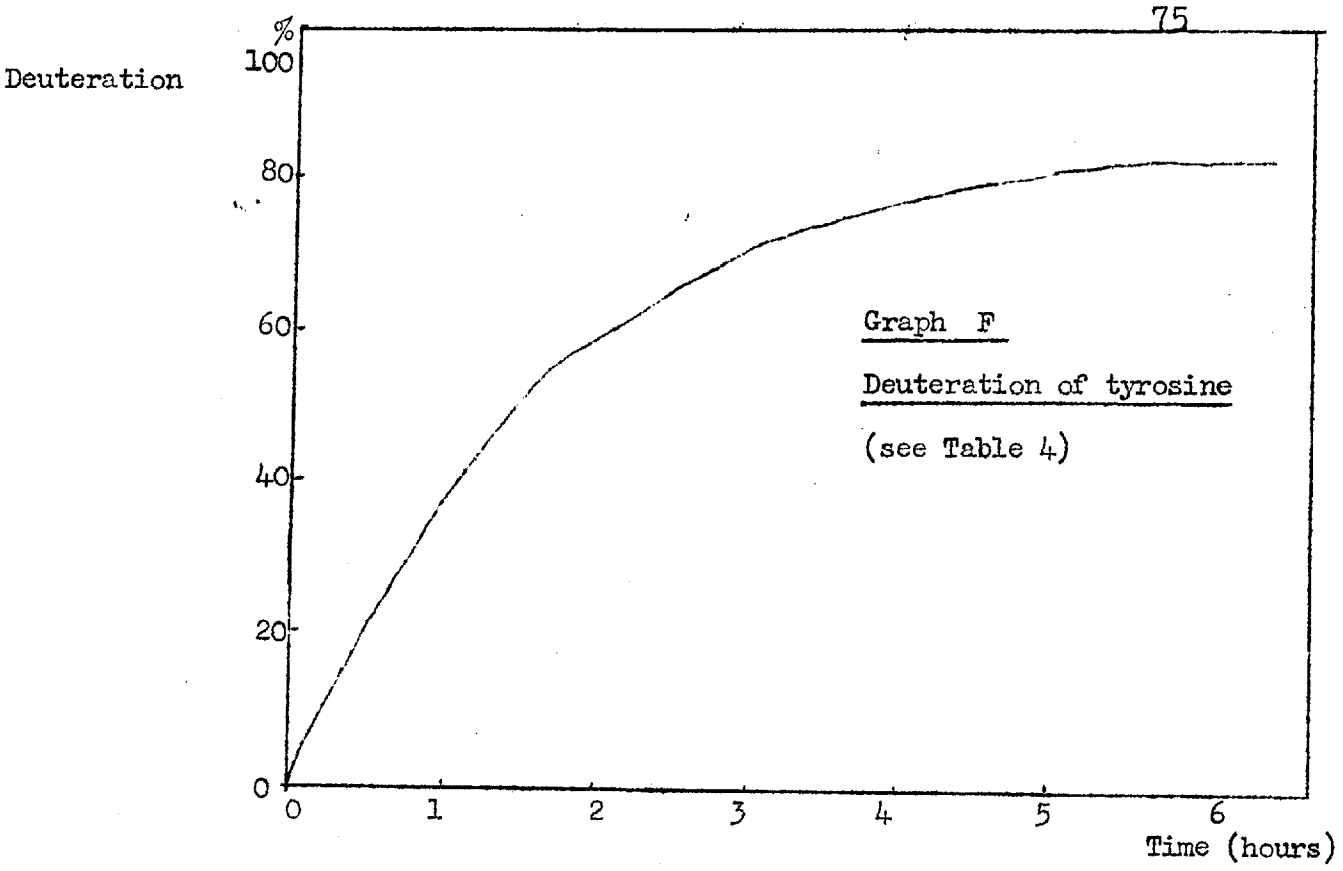
** Incomplete deuteration arose from introduction of atmospheric water during repeated sampling.




Graph E

Deuteration of dopa

(see Table 4)




Under more forcing conditions, for example, in 10 N-deuteriumchloride in a sealed tube at 165-170° exchange was found to be less specific. Not only had the $\alpha$-carbon hydrogens of the aromatic amino acids exchanged, as expected, in addition to the ortho and para hydrogens but also considerable "meta-exchange" had occurred in tyrosine. In the latter, the $\alpha$-carbon hydrogen exchanged faster than the meta hydrogens.

The following general observations were made.

(a) In aqueous systems, these exchange reactions occur specifically at positions ortho and para to the phenolic hydroxyl groups. However, acid-catalysed exchange reactions are generally less specific than the base catalysed reactions; the former also occur ortho and para to any electron releasing groups[1], for example, alkoxy and aryloxy groups.

(b) In tyrosine, acid catalysed exchange reaction proceeded much faster than the base catalysed. For example, in order to achieve a 67% exchange, about 43 hrs. heating was required under the optimum basic conditions whereas about 2½ hrs. sufficed under acidic conditions. Also rigorous exclusion of air is not so important for exchange under acidic rather than basic conditions.

(c) Exchange reaction catalysed by triethylamine occurred much faster than when catalysed by deuteroxide, (for example in vanillin).

Synthesis of Labelled 3,4-Dihydroxyphenylalanine

OO-Dibenzylprotocatechuic aldehyde (XIV; R=$PhCH_2$) was selected as the starting material for the synthesis of 3,4-dihydroxyphenylalanine (dopa) since we had shown above that catalytic debenzylation did not remove aryl deuterium or tritium.

The preparation of OO-dibenzyl-[5-$^2$H] protocatechuic aldehyde has already been described (see above); the correspondingly tritiated derivative was obtained in an analogous fashion. During this preparation the opportunity was taken to confirm the specificity of labelling in the intermediate 3-benzyloxy-4-hydroxy-[5-$^3$H] benzaldehyde (X; R=$PhCH_2$). Bromination of the latter, in the presence of sodium acetate, gave the corresponding 5-bromo-derivative containing less than 0.1% of the original activity.

Specific labelling of OO-dibenzylprotocatechuic aldehyde in the two remaining aryl positions proved more difficult. For this purpose, 2-bromo-[6-$^3$H]- and 6-bromo-[2-$^3$H] isovanillins were prepared (see below). Earlier attempts to obtain, from these bromoisovanillins, the OO-dibenzyl protocatechuic aldehyde by the way of veratraldehyde (XIV; R=$CH_3$) were discontinued because the latter is not demethylated to the catechol under the usual mild conditions[9].

2-Bromo-[6-$^3$H] isovanillin was demethylated (see above) and the resulting catechol converted into its dibenzyl ether. Benzylation was best effected with benzyl chloride in dimethylformamide in the presence of sodium hydride. Treatment with lithium aluminium hydride removed the bromine and reduced the aldehyde function. Oxidation of the dibenzyloxy benzyl alcohol with manganese dioxide gave the required OO-dibenzyl-[6-$^3$H] protocatechuic aldehyde. A similar series of reactions converted 2-bromo- [2-$^3$H] isovanillin into the corresponding protocatechuic aldehyde derivative. During these transformations, negligible loss of tritium was observed (see Table 2).

Table 2

Preparation of Tritiated OO-Dibenzylprotocatechuic Aldehyde

(Activities in $\mu$C per m.mole)

| Compound | Bromoisovanillin | Bromoprotocatechuic Aldehyde | OO-Dibenzylbromoprotocatechuic Aldehyde | OO-Dibenzylprotocatechuic Aldehyde |
|---|---|---|---|---|
| 2-Bromo-[6-$^3$H] | 24 | 24 | 25 | 25 |
| 6-Bromo-[2-$^3$H] | 62 | 59 | 60 | 58 |

6-Bromo-[2-$^3$H] isovanillin was most conveniently prepared by direct tritiation of the pure, inactive material in a manner analogous to the preparation of the deutero-derivative. A similar procedure with 2-bromoisovanillin was frustrated for solubility reasons; this compound also appeared rather sensitive under the exchange conditions. In order to obtain the other labelled isomer therefore, the following procedure was adopted. Isovanillin, tritiated in the presence of alkali, was brominated to give the highly crystalline 2-bromo-[6-$^3$H] isovanillin. The less readily purified 6-bromo-[2-$^3$H] isovanillin was also isolated from this reaction. Further bromination, in the presence of sodium acetate, of either isomer gave inactive 2,6-dibromoisovanillin in good yield. This confirms the position of labelling and removes any remaining doubt[14] concerning the structure of the dibromo-derivative.

The conversion of the labelled OO-dibenzylprotocatechuic aldehyde into racemic dopa (see Table 3) was accomplished by the hydantoin method[53,54]. This method also permitted the introduction of tritium into the side chain (see Scheme 3). The benzylidene hydantoin (XV; R=$PhCH_2$) was prepared in the usual way and reduced with sodium amalgam in dioxan containing

