STUDIES ON THE BIOSYNTHESIS AND STRUCTURE OF MELANIN

A thesis submitted by

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Abstract

The chemistry of melanin - a black, amorphous, polymeric pigment widely distributed in nature - is reviewed. The formation and reactions of melanin have been studied.

The oxidation of p-cresol to Pummerer's ketone and the addition of quinones to indoles were investigated radiochemically as model reactions for melanogenesis.

3,4-Dihydroxyphenyl[β-$^2H_2$]alanine was synthesized in order to discover the fate of the β-hydrogens in the oxidation of it to melanin. In connexion with this work various labelling techniques were investigated including a useful general reaction for the labelling of aldehydes.

The factors influencing the chemical and enzymic oxidation of 3,4-dihydroxyphenylalanine and tyrosine were studied spectroscopically and the evolution of carbon dioxide in melanogenesis followed quantitatively.

Pyrrole-2,3,5-tricarboxylic acid, the principal product of the oxidation of melanin, was estimated radiochemically and the amount of absorbed 3,4-dihydroxyphenylalanine in the melanin polymer measured.

Acknowledgements

The author would like to thank Professor D. H. R. Barton, F.R.S. for the opportunity of working in his laboratories, and also to express gratitude to Professor G. W. Kirby for his inspiration and interested guidance throughout this work.

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Melanin

Melanin (from the Greek μέλας - black) is the name given to a number of insoluble black pigments which occur widely throughout the plant and animal kingdoms. The black colouration of parts of many plants is frequently due to melanin and in animals the brown or black of skin, hair and feathers is caused by a melanin. Melanin is also produced in the mammalian melanoma and in the ink of the squid, octopus and sepia. According to Aristotle[1], 'Molluscs have no viscera, but they have what is called a mytis, and in it a vessel containing a thick black juice; in the sepia or cuttle-fish this vessel is the largest, and this juice is most abundant. All molluscs, when frightened, discharge such a juice, but the discharge is most copious in the cuttle-fish.'

Plant melanins are found in the black cuticle of many seeds and in the black of fungal spores (e.g. Ustilago - ergot). The blackening of fruit, such as the banana, when bruised is due to the formation of a melanin.

Since melanins are generally highly insoluble, infusable polymers of variable composition and unknown molecular weight, the problem of their chemistry and structure is particularly difficult. Natural melanins are contaminated by proteins and other substances which make purification difficult.

Even the composition of synthetic melanins depends, not only on the precursor, but also on the conditions employed in the preparation, isolation and drying. Apparently conflicting results may sometimes be explained by the differing conditions used for the preparation of the melanin.

The structure and synthesis of melanins has been extensively studied. Melanins are formed by the oxidation of a catechol or phenol by a number of oxygenases. Animal melanins are derived from 3,4-dihydroxyphenylalanine (dopa) by oxidation with molecular oxygen and a dopa oxidase. In plants, fungi, invertebrates and a bacterium another enzyme, tyrosinase, is present which is able both to hydroxylate tyrosine to 3,4-dihydroxyphenylalanine and to oxidise this in turn to melanin. Plant melanins are probably derived from several precursors since plants contain a wide variety of catechol derivatives.

Melanins may be synthesized chemically by the autoxidation or enzymic oxidation of many catechols. Catechol, 3,4-dihydroxyphenylalanine, adrenaline, 5,6-dihydroxyindole and 1,8-dihydroxynaphthaline all give melanins on oxidation. The oxidation of mono-phenols requires a hydoxylating enzyme, such as tyrosinase, although some copper complexes can catalyze this oxidation. In this way both phenol and tyrosine may be oxidized to a melanin.

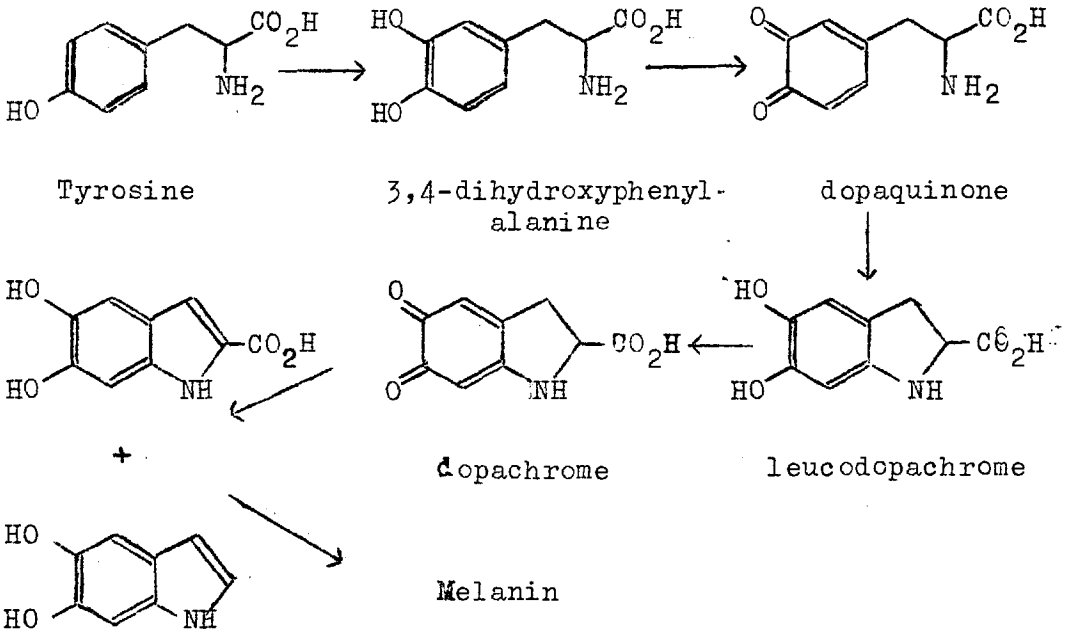
This review will describe the research carried out into the structure and synthesis of many melanins, but with particular emphasis on the melanins derived from tyrosine or 3,4-dihydroxyphenylalanine. Unless qualified the word 'melanin' will be used to describe this class of melanins. As far as possible those aspects of melanin and melanogenesis[2] and the enzymes involved in this reaction[3] which have already extensively reviewed will be avoided.

Intermediates involved in melanogenesis

Introduction

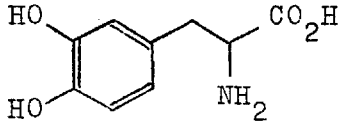
Since 1927 when Raper first proposed a scheme for the formation of melanin from tyrosine[4] much work has been directed towards checking the various reactions involved.

The Oxidation of Tyrosine to Melanin[4]



Each stage of this scheme, which is still accepted in broad outline, will be considered individually with reference to related compounds and reactions.

3,4-Dihydroxyphenylalanine (dopa)

Tyrosine 

3,4-dihydroxyphenylalanine

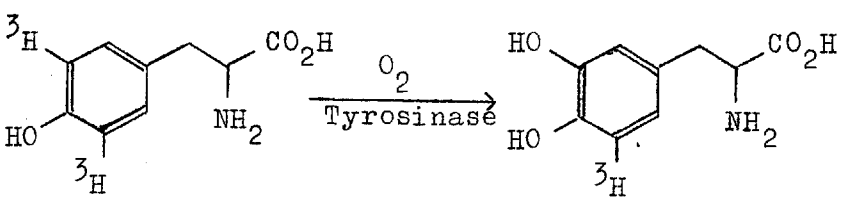
Early workers suggested that tyrosine was deaminated[5,6] but this was shown to be incorrect.[7,8] Onslow, in 1923, described the tyrosinase-tyrosine reaction and recognised that 3,4-dihydroxyphenylalanine was probably an intermediate.[9] Unlike tyrosine, which had been obtained from the alkali fusion of casein in 1846 [10] and synthesized by several routes,[11] 3,4-dihydroxyphenylalanine was not prepared until 1911 [12] although several routes were subsequently devised.[13] L-3,4-Dihydroxyphenylalanine was isolated from plants of the Leguminaceae (peaflower family)- in 1913 from vetch seedlings,[14] the pods of Vicia fabia (broad bean)[15] and Stirzolbium decringianum (Georgia velvet bean).[16] Yields as high as 15 g. per kg. were obtained from the seeds of Mucana pruriens.[17]

L-3,4-Dihydroxyphenylalanine was isolated in 10-20% yield from the enzymic oxidation of L-tyrosine, after the decomposition of the intermediate red coloured solution,[18,19,20] and also from the oxidation of [3-^{3}H]tyrosine by tyrosinase from the mammalian melanoma.[21] 3,4-Dihydroxyphenylalanine accumulated in the presence of ascorbic acid which reduced

dopaquinone as it was formed.[22] Irradiation of tyrosine was also reported to give 3,4-dihydroxyphenylalanine.[23]

Although most of the evidence points to the 3-hydroxylation of tyrosine some other intermediates have been proposed. 2,5-Dihydroxyphenylalanine can be formed from tyrosine,[23a] but since p-quinones are less reactive than o-quinones, this is unlikely to be an important precursor of melanin. Even oxidation of 2,3-dihydroxyphenylalanine gave a melanin very different from ordinary melanin.[24] An additional intermediate, detected by thin-layer chromatography of the oxidation products of tyrosine, was suggested to be a trihydroxyphenylalanine,[25,26] but this has been disputed.[27] 3,4-Dihydroxyphenylalanine could however be hydroxylated in the 6-position.[28,29]

The hydroxylation of tyrosine, using a mammalian tyrosinase, has been studied with [3-^{3}H]tyrosine.[30,31] This reaction was stopped with metaphosphoric acid and the tritium liberated into the water assayed. Tyrosine and 3,4-dihydroxyphenylalanine were isolated and assayed radiochemically. [5-^{3}H]-3,4-Dihydroxyphenylalanine was formed slightly faster than [^{3}H]water and an isotope effect of 1·03-1·09 was observed.

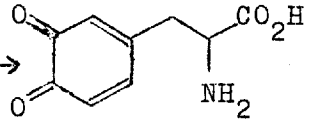


By using ^{18}O the source of the 3-hydroxyl group was shown to be molecular oxygen and not water.[32] This agreed with the results obtained from several other enzymic oxygenations.

A suggestion that the reaction of a molecule of tyrosine with one of dopachrome would give two of 3,4-dihydroxyphenylalanine[33] seems improbable since dopachrome is unlikely to undergo ring fission.

To summarise the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine is the best characterized step in melanogenesis. 3,4-Dihydroxyphenylalanine has been isolated from the reaction mixture and the mechanism of the hydroxylation has been partially elucidated by isotopic labelling. This reaction requires the enzyme tyrosinase, molecular oxygen and a hydrogen donor. The small amounts of other polyhydroxyphenylalanines which may possibly be formed are unlikely to contribute significantly to the structure of melanin.

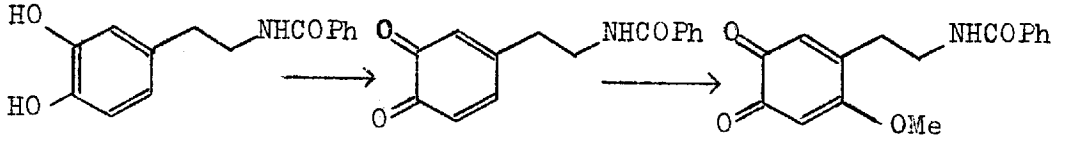
Dopaquinone

3,4-dihydroxyphenylalanine → 

Dopaquinone

Although dopaquinone is generally accepted as an

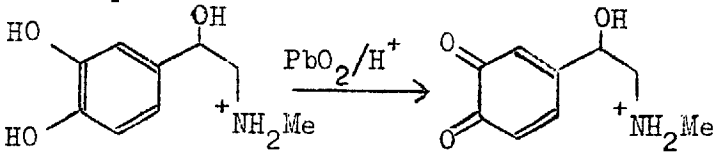
intermediate in the oxidation of 3,4-dihydroxyphenylalanine to dopachrome there is little direct evidence for it. Transient open-chain o-quinones were identified in the electrochemical oxidation of adrenaline and its derivatives and their rates of cyclisation could be measured.[34] Oxidation of tyrosine or 3,4-dihydroxyphenylalanine by tyrosinase in the presence of cysteine or reduced glutathione was reported to give addition compounds of dopaquinone.[35] By deactivating the amine group of 3,4-dihydroxyphenylalanine with a benzoyl group the open-chain o-quinone was stabilized sufficiently for it to be trapped as the methanol adduct.[29,36]



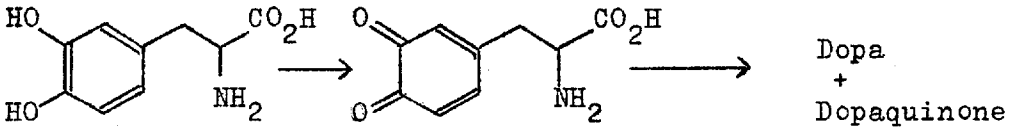
λ 375mμ

A N-acylated tyrosine was also oxidized to give a transient dopaquinone intermediate which had an ultra-violet absorption maximum at 390mμ.[37]

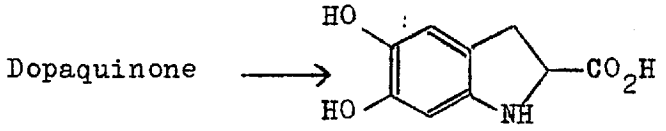
Oxidation of adrenaline with lead peroxide in strong acid gave an orange-yellow solution which was thought to be adrenalinequinone since it could be reduced to adrenaline.[38,39]



Oxidation of 3,4-dihydroxyphenylalanine and 3,4-dihydroxyphenylethylamine in strong acid gave similar results.[39] Both gave yellow quinones which decomposed on neutralization to give some red indolinequinone, more of which could be generated by further oxidation. Protonation of the amine function had prevented attack of it on the o-quinone.



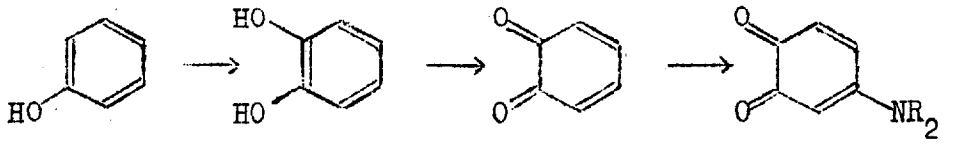
Leucodopachrome (5,6-dihydroxyindoline-2-carboxylic acid)



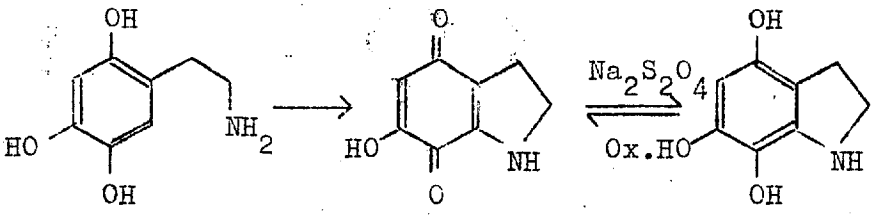
Leucodopachrome

Another elusive intermediate which has not been detected in the oxidation of 3,4-dihydroxyphenylalanine. Leucodopachrome is formed by the cyclisation of dopaquinone and is readily oxidized to dopachrome.

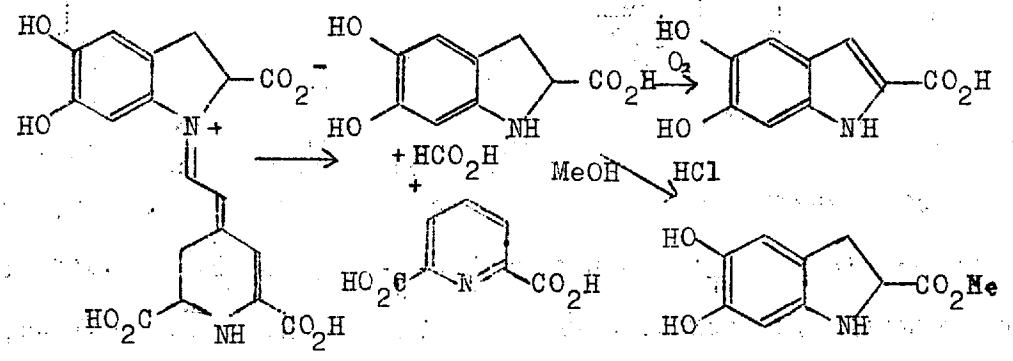
Oxidation of mono- and dihydric phenols by tyrosinase in the presence of an amine gave amino-quinones.[40]



Reduction of dopachrome by sulphur dioxide did not give the leuco derivative, but instead, catalyzed its rearrangement to 5,6-dihydroxyindole-2-carboxylic acid.[4] A related quinone could be prepared by reducing the oxidation product of 2,4,5-trihydroxyphenylalanine.[41]



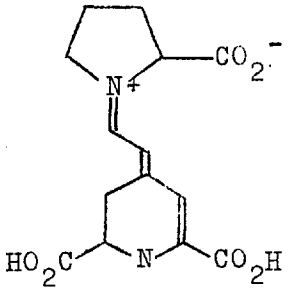
The study of betanin, the violet colouring of Beta vulgaris (beet),[42] has enabled leucodopachrome to be isolated. Alkaline degradation of its aglucone, betanidin, in the absence of oxygen gave an amino-acid.[43] Although very sensitive to oxygen it was stable in strong acid and could be methylated by methanolic hydrochloric acid. A crystalline optically-active triacetate was also prepared. Spectroscopic measurements proved that the amino-acid was leucodopachrome.



Betanidin

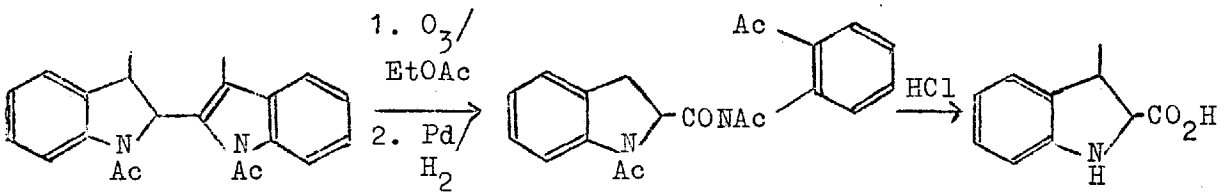
[Betanin- 5-O-Glucoside[44,45]]

Biosynthetically betanin may be derived from two molecules of 3,4-dihydroxyphenylalanine with ring fission to give the piperidine portion.[46] A related pigment, indicaxanthine, from the fruit of Opuntia ficus-indica (prickly pear) was shown by radiochemical feeding experiments to incorporate both proline and 3,4-dihydroxyphenylalanine, whereas betanin was found to be derived only from 3,4-dihydroxyphenylalanine.[47] Betanidin therefore represents an *in vitro* formation of leucodopachrome, although the enzymes involved may be very different from tyrosinase.



Indicaxanthin

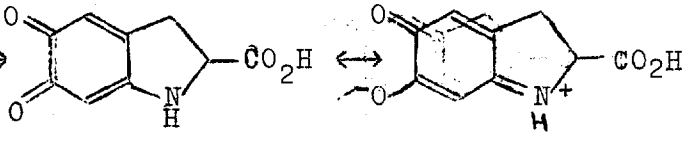
An indoline-2-carboxylic acid has been synthesized by the ozonolysis and hydrolysis of the skatole dimer.[48]



5,6-Dihydroxyindoline-2-carboxylic acid is the most probable precursor of dopachrome. It is unlikely to be isolated from the reaction since it is very readily oxidized

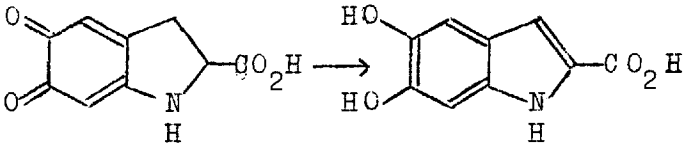
by dopaquinone or oxygen. It is formed from dopaquinone without enzyme catalysis. Since ascorbic acid is able to reduce dopaquinone to 3,4-dihydroxyphenylalanine and transient open-chain intermediates have been detected, this cyclization cannot be concerted with the oxidation of 3,4-dihydroxyphenylalanine.

Dopachrome (Indoline-5,6-quinone-2-carboxylic acid)

Leucodopachrome 

Dopachrome

Dopachrome is the red pigment which was recognized by Raper in 1923 as an intermediate in melanogenesis.[49] It is formed by the oxidation of leucodopachrome and is the first stable intermediate after 3,4-dihydroxyphenylalanine. Although the structure initially proposed[18] was most unlikely a revision based on its decomposition to 5,6-dihydroxyindole-2-carboxylic acid[4] has been generally accepted.