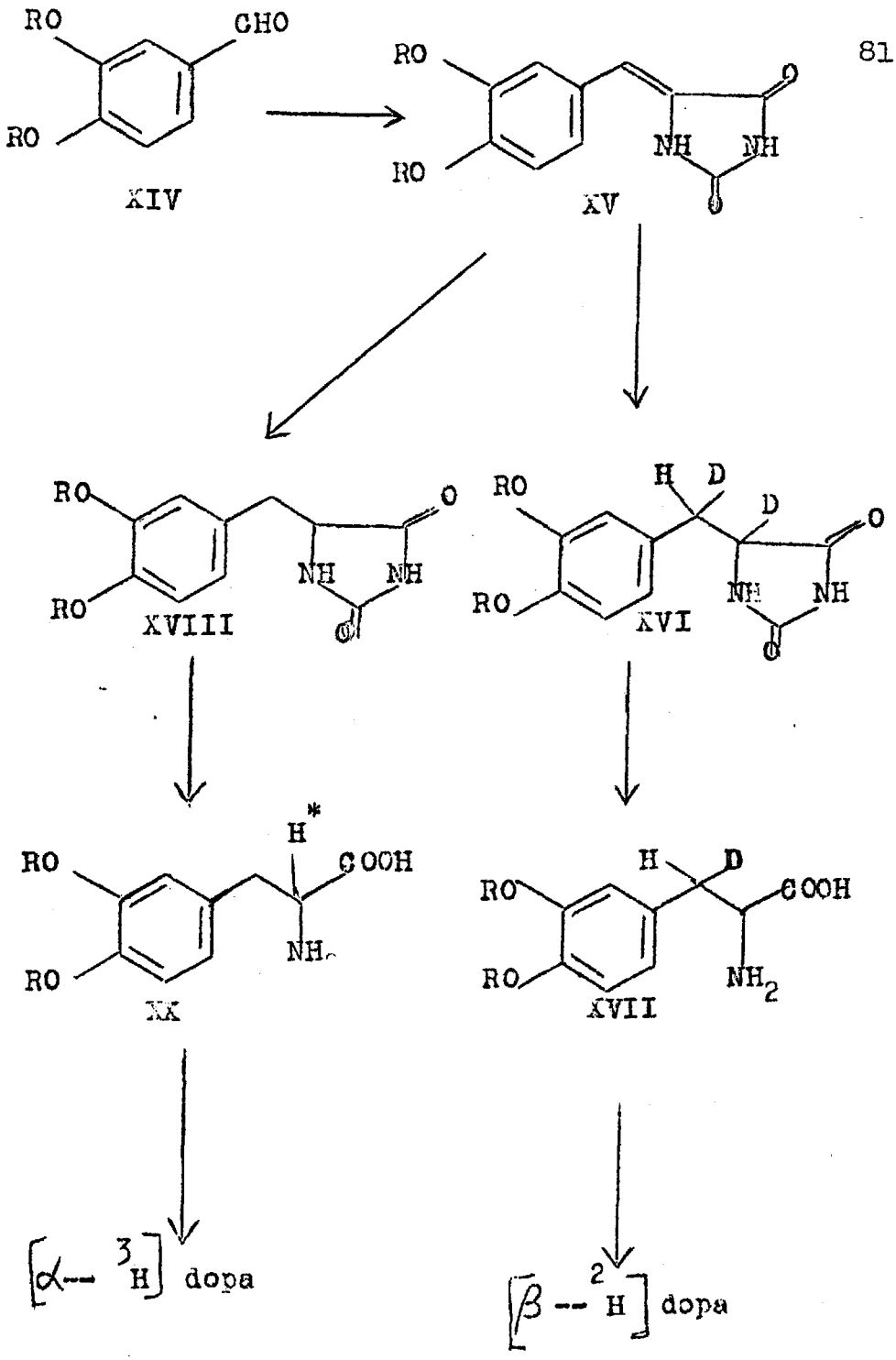
Table 3

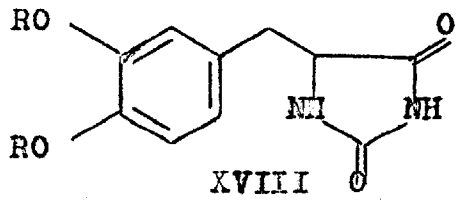
Preparation of Tritiated (+)-3,4-Dihydroxyphenylalanine (dopa)

Activities in $\mu$C per m.mole

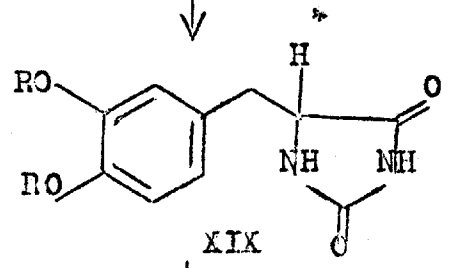
| Compound | at positions 2 | 5 | 6 | $\alpha$ | $\beta$ |
|---|---|---|---|---|---|
| OO-Dibenzylprotocatechuic Aldehyde | 58 | 54 | 26 | - | - |
| Dihydrohydantoin | 59 | 56 | 25 | - | 24* |
| OO-Dibenzyldopa | 58 | 53 | 24 | 17 | 15 |
| Dopa | 50 | 55 | 21 | 17 | 16 |

* Some of the $\alpha$-$^3$H was already lost during the isolation of this derivative. The value (24) represents both $\beta$(15) and $\alpha$ (9) tritium labelling.

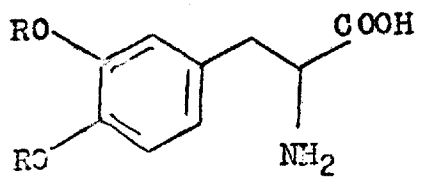

RO
CHO
RO
XIV
RO
O
NH
NH
RO
O
XV
RO
O
NH
NH
RO
O
XVIII
RO
H
D
D
O
RO
NH
NH
O
XVI
H*
RO
COOH
NH2
RO
XX
H
D
RO
COOH
RO
NH2
XVII
[α-- 3H] dopa
[β-- 2H] dopa




XVIII




XIX




XXI

deuterium oxide. The resulting dideuterated-derivative (XVI; R=$PhCH_2$) was hydrolysed with alkali to give dibenzyldopa (XVII; R=$PhCH_2$) which was converted by hydrogenolysis into deuterated dopa (XVII; R=H). The n.m.r. spectrum showed approximately one deuterium at the $\beta$-carbon; no detectable amounts of $\alpha$-deuterated species were present. Loss of deuterium from the 5-position during hydrolysis of the benzylhydantoin is not surprising since these compounds are known[55] to racemise very readily in alkali; but it was necessary to show that complete loss had occurred.

Benzylhydantoin (XVIII; R=$PhCH_2$) was treated with alkaline tritiated water at room temperature to give a specimen (XIX; R=$PhCH_2$) labelled with tritium at the 5-position. Hydrolysis in hot alkali in the usual way, gave the dibenzyl ether (XXI; R=$PhCH_2$) containing only 4% of the original activity. With the conditions for $\alpha$-exchange thus clearly defined, it was then possible to prepare samples of dopa labelled specifically at either the $\alpha$- or the $\beta$-position. Accordingly [$\beta$-$^3$H]dopa was prepared as described (see above) for the deutero- derivative. Hydrolysis of the inactive benzylhydantoin (XVIII; R=$PhCH_2$) in alkaline

tritiated water gave [$\alpha$-$^3$H] dibenzyldopa (XX; R=$PhCH_2$) which on hydrogenolysis gave the correspondingly labelled dopa.

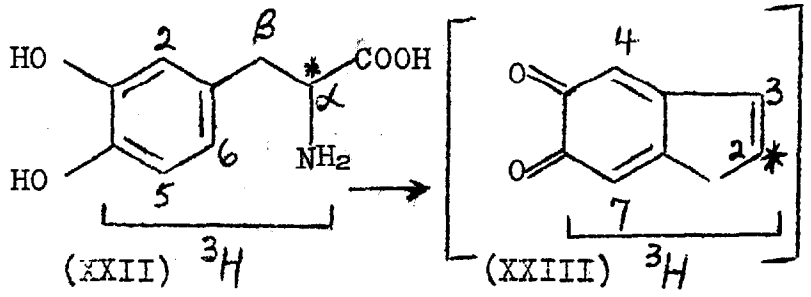
(±)- [2,5,6-$^3H_3$] Dopa was prepared by exchange of the unlabelled amino-acid in acidic tritiated water since rigorous exclusion of oxygen was not so important as it would be in a base-catalysed exchange reaction of this compound[15]. The preparation of [2,5,6-$^3H_3$]- and [6-$^3$H]dopa by the reduction of suitably brominated derivatives with tritium gas has been recently described[16].

## Study of Melanin Structure with Labelled (±)-3,4-Dihydroxyphenylalanine (dopa)

All the labelled dopas (see below) were oxidised to melanins under similar conditions in order to minimise errors due to variations in the experimental conditions. The enzymatic oxidations were conducted with crude mushroom tyrosinase and molecular oxygen in the presence of catalase in order to minimise the breakdown of benzene nuclei by hydrogen peroxide[10].

To estimate the loss of label from each position, the normal thing to do would have been to compare the molar activities of the dopas with those of the corresponding melanins. However this was not possible because the molecular weight or the empirical formula weight was not known. Elementary analyses are not sufficiently reliable owing to hydration and difficulty of combustion. Since the hydrogen content was so important an alternative method of estimation had to be found. Such a method had to be independent of variation in composition caused by hydration. Therefore each tritium labelled dopa was mixed with [α-$^{14}$C] dopa and the mixture recrystallised to give effectively the species (XXII) doubly labelled with $^{14}$C and $^{3}$H. This on oxidation would give doubly labelled melanin

written for convenience as a polymer of the indole-quinone unit (XXIII)




Previous tracer work by Swan and Wright[11] had shown that a negligible loss of the α-carbon of dopa occurred during melanin formation. Thus a comparison of the $^{3}H/^{14}C$ ratios of the precursor and of the corresponding melanin should give a direct value for the change in tritium content during melanin formation. The $^{14}C$ and $^{3}H$ contents of each doubly labelled melanin together with its elementary carbon content were determined[17], by combustion, on the same sample. This method had the additional advantage of eliminating any chance errors that might otherwise arise from the incomplete combustion of different samples on different runs.

In order to simplify the calculations, the $^{3}H/^{14}C$ ratio of each precursor has been reduced to a whole number (usually 1) representing the number of labelled hydrogens in the molecule. The $^{3}H/^{14}C$ ratio of the

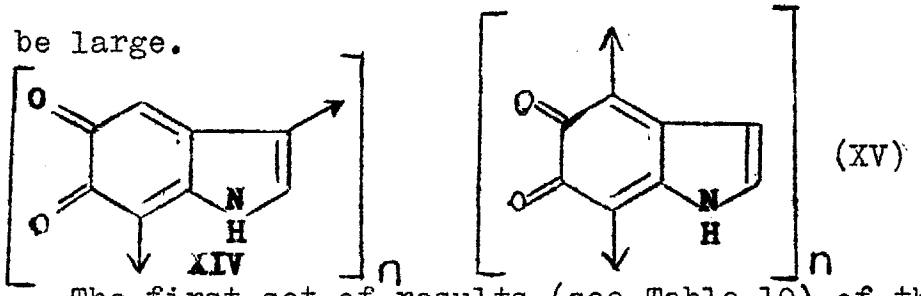
corresponding melanin is then expressed as a fraction of that of its precursor. Thus in the Tables (see below) the $^{3}H/^{14}C$ ratios recorded for melanins represent fractions of 1 tritium atom retained. The extent of loss of $^{14}C$ from the $\alpha$-carbon was checked by calculating the "$^{14}C$ ratio" of each melanin preparation. The "$^{14}C$ ratio" (see the Tables) is defined as

$$\frac{^{14}C \text{ activity of melanin per mg. atom of carbon}}{^{14}C \text{ activity of dopa per mg. atom of carbon.}}$$

Providing the % carbon in the melanin is known (from combustion analysis) this quantity can be calculated without knowledge of the molecular formula. If we assume complete decarboxylation of dopa and no loss of carbon from any other part of its molecule during melanogenesis, [that is, assuming a polymer of indole-quinone units (XXIII)], then each molecule of dopa (9 carbon atoms) will furnish an 8-carbon unit of melanin which will still contain all the original $^{14}C$ activity. Then the "$^{14}C$ ratio" will be equal to $\frac{9}{8}$ i.e. 1.125. Possible causes of variations in this figure are discussed below.

The "backbone structures" of melanin currently favoured are the 3-7 linked structure (XIV) of

Harley-Mason and his collaborators[44,49] and the 4-7 linked structure (XV) of various other workers[24]. In both these structures, n has always been assumed to be large.



XIV (XV)

The first set of results (see Table 10) of the present investigations rule out structure (XV). Of the three tritium atoms in the dopa 0.81 is retained in the melanin. Structure (XV) however would require the loss of most or all of the tritium label. It is interesting to note that similar results were obtained with melanin prepared by the autoxidation of dopa (see Table 10). Later investigations (see below) have shown that up to 13% of the melanin polymer units may be uncyclised. Taking this into account does not significantly alter the above interpretation.

The melanin in the above investigations were precipitated under strongly acidic conditions. Even though the pigment is highly insoluble, it is conceivable that some tritium might be lost during the acidic work-up. It was therefore necessary to

Table 10

$^{3}H$ and $^{14}C$ activities in dpm/mg.

(Melanins* precipitated with ca 2N hydrochloric acid; low activity tyrosinase used.)

| Compound | $^{3}H$ | $^{14}C$ | %C | $^{3}H/^{14}C$ | "$^{14}C$ ratio" |
|---|---|---|---|---|---|
| DL-[2,5,6-$^{3}H_3$] Dopa | 3183 | 727 | | 3 (3T) | |
| Melanin | 905 | 767 | 54.3 | 0.81 | 1.07 |
| DL-[2,5,6-$^{3}H_3$] Dopa | 2797 | 676 | | 3 (3T) | |
| ** Melanin | 815 | 670 | 50.1 | 0.88 | 1.09 |
| L-[3,5-$^{3}H_2$] Tyrosine | 3087 | 709 | | 2 (2T) | |
| Melanin | 497 | 570 | 53.0 | 0.40 | 1.10 |

* All melanins are enzymatic unless otherwise stated.

** Autoxidative

find a method of isolation under mild conditions. Indeed, these melanins are precipitated rapidly by the addition of a soluble inorganic salt[36]. Among the various salts tested, sodium chloride and ammonium nitrate proved the most effective. With sodium chloride as precipitant, the next set of results were obtained (see Table 11).

The melanin obtained from [5-$^{3}H$] dopa retained 42% of the precursor's tritium activity. This result

Table 11

$^{3}H$ and $^{14}C$ activities in dpmin./mg.
(Melanins precipitated with sodium chloride; low activity tyrosinase used.)

| Compound | $^{3}H$ | $^{14}C$ | %C | $^{3}H/^{14}C$ | "$^{14}C$ ratio" |
|---|---|---|---|---|---|
| DL-[5-$^{3}H$]Dopa | 3201 | 791 | | 1 | |
| Melanin | 1496 | 890 | 55.4 | 0.42 | 1.11 |
| DL-[$\beta$-$^{3}H$]Dopa | 3794 | 805 | | 1 | |
| Melanin | 1694 | 865 | 50.65 | 0.42 | 1.16 |

confirms our earlier rejection of the predominance of a 4-7 linkage in the melanin structure, and also excludes the possibility of the 3-7 linked backbone structure. Such a linkage would require the loss of all tritium from the 5-position of dopa. The result also agrees well with those obtained from our earlier investigations in which melanins obtained from [3,5-$^{3}H_2$] tyrosine retained ca 40% of the precursor activity (after allowing for the loss of 1 tritium atom in going from tyrosine to dopa).

In the above investigations with dopa, the "$^{14}C$ ratio" agreed, within experimental errors, with the expected value of 1.125. That no $\alpha$-carbon was lost as carbon dioxide during the oxidation of tyrosine and dopa was ascertained in the following

manner. [α-$^{14}$C] Tyrosine and [α-$^{14}$C] dopa were oxidised under the standard conditions and the carbon dioxide evolved during the oxidation was collected as barium carbonate. The latter invariably contained less than 1% of the precursor activity.

The above pattern of results has been generally supported by a more extensive series of experiments (see Tables 12 and 13). In this last series of oxidations, a new batch of enzyme, which was more active than the sample used in the earlier oxidations, was used. It was thought necessary therefore to retest the earlier precursors along with the new ones. The melanins which were prepared under similar conditions were isolated, under mild conditions, this time by the addition of ammonium nitrate. This salt was preferred because of its solubility in water.

In these results, the "$^{14}$C ratio" was generally ca 1.00 (that is 10% lower than usual) except in the isolated case of [2-$^{3}$H] dopa (which is rather surprising). This would imply either of the following possibilities: (a) no decarboxylation of dopa molecules during melanin formation, (b) loss of some of the labelled α-carbon. Assuming that all the dopachrome molecules aromatise to indole units (which seems reasonable),

Table 12

(Melanins were precipitated with ammonium nitrate; active enzyme used)

| Compound | Activities dpmin/mg | | | | |
|---|---|---|---|---|---|
| | $^{3}H$ | $^{14}C$ | %C | $^{3}H/_{14}C$ | "$^{14}C$ ratio" |
| DL-[2,5,6-$^{3}H_3$]Dopa | 2797 | 676 | | 3 (3T) | |
| Melanin | 816 | 636 | 50.8 | 0.94 | 1.01 |
| DL-[2-$^{3}H$]Dopa | 3801 | 602 | | 1 | |
| Melanin | 1551 | 563 | 55.3 | 0.44 | 0.93 |
| DL-[5-$^{3}H$]Dopa | 3201 | 791 | | 1 | |
| Melanin | 1465 | 755 | 52.8 | 0.48 | 1.00 |
| DL-[6-$^{3}H$]Dopa | 3472 | 489 | | 1 | |
| Melanin | 500 | 481 | 54.7 | 0.15 | 0.99 |
| DL-[$\alpha$-$^{3}H$]Dopa | 3509 | 645 | | 1 | |
| Melanin | 1853 | 655 | 54.6 | 0.52 | 1.02 |
| DL-[$\beta$-$^{3}H$]Dopa | 3794 | 805 | | 1 | |
| Melanin | 2054 | 800 | 53.8 | 0.54 | 1.01 |

Table 13

| Position of $^3H$ | Fraction of $^3H$ retained | Corrected value | 1st set* c.f.Tables 10,11 |
|---|---|---|---|
| 2 | 0.44 | 0.37 | |
| 5 | 0.48 | 0.42 | 0.42 |
| 6 | 0.15 | 0.13 | |
| 2,5,6 3T) | 0.94 | 0.86 | 0.81 |
| $\alpha$ | 0.52 | - | |
| $\beta$ | 0.54 | - | |

* In the 1st set of results, no correction was necessary since the "$^{14}C$ ratios" agree reasonably well with the theoretical value.

then the first possibility would imply complete dehydrogenation at the $\alpha$-carbon for aromatisation purposes. However a tracer study (see below) shows that 52% of $\alpha$-hydrogen is retained during melanogenesis. Thus the possibility of no decarboxylation is virtually ruled out. It would be good to test this further with $[^{14}CO_2H]$ dopa. It will appear therefore that some of the side chain is being degraded during melanogenesis in the presence of this new enzyme preparation. To correct for this in the results, the calculated tritium retention in each case was

multiplied by a factor, $\frac{\text{measured } "^{14}C \text{ ratio}"}{1.12}$, that is decreased by ca 10%. For example, for melanin from [2-$^{3}$H]dopa, the correction factor would be 0.92/1.12 and hence an observed tritium retention of 0.44 would be corrected to 0.37. This correction however has not significantly changed the general pattern of the results (see Table 13). It will be noticed that these corrected values agree reasonably well with the first set of results in which the "$^{14}C$ ratio" did not differ significantly from the theoretical value. Furthermore, the total loss of label from the dopas separately labelled at positions 2,5 and 6 came to 0.92. This value compares reasonably well with that, 0.86, obtained with the triply labelled [2,5,6-$^{3}H_{3}$]-precursor especially when it is realised that these two final values are the results of 16 independent determinations.

It will appear from these results that positions 4 and 7 of the indolequinone units are being used indiscriminately for coupling. This further disagrees with the postulate of Harley-Mason and his collaborators.

The result with [6-$^{3}$H]dopa is interesting. The melanin from this precursor, as expected, differed

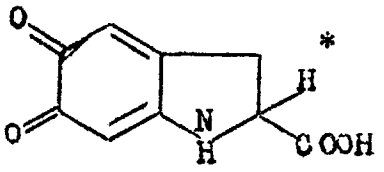
from all the others in that it lost most of the precursor tritium. However, a significant amount (13%) was retained. Clemo and his coworkers[52] had shown, by tracer studies, that melanin prepared from dopa still retained ca 1/6 of the original carboxyl groups, but could not interpret their results unambiguously. We can now present concrete evidence to prove that uncyclised entities like dopaquinone units may directly participate in the construction of the melanin polymer structure. The above idea has been propounded earlier by Nicolaus and his coworkers as a result of their degradative studies[43]. On the other hand, this result might be due to adsorption of unreacted dopa on the surface of the pigment. This later possibility can however be discounted for the following reason. Melanin derived from [$\alpha$-$^{14}$C] dopa was precipitated with ammonium nitrate in the usual manner. This material was then refluxed with 2N-hydrochloric acid for 24 hrs. Desorbed dopa was estimated after filtration by a radio dilution method. The amount of dopa desorbed was found to be only 1.5% by weight of the melanin. The result from the desorption experiment also removes any existing doubt as to whether or not the

adsorption of unreacted dopa might contribute significantly to the measured activities of the various melanins, especially those precipitated in the absence of an acid.

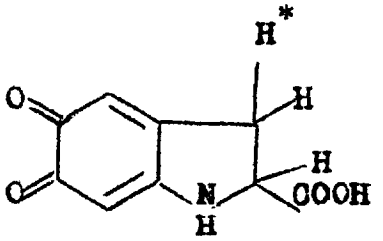
In interpreting these results, the possibility of tritium loss by reactions other than coupling of units must be considered. The fear of loss of label due to non-coupling reactions is inherent in studies of this nature. However we do know for certain that ordinary nuclear exchange of phenols do not take place under the experimental conditions. Moreover, if there is any loss due to non-coupling reactions, it is rather unlikely that such losses will be uniform in which case it will be highly improbable that similar tritium retentions are obtained from the autoxidation and enzymatic oxidation. Further work designed to test the above possibility is at present in progress in these laboratories. However we can be confident that tritium retention at a particular position disproves exclusive coupling at that position.