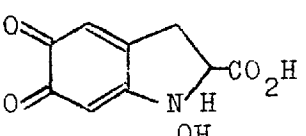


Dopachrome is very reactive and has not been isolated

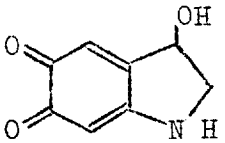
so that it has been characterized only by its reactions and spectral properties. A solution was stable for a few hours, but attempted isolation led to a brown solid which could not be characterized.[20] A report that dopachrome accumulated when 3,4-dihydroxyphenylalanine was autoxidized at 100° [50] seems unlikely in view of its known reactivity.

A pigment, hallachrome, isolated from the marine worm Halla parthenopeia[51] was thought to be identical with the red pigment from the oxidation of 3,4-dihydroxyphenylalanine.[52] When more precise data became available the pigments were shown to be quite different and the alternative name 'dopachrome' was given to the intermediate in melanogenesis.[53] Hallachrome may be related to the polyhydroxynaphthoquinone pigments of other marine animals.[54]

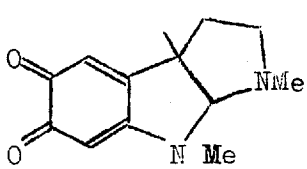
The absorption spectrum of dopachrome[37,53,55] was found to be similar to related compounds of known structure:



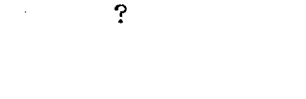
Dopachrome λ 305mμ (ε 9300 [55], 15,000 [37])
475mμ (ε 3500 , 6000)



Adrenochrome λ 300mμ (ε 9350)[56]
470mμ (ε 3900)

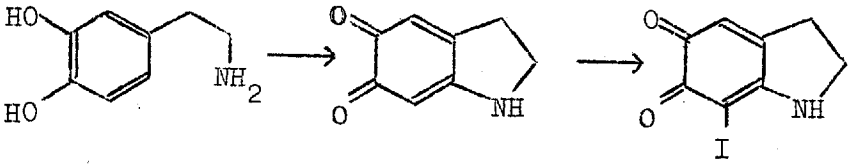


Rubreserine λ 300mμ (ε 7400)[57]
480mμ (ε 2800)



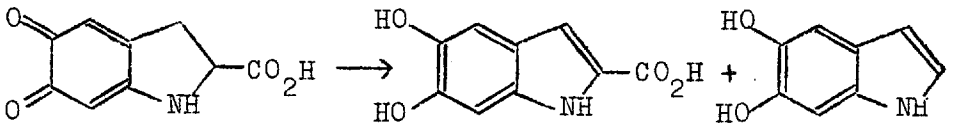
? Hallachrome λ 539mμ (ε 16,000)[55]

Periodate oxidation of various 3,4-dihydroxyphenylethylamines was thought to give 2-iodo[58] or 3-iodoindolinequinones[59,60] but these derivatives were later shown to be the 7-iodo-indolinequinones[61] formed by the iodination of the intermediate indolinequinone.[62]



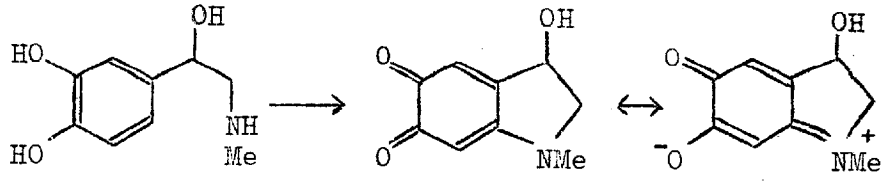
Oxidation of (3,4-dihydroxyphenylethyl)methylamine gave a red quinone, 1-methylindoline-5,6-quinone which readily rearranged to 5,6-dihydroxy-1-methylindole, but could be isolated as a mono-phenylhydrazone, oxime or semicarbazone.[60,63]

The dopachrome molecule contains both an oxidising group, the o-quinone, and an oxidizable function, the indoline. It was readily rearranged in acid or base[4] and this reaction was found to be catalyzed by zinc or, to a lesser extent, aluminium salts.[59,64,65]



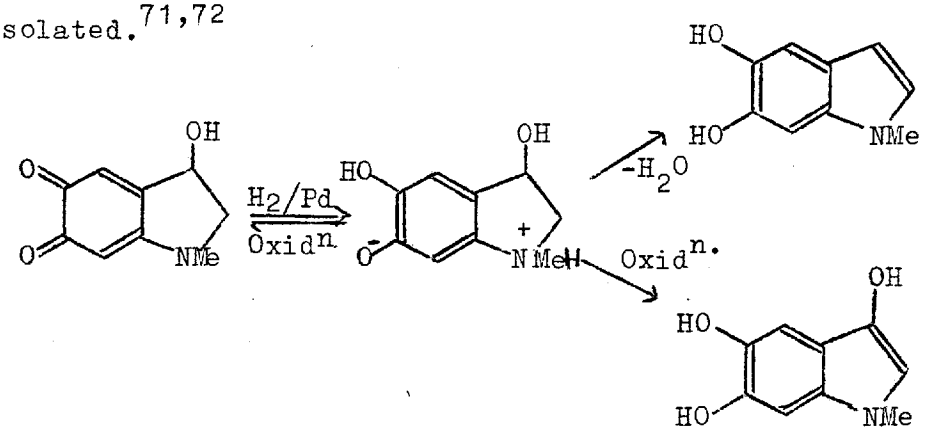
Oxidation of (3,4-dihydroxyphenyl-β-hydroxyethyl)methyl-amine, adrenaline, by a catechol oxidase,[66] siver oxide,[67]

or alkaline ferricyanide[36,68] gave the related indolinequinone, adrenochrome. Adrenochrome is more stable than dopachrome and hence its chemistry is better understood.[69]



Adrenaline Adrenochrome

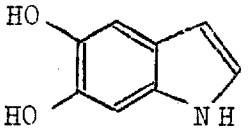
Adrenochrome could be reversibly reduced[70] and from the rearrangement of the reduced product both 5,6-dihydroxy-1-methylindole and 1-methyl-3,5,6-trihydroxindole were isolated.[71,72]



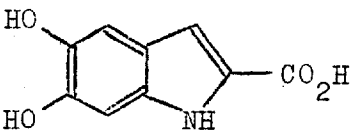
Acidification of a solution of adrenochrome discharged the red colour and eventually gave a black precipitate of a melanin.[69] In the absence of oxygen some 5,6-dihydroxy-1-methylindole could also be isolated from this reaction.[73] Adrenochrome was also rearranged by base or zinc acetate to 1-methyl-3,5,6-trihydroxyindole.[39,74] Like dopachrome, adrenochrome, gave only a mono-oxime and semicarbazone.[71]

From the data available indoline-5,6-quinone-2-carboxylic acid, or its mesomeric zwitterion, is the only reasonable structure for dopachrome but its structure still has to be confirmed. The formation of dopachrome from leucodopachrome may be catalyzed by tyrosinase, but its formation is so rapid that dopaquinone must be the principal oxidant.

Indolic intermediates



5,6-dihydroxyindole

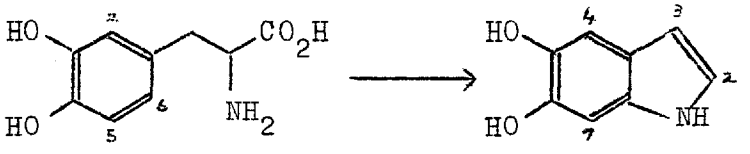


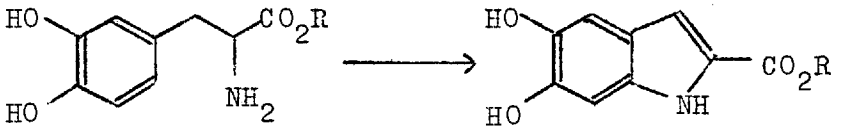
5,6-dihydroxyindole-2-carboxylic acid

Raper suggested that the colourless products formed by the decomposition of dopachrome were probably 5,6-dihydroxyindoles.[49] From the neutral and acid solutions of dopachrome 5,6-dihydroxyindole and 5,6-dihydroxyindole-2 carboxylic acid, respectively, were isolated as their dimethyl ethers.[4,75] 5,6-Dihydroxyindole could also be isolated, as the dimethyl ether, from the enzymic oxidation of 4-hydroxyphenylethylamine and 3,4-dihydroxyphenylethylamine.[76] The structures of these products have been confirmed by synthesis.[77] By chromatography and electrophoresis various hydroxylated indoles were detected as intermediates in melanogenesis.[78] 3,4-dihydroxyphenylalanine gave

5,6-dihydroxyindole and its 2-carboxylic acid, whilst 3,4-dihydroxyphenylethylamine gave only 5,6-dihydroxyindole and adrenaline was oxidized to 3,5,6-trihydroxyindole.

The oxidation of substituted dihydroxyphenylethylamine derivatives by alkaline ferricyanide, oxygen or Fremy salt is a useful synthesis of 5,6-dihydroxyindoles. Yields of 40-80% were frequently obtained.

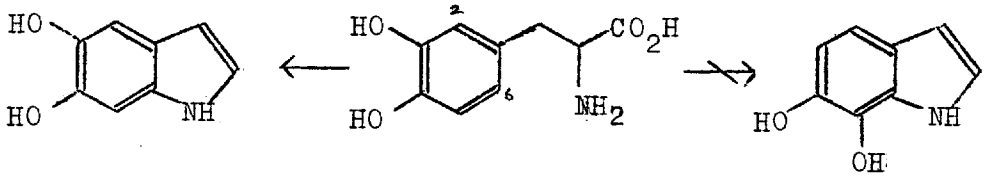




Oxidation of 3,4-dihydroxyphenylalanine and its methyl[79] or N-methyl[59] derivatives gave 5,6-dihydroxyindoles with the loss of the carboxyl group. The same products were obtained from the oxidation of the corresponding 3,4-dihydroxyphenylethylamines.[59,65] Esterification protected the carboxyl group so that an indole-2-carboxylic acid was produced.[45,59] Oxidation of 2,5-and 2,3-dihydroxyphenylalanine gave 5- and 7-hydroxyindole respectively.[80] With a similar loss of the 2-substituent 2,4,5-trihydroxyphenylethylamine and 2-amino-

4,5-dihydroxyphenylethylamine both gave 5,6-dihyroxyindole.[81] Bis(3,4-dihydroxyphenylethyl)amine was oxidized to 1-(3',4'-dihydroxyphenylethyl)-5,6-dihydroxyindole.[82] Adrenaline derivatives have also been oxidized to indoles.[39] Oxidations with potassium iodate were thought to give 3-iodoindoles,[61] but the products have been shown to be the 7-iodo isomers.[51,65]

The cyclization of 3,4-dihydroxyphenylalanine appears to be entirely at the 6-position. Oxidation of 2- and 5-methyl-3,4-dihydroxyphenylalanine to melanins proceeded normally whereas the 6-methyl isomer gave only a yellow solution.[79,83,84].



The alternative mode of cyclization has been suggested,[85] but in view of other unlikely structures proposed in that paper, this cannot be taken seriously.

Little is known about the relative importance of 5,6-dihydroxyindole and its 2-carboxylic acid as precursors for melanin. Both compounds are readily oxidized to melanin, although the carboxylic acid is comparatively stable.[81,86,87,75]

Elemental analysis of melanins suggested that there was a proportion of carboxyl groups in melanin, although results are of doubtful significance due to the variable composition of melanin. The interpretation will also be complicated by the presence of uncyclized amino-acid residues and degraded indoles. A melanin derived from the enzymic oxidation of tyrosine had an empirical formula $C_{16}H_{10}O_5N_2$ which was interpreted as a polymer of indolequinone and dihydroxyindole units with complete loss of the carboxyl group of tyrosine.[90] An analysis of natural melanins has showed that the proportion of carboxyl groups present was dependant on the method of isolation.[91] With vigorous conditions retention of 20% of the carboxyl groups was found, but using a milder procedure a figure as high as 50% could be obtained. Although carboxyl groups are present in melanin they need not be derived from the carboxyl group of tyrosine, but may originate from fission of the benzenoid portion.[87,91,92]

Both 5,6-dihydroxyindole and its 2-carboxylic acid are probably precursors of melanin. Their relative importance will depend on the oxidation conditions. Neutral rather than alkaline conditions favour the decarboxylation of dopachrome and a vigorous isolation will reduce the number of carboxyl groups in the melanin. Because of the lower reactivity of the indole-acid some workers have assumed that 5,6-dihydroxyindole was the principal intermediate.

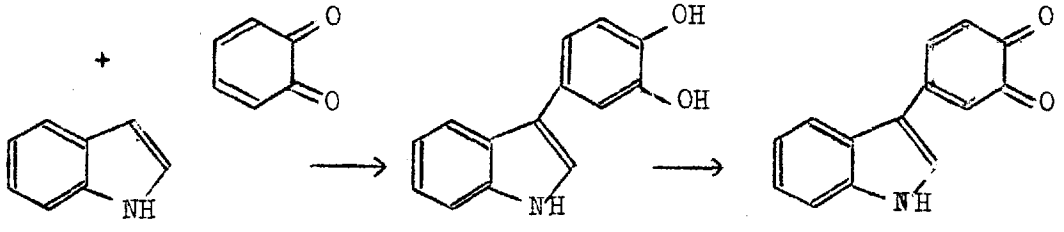
The formation of indoles from dopachrome does not appear to be enzymic although some metals catalyze the decomposition. This reaction, therefore, must be one of the slow stages in melanogenesis.

Final Stages of Melanogenesis

5,6-dihydroxyindole + 5,6-dihydroxyindole-2-carboxylic acid $\longrightarrow$ Dimer $\longrightarrow$ Oligimer $\longrightarrow$ Melanin

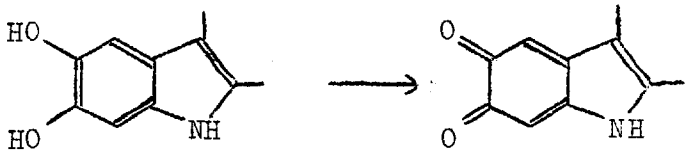
The final stages of melanogenesis represent the polymerisation of the simple derivatives of tyrosine with the eventual formation of the highly insoluble polymer melanin.

Addition of indole-5,6-quinone to other molecules of indole may give dimers similar to the deep purple adducts formed by the addition of simple indoles to quinones.[93]

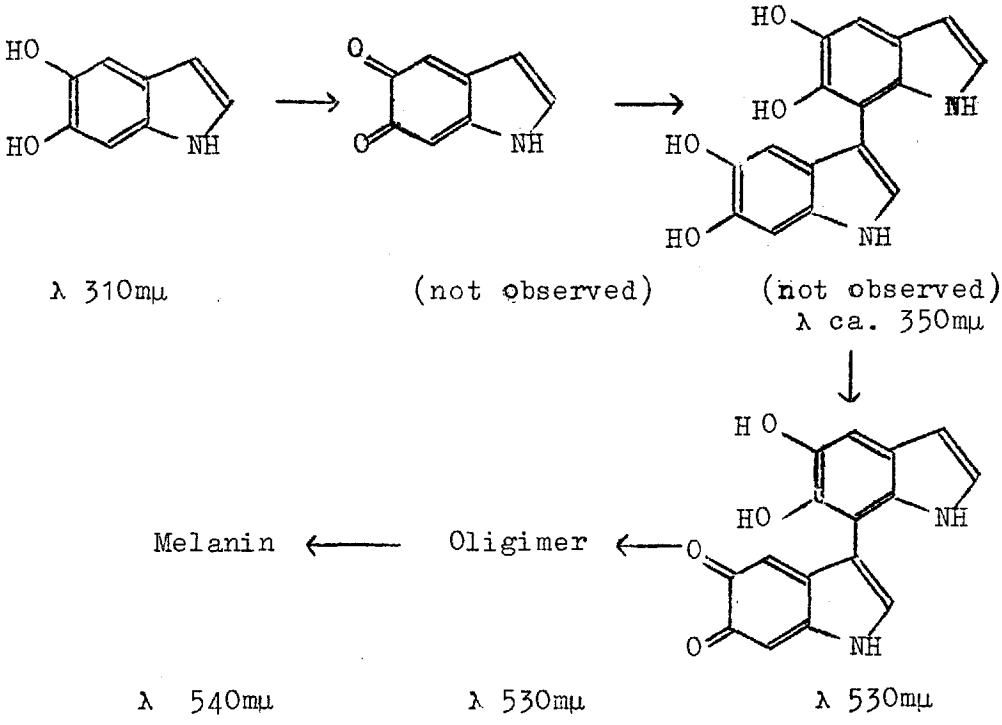


The 5,6-dioxy function will complicate this reaction by making the indole reactive in several positions, and no such dimers have been isolated in melanogenesis. Indole-5,6-quinone has also not been detected in the

oxidation although a related orange quinone, formed by the oxidation of 5,6-dihydroxy-2,3-dimethylindole, was fairly stable.[83,89,94]



By measuring the ultra-violet and fluorescence spectra, during the oxidation of 5,6-dihydroxyindole, the formation of dimers could be followed, although the linkages between the molecules could not be determined[88] (assumed 3-7' in diagram). The intermediate purple colour (λ 530mμ) was shown not to be due to indole-5,6-quinone as had been suggested previously.[63]



Similar dimers have also been suggested to be intermediates in the formation of adrenaline melanin.[73]

The purple colour (λ ca. 540mμ) formed in the final stages of melanogenesis was called melanochrome and suggested to be a number of linked indolequinone molecules.[94]

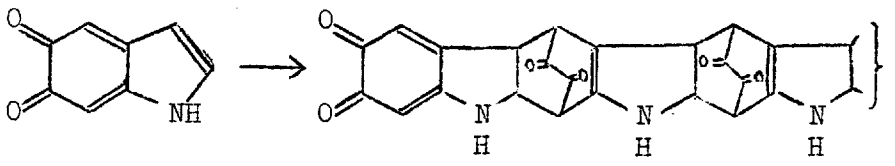
Both indole-5,6-quinone and 5,6-dihydroxyindole are reactive molecules and any position may be involved in the polymerization. The resulting polymer would be expected to be highly irregular in the absence of something to direct the coupling. If a proportion of uncyclized units and degraded indoles, such as pyrrole-acids, and, in natural melanins, protein and amino-acid residues are included, the structure of melanin would be exceedingly complex. Not only is every preparation of melanin likely to give a different product but the structure may vary with time due to continued oxidation or hydration. There is no evidence that melanin is a regular polymer, though this cannot be excluded in all melanins.

The Structure of Melanin

Oxidative processes

Hopes that melanin was a simple discrete molecule proved unfounded. A suggestion that melanin was an indigo-type dimer[95] was disproved by the synthesis of tetrahydroxyindigo.[96] 1-Methyl-3,5,6-trihydroxyindole (1-methyl-5,6-dihydroxyindoxyl), formed in the oxidation of adrenaline, had an activated 2-position[97] and could be oxidized to an indigo derivative.[72,98] 3-Hydroxylation of 5,6-dihydroxyindole, an intermediate in the formation of tyrosine melanin has been proposed,[99] but this was shown to be unlikely.[93,98]

A different type of polymer could possibly be formed by the Diels-Alder condensation of indole-5,6-quinone.[100]



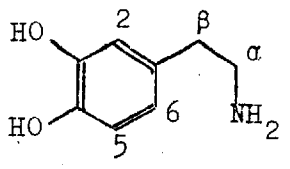
Early work by Angeli showed that the properties of melanin were similar to those of pyrrole black, a pigment prepared by the oxidative polymerisation of pyrrole.[101] Melanin was proposed to be a pyrrole rather than an indole polymer. Oxidation of tyrosine was suggested to result in ring fission of the aromatic portion to give a pyrrole[102] and any para-oxygenated phenylethylamine derivative ~~was~~

postulated should give a melanin on oxidation.[103] Degradation of melanin has proved that it is quite different from pyrrole black.[87]

Oxidation of catechol derivatives can result in fission of the aromatic ring. For instance, oxidation of catechol with peracid gave initially o-benzoquinone which was further oxidized to muconic acid.[104] and in the presence of metals hydrogen peroxide also produced muconic acid.[105] Enzymes in Pseudomonas could also degrade catechol[106] and protocatechuic acid.[107]

Autoxidation of both catechol and 5,6-dihydroxyindole liberated carbon dioxide by ring fission.[108] The fission of 5,6-dihydroxyindole across the 5-6 bond to give a diacid[109] could be followed by recyclization at another position, which would result in a highly irregular polymer.[110]

Enzymic oxidation of [carboxyl-^{14}C]tyrosine and [carboxyl-^{14}C]-3,4-dihydroxyphenylalanine to melanin confirmed that much of the carbon dioxide came from the carboxyl group.[111,112] Using specifically labelled [^{14}C]-3,4-dihydroxyphenylethylamine the loss of carbon dioxide from other positions could be measured.[113,114]



Position	CO_2 evolved
α	1·9%
β	2·3
3	29·8
4	27·2
5	3·5
1,2 and 6	35·3 (c.a. 30% from 2-position)

These results confirm that much of the carbon dioxide came from the 3- and 4-oxygenated positions. If a pyrrole were formed the α-, β-, 1- and 6-carbon atoms would be retained, with possibly the 2- and 5-carbons as carboxyl groups.