The results with the $\alpha$- and $\beta$-labelled precursors are a bit more difficult to interpret. [$\alpha$-$^3$H] Dopa should give the dopachrome (XVI) labelled as

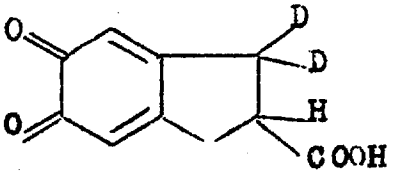
shown.



XVI



XVII



XVIII

* 3 H = H

This entity might aromatise before coupling by the loss of either the carboxyl or the tritium label, though the loss of the carboxyl is more likely. The effective loss of tritium due to coupling at that position may therefore be less than the 48% observed. A quantitative estimation of carbon dioxide evolved during the oxidation of dopa should throw some light on this.

$\beta$-Labelled dopa on oxidation would give the dopachrome unit (XVII). In this species there exists the clear possibility of a mass effect since either the hydrogen or tritium must be lost to aromatise the ring before coupling. If there is a large mass effect, then the observed tritium loss (46%) will be due

almost entirely to coupling. If on the other hand, there is no mass effect at all, then the hydrogen and tritium react indiscriminately, and the effective loss due to coupling would be essentially zero (actually - 4%!). Thus, whichever way one looks at it, the amount of coupling at this position cannot be large. This result further disproves the Harley-Mason structure (XIV). The possibility of a mass effect at this position is at present being investigated in these laboratories using fully deuterated [$\beta$-$^2H_2$] dopa which would give the species (XVIII) on oxidation.

The general conclusion from the present work is that melanin obtained by the enzymatic oxidation of dopa appears to be quite irregular and simple structures are definitely excluded. With supplies of all labelled dopas available it will be possible to compare in a quantitative manner the structures of melanin produced under different conditions. It is unlikely that autoxidation would give a more regular polymer than enzymatic oxidation; the one result obtained so far (see Table 10) suggests similarity between these two melanins. However it will be interesting to discover whether melanin formed in an intact organism is fundamentally different from that produced in vitro.

# EXPERIMENTAL

## 1) GENERAL

Melting points were taken on a Kofler block and were uncorrected. All n.m.r. spectra were recorded by Mrs. I. Boston on a Varian A-60 spectrometer on permanent loan to Professor D.H.R. Barton F.R.S. from the Wellcome Trust. Ultraviolet and infrared spectra were recorded on Unicam SP 700 and SP 200 spectrophotometers respectively. Microanalyses were carried out by Miss J. Cuckney and her staff. Deuterium oxide (99.7%) and tritiated water (200 mc per ml.) were used in the exchange experiments. Petroleum ether refers to the fraction boiling point 60-80°.

### Counting Methods

Tritiated and $^{14}C$ compounds were counted with a Tritium Scintillation Counter (Isotope Development Ltd., Type 6012 A) using liquid scintillator (Nuclear Enterprises Ltd., Type N.E. 213). The counting procedure was standardised using $[1,2\text{-}^{3}H_2]$-n-hexadecane and $[1\text{-}^{14}C]$-n-hexadecane of known activity. Reproducibilities of *ca* 3% were customary. Most of the compounds encountered were insoluble in the scintillation liquid. Hence compound typically (0.40-

0.50 mg.) in dimethylformamide (0.1 ml.) was counted in the liquid scintillator (1.2 ml.). Use of dimethylformamide had a quenching effect with consequent lowering of counting efficiency. Many compounds especially those containing free phenolic groups also had significant quenching effects at concentrations above ca 1 mg per ml. In such cases, internal standardisation was necessary. Dimethylformamide (DMF) was best dried[33], with purification, by azeotropic distillation of the contained water with benzene followed by distillation of the pure dry solvent under reduced pressure. Dimethylsulphoxide (DMSO) has also been used as a solvent for counting [$^{14}$C]-dopa in the form of its hydrochloride. Typical counting efficiencies are tabulated below:

$^{3}$H and $^{14}$C counting efficiencies (%) in 1.2 ml. or 1.0 ml. scintillator

| Added Vol. of DMF in ml. | 0.00 | 0.10 | 0.20 | | | | |
|---|---|---|---|---|---|---|---|
| Added vol. of DMSO in ml. | | | | 0.00 | 0.10 | 0.20 | 0.40 |
| $^{3}$H | 23.2 | 20.5 | 20.0 | | | | |
| $^{14}$C | | | | 78.5 | 72.5 | 69.5 | 56 |

$Ba[^{14}C]CO_3$ (5 mg.) was decomposed with N-perchloric acid (0.5 ml.) in a special piece of equipment, and the evolved $^{14}CO_2$ absorbed quantitatively in 0.5N-hyamine base[34] in methanol (0.5 ml.) and the solution counted in liquid scintillator (4.0 ml.) at an efficiency of 48.5%. This counting procedure was standardised using $[^{14}C]$perspex (60% carbon of known activity) which was burnt and the $^{14}CO_2$ formed collected as $Ba[^{14}C]CO_3$ which was counted. Tyrosine and dopa are insoluble in dimethylformamide and dimethylsulphoxide and hence were counted in the form of their ester hydrochloride and hydrochloride respectively. Tyrosine was treated with excess cold ethanolic hydrogen chloride at room temperature over 24 hours, and the product, which crystallised out upon the addition of ether, was recrystallised from ethanol/ether and counted. To dopa (1.00-3.00 mg. accurately weighed out) in a 5 ml.-volumetric flask was added methanolic hydrogen chloride (ca 0.2-0.4 ml.) to dissolve completely at room temperature. The solution was evaporated to dryness during 30 minutes at room temperature in vacuo over phosphorous pentoxide and potassium hydroxide. The residue was made up to 5 ml. in dimethylformamide and aliquot portions

(0.1 ml.) counted as usual. Counting at this low concentration, apart from improving its solubility, eliminated any quenching effect which the solute might otherwise exert. Some monohydric and dihydric phenols were counted by this method. [$^{14}$C]-dopa was counted in dimethylsulphoxide in the following manner: To dopa (0.30-0.60 mg) in a counting tube was added methanolic hydrogen chloride (0.1-0.2 ml.) to dissolve completely and the solution evaporated to dryness during 30 minutes at room temperature *in vacuo* over phosphorous pentoxide and potassium hydroxide. Dimethylsulphoxide (0.4 ml.) was added to dissolve properly and then liquid scintillator (1.0 ml.). Use of 1.0 ml. instead of 1.2 ml. of liquid scintillator made no significant change in counting efficiency. More accurate activity measurements on tyrosine and dopa were kindly made by Dr. H. Simon (Munich) using a combustion method[17]. Dr. Simon also counted melanin by the same method.

Radiochemical purification of tritium labelled compounds.

The compounds labelled with tritium, using tritiated water, usually contain, when first isolated, some undesirable radioactivity due to exchange of

their more labile protons, for example, protons on phenolic, carboxyl, amino and imido groups. It was therefore always necessary to ascertain the radiochemical purity of such compounds by successive recrystallisations or washings, with dilution with authentic inactive sample if possible, in some hydrolytic solvent to constant activity. Fortunately, since these protons are so labile, one or two recrystallisations usually suffice. All activities recorded in the script for such compounds were obtained after such treatments.

## 2) DEUTERIUM AND TRITIUM EXCHANGE REACTIONS OF PHENOLS AND THE SYNTHESIS OF LABELLED 3,4-DIHYDROXYPHENYLALANINE

Deuteration of p-Cresol:

a) With triethylamine: p-cresol (100 mg.) in deuterium oxide (0.3 ml.) (a two-phase system) was heated under reflux (101-103°) with varying amounts of triethylamine for varying periods of time (see Table 1). The reaction vessel was open to the air but protected from atmospheric moisture. The reaction mixture was cooled rapidly, homogenised by addition of minimum amount of dioxan and examined by n.m.r. spectroscopy. The doublet, 3.38 $\tau$ (J = 9 cps) corresponding to the protons ortho to the phenolic hydroxylic group diminished in intensity as deuteration proceeded and the meta-proton doublet 3.10 $\tau$ was correspondingly replaced by a broad singlet. The extent of deuteration (% exchange in Table 1) was obtained from the difference in integrated areas of the ortho- and meta-proton signals.

b) With deuteroxide: Exchange was effected in a similar way using potassium t-butoxide (0.5 mole per mole of phenol). The reaction mixture (a one phase system in this case) was refluxed (100°) under nitrogen.

Deuteration of Isoeugenol

Exchange in deuterium oxide was carried out with triethylamine or deuteroxide exactly as described for p-cresol (see above). The aryl protons gave a broad signal (n.m.r.) at 3.28 $\tau$ which diminished in area by one third upon deuteration. The splitting of the C-methyl doublet, 8.18 $\tau$ (J = 5 cps) was unaffected by deuteration. A comparison of the integrated areas of the methoxyl (6.22 $\tau$ ) and C-methyl signals on several spectra showed differences of up to 10%. This provides an indication of the uncertainty involved in our measurement of aryl deuteration.

Deuteration of Morphine:

A solution of morphine (200 mg.) in dimethylformamide (2 ml.) or dimethylsulphoxide (2 ml.) containing deuterium oxide (0.5 ml.) was heated at 100° in a nitrogen-filled sealed Carius tube. The exchange was slower (ca. 70% in 66 hrs.) than that observed (above) with p-cresol and triethylamine. The solution was diluted with water (10 ml.) when the product, [2 - $^2$H] morphine (160 mgs.) crystallised out rapidly and was collected by filtration. An experiment in dimethylformamide, under these conditions, but using morphine hydrochloride instead of morphine, gave undeuterated product.

Acetylation of Morphine:

A solution of morphine (50 mgs.) in pyridine (0.5 ml.) and acetic anhydride (0.75 ml.) was heated at 100° (30 mins.). The solvent was removed *in vacuo* and the crystalline residue was recrystallised from benzene/petroleum ether to give diacetylmorphine m.p. 171-173° (lit[21]., 173).