Evolution of carbon dioxide, formed by the attack of the hydrogen peroxide produced in the oxidation,[115a] could be prevented by the addition of the enzyme, catalase which specifically decomposed hydrogen peroxide.[116a]

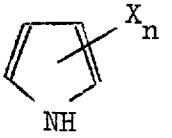
Degradation of melanin

Although chemical degradation of melanin is difficult a few reactions have given useful results. Both natural and synthetic melanins were reduced by sodium dithionite to less coloured compounds which could be reoxidized[117a] and melanin was bleached by various reagents.[118a]

Low yields of simpler compounds have been obtained from the vigorous oxidation and alkali fusion of melanins. Angeli was able to oxidize pyrrole black to a pyrrole or indole acid,[119a] and obtained pyrrole by alkali fusion.[120a]

These degradations were later used extensively by Nicolaus and co-workers to characterize melanins. Sepia and Ustilago melanins were chosen as typical animal and plant-fungal melanins.

Oxidation of Melanins by alkaline permanganate or hydrogen peroxide to Pyrrole acids

Melanin ⟶  X = CO_2H

Pyrrole acid	2-X	2,3-X_2	2,4-X_2	2,5-X_2	2,3,5-X_3	2,3,4,5-X_4	Reference
Sepia melanin		+			+++	+	91,92,115-120
Sepia melanin methylated with CH_2N_2					3and5-methyl esters		87,92, 116,120
Sepia melanin decarboxylated at 200°		++	+	+	+++	+	92,117, 120,121
Pyrrole black	+	+		+++	+		92,117.

Alkali fusion of Melanins at 200-250°

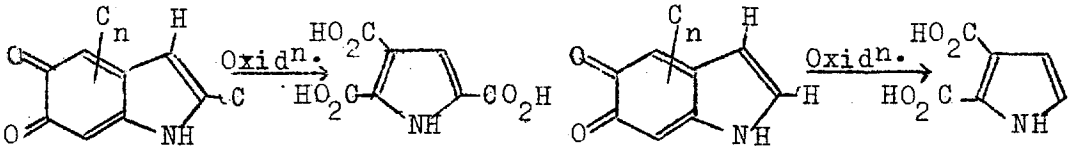
Pyrrole	2,3-X_2	2,4-X_2	2,5-X_2		Reference
Sepia melanin	+	+	+	and 5,6$(OH)_2$-indole 5,6$(OH)_2$-indole-2-CO_2H 5,6$(OH)_2$-indole-4,7$(CO_2H)_2$ 4-methylcatechol	87,118 120
Ustilago melanin				Catechol, salicylic acid protocatechuic acid	118,120 122.

Hydrogenation of Melanin over Palladium at 150-200°

Sepia melanin	5,6-dihydroxyindole	120
	5,6,5',6'-tetrahydroxy-4,7'-biindolyl	
Ustilago melanin	Catechol	
	3,4,3',4'-tetrahydroxybiphenyl	120

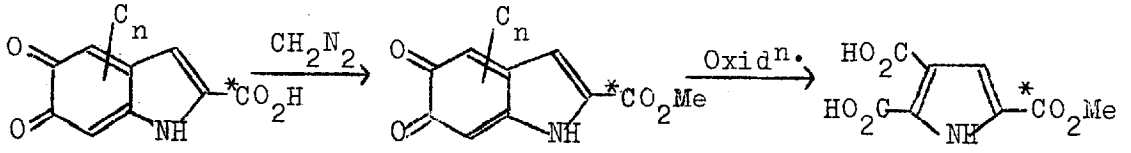
In spite of the very low yields obtained in these degradations the products were used to interpret the structure of melanin. The principal oxidation product of sepia melanin was pyrrole-2,3,5-tricarboxylic acid. Repeated mild oxidation with neutral hydrogen peroxide gave a 6·5% yield,[120] although only 1-2% was obtained from the permanganate oxidation.[91] Radiochemical dilution showed that only 0·2% of pyrrole-2,3-dicarboxylic acid was formed from the permanganate oxidation of synthetic dopa melanin.[119]

The formation of pyrrole-2,3,5-tricarboxylic acid from sepia melanin would be expected from the degradation of a 2-substituted or 2-carboxyindole, whereas pyrrole-2,3-dicarboxylic acid would require an indole with both the 2- and 3-positions free.



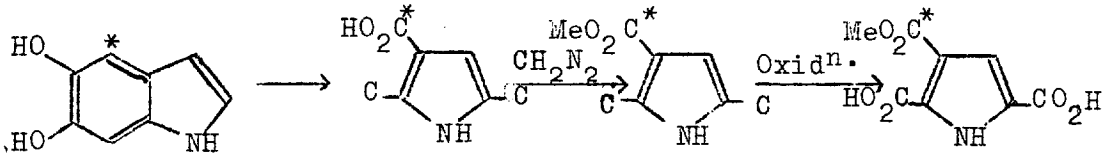
Melanin Melanin

Methylation of the pigment with diazomethane introduced about 20% methoxyl groups.[91,116] Oxidation of this derivative gave two mono-esters of pyrrole-2,3,5-tricarboxylic acid. The 5-methyl ester should be derived from a 2-carboxyl group of a melanin indole unit, probably originating from the carboxyl group of tyrosine.[87]



Melanin

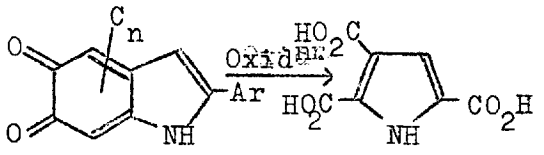
The 3-methyl ester was suggested to originate from a pyrrole unit in the melanin polymer, formed by the degradation of an indole in melanogenesis.



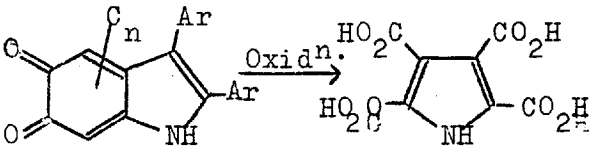
Melanin

Melanin lost about 10% carbon dioxide on heating to 200°, presumably from the loss of free carboxyl groups.[120] This was reflected in the pyrrole acids, formed on oxidation, by an increased amount of pyrrole-2,3-dicarboxylic acid and the production of the 2,4- and 2,5-isomers. Melanin, from the oxidation of 5,6-dihydroxyindole-2-carboxylic acid, on heating to 200°, gave off 6% carbon dioxide but oxidation

of the decarboxylated melanin gave only pyrrole-2,3,5-tri- and2,3,4,5-tetracarboxylic acids. The lack of the 2,3-di- and 2,3,4-tricarboxylic acids suggested that the carbon dioxide came from the degradation of a benzenoid portion and not from a 2-carboxyl group.[87]



Melanin



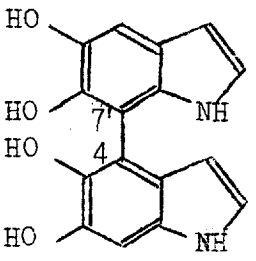
Melanin

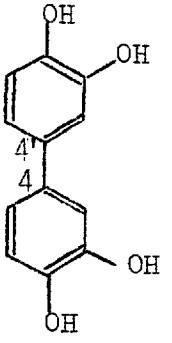
Alkali fusion provided the first proof of an indole unit in melanin.[87] Degradation of sepia melanin gave 5,6-dihydroxyindole and two derivatives of it.

Neither pyrrole acids nor indoles are formed from the degradation of Ustilago melanin, although alkali fusion gave some catechol derivatives. Ustilago melanin, and many plant melanins, were suggested to be catechol polymers.[120,122] There are a wide variety of catechol derivatives present in plants which may act as precursors for melanins. Blackening of bananas was shown to be due to 3,4-dihydroxyphenylethylamine.[123] Since 3,4-dihydroxyphenylalanine[14,15,16] and its derivatives[124] occur in several plants it is possible that some plant melanins may be related to tyrosine melanin.

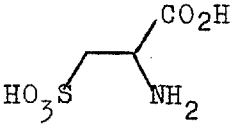
The only clue about the linkage of the units in melanins has come from the hydrogenation of melanin.[120] Some

4,7'-linked 5,6-dihydroxyindole dimer was isolated from sepia melanin and 4,4'-linked catechol dimer from Ustilago melanin.

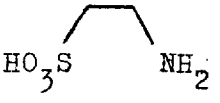




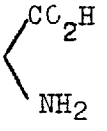
Purified sepia melanin contained 0·3% sulphur[118] and oxidation of it with peracetic acid gave some sulphur containing amino-acids - cysteic acid and taurine - as well as glycine and aspartic acid.[120]



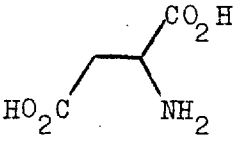
Cysteic acid



Taurine



Glycine



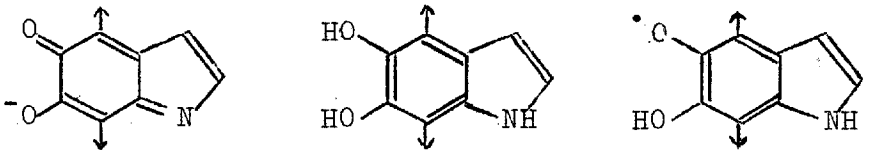
Aspartic acid

Oxidation of tyrosine or 3,4-dihydroxyphenylalanine in the presence of cysteine has been shown to give addition compounds of the amino-acid with dopaquinone.[35]

Physical Properties

A study of the physical properties of melanins has given some useful information. Electron micrographs of natural melanins revealed rounded particles of 1000-5000Å diameter.[125]

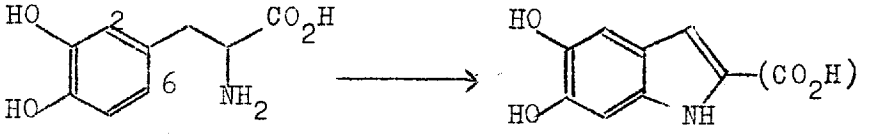
X-ray diffraction, infra-red spectra and basicity data have been used to characterize melanins.[126,127] However the ultra-violet spectra showed no definite absorption maxima and the infra-red spectra were insufficiently resolved to be very useful.[28] Electron spin resonance showed that melanin contained a large number of free-radicals.[129,130] The results were interpreted in terms of three sorts of indole unit in melanin:[130]



The presence of unpaired electrons in melanin has also been explained by supposing melanin to be a one-dimensional semi-conductor with any bound proton acting as an electron trap.[131]

Oxidation of Substituted Precursors

Oxidation of methyl substituted 3,4-dihydroxyphenylalanines has shown that cyclization occurred principally at the 6-position to give a 5,6-dihydroxyindole.[79,83,84]



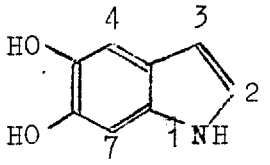
Extensive work with variously substituted indoles was designed to elucidate the mode of polymerization of the units in melanin. Early work showed that 5,6-dihydroxyindole was very sensitive to oxidizing agents and rapidly darkened in air.[88] By contrast 5,6-dihydroxyindole-2-carboxylic acid was relatively inert,[89] although it was still oxidized to a melanin.[87] Monohydoxyindoles were also found to be unstable to air, particularly in alkaline solution.[86,132]

Many substituted 5,6-dihydroxyindoles have been synthesized and the results obtained from their autoxidation are tabulated below (page 37).

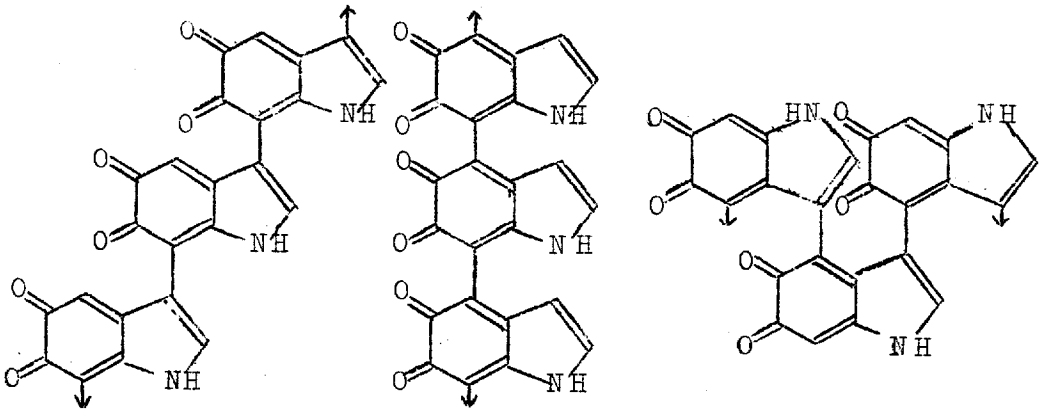
From those derivatives which failed to give a proper melanin the positions involved in the coupling could be determined. Chromartie and Harley-Mason suggested that the 3-position and 4- or 7-positions were essential for melanin formation.[83] Beer and Robertson suggested that although the 3-position was not required for melanin formation it deflected the course of the reaction.[94] These results have been used to support theories of the modes of linkage of the indole units in melanin. Since a 3-4' linked polymer, for steric reasons could not be planar, Harley-Mason favoured a 3-7' linkage with some 2-4' or 2-7' cross-links.[84,98] Other workers have suggested 3-4' or 3-7' linkages,[110] 4-7' linkages,[99,130,133] or 3,4' and 7" linkages.[127]

Autoxidation of 5,6-dihydoxyindoles

Precursor	Intermediate	Melanin	Reference
56$(OH)_2$indole	Purple	Black, insoluble	84,88,93,94,108
1-Methyl	Pink	Slight black ppt.	83,84,94,133
2-Methyl	Brown	Black, insoluble	83,89,99
3-Methyl	Blue	Blue ppt. soluble	83,84,89,93,94
4-Methyl		Black ppt.	83,84
4-Propyl	Violet	Black ppt.	134
7-Methyl		Black ppt.	83
7-Propyl	Purple	Blue-violet ppt.	94,134
1,2-Dimethyl		Black ppt.	93
2,3-Dimethyl	Stable-orange	No ppt.	83,84,89,94
2,3-$(CH_2)_2$	Brown	No ppt.	89
3-Me-4-Pr		Blue ppt.	134
3-Me-7-Pr	Blue	Dark blue, ?ppt.	94,134
4,7-Dimethyl		Purple ppt.	84
2,4,7-Me_3	Red-purple	Red no ppt.	94



⟶ Melanin



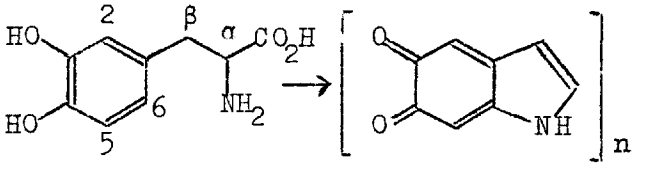
3-7' linkage 4-7' linkage 3-4' linkage

The failure of a particular precursor to give a melanin indicates that the steric or chemical requirements for melanogenesis have been affected. This does not, however, necessarily prove that condensation would have occurred at a blocked position. The structure of the melanin from those derivatives which were apparently oxidized normally may also differ extensively from ordinary melanin. The results provide an indication that several positions can be involved in the polymerization and suggest an irregular polymer. In order to elucidate the coupling of the units in melanin only an unsubstituted precursor can be used, since only this will give a normal melanin.

The Oxidation of Tritium and Deuterium Labelled Precursors

Blocking groups have the obvious disadvantage that a substituent not only blocks a position, but also has steric requirements, changes the reactivity of the molecule and modifies the structure of the resulting melanin. The use of deuterium and tritium labelling has enabled a direct measurement of the extent of coupling at each position to be made in a normal melanin.

The loss of label during the oxidation of specifically labelled 3,4-dihydroxyphenylalanine has been used in this department by Kirby and Ogunkoya to measure the coupling at each position.[119,135] The difficulties associated with an uncharacterized product of variable composition were obviated by double-labelling using 3,4-dihydroxyphenyl[α-^{14}C]alanine as an internal reference, since little of the α-carbon was lost on oxidation,[114] and by relating the incorporations to the C and H analyses. The fractional retention of tritium, in melanins derived from 3,4-dihydroxyphenylalanine specifically labelled at each position, was calculated.



α 0·52 retention
β 0·54 (=1·08 ^{3}H)
2 0·37
5 0·42
6 0·13

Melanin

In a similar oxidation [3-^{3}H]tyrosine retained 0·40 H from the

two labelled hydrogens, which agreed with the figure from the oxidation of 3,4-dihydroxy[5-^{3}H]phenylalanine (0·42).[119]

If an indolic intermediate was involved complete loss of the 6-hydrogen would be expected. The retention of 13% could be due to unreacted precursor absorbed on the polymer. This was discounted since refluxing the melanin with strong acid liberated only 1·4% of 3,4-dihydroxyphenylalanine. Either there were uncyclized units chemically bonded in the polymer or some of the indole units were cyclized onto the 2-position of 3,4-dihydroxyphenylalanine. The retention of 0·52 hydrogens from the α-position suggests either indoline units in melanin or that decarboxylation of dopachrome to 5,6-dihydroxyindole does not require loss of the α-tritium. Any indole-2-carboxylic acid units in melanin must lose the α-tritium. This retention could also be due to uncyclized units. The loss of tritium from the α, β, 2- and 5-positions suggested a highly irregular polymer, although β-loss may be complicated by a large isotope effect in the formation of the indole.

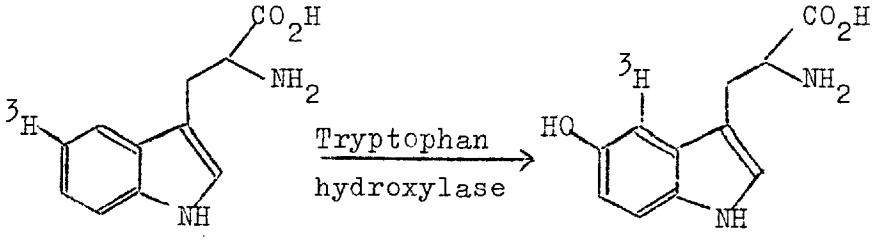
Melanins formed in other ways may differ significantly from this melanin which was prepared using a mixture of tyrosinase and catalase at pH 6·8. This technique has provided a quantitative method for comparing melanins produced under different conditions.

Other work using α- and β-deuterium labelled 3,4-dihydroxyphenylalanine was directed along similar lines and analogous results were obtained.[112,136] These labelled derivatives were either autoxidized or enzymically oxidized using tyrosinase with or without added catalase. More deuterium was retained during the autoxidation (α 97%, β 82%) than during the enzymic oxidation (α 68%, β 70%). The results were interpreted in terms of the types of unit in the melanin.

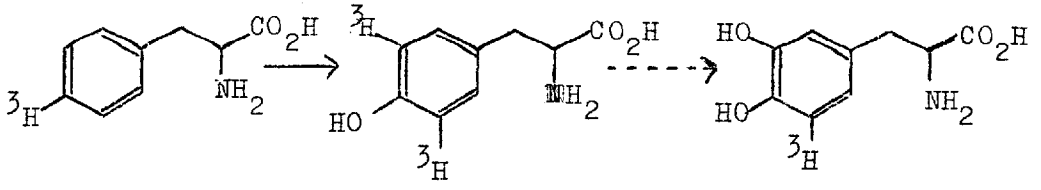
Unit		Autoxidation	Enzymic
2-linked	Indole-5,6-quinone	34·5%	35%
3-linked		24·5	16·5
2- and 3-linked		17·5	32
Dopachrome		23·5	16·5

Tritiated 3,4-dihydroxyphenylalanine was also found to be incorporated into a mouse melanoma.[137]

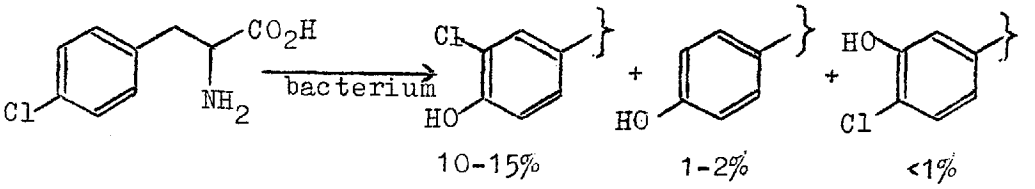
Care must be taken in the interpretation of isotopic labelling experiments. Apart from such obvious sources of error as a large isotope effect some anomalous incorporations have been reported. Hydroxylation of [5-^{3}H]tryptophan by Chromobacterium violaceum in the 5-position should remove all the tritium, but in fact 90% was retained.[138]



Similarly hydroxylation of [4-^{3}H]phenylalanine by the hydroxylase from Pseudomonas sp. or rat liver gave tyrosine with 70% retention of label.[139]



Other anomalous 4- to 3-shifts have been observed in the hydroxylation of acetanilide[140] and even in the hydroxylation of 4-chlorophenylalanine.[141]



A study of the hydroxylation of tyrosine by a mammalian tyrosinase showed no such anomalies,[31] which suggests that the mechanism of hydroxylation of an aromatic ring may be different in the presence of a hydroxyl group.

The Enzymes involved in Melanogenesis

Early work on enzymes identified a number of oxidases. In 1883 Yoshida discovered 'laccase' the enzyme thought to be responsible for the hardening of lacquer.[142] This was the first step towards elucidating the formation of Chinese lacquer first described in the West in 1760.[143] Laccase polymerized urushiol, from the latex of Rhus vernicifera, which is a mixture of 3-pentadecylcatechol and its mono-, di- and tri-olefins.[144] By careful control of moisture and oxygen the lacquer polymerizes with a drying oil, tung oil, to give a colourless, infusible and chemically inert polymer.

In 1896 Bertrand discovered that an enzyme, present in various plants, could, unlike laccase, oxidize a solution of tyrosine red, then black, with the eventual precipitation of a black pigment.[145] This oxidase, which he called 'tyrosinase', occurred not only in dahlia tubers and beet which contained tyrosine, but also in mushrooms which did not. Extensive work on tyrosinase over the following ten years[147] showed that tyrosinase was widely distributed in nature and occurred not only in many plants and fungi, but also in some of the lower animals. Tyrosinase has also been identified in the melanomas of horse,[148] and man[149] and in the ink of the squid.[91,150] Tyrosinase has even been used with polyhydric

aromatic compounds to colour human hair.[151]

Raper showed that the enzyme required oxygen to oxidize tyrosine.[49] Tyrosinase was also found to catalyze the oxidation of p-cresol, resorcinol, quinol, catechol and other phenols to o-quinones.[7,40] Plant and animal tyrosinases from potatoes and meal worms had similar properties.[152]

The presence of tyrosinase in mammals has not been easy to demonstrate, but tyrosinase was reported in coloured rabbits[153] and a dopa oxidase, which was unable to oxidize tyrosine, was present in many mammals.[23,154] Both tyrosinase and a dopa oxidase were isolated from a mouse melanoma.[154]

Although the reactions of tyrosinase have been extensively investigated little work was carried out into the nature of the enzyme until techniques for the handling of macro-molecules had been developed. In 1916 tyrosinase was suggested to be a zinc containing enzyme,[6] but Kubowitz, in 1938, isolated a tyrosinase and showed it to be a copper based enzyme (0·2% Cu).[155] Five different tyrosinases were isolated, by electrophoresis, from Psalliota campestris (common mushroom) and one was shown to have a molecular weight of about 100,000 with 4 copper atoms per molecule (0·25% Cu).[156] The two types of enzyme activity shown by tyrosinase, the tyrosine hydroxylase and the dopa oxidase, could not be separated.

Manometric and spectroscopic methods for the determination of the activity of tyrosinases[157,158] have enabled the hydroxylating (cresolase) and dehydrogenating (catecholase) activities to be determined. Extreme values for the ratio catecholase:cresolase were 250 [159] and 1·6 [160] but a more usual ratio was 5-20. The five components of a mushroom tyrosinase were found to have the catecholase:cresolase ratio proportional to the enzyme's copper content.[156,160,161]

Due to the instability and variability of tyrosinase some conflicting data have been published. One preparation had a molecular weight of 34,500 with 1 copper/molecule[162] another was reported to have a molecular weight of 100,000, but contained only 2 copper/molecule.[161,163] Recent workers have obtained more consistent results. Kubowitz's[164,165] molecular weight of 126,000 for mushroom tyrosinase was confirmed by Kuson's[166] figure of 119,000 and both enzymes contained 4 copper/molecule. One tyrosinase was found to dissociate to give 33% of a light component which had a molecular weight of 34,500 and contained 1 copper/molecule.[166] This was presumably similar to one of the anomalous enzymes. Although this dissociation was found to be partially reversible the enzyme permanently deactivated.[165]

The multiple forms of tyrosinase were considered to occur naturally and not as a result of degradation of a

single enzyme.[161] If the tyrosinases were built up of sub-units, the recombination of identical units in different ways could lead to the observed multiplicity. Alternatively two or more kinds of sub-unit could exist, each possessing cresolase and catecholase activity, and their recombination would give the hybrid tyrosinases (isoenzymes).[166] These isoenzymes were found to be partially interconvertible.[167]

Four forms of another fungal tyrosinase (from Neurospora crassa) had a molecular weight over the range 33,000 to 120,000.[168] This enzyme contained 1 copper for each 33,000 units of molecular weight (i.e. 1-4 Cu/molecule) suggesting a dissociation similar to that observed for mushroom tyrosinase. Like mushroom tyrosinase this enzyme oxidized a wide variety of mono- and dihydric phenols.[169]

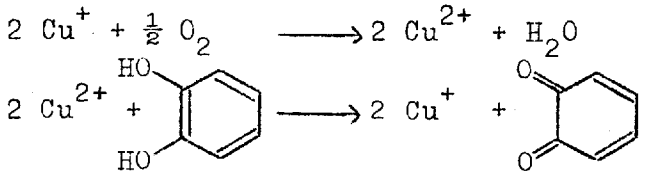
A mammalian tyrosinase from the mouse melanoma was separated into three components containing 0·22-0·25% copper[170] and two tyrosinases have been isolated from a hampster melanoma.[171]

Other tyrosinases which have been studied include that from the Calliphora lava,[172] which had a molecular weight of 530,000 but was unable to oxidize monophenols, a potato tyrosinase and a broad bean tyrosinase both of which could be separated into two components.[172,173,174]

The oxidations catalyzed by tyrosinase make use of

the oxidation and reduction of the copper complex in the enzyme molecule. The copper in the free enzyme was predominently in the cuprous state.[155,165,166,168,175] Ageing of the enzyme has been attributed to partial oxidation of the copper[165] and cuprous ion reagents also inactivated the enzyme.[176]

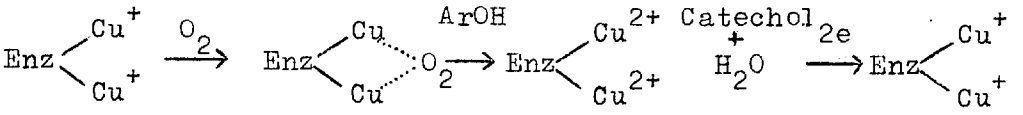
Molecular oxygen oxidized the cuprous complex to cupric which then oxidized the catechol to an o-quinone.[177]



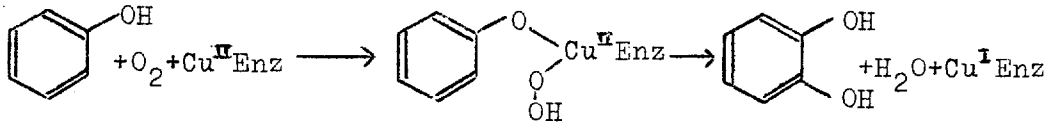
Copper(III) has been postulated to effect hydroxylation in the oxidation of 2,6-dimethylphenol by aqueous copper sulphate and oxygen.[178] In the presence of periodate or tellurate copper(III) complexes could be isolated.[179]

Kinetic studies have given rise to several theories for the mechanism of the hydroxylation and dehydrogenating actions of tyrosinase.[180] Hydroxylation proceeeds only in the presence of a reducing agent.[32,164,181,182] In the absence of oxidizable compounds such as catechol, 3,4-dihydroxyphenylalanine or ascorbic acid there is a long induction period.

Since tyrosinase combined with carbon monoxide in the ratio 2 Cu:1 CO,[183] two copper atoms were suggested to be involved in the oxidation.[32,125,184]



The hydroxylation of phenols has been suggested to involve a copper(II) hydroperoxide complex.[185]



A model for tyrosinase action was found with the charge transfer complex, formed by dissolving cuprous chloride in pyridine.[186] This was able to catalyze the hydroxylation of monophenols with a free o-position to o-quinones, and those with substituted o-positions to p-quinones. Cupric acetate in methanol containing morpholine had a similar effect.[40]

Laccase (E.C. 1,10.3.2.) is an enzyme closely related to tyrosinase (E.C. 1.10.3.1.). Whereas tyrosinase oxidized o-dihydroxyphenols, laccase preferentially oxidized p-dihydroxyphenols. Laccase from Rhus vernicifera was reported to have a molecular weight of 120,000 [187] or 141,000 [188] with either 4 [187] or 6 [188] copper atoms per molecule. Unlike tyrosinase the copper in laccase was in the cupric state[189] colouring the enzyme blue.[190] Laccase from the fungus, Polyporus versicolor had a molecular weight

of 62,000 [191] or 114,000 [192] with 4 copper/molecule. Two of these copper atoms were permanently in the cuprous state whereas the active sites were cupric.[193] Lacquer laccase in the reduced, cuprous, form exchanged cuprous copper with $^{64}CuNO_3$ but not with $^{64}Cu(NO_3)_2$.[194] The oxidized enzyme did not exchange copper with either, but during the oxidation of hydroquinone slow exchange occurred.

Discussion of Experimental Work

Introduction

Melanin resists the usual techniques for structural determination since it is completely insoluble and very stable. Many indirect methods have been used in order to try to solve this problem.

When this project was started, work was in progress in this department on the oxidation of tritium-labelled tyrosine and 3,4-dihydroxyphenylalanine. The results of this work provided the first quantitative estimate of the linkage positions in melanin.[119,135] Loss of tritium would only indicate the positions accurately if there were no exchange of the label and no isotope effect in the oxidation. In order to check these assumptions two model reactions were studied. The dimerisation of p-cresol to Pummerer's ketone[195] is the best known example of phenol oxidation, but the oxidation has not been studied using isotopic labels. The addition of indoles to ortho and para-quinones to give the intensely coloured 3-indolylquinones[93] provides a model for the final stages of melanogenesis - the polymerisation of 5,6-dihydroxyindole. Both free-radical and ionic reactions are probably involved in melanogenesis. The intense colour of melanin indicates that there are conjugated

quinones present, and e.s.r. studies have shown the presence of free-radicals.

Oxidation of 3,4-dihydroxyphenyl[β-^{3}H]alanine to melanin may give ambiguous results since the loss of tritium to give the intermediate indole could have a large isotope effect. In order to avoid this possibility a fully substituted derivative is required. 3,4-Dihydroxyphenyl-[β-$^{2}H_{2}$]alanine was synthesized and oxidized to melanin. This synthesis proved more complicated than was at first thought. The hydantoin synthesis of 3,4-dihydroxyphenyl-alanine[196,197] was modified in order to introduce the two deuteriums into the β-position. Unfortunately the hydantoin condensation resulted in exchange of the label which could not be prevented. An alternative synthesis using diethyl acetamidomalonate worked well and the β-deuterated derivative was prepared in good yield with high deuterium incorporation. In connexion with these syntheses a useful general preparation of formyl-labelled aromatic aldehydes was investigated and the usual conditions for the Cannizzaro reaction were modified in order to use this reaction for preparing α-dideuterobenzyl alcohols from the deuteroaldehydes.

The hydroxylation of tyrosine to 3,4-dihydroxyphenyl-alanine has been studied using a mammalian tyrosinase.[30,31] The factors influencing the oxidation of 3,4-dihydroxyphenyl-

alanine, and the hydroxylation and oxidation of tyrosine by mushroom tyrosinase were investigated spectroscopically by following the formation of dopachrome, or the oxidation of ascorbic acid.

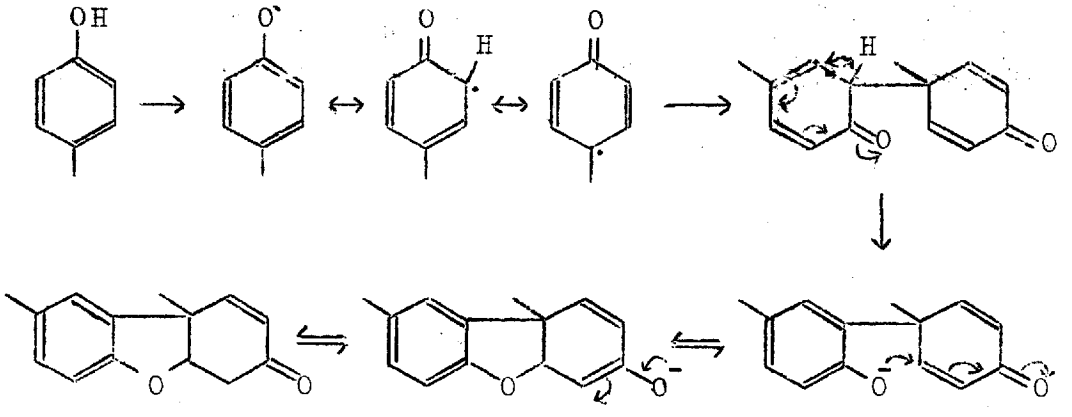
Dopachrome is thought to be indoline-5,6-quinone-2-carboxylic acid. In order to try to confirm this structure its formation and isolation were investigated.

Carbon dioxide is evolved in melanogenesis even in the presence of catalase. The evolution of carbon dioxide was followed quantitatively by using a modified microanalytical apparatus to collect carbon dioxide.

The main product of the oxidative degradation of melanin is pyrrole-3,3,5-tricarboxylic acid, although its yield is uncertain. In view of the small amounts of this acid which were isolated little significance can be given to the interpretation of this degradation. In order to measure the quantity actually formed the acid was isolated by radiochemical dilution by a method similar to that used to determine the yield of pyrrole-2,3-dicarboxylic acid in the same oxidation of melanin.[119]

Preparation and Reactions of Pummerer's Ketone

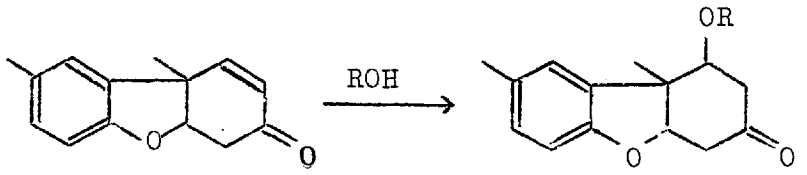
Pummerer showed that the oxidation of p-cresol with alkaline ferricyanide gave a mixture of polymeric phenols together with about 20% of a neutral compound.[195] The structure of this compound, Pummerer's ketone, was correctly formulated when the theory of the coupling of phenol radicals was proposed.[198]



Pummerer's ketone

Aqueous potassium ferricyanide oxidized p-cresol in the presence of sodium carbonate at 0° to give a sticky white precipitate. Chromatography of this on alumina grade III in benzene gave 23% of Pummerer's ketone mixed with a trace of an oil which could be removed by recrystallization from benzene-petroleum ether. This oil could possibly have been the stereoisomer of Pummerer's ketone, so a sample of the purified ketone was equilibrated in basic methanol. Thin-layer chromatography indicated that a compound with a R_F similar

to that of the oil was formed rapidly to the extent of about 15%. The ultra-violet spectrum of this compound showed that the absorption maximum of the unsaturated ketone in Pummerer's ketone at 222mμ had shifted to 205mμ which suggested a methanol adduct (R = Me).



Proton magnetic resonance confirmed the presence of a methoxyl group and the infra-red spectrum showed a saturated ketone at 1723 cm^{-1}. The oil, formed during the preparation of Pummerer's ketone, was presumably the hydrate (R = H). This methanol adduct has recently been synthesized photochemically.[199]

An alternative chromatography of the crude oxidation product of p-cresol in benzene on silica gel gave a mixture of phenols; Pummerer's ketone required a stronger solvent and was eluted mixed with some polymeric phenols. Vacuum sublimation of the crude oxidation product gave fairly pure Pummerer's ketone but only in low yield.

Aqueous ferric chloride[200] did not oxidize p-cresol at room temperature, but in the presence of excess sodium acetate some Pummerer's ketone was formed.

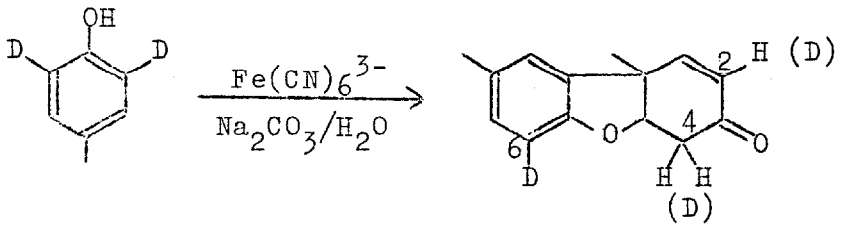
The enzyme peroxidase in the presence of very dilute hydrogen peroxide oxidized p-cresol to give 24% of Pummerer's ketone (32% based on reacted p-cresol).[201]

For the model reaction p-cresol was labelled in the 2,6-positions. Refluxing p-cresol with deuterium oxide, in the presence of $\frac{1}{2}$ mole of triethylamine, exchanged the protons ortho to the hydroxyl group rapidly.[119,202] This phenol, however, exchanged so rapidly that storage was difficult. Even at 0° the activity of pure, liquid, [2-^{3}H]-p-cresol (super-cooled) had dropped to 75% after 6 months. With 3 exchangeable protons an equilibrium activity of 67% would be expected since the phenolic hydrogen is lost in the preparation of the benzoate for counting. Chromatography of [2-^{3}H]-p-cresol on alumina grade V in benzene removed 21% of the label.

Oxidation of [2,6-$^2H_2$]-p-cresol could give Pummerer's ketone with up to three deuteriums. The loss of one deuterium in the aromatization of the intermediate dienone must almost certainly occur although a few enzyme systems have given anomalous incorporations (see pages 41-42).

Pummerer's ketone was prepared by the ferricyanide oxidation of [2-$^2H_2$]-p-cresol and isolated by chromatography on alumina grade III. Proton magnetic resonance showed

only a small amount of deuterium (ca. 0·16 each) in the 2- and 4-positions, but the aromatic region had been reduced from three protons to two.



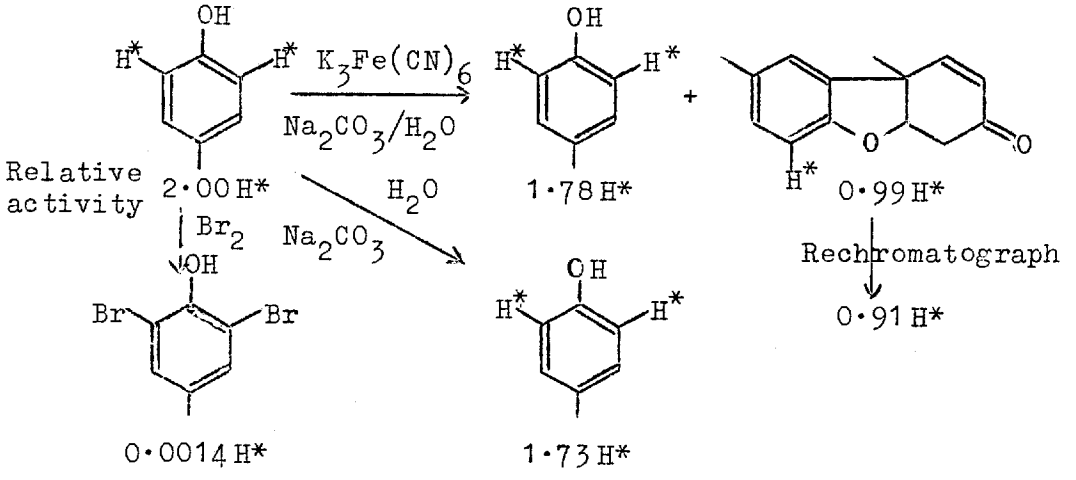
This experiment showed that the aromatic proton survived the oxidation, but did not give any information about the others.

The three protons of Pummerer's ketone α to the carbonyl group were readily exchanged.[203] 15 minutes refluxing in deuterium oxide and dimethylformamide in the presence of triethylamine gave [2,4,4-$^{2}H_{3}$]Pummerer's ketone. Even after more vigorous exchange (110° for 40 hours) no deuterium could be detected in the aromatic ring. Chromatography of the trideutero derivative on alumina grade III in benzene removed most of the deuterium. This proved that the isolation procedure could have been responsible for the loss of the label in the oxidation of [2,6-$^{2}H_{2}$]-p-cresol.

Alkaline exchange of p-cresol with tritiated water (0·2 C/ml) gave [2-^{3}H]-p-cresol. Though the pure liquid could be counted directly in a liquid scintillator it was more convenient to prepare the crystalline benzoate. The Schotter-Baumann method in dilute alkali did not exchange the p-cresol.

Bromination of [2-^{3}H]-p-cresol in ethanol gave 2,6-dibromo-p-cresol. After repeated recrystallization from petroleum ether its activity was only 0·07% of the p-cresol proving that not less than 99·93% of the activity had been in the positions ortho to the hydroxyl group. Bromination of p-cresol in carbon tetrachloride gave a monobromo derivative and in water a tetrabromo derivative.