Deuteration and Tritiation of Tyrosine

Deuteration: A solution of the sodium salt of tyrosine (94 mg.) (prepared as a highly hygroscopic yellowish white solid from tyrosine and 1.7 molar equivalent of a methanolic solution of sodium methoxide) in deuterium oxide (0.3 ml.) was heated at 100° (72 hr.) in a nitrogen-filled Carius tube. The slightly coloured solution was diluted with water and carefully neutralised with 6N hydrochloric acid to *ca.* pH 6 when [3,5-$^2H_2$] tyrosine rapidly crystallised out and was collected by centrifugation and washed with water and acetone, (recovery 67%). A solution of its hydrochloride in deuterium oxide, containing *t*-butanol (8.72$\tau$) as an internal standard was examined by n.m.r. spectroscopy. The doublet 3.10$\tau$ (J = 9 cps) corresponding to the protons *ortho* to the phenolic hydroxylic group had practically disappeared while the meta-proton doublet 2.78$\tau$

(J = 9 cps) had been replaced by a broad singlet. The rest of the spectrum consisting of an approximate triplet ca. 5.65 $\tau$ due to the methine proton and an approximate doublet ca 6.78 $\tau$ due to the methylene protons (an ABC pattern) remained unchanged.

Chlorination of [3,5-$^2H_2$]Tyrosine[26] [3,5-$^2H_2$]Tyrosine in acetic acid was treated at room temperature with a solution of thionyl chloride in glacial acetic acid. Tyrosine dissolved exothermally and the product 3-Cl- [5-$^2H$] tyrosine slowly crystallised out. It was recrystallised from glacial acetic acid to plates m.p. 138-140° recrystallising and remelting 240-242°. (lit[26]., 140,240). An n.m.r. spectrum of this compound in deuterium oxide showed a pair of doublets 2.65 $\tau$ (J = 2 cps), 2.85 $\tau$ (J = 2 cps) equivalent to two protons showing the expected meta-splitting pattern.

Tritiation of Tyrosine: [3,5-$^3H_2$] Tyrosine (activity 2.48 mC per m.mole) was prepared in the above manner using tritiated water (activity 1.8 mC per mg. atom hydrogen). The radiochemical yield was 69%. A specimen was diluted 162 times with inactive sample and recrystallised to a steady activity ($5.65 \times 10^5$ dps per m.mole). The activity of its 3-monochloro

derivative was 54% of this value.

Acid Catalysed Deuteration of Tyrosine and Dopa and Tritiation of Dopa

Deuteration: A solution of the amino acid (104 mg.) in 4.1 N-deuterium chloride (0.4 ml.) was heated at 100° in a stoppered vessel, the heating being interrupted from time to time while the degree of exchange was determined (n.m.r.) (see Table 4). The n.m.r. spectra of tyrosine and dopa in the region 5-7τ are very similar. Deuterium chloride was prepared by allowing thionyl chloride (0.065 ml.) to react with deuterium oxide (0.4 ml.), liberated sulphur dioxide was swept out with a stream of dry nitrogen. The two ortho hydrogens in tyrosine and all the three aryl hydrogens in dopa were eventually replaced by deuterium. There was no significant change at other positions under these conditions. However, the amino acid, in the same deuterating medium at the same concentration, left in the thermostat at 20° for 6 days, showed no significant exchange in its aromatic nucleus (n.m.r. control).

Tritiation of Dopa. This was carried out in a similar manner (described above) using tritiated water (3.6 mC per m.mole) to give [2,5,6-$^{3}H_{3}$] dopa of activity,

determined by appropriate dilution (c/f tyrosine), 3.92 mC per m.mole. The radiochemical yield was 72.5%.

Exchange under Forcing Acidic Conditions. Under more forcing conditions however, exchange became less specific. A solution of the amino acid (200 mg.) in ca. 10N-deuterium chloride (0.7 ml.) was heated in a nitrogen-filled Carius tube at 165-170° for 39 hrs. The deuterated amino acid was recovered in the usual manner (see below) and examined by n.m.r. spectroscopy. All the aryl signals had collapsed (practically completely in dopa and almost so in tyrosine), the approximate triplet at 5.65 $\tau$ of the methine proton in the undeuterated species had disappeared while a broad singlet had appeared at 6.78 $\tau$ ; all these facts being consistent with the formation of $[2,5,6,\alpha\text{-}^2H_4]$ dopa and $[2,3,5,6,\alpha\text{-}^2H_5]$ tyrosine. L-Tyrosine treated under these conditions in hydrochloric acid was completely racemised (followed by optical rotation measurements on 2% solutions in N-HCl). Thus, under the above conditions, there has been exchange at positions meta to the phenolic hydroxyl group in tyrosine. When these deuterated products were heated at 100° in 6N-hydrochloric acid for 3 hours, only the aryl deuterons ortho and para to the phenolic

hydroxyl group re-exchanged for hydrogens (n.m.r. control) to give the species [α-$^2$H] dopa and [2,6,α-$^2H_3$] tyrosine. The amino acids were generally recovered from the strongly acidic solutions by careful neutralisation with aqueous ammonia to pH 4-5 (in case of dopa) and pH 6-7 (in case of tyrosine).

Deuteration and Tritiation of Vanillin and 3-O-Benzylprotocatechuic Aldehyde[22]

With deuteroxide: A solution of the aldehyde (1 m.mole) and potassium t-butoxide (0.5 m.mole) in deuterium oxide (0.3 ml.) was heated at 100° (72 hrs.) in a nitrogen-filled Carius tube. The solution was acidified when 3-O-benzyl-[5-$^2$H] protocatechuic aldehyde m.p. 112-114° crystallised out. This compound lacked the doublet at 2.95 τ (J = 8 cps) characteristic of proton at C-5 in the starting material. [5-$^2$H] vanillin m.p. 79-81° (from water) was obtained after extraction into ether, drying the extract (sodium sulphate) and evaporation to dryness; this compound showed (n.m.r.) two aryl protons having the expected meta-relationship. In deuterium oxide, containing triethylamine, these protons gave a pair of doublets (J = 2 cps) at 2.07 and 2.20 ppm upfield from the aldehyde singlet. The

identities of these deuterated compounds were also supported by their infrared absorption spectra.

With triethylamine: A solution of vanillin (1 m. mole) and triethylamine (1 m.mole) in deuterium oxide (0.3 ml.) was heated in a stoppered vessel at 100° (14½ hrs.) and the product, [5-$^2$H] vanillin, isolated and characterised as above.

[5-$^2$H] protocatechuic aldehyde. [5-$^2$H] vanillin was demethylated under mild conditions[9] with aluminium chloride and pyridine and the product [5-$^2$H] protocatechuic aldehyde m.p. 151-153° showed two aryl protons with the expected meta-relationship and positioning similar to the starting material.

3,4-Dihydroxy- [5-$^2$H] toluene. 3-O-Benzylprotocatechuic aldehyde[22] (254 mg.) in acetone (10 ml.) containing benzyl chloride (0.5 ml.) and anhydrous potassium carbonate (151 mg.) was refluxed for 20 hrs. under nitrogen with stirring. The reaction mixture was filtered and excess benzyl chloride removed from the product by steam distillation. An ether extract of the involatile residue was washed with sodium hydroxide, then water, dried (sodium sulphate) and evaporated to give the required dibenzylether (263 mg.) m.p. 88-90°. This material

(195 mg.) suspended in ethanol (30 ml.) containing 6N-hydrochloric acid (0.1 ml.) was hydrogenated over 10% palladium-carbon (21 mg.) for one hour. Filtration and evaporation gave 3,4-dihydroxy-[5-$^{2}$H] toluene (60 mg.) which crystallised from benzene as prisms m.p. 62-64. Its n.m.r. spectrum run in deuterium oxide containing t-butanol (8.77$\tau$) as an internal standard showed bands at 3.22$\tau$ and 3.41$\tau$ arising from protons at C-2 and C-6 respectively; as expected the low field band was a sharp doublet (J = 2 cps) while the other was broadened by the neighbouring deuterium atom.

3-O-Benzyl-[5-$^{3}$H]-protocatechuic aldehyde: This was obtained by tritiating the inactive compound under the conditions described for deuteration, using tritiated water (0.8 mC per mg. atom hydrogen) to give product m.p. 114-116$^{o}$ and activity, determined by dilution as usual, 0.53 mC. per m.mole; that is, a radiochemical yield of 66%. Bromination of a diluted specimen (as described below) gave the corresponding 5-bromo derivative containing less than 0.1% of the original activity. Activity of starting material was 2.72 x $10^{5}$ cps per m.mole while that of its 5-bromo derivative was 0.03 x $10^{5}$ cps per m.mole.

3-O-Benzyl-5-bromoprotocatechuic aldehyde. A solution of 3-O-benzylprotocatechuic aldehyde[22] (109 mg.) in acetic acid (2 ml.) containing anhydrous sodium acetate (80 mg.) was treated (1 hr.) with bromine (100 mg.) at room temperature. The 5-bromo derivative (c/f bromination of vanillin[37]) separated rapidly as colorless plates (103 mg.) and was recrystallised from ethanol to give material m.p. 163-165°. (Found: C, 54.7; H, 3.7. $C_{14}H_{11}BrO_3$ requires C, 54.7; H, 3.6%).

OO-Dibenzyl-[5-$^3$H] protocatechuic aldehyde[23]. This was obtained, without loss of tritium, by benzylating the correspondingly labelled monobenzyl ether above with benzyl chloride in refluxing acetone containing excess potassium carbonate, as described under 3,4-dihydroxy-[5-$^2$H] toluene, m.p. 89-91°.

Deuteration and Tritiation of Isovanillin. This was carried out with deuteroxide as catalyst as described for vanillin; the yellow solution of isovanillin in the basic medium went completely black after a few hours heating. The product, isolated in a similar manner, was chromatographed on alumina (Grade V) to remove dark impurities. Elution with benzene-chloroform (2:1) gave crystalline partially deuterated

$[2,6\text{-}^{2}H_2]$ isovanillin (60% yield) m.p. 113-115°. The n.m.r. spectrum of isovanillin (in dimethylsulphoxide) showed the following aryl signals: 2.59 $\tau$ ($H_6$; double doublet, J = 8 and 2 cps), 2.69$\tau$($H_2$ doublet J = 2 cps) and 2.88$\tau$($H_5$; doublet J = 8 cps). Deuteration proceeded only slowly, both exchangable protons ($H_2$ and $H_6$) being at positions ortho to the aldehyde group. Exchange occurred more rapidly at position 2 rather than at position 6 (n.m.r control) but it was not possible to effect exchange exclusively at the former position.

Tritiation was carried out, under the same conditions, using tritiated water (1.8 mC. per mg. atom hydrogen) to give $[2,6\text{-}^{3}H_2]$ isovanillin (2.1 mC per m.mole). Bromination experiments (see below) on several batches of labelled material showed the ratio of activities at positions 2 and 6 to be ca. 2:1.