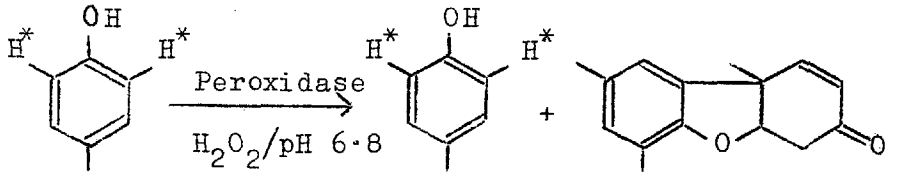
Oxidation of [2-^{3}H]-p-cresol in aqueous sodium carbonate with potassium ferricyanide was carried out under similar conditions to the oxidation of [2,6-$^2H_2$]-p-cresol. The unreacted p-cresol was recovered from the reaction mixture and [2-^{3}H]-p-cresol was checked for exchange in aqueous sodium carbonate. The results are summarized in the table:



These results not only show that the 2- and 4-tritiums are lost in Pummerer's ketone but also indicate that p-cresol

was exchanged under the reaction conditions.

A similar series of experiments was carried out using horseradish peroxidase-hydrogen peroxide in neutral phosphate buffer to oxidize [2-^{3}H]-p-cresol. Rapid chromatography of the crude oxidation product on alumina grade III gave Pummerer's ketone with significantly higher activity than previously, suggesting a partial retention of the label in the 2- and 4-positions.



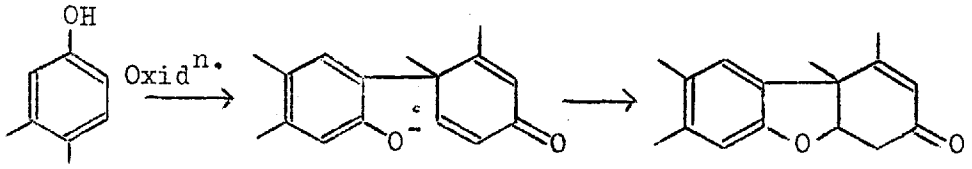
Relative activity 2·00 H* 2·03 H* 2·19 H*

Rechromatograph ↓ 1·09 H*

Chromatography was not essential for the isolation of Pummerer's ketone since it was the only neutral product of the oxidation. Westerfield and Lowe obtained it by extraction of an ethereal solution of the oxidation products with alkali.[201] In order to check whether this procedure were suitable a trial extraction was carried out with [2,4-$^{3}H_2$]Pummerer's ketone. A solution of the labelled ketone was shaken alternately with 5% sodium hydroxide and water for a total of 6 washings. The recovered Pummerer's ketone retained 92% of the activity proving that little exchange had occurred and that if the enzyme gave optically active Pummerer's ketone it would not

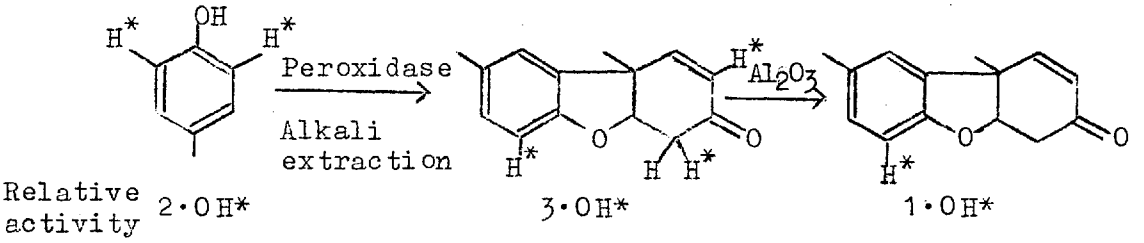
racemize, unless enolization involved only one of the 4-hydrogens.

From a second oxidation of [2-^{3}H]-p-cresol by peroxidase-hydrogen peroxide 24% of a neutral oil was obtained after extraction with alkali. Since this proved difficult to purify it was chromatographed rapidly on alumina grade V in benzene. The resulting Pummerer's ketone had retained 3 tritium atoms, but was optically inactive ($[\alpha]_D^{27}$ <0·4°). Oxidation of 3,4-dimethylphenol to a Pummerer's ketone would, if the enzyme were specific, give an optically active product since the intermediate dienone has no plane of symmetry. This oxidation, using horseradish peroxidase, also gave an inactive product.[204]

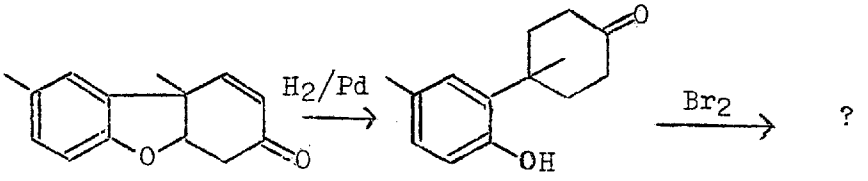


There was some ambiguity in the counting results as the work was interrupted by a vacation. Not only did [2-^{3}H]-p-cresol exchange on storage but even crystalline [2-^{3}H]-p-cresol benzoate lost activity. After 1 month p-cresol lost 6% of its activity whereas the benzoate had lost 10%. Although the activity of the Pummerer's ketone was high the ratio of its activity to that of the Pummerer's ketone after removal of the 2-and 4-tritiums was the expected 3:1.

When the activities were corrected for the known exchange complete retention of 3 tritiums was found.



Pummerer's ketone was degraded in order to prove whether the label in the aromatic ring were entirely in the 6-position. Direct bromination of Pummerer's ketone gave a complex mixture of products, so the ketone was hydrogenated to 2-(1'-methyl-4'-oxocyclohexyl)-4-methylphenol.[198] Bromination of this keto-phenol also gave a mixture of products.



When the keto group was protected as the ethylene ketal, bromination gave only two products, which could be separated by chromatography on alumina. The first fraction had a phenolic absorption in the infra-red at 3510 cm^{-1} (c.f. 2-bromo-p-cresol, 3490 cm^{-1}), whereas the second fraction had the phenolic absorption at 3600 cm^{-1} (c.f. the keto phenol 3600 cm^{-1}). The first fraction was therefore the required bromo derivative, but in view of the low yield obtained this

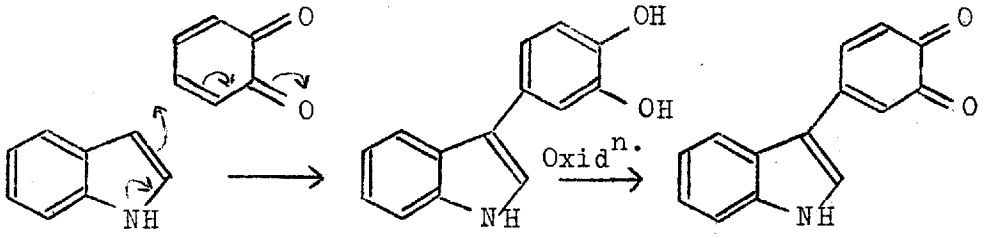
degradation was not pursued.

The 6-proton of the keto-phenol could be exchanged in the presence of base. A sample of [6-^{3}H]Pummerer's ketone, prepared by the oxidation of [2-^{3}H]-p-cresol, was hydrogenated to the keto-phenol and the label exchanged out by equilibrating with a large excess of water. The recovered keto phenol had an activity 3% of the Pummerer's ketone. There was insufficient material to repeat the exchange so this retention must be regarded as a maximum.

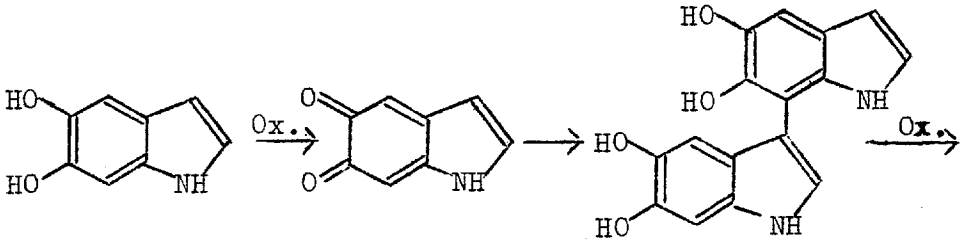
These experiments with Pummerer's ketone show that a tritium label is not lost in the free radical oxidation, although the label is labile and may be lost in the work-up. Since the oxidation of tyrosine or 3,4-dihydroxyphenylalanine is done under similarly mild conditions there is little danger of losing the label in the enzymic reaction. In order to ensure that exchange did not occur in the isolation procedure, an alternative to the concentrated hydrochloric acid precipitation of melanin was investigated (see page 68).

The Preparation and Reactions of Indolyl-quinones

Simple indoles react with ortho-and para-quinones to give the intensely coloured 3-indolylquinones.[93,205,206]



This reaction provided a closer model for the formation of melanin than the Pummerer's ketone reaction. The intermediate in melanogenesis, 5,6-dihydroxyindole, probably condenses with its quinone, indole-5,6-quinone. Such a condensation would lead to a 3-7' or 3-4' linked polymer.[93]

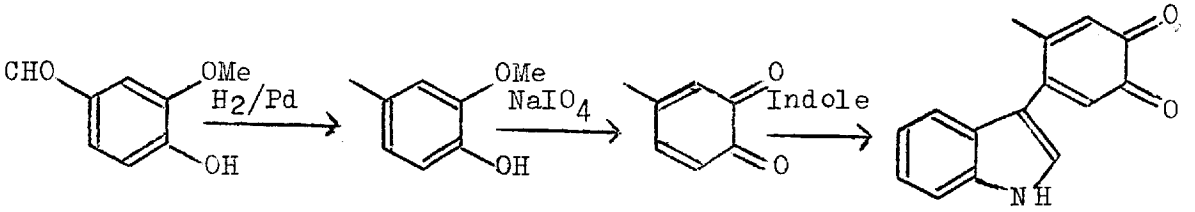


Both 5,6-dihydroxyindole and indole-5,6-quinone have more reactive positions than simple indoles or quinones so their reaction would be expected to be more complex. The preparation of indolylquinones does, however, provide a good model for the final stages of melanogenesis.

Several indolylquinones were prepared in order to find a suitable model reaction. The original preparation by

Bu'Lock and Harley-Mason involved the reaction of an indole with a quinone in aqueous ethanol.[93] Since o-quinones are very unstable an alternative procedure was investigated.

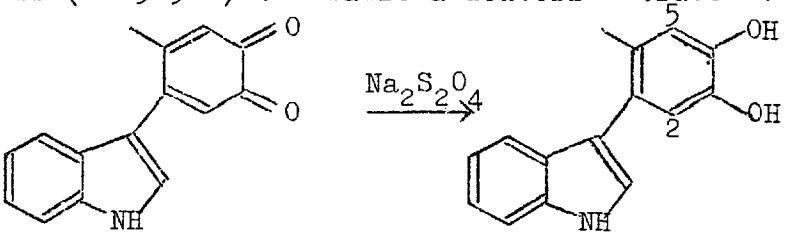
2-Methoxy-4-methylphenol, creosol, was prepared by the hydrogenolysis of vanillin. Oxidation of creosol with sodium metaperiodate gave 4-methyl-o-benzoquinone in good yield.[207] Though still very reactive, this quinone was more stable than o-benzoquinone.



4-Methyl-o-benzoquinone reacted with indole in aqueous ethanol or dioxan to give 4-(indol-3-yl)-5-methyl-o-benzoquinone. In this reaction an additional mole of quinone is required in order to oxidize the intermediate indolylcatechol. By oxidizing the catechol in the presence of indole these difficulties could be avoided.

Oxidation of a mixture of creosol and indole with sodium metaperiodate in aqueous dioxan gave 4-(indol-3'-yl)-5-methyl-o-benzoquinone which was readily purified by prolonged extraction with ether (40% yield). This deep purple, sparingly soluble, quinone was unsuitable for deuterium or tritium tracer studies, but it could be reduced to the colourless indolylcatechol by aqueous dithionite.

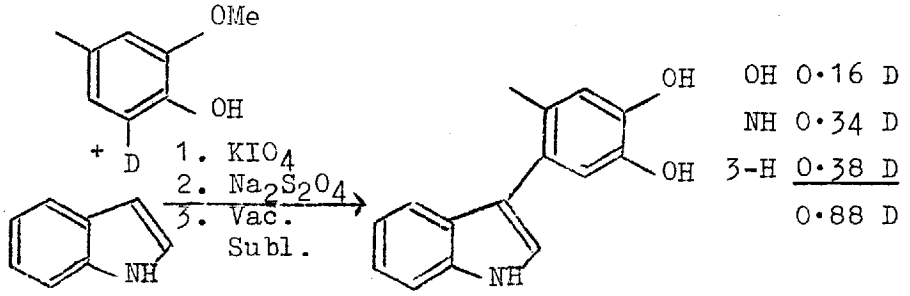
The n.m.r. spectrum of the catechol showed two singlets at $\tau 3{\cdot}17$ and $\tau 3{\cdot}23$ sufficiently well separated from the indolic protons ($\tau 2{\cdot}5$-$3{\cdot}1$) to enable a deuterium tracer to be used.



5-H τ 3·23
2-H τ 3·17

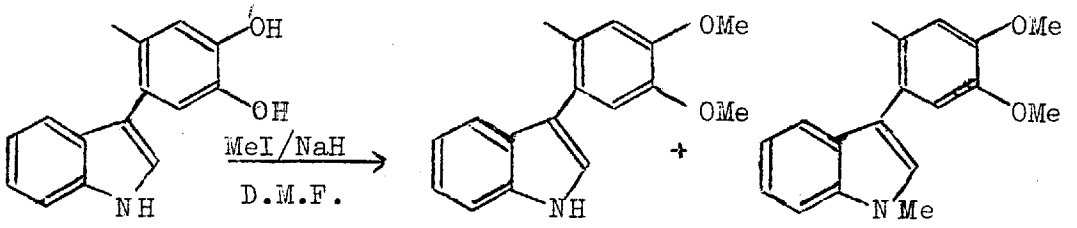
Creosol was labelled by the usual alkaline exchange with deuterium oxide and triethylamine. Oxidation of [6-^{2}H]creosol to 4-methyl[6-^{2}H]-o-benzoquinone gave pale yellow crystals (m.p. 69-70°) which had a complex n.m.r. spectrum which suggested a dimer. 4-Methyl-o-benzoquinone was prepared by a similar method and it too had a complex n.m.r. spectrum although its infra-red spectrum was similar to the published one.[208]

Oxidation of [6-^{2}H]creosol in the presence of indole gave 4(indol-3-yl)-5-methyl[3-^{2}H]-o-benzoquinone which was reduced by sodium dithionite in aqueous acetone and vacuum sublimed at 150°. The n.m.r. spectrum of the product showed that little deuterium had been lost, but the label had been scattered, by exchange with the OH and NH protons.



Vacuum sublimation was shown to be the cause of this scattering since sublimation of the [N,O,O-$^{2}H_{3}$]-derivative resulted in an increase of the OH and NH signals in the n.m.r. spectrum to 0·71 H and 0·47 H with a corresponding reduction of the aromatic protons.

In order to get a crystalline derivative, suitable for tritium tracer studies, the crude indolylcatechol was methylated. Using methyl iodide and sodium hydride in dimethylformamide two products were obtained which could be separated by chromatography on alumina grade I in benzene. The first fraction (50%) showed neither NH nor OH absorptions in the infra-red spectrum which suggested a trimethyl derivative. The second fraction (28%) had a sharp NH absorption at 3490 cm^{-1} and was the dimethyl ether. Analyses and the n.m.r. spectra confirmed these structures.



Like some other indoles these derivatives gave characteristic colours on a thin-layer chromatoplate, after exposure to iodine vapour or standing in air.

T.l.c. on Silica gel GF_{254} in 15% ethyl acetate-benzene

Indole	R_F 0·61	Green
2-Methylindole	0·65	Reddish-brown
3-Methylindole	0·65	Orange-brown
Indole-2-carboxylic acid	0·60	Yellow
4-Indolyl-5-methylcatechol	0·16	Black (quinone)
dimethyl derivative	0·56	Orange
trimethyl derivative	0·64	Green

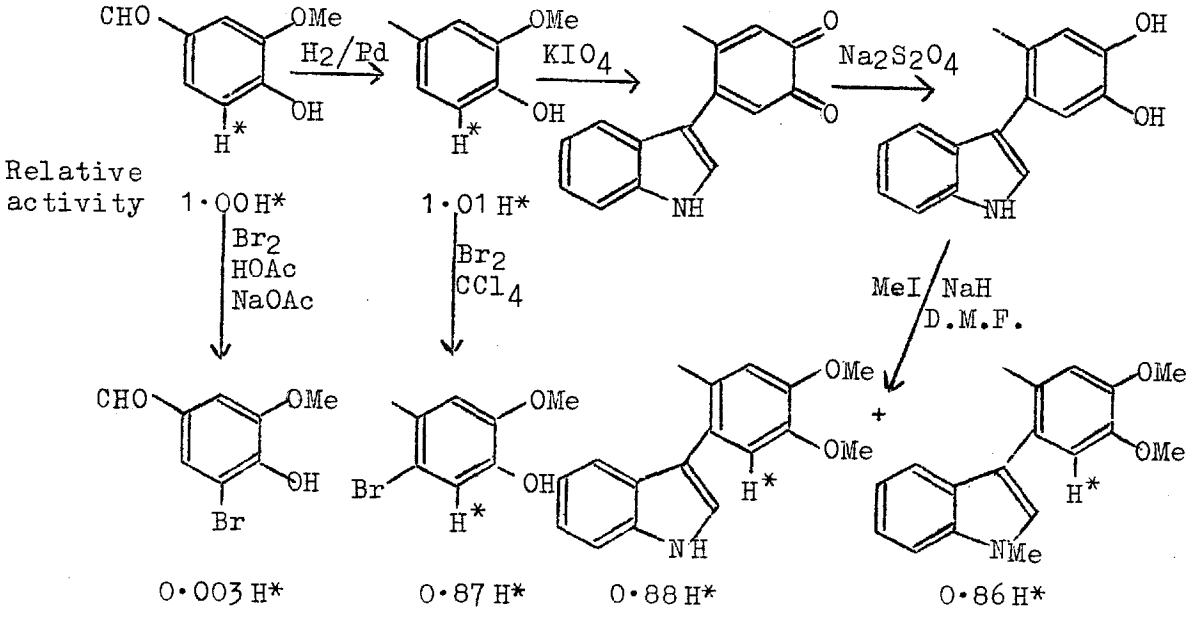
Methylation of the indolylcatechol with diazomethane in ether, using boron trifluoride as a catalyst, was slow and the product was a mixture of the dimethyl- and a monomethyl ethers.

For the study of this reaction with a tritium tracer [5-^{3}H]vanillin, prepared by base catalyzed exchange in tritiated water, was hydrogenolyzed to [6-^{3}H]creosol. Oxidation of this with periodate in the presence of indole gave 31% of 4-(indol-3'-yl)-5-methyl[3-^{3}H]-o-benzoquinone. This was reduced by sodium dithionite and methylated with methyl iodide and sodium hydride in dimethylformamide. The two ethers were separated by chromatography and purified for counting in liquid scintillator.

In order to check the position of the label in the precursor [5-^{3}H]vanillin was brominated to 5-bromovanillin.

Rigorous purification of it reduced the activity to 0·34% showing that not less than 99·7% of the activity was in the 5-position. Bromination of [6-^{3}H]creosol in carbon tetrachloride gave 5-bromo[6-^{3}H]creosol with some loss of activity. Creosol could not be brominated in the 6-position and even using bromine in acetic acid-sodium acetate 5-bromocreosol was the main product.

The results obtained using a tritium label are summarized:



These results indicate that little activity is lost in the condensation. The loss of 14% activity is significant but in view of the large number of different reactions involved in the preparation some loss of label is not surprising. The loss is not large enough to invalidate

the results which had been obtained from the oxidation of specifically labelled 3,4-dihydroxyphenylalanines.[119,135] As in the Pummerer's ketone reaction, the results indicate that it was advisable to keep the conditions of the oxidation as mild as possible.

Isolation of melanin

In the preparation of melanin the initial suspension was precipitated from the phosphate buffer by the addition of concentrated hydrochloric acid.[119] In view of the above results and the known exchange of indoles in acid[209] a milder method was sought. A strong solution of acid or neutral salts precipitated melanin readily, but mild alkali changed the suspension into a clear solution. Unlike the usually featureless spectrum of melanin this solution had maxima at 295 and 410mμ. Ammonium nitrate and sodium chloride were found to be particularly suitable for the precipitation of melanin. Providing the melanin was given plenty of time to precipitate it showed little tendency to redisperse during the subsequent washings. The precipitated melanin was centrifuged off and washed four times with water, then three times with acetone and dried *in vacuo*. A melanin precipitated with sodium chloride contained only 2·3% ash after repeated ignition indicating that the washings were

sufficient to remove the salt. This salt precipitation was used for the oxidations of specifically labelled 3,4-dihydroxyphenylalanines[119,135] and in the melanin preparations in this work.