Proof of labelling pattern in $[2,6\text{-}^{3}H_2]$ isovanillin

A solution of the labelled isovanillin (270 mg., 29 m C per m.mole) in dimethylformamide (2 ml.) containing benzyl chloride (1 ml.) was treated under nitrogen with sodium hydride (53% dispersion in mineral oil, 100 mg.) at room temperature for 30 mins. The reaction mixture was then heated at 100° for 1 hr. and steam distilled to remove excess benzyl chloride. The

neutral product, isolated by extraction into chloroform washed with aqueous alkali followed by water, dried (sodium sulphate) and evaporated, was recrystallised from methanol to give O-benzyl-[2,6-$^3H_2$] isovanillin (29$\mu$ C per m.mole) as needles (275 mg.) m.p. 61-63° (lit[18]., 62-63°). Oxidation of this material with potassium permanganate in acetone[19] gave the correspondingly labelled O-benzyl-isovanillic acid (28$\mu$ C per m.mole) m.p. 177-178° (lit[19]., 177-178°).

Demethylation of the labelled isovanillin gave [2,6-$^3H_2$] protocatechuic aldehyde (29$\mu$ C per m.mole). Bromination gave 5-bromo- [2,6-$^3H_2$] protocatechuic aldehyde (29$\mu$C per m.mole) which crystallised from aqueous ethanol as needles m.p. 228-230° (lit[20].,230°). The n.m.r. spectrum of 5-bromoprotocatechuic aldehyde (in dimethylsulphoxide) showed aryl proton signals at 2.42$\tau$ and 2.72$\tau$ . Both were well resolved doublets (J = 2 cps) confirming the <u>meta</u> orientation of the corresponding protons.

<u>Bromination of Isovanillin</u>.

This was carried out in acetic acid as described by Henry and Sharpe[13] with similar results; the

purification of the isomers by fractional crystallisation was followed by thin layer chromatography in which the isomers separate well enough for easy identification but not for preparative purposes. The 2-bromo derivative was very readily purified. 6-Bromoisovanillin, even when obtained as well formed crystals was shown by thin layer chromatography to contain traces of the 2-isomer. On silica gel (Merck) plates developed in chloroform the 2 and 6-bromo isomers (revealed by iodine vapour) had Rf values of 0.7 and 0.6 respectively. Repeated recrystallisation of 6-bromoisovanillin monohydrate from aqueous ethanol eventually gave pure material. The n.m.r. spectrum (in dimethylsulphoxide) of the 2-bromo isomer showed the characteristic AB quartet of the aryl protons (2 doublets at 2.64 and 2.96 $\tau$ J = 9 cps) while the aryl protons of the 6-isomer showed 2 singlets (2.66 and 2.71 $\tau$ J = 3 cps).

2-Bromo-[6-$^3$H] isovanillin

[2,6-$^3H_2$] isovanillin was brominated as above[13] to give crude 2-bromo isomer whose activity dropped to a steady value after one recrystallisation from ethanol. Separate samples of [2,6-$^3$H] isovanillins

(18.1 and 28.6 $\mu$ C per m.mole) gave corresponding samples of 2-bromo-[6-$^3$H]isovanillin (5.9 and 9.5 $\mu$C per m.mole). Higher activities were employed for the conversion of the labelled bromo compound into OO-dibenzyl-[6-$^3$H] protocatechuic aldehyde (see Table 2).

Bromination of 2-bromo-[6-$^3$H] isovanillin: 2-Bromo-[6-$^3$H]isovanillin ($2.40 \times 10^5$ cps per m.mole, 97 mg.) in dimethylformamide (1 ml.) and acetic acid (1 ml.) containing anhydrous sodium acetate (60 mg.) was treated with bromine (70 mg.) with stirring (40 min.) at room temperature. Water (10 ml.) was added, and after 1 hr. the crude product (79 mg.) was collected. crystallisation from aqueous ethanol gave 2,6-dibromo-isovanillin ($0.08 \times 10^5$ cps per m.mole) as needles m.p. 162-164° (lit[14]., 160-161°).

Deuteration and Tritiation of 6-bromoisovanillin. A solution of 6-bromoisovanillin monohydrate[13] (102 mg.) in deuterium oxide (0.2 ml.) containing triethyl-amine (43 mg.) was heated at 100° for 14½ hrs. Work up in the usual manner (see under isovanillin) gave anhydrous 6-bromo-[2-$^2$H]isovanillin (68 mg.) m.p. 110-112. The n.m.r. spectrum, in dimethylsulphoxide, showed a singlet at 2.71 $\tau$ (IH) and a very weak line

at 2.66$\tau$. With the undeuterated material, both signals were of similar integrated intensity although the high field signal (from $H_5$) was broader and therefore lower than its companion.

In a similar way, 6-bromo-[2-$^3$H]isovanillin (1.3 mC per m.mole) was prepared using tritiated water (1.8 mC per mg. atom hydrogen). Conversion of a diluted specimen into OO-dibenzyl-[2-$^3$H]protocatechuic aldehyde is recorded in Table 2.

Bromination of 6-bromo-[2-$^3$H]isovanillin. A solution of 6-bromo-[2-$^3$H]isovanillin ($5.46 \times 10^5$ cps per m. mole, 100 mg.) in glacial acetic acid (1 ml.) containing anhydrous sodium acetate (60 mg.) was treated with bromine (65 mg.) at room temperature when product (65 mg.) rapidly crystallised out. Recrystallisation from ethanol or aqueous ethanol gave 2,6-dibromoisovanillin ($0.02 \times 10^5$ cps per m. mole) m.p. 162-164° (lit[14]., 160-161°). The n.m.r. spectrum (in dimethylsulphoxide) showed a singlet at 2.63$\tau$ corresponding to one aryl proton.

Conversion of 2-Bromoisovanillin into OO-Dibenzyl-protocatechuic Aldehyde.

2-Bromoisovanillin (100 mg.) was demethylated[9] by refluxing in methylene chloride (7.5 ml.) and

pyridine (1.5 ml.) containing anhydrous aluminium chloride (65 mg.) for 24 hrs. This cooled reaction mixture was decomposed in the usual way with 6N-hydrochloric acid. Extraction of the aqueous phase with ether gave 2-bromoprotocatechuic aldehyde (86%) which crystallised from water as long needles m.p. 183-185° (after drying in vacuo at room temperature). (Found: C, 38.6; H, 2.1. $C_7H_5BrO_3$ requires C, 38.7; H, 2.3%). The n.m.r. spectrum (in dimethylsulphoxide) showed the expected AB quartet at 2.64 and 3.03 $\tau$ ($J$ = 8 cps).

Benzylation was carried out in dimethylformamide with benzyl chloride and sodium hydride (see above). The crude neutral product was chromatographed on alumina (Grade 3), elution with benzene gave 00-dibenzyl-2-bromoprotocatechuic aldehyde (75%) which crystallised from ethanol as needles, m.p. 142-144°. (Found: C, 63.2; H, 4.15. $C_{21}H_{17}BrO_3$ requires C, 63.5; H, 4.25%).

The dibenzylether (500 mg.) in tetrahydrofuran (15 ml.) was refluxed (19 hrs) with lithium aluminium hydride (250 mg.). The reaction mixture was decomposed with water, acidified with excess 6N-hydrochloric acid, and the product extracted into chloroform. The extract was dried (sodium sulphate)

and evaporated to give crude 3,4-dibenzyloxybenzyl alcohol (440 mg.).

The benzylalcohol was shaken in benzene (20 ml.) with manganese dioxide[25] (4 gm.) for 2 hrs. Filtration and evaporation gave OO-dibenzylprotocatechuic aldehyde which was recrystallised from methanol to give material (230 mg.) m.p. and mixed m.p. 89-91° (lit[23]., 91).

Conversion of 6-Bromoisovanillin into OO-Dibenzylprotocatechuic Aldehyde.

The procedure followed in detail that given above for the 2-isomer to give similar yields of intermediates. 6-bromoprotocatechuic aldehyde crystallised from water as needles m.p. 225-227° (decomp.). (Found: C, 38.8; H, 2.85. $C_7H_5BrO_3$ requires C, 38.7; H, 2.3%).

OO-Dibenzyl-6-bromoprotocatechuic aldehyde was obtained from ethanol as needles m.p. 105-107°. (Found: C, 63.3; H, 4.2. $C_{21}H_{17}BrO_3$ requires C, 63.5; H, 4.25%.) Thin layer chromatography, on alumina (Grade 3) developed in benzene, showed this compound, the 2-bromo isomer and OO-dibenzylprotocatechuic aldehyde as well resolved spots (revealed by iodine

vapour) with Rf values 0.57, 0.44, and 0.34 respectively; the spots are sufficiently well separated for preparative and purification purposes.

5-(3,4-Dibenzyloxybenzylidene) hydantoin.

Hydantoin was prepared[39] from glycine and potassium cyanate in very good yield. 3,4-Dibenzyloxybenzaldehyde[23] (1 gm.) and hydantoin (450 mg.) in acetic anhydride (9 ml.) were refluxed (70 min.) in presence of anhydrous sodium acetate (450 mg.). The hot reaction mixture was poured into excess hot water and the resulting suspension heated for 10 mins. and then filtered hot. The dark yellow product (1.3 gm.), after washing with hot water, was sufficiently pure for the next step (see below). Repeated crystallisation from ethyl acetate gave yellow needles of the benzylidene hydantoin m.p. 197-200° $\nu_{max}$ (in chloroform) 1780, 1750, 1710, 1660 $cm^{-1}$. (Found: C, 72.4; H, 5.3. $C_{24}H_{20}N_2O_4$ requires C, 72; H, 5.0%).

5-(3,4-Dibenzyloxybenzyl)hydantoin:

The corresponding benzylidenehydantoin (1 gm.) in dioxan (10 ml.) and water (2 ml.) was treated at room temperature with successive small portions of 3% sodium amalgam (10 gm.) during 2 hrs. with stirring. After a further 1 hr. the solution, whose

original dark brown colour had become much lighter, was decanted off the mercury, diluted with water (100 ml.), filtered, and acidified with 6N-hydrochloric acid, when the product (800 mg.) separated immediately. After 1 hr. at 0°, it was collected, dried and crystallised from benzene to give the benzylhydantoin as colourless plates m.p. 136-138° $\nu_{max}$ (in chloroform): 3450, 1775, 1730 $cm^{-1}$. (Found: C, 71.9; H, 5.5. $C_{24}H_{22}N_2O_4$ requires C, 71.6; H, 5.5%).