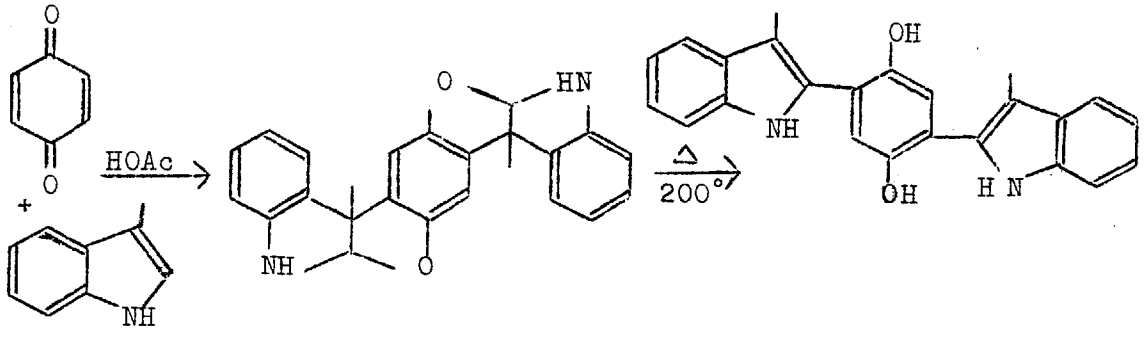
The reaction of quinones with 3-methylindole

Whereas indole and 2-methylindole could react with quinones to give the corresponding indolylquinones, 3-methylindole lacks a 3-hydrogen and cannot give the same type of adduct. In acetic acid, 3-methylindole reacted with p-benzoquinone to give a colourless solid which was found to have a formula $C_{24}H_{20}N_2O_2$ and to give a diacetyl derivative.[93] These compounds were presumably formed from two molecules of indole and one of quinone. For simplicity this compound will be referred to as the 2:1 adduct.

Although the 2:1 adduct was sparingly soluble in organic solvents (2% w/v in dimethylsulphoxide) it was more soluble in strong acid (10% w/v in trifluoroacetic acid, 15% in sulphuric acid). The n.m.r. spectrum of it in trifluoroacetic acid showed that the adduct had been partially protonated in a manner similar to that reported for several indole derivatives.[210] The infra-red absorption at 3440 cm^{-1} disappeared on acetylation to give a N-acetyl derivative (ν 1679 cm^{-1}).

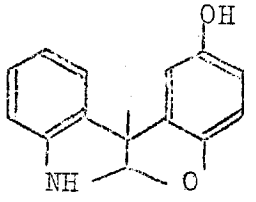
The 2:1 adduct was converted into a pale yellow isomer, which fluoresced strongly, by vacuum sublimation at 200°. This compound was soluble in alkali and could be oxidized to a coloured compound, which suggested a hydroquinone. The n.m.r. spectrum, in dimethyl sulphoxide, showed peaks at τ2·3-3·1 (ArH), τ0·75 (OH) and τ-0·80 (NH) and the infra-red absorption at 3490 cm^{-1} shifted on acetylation to 3420 cm^{-1} with the formation of a phenyl acetate (ν 1742 cm^{-1})

These data were best interpreted as indicating the reaction of two molecules of 3-methylindole to p-benzoquinone to give 7b,14b-dimethyl-5a,7b,12a,14b-tetrahydrobisindolo-[2,3-b;2',3'-b']benzo[1,2-d;4,5-d']difuran which was thermally isomerized to 2,5-di(3'-methylindol-2'-yl)hydroquinone.



In order to confirm these structures, the 1:1 adduct was investigated. 3-Methylindole did not react with p-benzo-quinone in acetone containing a trace of acetic acid, but

the addition of hydrochloric acid catalyzed a rapid reaction which gave a complex mixture of products. The principal component was separated by chromatography on alumina grade III and silica gel, and purified by chromatographing twice more on alumina. The product was an unstable oil which failed to crystallize. The n.m.r. spectrum of this compound showed it to be the required 1:1 adduct.



τ8·55 C-Me
4·97 OH, NH
4·12 O-CH-N
2·9-3·7 7ArH.

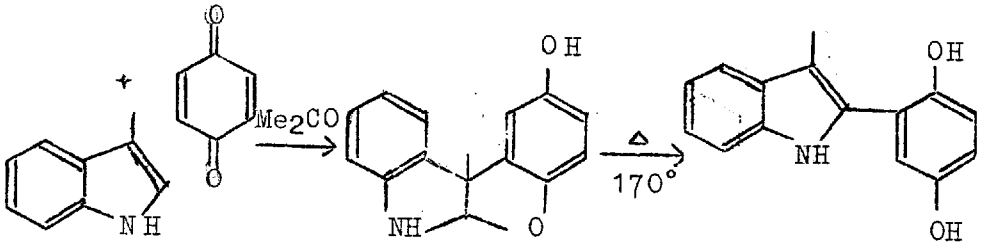
The infra-red spectrum of it showed both phenolic (3630 cm^{-1}) and amine (3460-3400 cm^{-1}) absorptions.

This 1:1 adduct isomerized slowly at room temperature, and rapidly on vacuum sublimation at 170°, to give another strongly fluorescent, crystalline quinol (phenyl acetate ν 1757 cm^{-1}). The n.m.r. spectrum of it showed it to be analogous to the isomer of the 2:1 adduct.

2:1 Isomer	1:1 Isomer
Obscured	τ7·82 C-Me
τ2·3-3·1 ArH*	2·4-3·3 7ArH
0·75 2OH	1·18,1·27 2OH
-0·80 2NH	-0·61 NH

* inaccurate integral measured 11·3, 11·5 H, required 10 H.

The 1:1 adduct was therefore 5a,10b-dihydro-2-hydroxy-10b-methylindolo[2,3-b]benzo[d]furan which isomerized to 2-(3'-methylindol-2'-yl)-hydroquinone.

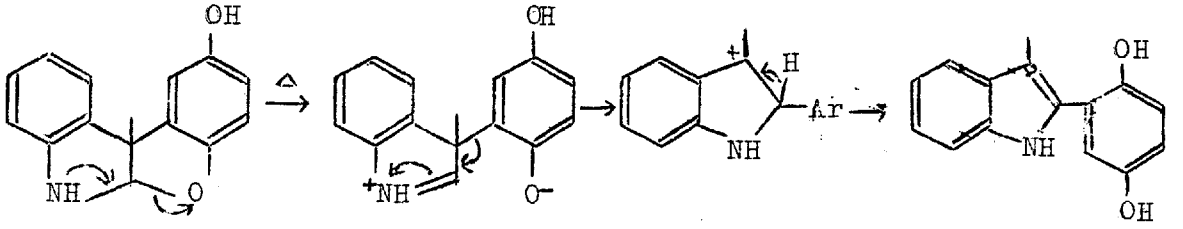


The ultra-violet spectra of the two isomerized adducts were characteristic of 2- rather than 3-arylindoles:

1:1 Isomer	2:1 Isomer	Indol-3-yl-hydroquinone *	Indol-2-yl hydroquinone ‡
λ226mμ (ε33,000)	227·5mμ(ε46,700)	222mμ(ε35,500)	224mμ(ε34,700)
244infl(ε16,000)	251infl(ε19,500)	246 (ε18,000)	267 (ε9800)
304infl(ε9500)	307 (ε16,000)	302·5(ε13,000)	291 (ε7800)
321 (ε10,200)	353 (ε26,000)	335 (ε20,000)	309 (ε9100)

* Ref. 205. ‡ Ref. 206

The formation of a 3-alkyl-2-arylindole would be expected since the aromatic group is more likely to migrate. 3-arylindoles are known to rearrange to give the 2-isomers.[211]



An abstract[212] and paper[213] recently published proved

the structure of the 2:1 adduct and its isomer, although the 1:1 adduct was not prepared. A number of related derivatives were prepared from other 3-substituted indoles.

Quenching

In order to use tritium for tracer studies it is necessary to be able to measure the activity accurately. All radioactive compounds were assayed in liquid scintillator. At high count rates (1000 c/s) the efficiency of the counter was lowered, and an empirical formula $a_o = a(1 + 0{\cdot}0083c^2)$ was derived, where c was the observed count rate in 1000 c/s.

Quenching of the scintillator by the compound being assayed was determined by measuring the activity of the tritium standard in the presence of inactive compound. Data for Pummerer's ketone fitted the empirical equation,

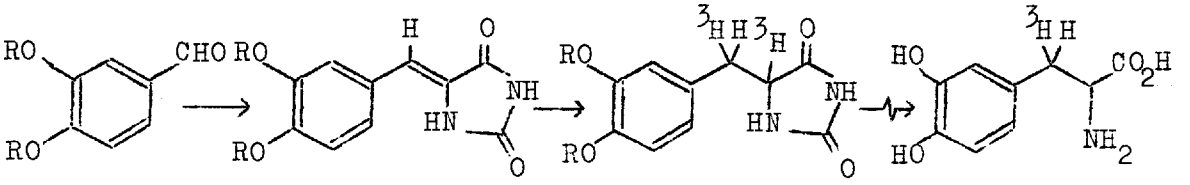
$\log a_o = \log a + Q.w$ where w is the weight of compound added (mg.) and Q the quenching constant.

p-Cresol benzoate	Q	0·000/mg.
Pummerer's ketone		0·049
Creosol		0·050
Creosol benzoate		0·013
5-Bromocreosol		0·038
Vanillin		0·115
5-bromovanillin		0·131
3-(3',4'-dimethoxy-6'-methylphenyl)indole		0·106
3-(3',4'-dimethoxy-6'-methylphenyl)-1-methylindole		0·050

The Synthesis of 3,4-Dihydroxyphenyl[β-$^2H_2$]alanine

Work in this department on the oxidation of tritium labelled 3,4-dihydroxyphenylalanine derivatives had enabled the extent of coupling at each position to be measured.[119,135] The interpretation of tritium loss from 3,4-dihydroxyphenyl-[β-^{3}H]alanine is difficult since there could be a large isotope effect in the loss of tritium to give the indolic intermediate. In order to remove this ambiguity 3,4-dihydroxyphenyl[β-$^2H_2$]alanine was synthesized.

The preparation of 3,4-dihydroxyphenyl[β-^{3}H]alanine, for the tritium tracer studies of melanogenesis, provided a reaction whereby one β-deuterium could be introduced.[119]



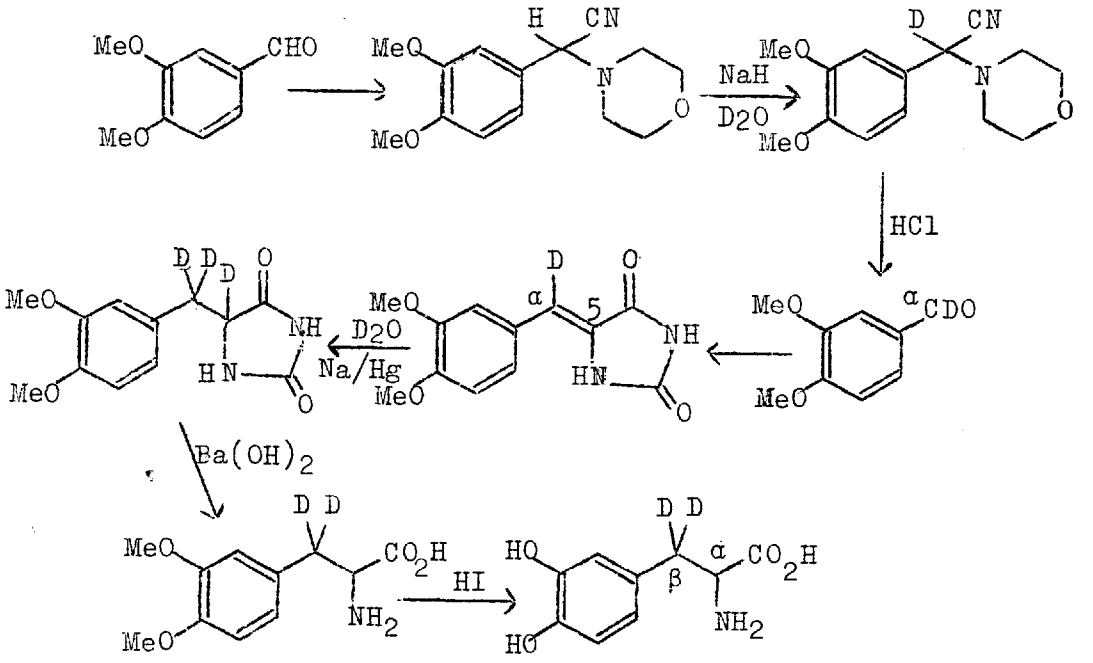
R = $C_6H_5CH_2$-

In this synthesis the reduction of 3,4-dibenzyloxybenzal-hydantoin with sodium amalgam in dioxan in tritiated water gave the [α,β-$^3H_2$] derivative which lost 96% of the α-tritium on alkaline hydrolysis. To extend this synthesis to introduce two β-deuteriums it is necessary to start with a deuteroaldehyde.

A technique for the labelling of the formyl hydrogen of aromatic aldehydes has been devised in this department,

and used to prepare [α-^{2}H] and [α-^{3}H]vanillin.[214] Veratraldehyde was used as the starting point for the synthesis since the intermediates were more convenient than those of 3,4-dibenzyloxybenzaldehyde.

The complete scheme for the preparation of 3,4-dihydroxyphenyl[β-$^{2}H_2$]alanine is shown below:-



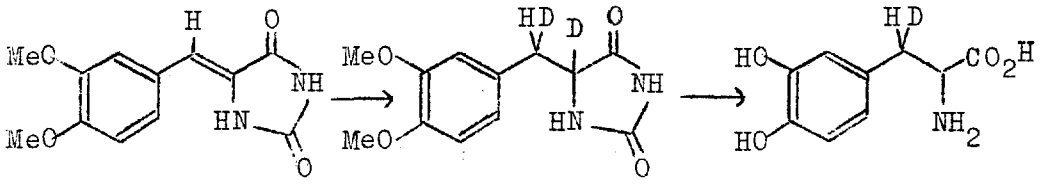
3,4-dihydroxyphenylalanine has been synthesized by a similar route starting from veratraldehyde,[196] and the synthesis was extended in order to prepare 3,4-dihydroxyphenyl-[β-^{14}C]alanine.[197]

Veratraldehyde was converted into 3,4-dimethoxyphenylmorpholineacetonitrile by reacting the aldehyde with

morpholine perchlorate and sodium cyanide in morpholine. The benzylic proton of this derivative could be removed by sodium hydride in dimethylformamide and quenching of it with deuterium oxide gave the [α-^{2}H] derivative. Hydrolysis in dilute acid gave [α-^{2}H]veratraldehyde with up to 99% deuteration and in an overall yield of 90%.

Veratraldehyde condensed with hydantoin when refluxed with acetic anhydride containing sodium acetate to give 75% of veratralhydantoin. The reduction of this by sodium amalgam in dilute alkali was modified for the deuterogenation, in order to economize on the amount of deuterium oxide used. In a mixture of dioxan and water at 40° the reduction took a week. Neither acetic acid in dioxan nor acetic anhydride proved satisfactory. By using a limited volume of dioxan and starting the reduction at 80° the reaction was complete in 2 hours and gave a 90% yield of veratrylhydantoin.

Reduction of veratralhydantoin by sodium amalgam in dioxan and deuterium oxide gave [α,5-^{2}H$_2$]veratrylhydantoin. Hydrolysis of this with base and acid gave 3,4-dihydroxyphenyl[β-^{2}H]alanine. The mass spectrum of its ethyl ester indicated 0·96 deuterium atoms in the β-positions and a residue of 0·09 deuteriums in the α-position.



In a similar manner [α-^{2}H]veratraldehyde was condensed with hydantoin, reduced with sodium amalgam in deuterium oxide and dioxan, then hydrolyzed to 3,4-dihydroxyphenyl [β-$^2H_2$]alanine:-

	Theory D	Measured D	Yield
Veratraldehyde	1	0·94 (n.m.r.,m.s.)	83%
Veratralhydantoin	1		80
Veratrylhydantoin	3	2·37 (n.m.r.)	68
Dimethyldopa	2		93
Dopa	2	1·31 (m.s.)	54
			Overall 23%

These results indicate the failure of one of the stages of the synthesis. Since the monodeutero series had given satisfactory results the deuterium must have been lost in the hydantoin condensation. The mass spectrum of veratralhydanoin prepared from [α-^{2}H]veratraldehyde showed the retention of only 66% of the label. An alternative synthesis of veratralhydantoin using pyridine-diethylamine[215] as a solvent also resulted in considerable loss of deuterium. In order to distinguish between a hydride transfer and an exchange mechanism the condensation was repeated using a tritium label and taking samples at intervals. The results showed an approximately linear decrease of activity of the product at a rate of about 10%. This suggested that slow exchange was occurring and that a reaction time shorter than

the usual 2 hours would help.

In an attempt to remove all exchangeable protons, hydantoin was deuterated, by refluxing with acidified deuterium oxide in dimethylformamide, and the acetic anhydride dried. The [$^{2}H_{4}$]hydantoin was condensed with [α-^{2}H]veratraldehyde with a reaction time of 1 hour. Veratralhydantoin was obtained in good yield but the mass spectrum indicated that only 0·57 deuterium atoms had been retained. The mass spectrum of the ethyl ester of the resulting 3,4-dihydroxy-phenylalanine comfirmed this and showed that there were 0·96 and 0·59 deuteriums in the two β-positions and 0·09 in the α-position. This meant that the methyl protons of the acetic anhydride also exchanged in the hydantoin condensation. In view of the difficulty of preventing this without using perdeutero-acetic anhydride the synthesis was abandoned. The retention of 9% deuterium in the α-position was also undesirable.

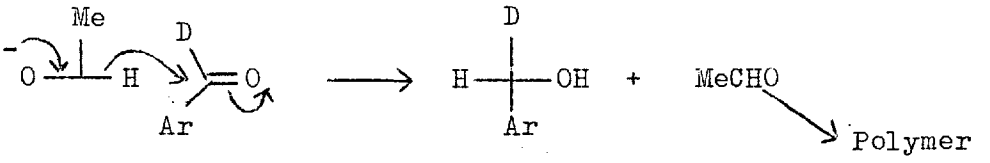
Synthesis of 3,4-Dihydroxyphenyl[β-$^2H_2$]alanine - 2

The diethyl acetamidomalonate synthesis of amino-acids has been used to prepare some substituted 3,4-dihydroxyphenylalanine derivatives.[216] For the synthesis of the [β-$^2H_2$] derivative a [α-$^2H_2$]veratryl derivative would be required. [α-$^2H_2$]veratryl alcohol can conveniently be prepared from [α-^2H]veratraldehyde by the Cannizzaro reaction. An early preparation of veratryl alcohol used this reaction,[217] although oxygenated benzaldehydes were found to react slowly.[218] The Cannizzaro reaction on [α-^2H]benzaldehyde retained at least 98% of the deuterium,[219,220] although there was a large isotope effect.[219,221]

Veratryl alcohol was obtained in quantitative yield from veratraldehyde when refluxed with aqueous ethanolic sodium hydroxide for 2 hours. A similar reaction on [α-^2H]veratraldehyde was very much slower, and neither a silver catalyst[219,222] nor hydrogen peroxide[223] had any obvious effect. At 90° this reaction took 5 hours but the resulting [α-$^2H_2$]veratryl alcohol had lost 12% of the deuterium. At 40° the reaction required 120 hours and only 5% of the deuterium was lost. Exchange of the deuterium in [α-^2H]veratraldehyde had not occurred since recovered aldehyde (2%) after the reaction contained more deuterium (98·2%) than the starting material (97·7%), due to

the large isotope effect. The [α-$^2H_2$]veratryl alcohol was unlikely to exchange and benzyl alcohol did not exchange after prolonged heating with deutero-base.

A possible source of contamination of the [α-$^2H_2$]veratryl alcohol could be the reduction of the aldehyde by the ethoxide ion.



In order to avoid this, an alternative solvent system was sought. The reaction was slow in refluxing aqueous dioxan since the reactants were not homogeneous. Sulpholan was a sufficiently good solvent, but was difficult to separate from the product.

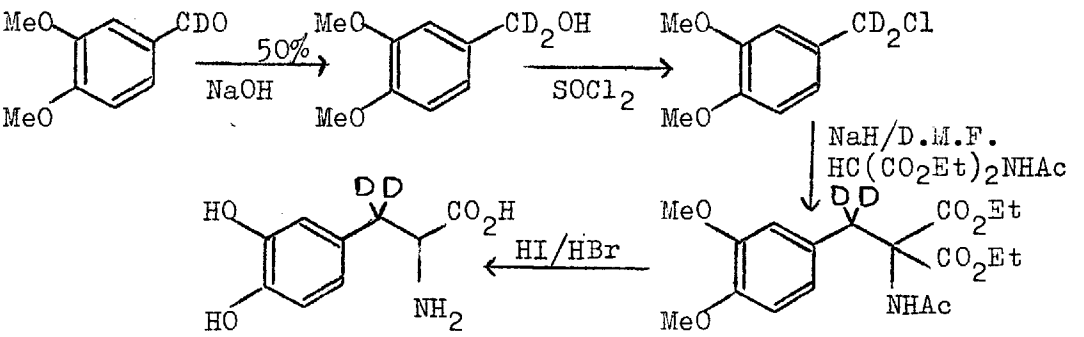
Although veratraldehyde was only sparingly soluble in water, aqueous sodium hydroxide was found to be the best reagent.