(+)-3,4-Dibenzyloxyphenylalanine:

A solution of the corresponding benzylhydantoin (400 mg.) in 2-methoxyethanol (6 ml.) and water (2 ml.) containing potassium hydroxide (1 gm.) was refluxed for 15 hrs. The solvent was evaporated *in vacuo* and the residue dissolved in water (50 ml.). Acidification with 6N-hydrochloric acid gave a precipitate (390 mg.) of the required amino acid hydrochloride. After treatment with charcoal (in ethanol) (+)-3,4-dibenzyloxyphenylalanine hydrochloride crystallised from ethanol-ether as small prisms m.p. 182-185° $\nu_{max}$ (in Nujol mull) 1752 $cm^{-1}$ (Found: C, 67.2; H, 5.7. $C_{23}H_{24}ClNO_3$ requires C, 66.7; H, 5.8%).

Hydrolysis of the benzylhydantoin with potassium hydroxide in aqueous ethanol gave the corresponding 3,4-dibenzyloxybenzylhydantoic acid which crystallised from acetonitrile as needles m.p. 175-176° $\nu_{max}$ (in Nujol mull) 3460, 3350, 1680, 1635 $cm^{-1}$ (Found: C, 68.2; H, 6.1. $C_{24}H_{24}N_2O_5$ requires C, 68.6; H, 5.75%).

(±)-3,4-Dihydroxyphenylalanine (dopa)

The corresponding dibenzylether (212 mg.) in ethanol (20 ml.) containing 6N-hydrochloric acid (0.2 ml.) was hydrogenated over 10% palladium-charcoal at room temperature (50 min.). After removal of the catalyst and evaporation of the solvent at room temperature, the residue was dissolved in water (0.5 ml.). The solution was adjusted to pH 4-5 by slow and careful addition of aqueous ammonia at 0°. The (±)-3,4-dihydroxyphenylalanine which crystallised out on standing at 0° (2 hrs.) was collected, washed with ethanol and acetone, and dried in vacuo to give material (60 mg.) m.p. and mixed m.p. 272-275°. The infrared spectrum in Nujol mull was identical with that of authentic (±) dopa, so also was the behaviour on paper chromatograph developed in n-butanol: acetic acid:water = 3:1:1 and sprayed with 0.1% nihydrin in n-butanol.

Hydrolysis of 5-(3,4-Dibenzyloxybenzyl)-[5-$^3$H]hydantoin

A solution of the unlabelled benzylhydantoin (207 mg.) in 2-methoxyethanol (3 ml.) and tritiated water (1 ml. 10 mC) containing potassium hydroxide (504 mg.) was kept at room temperature (24 hr.). The solvent was removed in vacuo and the residue dissolved in water (20 ml.). Acidification, in the usual way, gave [5-$^3$H] hydantoin (160 mg.). Crystallisation from benzene gave plates (124 mg., activity $4.80 \times 10^4$ dps per m.mole) m.p. 136-138°. The activity of this material was not significantly altered by repeated addition and evaporation of methanol. Dilution, with recrystallisation, of this labelled hydantoin with authentic inactive hydantoin gave a product with the expected activity. Hydrolysis in refluxing 2-methoxyethanol, as above, gave 3,4-dibenzyloxyphenylalanine hydrochloride ($0.19 \times 10^4$ dps per m.mole) containing 3.9% of the original activity. A duplicate experiment gave a 4.1% retention of activity.

Preparation of Tritium Labelled (±)-3,4-Dihydroxyphenylalanine (dopa)

[2-$^3$H]-; [5-$^3$H]-; [6-$^3$H]dopa: Conversion of the three labelled specimens of OO-dibenzylprotocatechuic aldehyde into the desired amino acid was carried out, as described above, with the results given in Table 3.

[2,5,6-$^3H_3$] dopa was obtained by acid catalysed tritiation of dopa (see above).

[$\alpha$-$^3H$]-; [$\beta$-$^3H$] dopa. Reduction of the benzylidene hydantoin with sodium amalgam in dioxan containing tritiated water (see above) and hydrolysis of the resulting benzylhydantoin gave $\beta$-labelled OO-dibenzyl-dopa. Hydrogenation then gave (±)-3,4-dihydroxy-[$\beta$-$^3H$] phenylalanine ([$\beta$-$^3H$] dopa) (see Table 3). Hydrolysis of the non-radioactive benzylhydantoin in the presence of tritiated water and hydrogenation of the product afforded (±)-3,4-dihydroxy-[$\alpha$-$^3H$] phenylalanine ([$\alpha$-$^3H$] dopa). The chemical nature of each labelled dopa was established as described above. Furthermore, successive dilutions, with recrystallisations, of each labelled dopa with known weights of inactive authentic (±) dopa caused no significant alterations in the expected activities; this further confirms the chemical purity of the labelled precursor.

Conversion of 2-Bromoisovanillin into Veratraldehyde

A solution 2-bromoisovanillin (95 mg.) in dimethylformamide (2 ml.) was treated slowly with sodium hydride (29.4 mg.) and methyl iodide (315 mg.). The solution after 1 hr. at 100°, was diluted with cold water when 2-bromoveratraldehyde (88 mg.) separated as leaflets m.p. 85-86° (lit[30]., 85-85.5).

A solution 2-bromoisovanillin (200 mg.) in

methanol (8 ml.) containing N-sodium hydroxide (1 ml.) was treated with sodium borohydride (27 mg.). The solution, after 1 hr. at 100°, was evaporated and the residue dissolved in water (10 ml.) and filtered. The filtrate on acidification with 6N-hydrochloric acid gave 2-bromoisovanillyl alcohol (163 mg.) as needles m.p. 160-162° (lit[30]., 156-158). This alcohol was methylated in the usual manner (see above) to give 2-bromoveratryl alcohol (isolated by extraction into chloroform) which crystallised from benzene-petroleum ether as needles m.p. 78-80° (lit[30]., 82.5°).

A solution of the bromoveratraldehyde or bromoveratryl alcohol (335 mg.) in tetrahydrofuran (20 ml.) was refluxed (17 hrs.) with lithium aluminium hydride (155 mg.) in an apparatus protected from moisture. The mixture was decomposed with water, acidified and extracted with ether. The extract was dried (sodium sulphate) and evaporated to give crude veratryl alcohol (242 mg.) which was characterised as its phenylurethan derivative m.p. 116-118° (lit[31]., 118).

Oxidation of this alcohol (130 mg.) by shaking in benzene (10 ml.) with ten times its weight of activated manganese dioxide in the usual manner gave crude veratraldehyde which was chromatographed over

alumina (Grade 3). Elution with benzene gave product m.p. 39-41° (lit[32]; 42-43).

## 3) MELANIN DEGRADATION

### Oxidation of dopa and tyrosine to melanin

Preliminary investigations were carried out to confirm that the oxidation of tyrosine and dopa is catalysed by the enzyme tyrosinase, and that the yield of melanin is improved by the presence of catalase. Tyrosine was found to undergo little or no oxidation at pH 8 in the absence of tyrosinase; dopa under the same conditions was oxidised slowly; addition of tyrosinase however accelerated the rate of oxidation considerably.

Oxidation: Into a solution of amino acid (100 mg.) in M/15 phosphate buffer (20 ml. for dopa; 200 ml. for tyrosine) at pH 6.8 containing mushroom tyrosinase[35] (3.5 mg.) and catalase (ex beef liver 14 mg.) was bubbled oxygen slowly for 8 hrs. at room temperature. The black pigment which could not be isolated by filtration or centrifugation of the colloidal solution was obtained by the following methods:

Precipitation by acid: The colloidal solution was acidified with 8N-hydrochloric acid (5 ml.) when melanin precipitated on standing. It was collected by centrifugation, and washed with more acid till the supernatant liquid was almost colourless, then with

water till neutral to litmus and finally acetone. After air-drying (12 hrs.) it was ground to a fine powder and dried at room temperature in vacuo over phosphorous pentoxide to constant weight (52-60 mg.). Samples were usually left to equilibrate in air before analyses.

Precipitation by salt:[36] To the colloidal solution was added ammonium nitrate (5.1 gm.) (sodium chloride (3.9 gm.) has also been used) and well stirred to dissolve the salt when melanin precipitated on standing and was collected by centrifugation. It was washed with water (4 x 25 ml. portions), then acetone (2 x 25 ml. portions) and dried as above to give 66-78 mg. of product.

Autoxidation of dopa: Dopa (100 mgs.) in M/15 phosphate buffer (20 ml.) at pH 8 was autoxidised by bubbling oxygen through for 38 hrs. and the melanin isolated by the acid method of precipitation.

Properties: These melanins are black infusible compounds (not melting up to 360°) insoluble in strong acids and bases and in all common organic solvents including trichloroethylene, trifluoroacetic acid, dimethylsulphoxide, dimethylformamide, tetrahydrofuran, sulpholane, cellosolve and nitromethane. They are very hygroscopic and their infrared spectra

in Nujol mull revealed no distinguishing features. Difficulty of combustion coupled with their hygroscopic nature have made elementary analyses on melanins rather difficult to rely on rigidly. The following are typical analytical results:

| Tyrosine Melanins | | | Dopa Melanins (enzymatic) | | | Dopa Melanins autoxidative | | |
|---|---|---|---|---|---|---|---|---|
| C | H | Ash | C | H | Ash | C | H | Ash |
| 54.63 | 4.16 | 2% | 55.33 | 3.66 | 4% | 49.98 | 4.42 | - |
| | | | | | | 50.27 | 3.92 | 0.5% |
| 56.85 | 3.11 | 1.78 | 56.13 | 5.36 | - | | | |
| | | | 52.48 | 4.48 | - | | | |
| 53.73 | 4.63 | - | | | | | | |
| 52.65 | 4.93 | - | | | | | | |

The melanin samples above were obtained by the acid method of isolation. For the samples isolated by the salt method carbon contents vary from 50.22 to 55.26% (all being prepared enzymatically).

Synthesis of pyrrole-2,3-dicarboxylic acid.

Amino acetaldehyde hydrochloride[27] prepared by sodium amalgam reduction of glycine ester hydrochloride in water, was treated[28] with the sodium salt of ethyl oxaloacetate to give 3-ethoxycarbonyl-pyrrole-2-carboxylic acid m.p. 145-147° (lit[28]., 146-147°).

(Found: C, 52.6; H, 4.85. Calculated for $C_8H_9NO_4$: C, 52.5; H, 4.95%).

This on hydrolysis[29] in refluxing ethanolic potassium hydroxide, gave pyrrole-2,3-dicarboxylic acid m.p. 227-228° (after drying in vacuo at 100°) (lit[29]., 225). (Found: C, 46.2; H, 3.25. Calculated for $C_6H_5NO_4$: C, 46.5; H, 3.25). (EtOH $\lambda_{max}$ 243, 282mμ; $\epsilon$ 8,900 and 11,800. Lit[29]., 243 and 282 mμ ; $\epsilon$ 2,900 and 4,200).

Esterification of pyrrole-2,3-dicarboxylic acid.

When an ethereal solution of the above dicarboxylic acid was treated briefly at room temperature with an ethereal solution of diazomethane, 3-methoxycarbonyl-pyrrole-2-carboxylic acid separated and was crystallised from water as needles m.p. 200-202° (lit[28]., 201). (Found: C, 49.7; H, 4.2. Calculated for $C_7H_7NO_4$: C, 49.7; H, 4.2). However, a methanolic solution of the dicarboxylic acid treated with an ethereal solution of diazomethane at room temperature for 3 hrs. gave, after evaporation, 2,3-dimethoxycarbonyl pyrrole which crystallised from benzene-petroleum ether (60-80°) as plates m.p. 71-72° (lit[38]., 72-73).

Rate of Oxidation of Melanin in the Absence and the Presence of added Pyrrole-2,3-dicarboxylic acid and the Consumption of Oxidant by Melanin and Dicarboxylic acid.

Stability of alkaline permanganate: The stability of alkaline permanganate was checked by shaking a mixture of N-potassium permanganate (10 ml.) and 2N-potassium carbonate (0.6 ml.) at room temperature over 72 hrs. during which aliquot portions (0.5 ml.) were withdrawn at intervals and titrated in 6N-sulphuric acid (10 ml.) against standard ferrous ammonium sulphate. A graph of titrant against time showed that the permanganate under these conditions was still at least 94% pure after 48 hrs. (See Table 5).

Oxidation: A suspension of finely powdered melanin (22.2 mg.) in freshly made N-potassium permanganate (10 ml.) and 2N-potassium carbonate (0.6 ml.) was shaken vigorously in a stoppered vessel at room temperature during 24 hrs. The following operations were carried out:

(a) Aliquot portions (0.5 ml.), withdrawn at intervals, were titrated in 6N-sulphuric acid (10 ml.) against N/40-ferrous ammonium sulphate (see Table 6). A graph of titrant against time showed that oxidation was practically complete within 2 hrs.

(b) The above procedure (a) was repeated in the presence of added pyrrole-2,3-dicarboxylic acid using the following quantities: melanin (18.2 mg.); dicarboxylic acid (20.8 mg.); N-permanganate (10 ml.); 2N-potassium carbonate (0.6 ml.). The results (see Table 7) showed that the added pyrrole carboxylic acid consumed a significant quantity of oxidant and confirmed that oxidation was practically complete within 2 hrs.

(c) Oxidation was carried out with 5 ml. of permanganate instead of 10 ml. The contents of the reaction vessel, at the end of oxidation, were back-titrated in 6N-sulphuric acid (10 ml.) against N/4-ferrous ammonium sulphate. The consumption of N-potassium permanganate per mg. of melanin obtained from three such oxidations were 0.18; 0.19; 0.18.

(d) The above procedure (c) was repeated in the presence of added pyrrole dicarboxylic acid using the following quantities: melanin (18.0 mg.); pyrrole dicarboxylic acid (23.2 mg.); N-permanganate (10 ml.); 2N-potassium carbonate (0.6 ml.). After subtracting the consumption by melanin (as determined above (c)) the consumption of N-potassium permanganate per mg. of pyrrole-2,3-dicarboxylic acid, obtained from three separate oxidations, was 0.11; 0.09;

0.10 ml. At the end of oxidations in (a) and (b) and titrations in (c) and (d), sulphur dioxide was bubbled through the contents of the vessel. In this way, manganese dioxide formed during the oxidation dissolved and a clear solution was obtained in each case showing that all the melanin had been oxidised under these conditions.

(e) The oxidations in (c) and (d) were repeated; this time over 1 hr. Similar results were obtained.

Oxidation of melanin in the presence of added pyrrole-2,3-dicarboxylic acid followed by the isolation of the Dicarboxylic acid.

A suspension of finely powdered melanin (40.6 mg.) and pyrroledicarboxylic acid (51.6 mg.) in freshly made N-potassium permanganate (11.86 ml.) and 2N-potassium carbonate (0.6 ml.) was shaken vigorously at room temperature for 1 hr. at the end of which the colour of the permanganate had been lost and the reaction mixture had gone dark brown. Sulphur dioxide was bubbled through to give a clear solution which was acidified strongly with 8N-hydrochloric acid (2 ml.) and extracted with ether (4 x 50 ml. portions). The extract was dried (sodium sulphate) and evaporated to give dirty white solid residue (33 mg.) m.p. 213-220°,

EtOH $\lambda_{max}$ 242,284 mμ , and an infrared spectrum similar to that of pyrrole-2,3-dicarboxylic acid. A methanolic solution of this residue was treated with an ethereal solution of diazomethane at room temperature. The solvent was removed and the residue chromatographed over alumina (Grade 5). Elution with benzene gave 2,3-dimethoxycarbonyl pyrrole (14.6 mg.) which crystallised from benzene-petroleum ether as plates m.p. and mixed m.p. 70.5-72° (lit[29]., 72-73).

Study of Melanin Degradation by Isotope Dilution Method.

The above procedure consisting of oxidation and followed by the recovery of pyrroledicarboxylic acid was repeated using varying amounts of oxidant (see Table 9). The same procedure was finally carried out on melanin obtained from enzymatic oxidation of (±) (3,4-dihydroxyphenyl)-[2-$^{14}$C] alanine (activity ca. 10 μC per m.mole). The results of two such oxidations are recorded in Table 8.

Table 8

Isotope Dilution Study of [$^{14}$C] Melanin Degradation

| | Oxidation I | Oxidation II |
|---|---|---|
| Wt. of melanin (activity 87749 dpm per mg.) | 40.6 mgs. | 43.8 mgs. |
| Vol. of N-potassium permanganate | 11.86 ml. | 12.55 ml. |
| Vol. of 2N-potassium carbonate | 0.6 ml. | 0.6 ml. |
| Wt. of pyrroledicarboxylic acid added | 51.6 mgs. | 53 mgs. |
| Wt. of Crude extract | 40.6 mgs. | 48 mgs. |
| Activity of Crude extract | 188 dps/mg | 214 dps/mg. |
| Chromatographic dimethyl ester (of pyrrole 2,3-dicarboxylic) acid | 14.6 mgs. | 10.5 mgs. |
| m.p. | 67-69° | 68-69° |
| Activity | 23.9 dps/mg | 9.2 dps/mg |
| Dimethyl ester after 1 recrystallisation m.p. | 69-71° | 69-70.5° |
| Activity | 3.2 dps/mg | 2.6 dps/mg. |
| Dimethyl ester after 2 recrystallisations m.p. | 70.5-72° | 71-72° |
| Activity | 2.0 dps/mg | 2.9 dps/mg |
| Dimethyl ester after 3 recrystallisations m.p. | 70.5-72° | 71-72° |
| Activity | 2.0 dps/mg | 2.8 dps/mg |
| Total activity (dpm) of melanin oxidised | $3.56 \times 10^6$ | $3.84 \times 10^6$ |
| Maximum total activity of pyrrole dicarboxylic acid produced | $7.30 \times 10^3$ | $1.02 \times 10^4$ |
| Maximum yield of dicarboxylic acid from oxidation of melanin | 0.2% | 0.3% |

Collection and assay of carbon dioxide evolved during the oxidation of [$\alpha$-$^{14}$C] dopa and of [$\alpha$-$^{14}$C] tyrosine to melanin.

The quantities of amino acid, enzymes and buffer solutions used were as stated above (see Oxidation of dopa and tyrosine to melanins). The apparatus consisted of a potassium hydroxide tower followed by tubes 1, 2, 3, 4, 5 and 6 in that order; 2 was the reaction tube, 4 was the absorption tube, 3 and 5 were traps while 1 and 6 contained test solutions of barium hydroxide. The whole apparatus was first swept for 30 mins. with oxygen which entered through the potassium hydroxide tower. Clear saturated aqueous barium hydroxide (35 ml.) was introduced into each of tubes 1 and 6 and phosphate buffer into tube 2. The apparatus was swept out again with oxygen for 2 hrs. Saturated aqueous barium hydroxide (20 ml.) was put in the absorption tube 4 immediately after which the amino acid with or without the enzyme was put in the reaction tube 2 which was swirled round to dissolve its contents. Oxygen was bubbled through for the necessary length of time. The solution in the absorption tube remained clear until the formation of the black pigment commenced.

The test solutions remained clear throughout the operation at the end of which the precipitated barium carbonate was rapidly filtered off into a sintered crucible (of known constant dry weight). It was dried *in vacuo* at 140-150° for 3 hrs, cooled and reweighed and the process was repeated until the weight was constant; 3-6 hrs. drying usually sufficed. It was stored in a vacuum desiccator and countered within 24 hrs. The results of the radiochemical assay of the barium carbonate collected in several experiments are tabulated below.

Activities in dps/m.mole x $10^{-3}$

| Compound | Enzymatic Oxidation | | Autoxidation |
|---|---|---|---|
| | 1st Run | 2nd Run | |
| DL-Dopa | 2.60 | 2.50 | 2.46 |
| $BaCO_3$ | 0.02 | 0.00 | 0.02 |
| L-Tyrosine | 2.15 | 2.15 | |
| $BaCO_3$ | 0.00 | 0.00 | |

## Desorption of Melanin

Melanin (obtained from dopa of activity 540 dps/mg., 31.5 mg.) was refluxed in 2N-hydrochloric

acid (0.5 ml.) for 3½ hrs. The solution of desorbed dopa was separated from the suspension by centrifugation. The residue was washed with water (2 ml.) and the washings added to the main bulk of solution. This solution was then assayed for dopa by a radio dilution method in the following manner. Inactive dopa (53.8 mg.) was added and the homogeneous solution treated with aqueous ammonia to pH 4-5 when dopa (43.8 mg. activity, 4.4 dps/mg.) crystallised out on cooling. The activity of the dopa was not altered significantly after two recrystallisations. When the above procedure was repeated, this time with 24 hrs. refluxing and continuous stirring, similar results were obtained.

Thus the total amount of dopa desorbed from 31.06 mg. of melanin in this way is equal to (4.4 x 53.8)/540 that is 0.44 mg. Thus the melanin contains 1.4% by weight of adsorbed dopa.

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