Strength NaOH	Conditions	Temperature	Time	Conversion
20%	Reflux	105°	4 hrs	80%
50%	Carius tube	155°	30 min	80%
50%	Reflux	120°	20 min	85%
Saturated	Reflux	173°	5 min	97%

Refluxing [α-^2H]veratraldehyde (0·977 D) with 50% sodium hydroxide for 1½ hours gave [α-$^2H_2$]veratryl alcohol (1·955 D)

in excellent yield. In the absence of ethanol no acetaldehyde was formed as a by-product, so a pure, colourless product was obtained. This reduction by ethoxide was important with veratraldehyde because the reaction required far more vigorous conditions than benzaldehyde.

3,4-dihydroxyphenyl[β-$^2H_2$]alanine was synthesized from [α-$^2H_2$]veratryl alcohol by the following route:-



Thionyl chloride in ether chlorinated [α-$^2H_2$]veratryl alcohol at room temperature to give [α-$^2H_2$]veratryl chloride which was condensed with diethyl acetamidomalonate without purification. This condensation was best carried out using sodium hydride in dimethylformamide rather than the classical method of sodium ethoxide in anhydrous alcohol.[112,216] Diethyl acetamido[α-$^2H_2$]veratrylmalonate was obtained pure, and in excellent yield, by this method. Hydrolysis in a mixture of hydriodic and hydrobromic acids gave 3,4-dihydroxyphenyl[β-$^2H_2$]alanine in good yield, and the mass spectrum of its

ethyl ester indicated not less than 95% deuteration (1·90 D).

Oxidation of 3,4-dihydroxyphenyl[β-$^2H_2$]alanine to melanin

In order that the results could be compared with those obtained from the other specifically labelled 3,4-dihydroxyphenylalanines, the [β-$^2H_2$] derivative was oxidized to melanin and isolated under identical conditions.[119,135] A 0·5% solution of 3,4-dihydroxyphenyl[β-$^2H_2$]alanine was oxidized for 8 hours by oxygen in the presence of tyrosinase and catalase. The melanin was isolated by precipitation with ammonium nitrate, and washed with water and acetone. After drying *in vacuo* over phosphorous pentoxide a 67% w/w yield was obtained. Unfortunately the deuterium analysis of this melanin is not yet available, but the results will be included if possible.

The Deuteration and Tritiation of Aldehydes

The methods available for the synthesis of formyl-labelled aldehydes are of limited application:-

a) The deuterolysis of deuteroacetylene to [$^2H_4$]acetaldehyde.[224]

b) The partial deuterogenation of aryl-acid chlorides.[225]

c) The lithium aluminium deuteride reduction of benzils, followed by lead tetraacetate cleavage.[226]

d) The deuterogenation of nitriles, followed by oxidation.[227]

e) The decarboxylation of deutero α-ketoacids.[228]

f) The deuterolysis of the 1,3-dithiane derivative of an aldehyde.[229]

The procedure developed in these laboratories makes use of the readily accessible morpholino-nitriles and enables an aldehyde to be labelled in the formyl-group in three steps, with good overall yield and high incorporation.

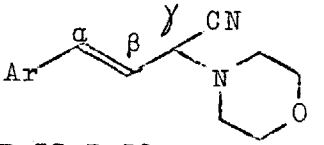
Benzylvanillin had been deuterated and tritiated by this method,[214] and [α-^{2}H]veratraldehyde was required for the present work. A detailed investigation of this reaction was made since it appeared to be a useful general method. A number of morpholino-nitriles were prepared jointly with Dr. D. J. Bennett and a start was made on their deuteration. The experiments performed jointly are indicated in the experimental section. A preliminary communication describing some of the results has recently been published.[230]

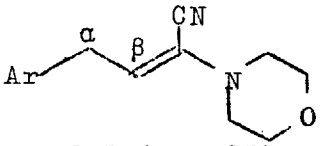
By converting an aromatic aldehyde to its morpholino-nitrile, a α-phenylmorpholineacetonitrile, the formyl hydrogen is activated and can be removed or exchanged. Hydrolysis of the labelled morpholinonitrile regenerates the aldehyde with a formyl label.

The preparation of morpholino-nitriles

The morpholinonitrile derivatives were prepared by a modification of standard procedures.[231,232,233] The aldehyde was treated with a slight excess of morpholine perchlorate in morpholine solution to form the tertiary iminium perchlorate, which was not isolated. Addition of aqueous sodium cyanide to the reaction mixture gave the morpholinonitrile which could be isolated in almost quantitative yield. Most of these derivatives were stable crystalline solids which were easy to purify. Only two aldehydes failed to give satisfactory derivatives by this method. The morpholino-nitrile of p-diethylaminobenzaldehyde was an oil which could not be crystallized, but the p-dimethylamino derivative was crystalline although it was sensitive to aerial oxidation. Cinnamaldehyde gave a mixture of products, but only a trace of the morpholino-nitrile. The principal product was

probably the isomer in which the double bond was conjugated with the cyanide group. The shift of the N-CH_2 resonance in the n.m.r. spectrum confirmed the structure.





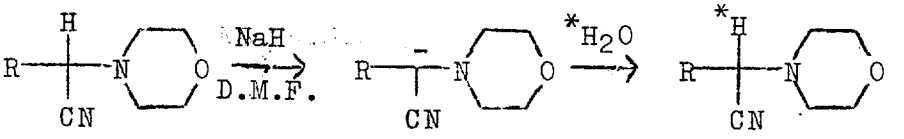
N.m.r.($CDCl_3$)		
τ7·35-7·50 m. $2NCH_2$	7·0-7·2 m. $2NCH_2$
6·15-6·4 m. $2OCH_2$	6·15-6·35 m. $2OCH_2$
5·60 dd. γ-CH	
3·87 dd. β-CH	4·61 t. β-CH
3·09 dd. α-CH	6·36 d. α-CH_2
2·61 s. 5ArH	2·74 s. 5ArH
$J_{\alpha\beta}$ 16·2 c/s	$J_{\alpha\beta}$ 8·0 c/s
$J_{\beta\gamma}$ 4·8 c/s	
$J_{\alpha\gamma}$ 1·7 c/s	

s singlet, d doublet, t triplet, m multiplet

Cinnamaldehyde morpholino-nitrile could be prepared from from its tertiary-iminium perchlorate, which was synthesized from cinnamaldehyde and morpholine perchlorate in ethanol containing a trace of triethylamine.[234] Addition of this salt to aqueous sodium cyanide gave a good yield of the required derivative.

Morpholino-nitriles did not show any cyanide absorptions in the infra-red. The benzylic proton showed the expected shifts in the n.m.r. spectra due to electronic effects- electron-donating τ+0·1, electron-withdrawing τ-0·1.

Labelling of morpholino-nitriles - Sodium hydride method



Most morpholino-nitriles could be deuterated or tritiated by a method similar to that used for deuterating veratraldehyde morpholino-nitrile. A solution of the morpholino-nitrile in anhydrous dimethylformamide was treated with excess sodium hydride at room temperature for ½ to 2 hours in order to generate benzylic anion. These anions were stable in the absence of oxygen and hence the benzylic proton could be completely removed. Addition of deuterium oxide or tritiated water quenched the anion to give the labelled morpholino-nitrile.

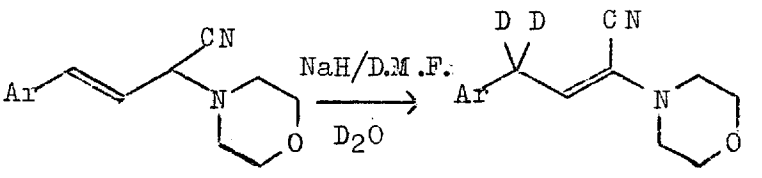
Arylmorpholineacetonitrile	Deuterium	Tritium	Yield
Phenyl	92%	89%	85%
p-Nitrophenyl	0		78
m-Nitrophenyl	75		77
p-Cyanophenyl	>95		91
p-Dimethylaminophenyl	94		80
3,4-Dimethoxyphenyl	99	97	94
4-Benzyloxy-3-methoxyphenyl*	100	81	95
2-Benzyloxyphenyl#	100		80
α-Naphthyl	97		95

* Ref. 214. # Ref. 235

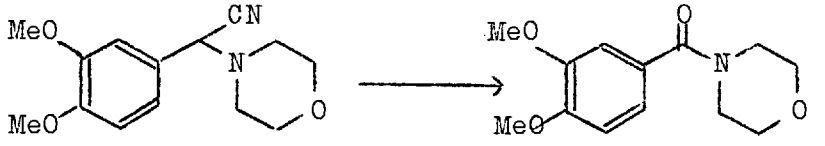
Both the nitro-derivatives required chromatography in order to purify the deutero-morpholino-nitrile. Both generated the anions so readily that considerable loss of label occurred. The p-nitrophenylmorpholineacetonitrile anion was formed so readily that it gave a permanganate-coloured solution in aqueous sodium carbonate.

After reacting α-naphthylmorpholineacetonitrile with sodium hydride for two hours only 79% incorporation was found. A longer reaction time and warming to 40° enabled the fully deuterated derivative to be made. 2-Benzyloxybenzaldehyde was also given a long reaction time. These two derivatives were the only ortho-substituted derivatives tried and steric hindrance was probably retarding the formation of the anion.

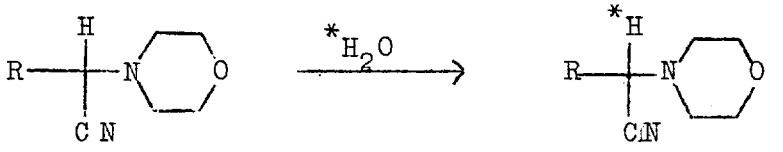
Cinnamaldehyde morpholino-nitrile was difficult to deuterate. Chromatography of the crude deuteration product on silica gel gave cinnamaldehyde, but the mass spectrum of its semicarbazone showed it to be a mixture of a mono- and a di-deutero species. Attempted isolation of the morpholino-nitrile gave an oil. The n.m.r. spectrum showed a broad singlet at τ4·75 corresponding to one proton.



All the anions were sensitive to oxygen, so they were prepared in oxygen-free nitrogen. 3,4-dimethoxyphenyl-morpholineacetonitrile was found to be particularly sensitive to oxidation. Unless the deuteration was carried out in the complete absence of oxygen a large proportion of an oil was formed. This oil was hydrolyzed to ethyl veratrate by ethanolic hydrochloric acid, and to veratric acid by dilute alkali. The n.m.r. spectrum showed that the oil was veratroylmorpholine. A carbonyl absorption at 1633 cm^{-1} confirmed the tertiary amide and the structure was proved by synthesis.



Labelling of morpholino-nitriles - Direct exchange



An alternative procedure for the labelling of morpholino-nitriles was found to be particularly suitable for small quantities (less than 1 mmole) in which aerial oxidation of the anion had been troublesome. The benzylic proton

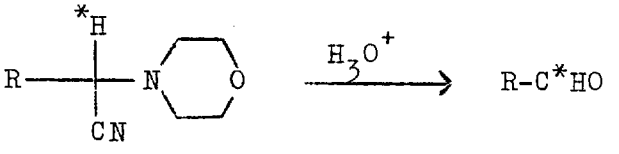
exchanged rapidly with deuterium oxide or tritiated water at 100°. In dimethylformamide at 100° phenylmorpholineacetonitrile had a half exchange time of 40 minutes, and in sulpholan 3,4-dimethoxyphenylmorpholineacetonitrile had a half exchange time of 100 minutes. A reaction time of 5 hours was sufficient to completely exchange the aromatic morpholinonitriles. The exchange of α-morpholinevaleronitrile, butyraldehyde morpholino-nitrile, was slower but it was completely tritiated after heating for 3 hours at 155°. Other aliphatic aldehydes were not investigated, so further work is required to find out if this technique is applicable. In the presence of base the two α-hydrogens of benzyl cyanide could also be exchanged.

Prolonged exchange of a morpholino-nitrile could result in substitution of the aromatic protons. In order to check this, some morpholino-nitriles were heated, for up to 75 hours, with tritiated water and the resulting aldehydes oxidized to the benzoic acids to remove the formyl hydrogen. The residual activity indicated less than 0·5% exchange in the aromatic ring. This must represent a maximum incorporation since the acids proved difficult to crystallize to constant activity.

Arylmorpholineacetonitrile	Time of exchange at 100°	Residual activity of acid
3,4-Dimethoxyphenyl	75 hours	0·006%
p-Nitrophenyl	15	0·5%
m-Nitrophenyl	15	0·07%

These results indicate that the direct exchange procedure is suitable for the labelling of most morpholino-nitriles, and that only the α-hydrogen is exchanged.

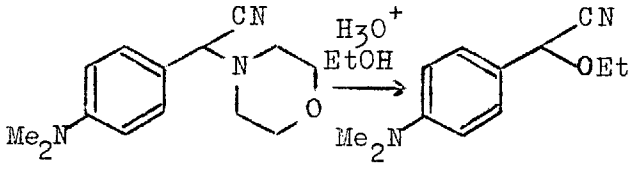
Hydrolysis of morpholino-nitriles to aldehydes

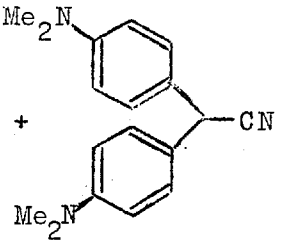


Having prepared the labelled morpholino-nitrile, by the sodium hydride method or direct exchange, it had to be hydrolyzed to the aldehyde without loss of label. Refluxing with a mixture of concentrated hydrochloric acid in ethanol and water hydrolyzed most morpholino-nitriles in an hour. The aldehydes were obtained in almost quantitative yields by evaporating the alcohol and extracting with chloroform or dichloromethane. Benzaldehyde was volatile and would be lost with the ethanol, but the hydrolysate gave a quantitative yield of the 2,4-dinitrophenylhydrazone. Hydrolysis in aqueous acid gave

an excellent yield of benzaldehyde.

Both p-diethyl and p-dimethylaminophenylmorpholineacetonitriles were hydrolyzed to give a mixture of products which contained only a trace of the aldehyde. The principal product was the ethoxycyanide of the aldehyde. Traces of compounds showing an ethoxy-group, which had previously been observed in the n.m.r. spectra of p-cyano[α-^{2}H]benzaldehyde and [α-^{2}H]naphthaldehyde, were presumably due to similar derivatives.



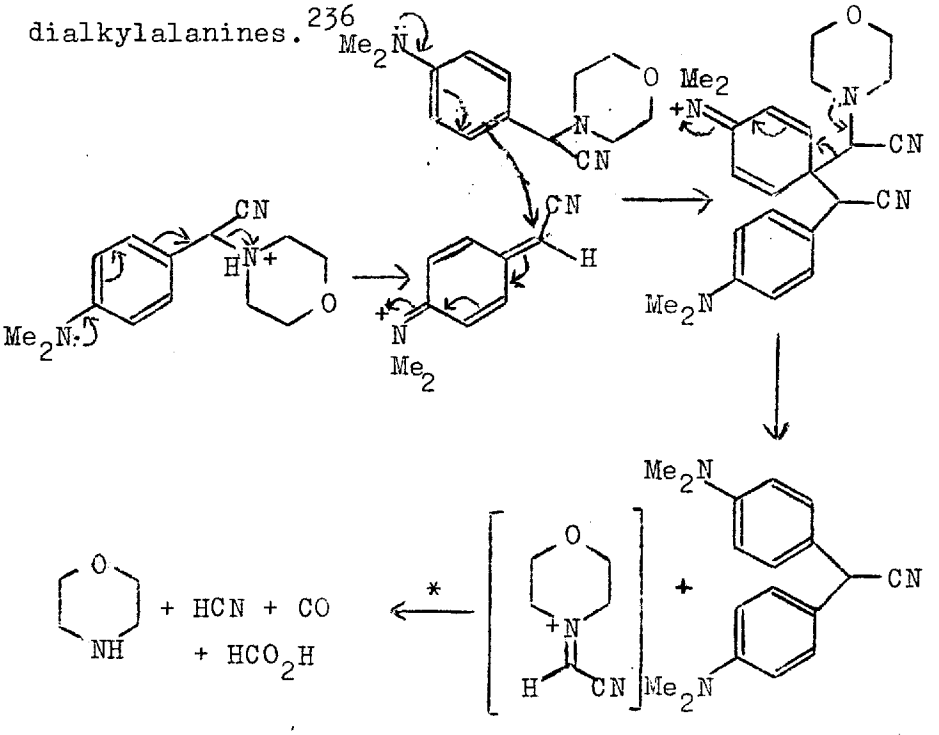


N.m.r. ($CDCl_3$)

τ7·44 m. $2NCH_2$	τ8·78 t. CH_3	
6·31 m. $2OCH_2$	6·45,6·41 2q. OCH_2	
5.30 s. α-CH	4·98 s. α-CH	τ5·02 s. α-CH
3·29 d. 2ArH	3·38 d. 2ArH	3·29 d. 4ArH
2·67 d. 2ArH J 8·8c/s	2·78 d. 2ArH J 8·9c/s	2·80 d. 4ArH J 8·7c/s

A trace of bis-p-dimethylaminophenylacetonitrile was formed during the hydrolysis, which could be detected by the green spot it gave on a thin-layer chromatoplate after exposure to iodine or air. Preparative-scale thin-layer chromatography of the hydrolysis product gave 3% yield. This compound was thought to be an oxidation product of p-dimethylaminophenylmorpholineacetonitrile (mass spectrum- molecular weight 34 units

greater than morpholinonitrile $=H_2O_2$). Treatment of the morpholinonitrile with cold dilute hydrogen peroxide in the presence of acid gave a 44% yield. With the larger quantity available the n.m.r. spectrum and analysis showed it to be bis-p-dimethylaminophenylacetonitrile. Parallel reactions with and without hydrogen peroxide showed that the reaction was acid catalyzed and did not involve oxidation. Though rapid in the presence of an equivalent of acid, excess inhibited the reaction. The mechanism may be similar to the displacement of the p-substituent of some other dialkylalanines.[236]



* Ref. 231.

Bis-p-dimethylaminophenylacetonitrile is a derivative of Michler's hydrol and would be expected to be oxidized to a dye. This structure would also explain the presence of strong doubly and triply charged ions in the mass spectrum. The molecular peak at m/e 279 was the base peak and there was a doubly charged peak at 139·5 (23%) and a triply charged peak at 93 (0·4% estimated from P+1 peak). A trace of Michler's ketone was also isolated from the hydrolysis.

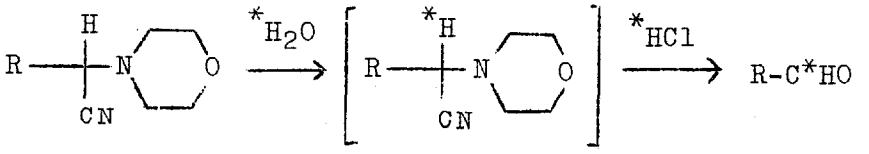
In order to avoid the formation of ethoxycyanides and to make the isolation of the aldehyde easier, the morpholino-nitriles were hydrolyzed in aqueous acid. Most morpholino-nitriles gave a quantitative yield of aldehyde by this means, but p-dimethylaminophenylmorpholineacetonitrile was hydrolyzed to a mixture of the aldehyde and its cyanohydrin.

Acid hydrolysis of labelled morpholino-nitriles gave the formyl labelled aldehyde with little loss of activity.

Arylmorpholineacetonitrile	Retention of label	Yield
Phenyl	97%	95%
p-Nitrophenyl		78
m-Nitrophenyl	30	100
p-Cyanophenyl	72	100
p-Dimethylaminophenyl	70	63
3,4-Dimethoxyphenyl	100	100
4-Benzyloxy-3-methoxyphenyl*	100	100
2-Benzyloxyphenyl‡	100	
α-Naphthyl	100	100

* Ref. 214. ‡ Ref. 235. Both were partially debenzylated.

Labelling of morpholino-nitriles - Direct exchange and in situ hydrolysis



Those morpholino-nitriles which lost label on isolation or in the hydrolysis were labelled under conditions in which exchange would not cause loss of label. Direct exchange of the morpholino-nitrile was carried out with deuterium oxide or tritiated water in sulpholan and the product hydrolyzed by the addition of thionyl chloride or sulphuryl chloride and heating. Sulpholan was used as a co-solvent because dimethylformamide was sensitive to acid. The technique used was to seal a small break-seal ampoule of the latent acid and a short glass rod inside the Carius tube containing the morpholino-nitrile, deuterium oxide and sulpholan. After exchange at 100° the reaction was acidified by shaking the tube to break the ampoule. Continued heating at 100° hydrolyzed the morpholino-nitrile in labelled acid, so that the deuteroaldehyde was isolated on opening the tube.

Arylmorpholineacetonitrile	Deuterium	Yield
p-Nitrophenyl	100%	48%
m-Nitrophenyl	100	76
p-Cyanophenyl	96	86

The Oxidation of 3,4-Dihydroxyphenylalanine

Dopachrome is an important intermediate in melanogenesis and is thought to be indoline-5,6-quinone-2-carboxylic acid although it has not been isolated. The oxidation of 3,4-dihydroxyphenylalanine to dopachrome was studied in order to try to prove the structure.

3,4-Dihydroxyphenylalanine was not oxidized by neutral potassium ferricyanide, but the addition of base led to the rapid formation of the red quinone. With up to 4 moles of ferricyanide the intensities of the two absorption maxima at 306 and 472 mμ were proportional to the amount of ferricyanide added and an excess of ferricyanide over 4 moles did not react. This was consistent with the proposed oxidation level of dopachrome. From the spectra there was no sign of any intermediate dopaquinone.

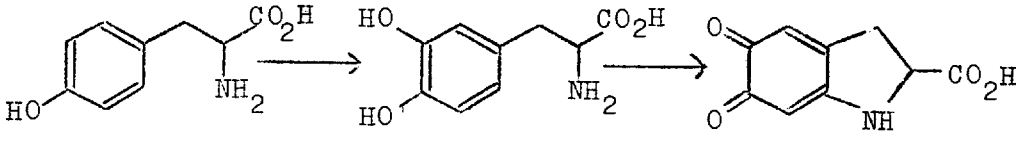
The spectra of dopachrome were determined in aqueous solution in 1 cm spectrometer cells by oxidising 3,4-dihydroxyphenylalanine with ferricyanide in the sample cell and balancing the background absorption by the addition of ferrocyanide to the reference cell. The reactions were complete in 5 minutes and the solutions of dopachrome were fairly stable (4% loss/hour). The absorption maxima in 0·0032M sodium hydrogen carbonate were at 306 mμ (ε 9500) and 472 mμ (ε 3850).

In order to try to isolate dopachrome 3,4-dihydroxyphenylalanine was oxidized with alkaline ferricyanide and the ferrocyanide precipitated with lead nitrate. Freeze drying the red solution gave a voluminous reddish-brown solid which had a similar spectrum to dopachrome. The n.m.r. spectrum in deuterium oxide showed a broad singlet at $\tau 3{\cdot}3$ and the CH and CH_2 as an unresolved multiplet at $\tau 6{\cdot}5\text{-}7{\cdot}2$. The integral corresponded approximately to the expected 2:3 ratio. The dopachrome prepared by this method was too unstable to be purified or separated from the large amount of potassium nitrate present.

The oxidation of3,4-dihydroxyphenylalanine by silver oxide and Fremy salt was also investigated, but no significant results were obtained.

Tyrosinase Oxidation of 3,4-Dihydroxyphenylalanine and Tyrosine

A mixture of mushroom tyrosinase and beef-liver catalase was used in all the preparations of melanin. The factors influencing the oxidation of 3,4-dihydroxyphenylalanine and the hydroxylation and oxidation of tyrosine were investigated spectroscopically by measuring the formation of the visible absorption maximum of dopachrome at 472 mμ (ε 3850). These experiments were conveniently followed in the 1 cm cells of a recording spectrometer set at the absorption maximum.



Tyrosine Dopa Dopachrome

Tyrosine or 3,4-dihydroxyphenylalanine ($1\text{-}5 \times 10^{-4}$M) in phosphate buffer at pH 6·8 (0-0·1M) was oxidized by dissolved air or oxygen in the presence of tyrosinase (0-0·1%) and catalase (0-0·6%). In order to obtain reproducible results all solutions were made with water redistilled in an all glass apparatus under nitrogen. Solutions of tyrosinase (0·28%) were found to lose only 2% activity/day at 0°.

In a typical experiment the 1 cm spectrometer cells were filled as follows:-

	Sample cell	Reference cell
M Phosphate buffer	0·25ml	0·25ml
Water	0·75	1·75
0·001M Dopa	0·5	0·5
0·28% Tyrosinase	1·0	-

The cells were saturated with oxygen before adding the enzyme to start the reaction. The presence of 3,4-dihydroxyphenylalanine in both cells automatically allowed for autoxidation. The concentration of dopachrome increased linearly for about 5 minutes, then the rate of reaction decreased as the dissolved oxygen was exhausted and the dopachrome decomposed. Tyrosine was oxidized more slowly and the reaction was preceded by an induction period of 3 to 10 minutes during which time little dopachrome was formed. From the maximum rate of formation of dopachrome, the specific activity of the enzyme was calculated.

The units used to measure enzyme activity are rather confused and depend on the method and substrate used. Manometric results are usually expressed in terms of the volume of oxygen absorbed, whereas spectroscopic methods give rise to a unit based on change of absorbance or moles of product formed. The enzyme activities given in this thesis are expressed in international units which are defined as the amount of enzyme (mg) which can convert 1μmole of substrate per minute.[146]

The activities of the various batches of mushroom tyrosinase, used in the oxidation of specifically-labelled precursors,[119] were compared. The newer batches were 70% more active than those which had been kept for 2 years (at 0°).

The specific activity of tyrosinase did not depend on the concentration of the phosphate buffer:

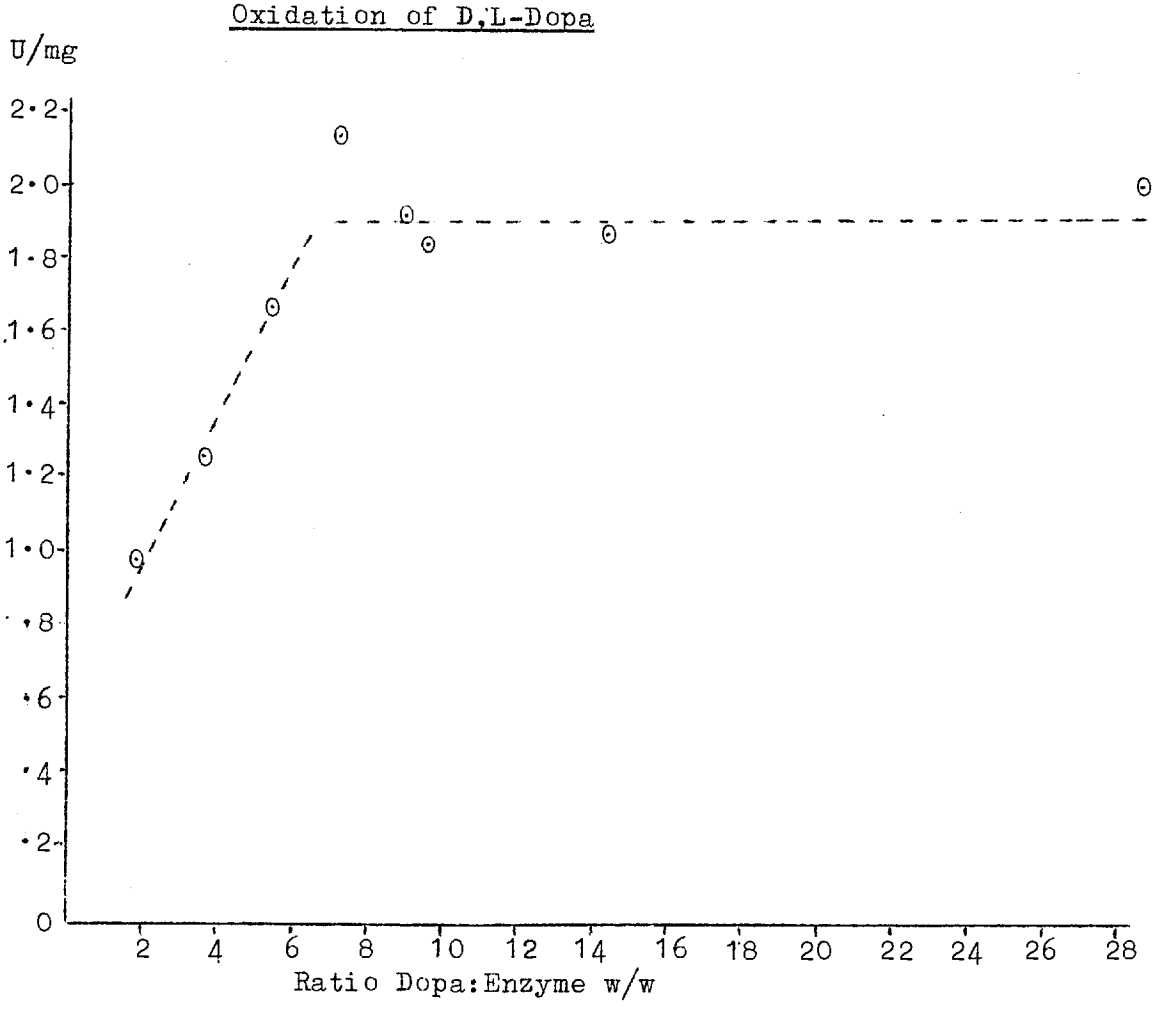
Water	1·41 U/mg
0·027M buffer	1·21
0·1M buffer	1·26

Catalase produced a small increase in tyrosinase activity, but catalase oxidized 3,4-dihydroxyphenylalanine at a rate less than 1% that of tyrosinase.

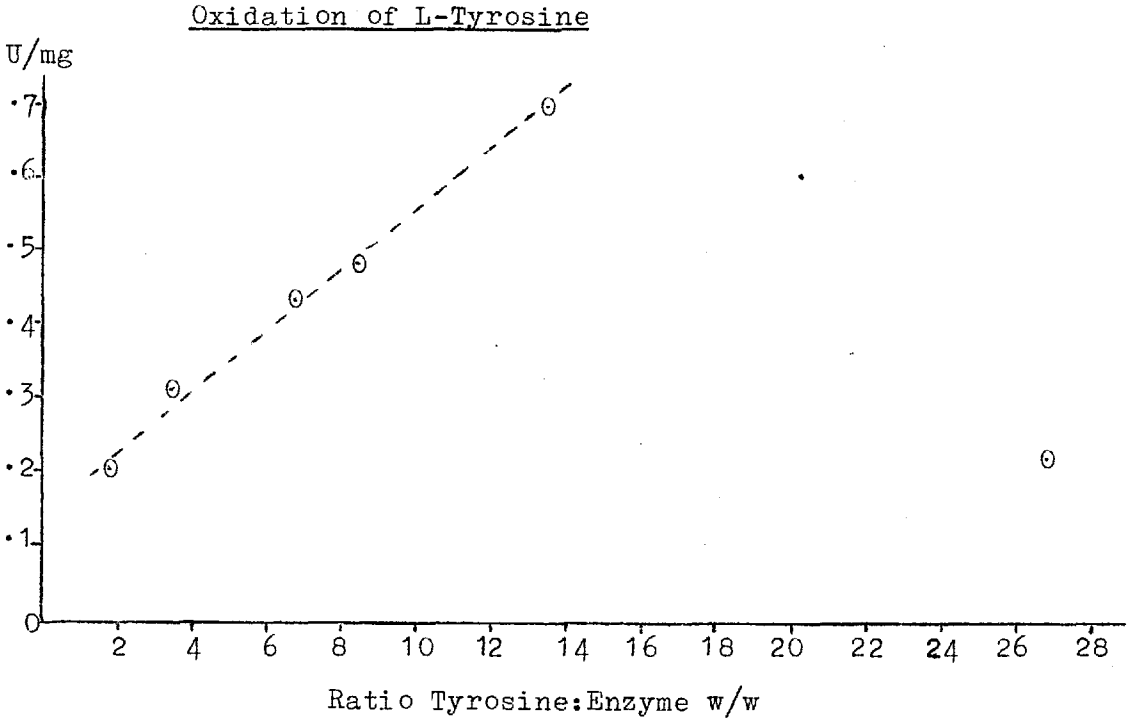
Mushroom tyrosinase oxidized both D-and L-3,4-dihydroxyphenylalanine, but L was oxidized 2·2 times faster than D:

L-Dopa	1·66 U/mg	Average 1·20 U/mg.
D-Dopa	0·75 U/mg	
D,L-Dopa	1·21 U/mg	

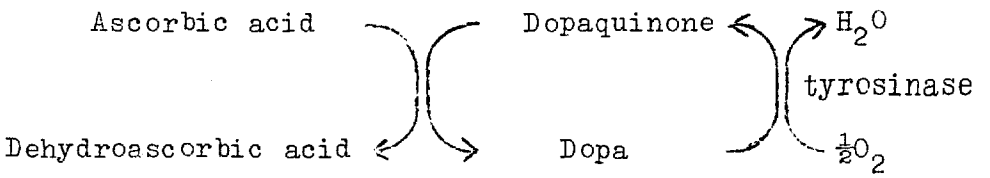
The effect of enzyme and substrate concentration on the specific activity was more complex. The activity depended on the substrate:enzyme ratio, rather than their concentrations. With increasing concentration of 3,4-dihydroxyphenylalanine the activity increased linearly to a maximum then remained constant.



The activity of tyrosinase using L-tyrosine as the substrate increased linearly with increasing tyrosine concentration for all results except one, which was carried out with a high substrate concentration and little enzyme, in which the enzyme was largely deactivated. Substrate inhibition of a mammalian tyrosinase has been observed.[30]



The estimation of enzyme activity by measuring the formation of dopachrome was not very satisfactory since several oxidations were involved. With tyrosine as the substrate the activity measured represents the overall activity of hydroxylation and dopa oxidation. In order to simplify this system dopaquinone was reduced, as it was formed, back to 3,4-dihydroxyphenylalanine. Ascorbic acid, vitamin C, which is not oxidized by tyrosinase, will reduce dopaquinone.[158,238]



The net result of this reaction is the enzymic reduction of $\frac{1}{2}$ mole of oxygen by 1 mole of ascorbic acid, and hence the rate of loss of ascorbate provides a direct measure of the rate of formation of dopaquinone.

Oxidations in the presence of ascorbic acid were carried out spectroscopically by a method similar to that used to measure dopachrome formation. The loss of ascorbic acid was followed by recording the intensity of the absorption maximum at 266 mμ (ε 14,400). Since ascorbic acid was readily oxidized the standard solution was stored under nitrogen at 0° with added ethylenediaminetetraacetic acid (E.D.T.A.) to remove metal ions. Addition of ascorbic acid to both cells allowed for autoxidation and enabled the loss of ascorbic acid to be measured directly. Since two moles of dopaquinone react to give one mole of dopachrome, the enzyme activities would be expected to be doubled.[168] The enzyme activities measured by the ascorbate method were twice to three times those obtained by the dopachrome method.

Oxidation of tyrosine in the presence of ascorbic acid was more complicated since 3,4-dihydroxyphenylalanine was being continuously formed and would therefore accumulate. This resulted in an accelerating rate of reaction, after the induction period of about 2 minutes. From the results obtained by the dopachrome method the dopa-oxidase activity

of the enzyme was proportional to the concentration of dopa. The concentration of 3,4-dihydroxyphenylalanine would therefore be proportional to the rate of loss of ascorbic acid, and hence:

$$a_T = \frac{-1}{w_e^2 k} \cdot \frac{d^2[\text{Ascorbate}]}{dt^2}$$

Where: a_T - tyrosine-hydoxylase activity

w_e - weight of enzyme

k - rate of dopa-oxidase activity change with dopa concentration i.e. $\frac{d[a_D]}{d[\text{Dopa}]}$

Although $\frac{d^2[\text{Asc}]}{dt^2}$ could not be measured accurately it was found to increase linearly for 10 minutes to a maximum equivalent tc about 10^{-6}M dopa (tyrosine 2 x 10^{-4}M). The results indicated an L-tyrosine-hydroxylase activity (a_T) of 0·4 U/mg which was consistent with the overall rate of 0·25 U/mg measured by the dopachrome method (≡0·5-0·75 U/mg on the dopaquinone scale). These activities were much lower than those found for L-3,4-dihydroxyphenylalanine at the the same concentration (2·16 U/mg, dopaquinone scale). However since several assumptions are required for this calculation little significance can be given to the results.

The hydroxylation of tyrosine has been studied, using a tritium label, in the presence of a mammalian tyrosinase.[31] Only a small isotope effect (1·03-1·09) was observed in the

hydroxylation of [3-^{3}H]tyrosine. [3,5-$^{2}H_{2}$]tyrosine was prepared by the alkaline exchange of L-tyrosine with deuterium oxide. Oxidation of [3,5-$^{2}H_{2}$]tyrosine by mushroom tyrosinase was significantly slower than tyrosine. Using the dopachrome method, an isotope effect of 1·22 was measured. The ascorbate method for measuring hydroxylase activity (a_T) gave a larger figure (1·4).

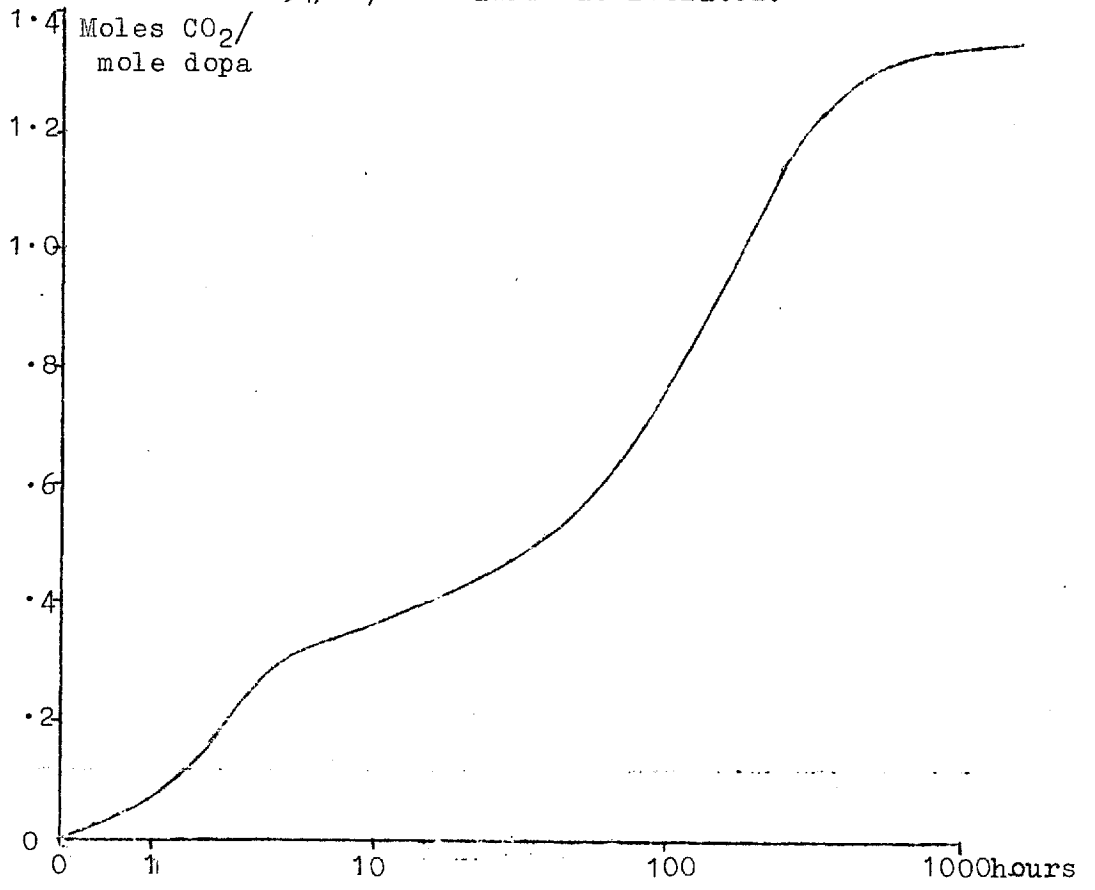
Since tyrosinase appeared able to oxidize most phenols, a check was made to see whether the enzyme was capable of catalyzing a free-radical oxidation. [2-^{3}H]-p-cresol was oxidized by tyrosinase with oxygen and any Pummerer's ketone formed isolated by isotope dilution. After rigorous purification a maximum yield of 0·02% was found. Tyrosinase therefore has little tendency to couple phenols, that is, it does not possess any peroxidase activity.

Estimation of Carbon Dioxide in Melanogenesis

Considerable quantities of carbon dioxide are evolved in the oxidation of tyrosine or 3,4-dihydroxyphenylalanine to melanin. Even in the presence of catalase, which prevents the degradation by hydrogen peroxide, an appreciable amount is evolved. In order to estimate it quantitatively an apparatus similar to that used for the micro-analytical determination of C and H was used. Pure oxygen was bubbled through the reaction vessel and the exit gases dried by passing through a large anhydrone tube. The residual water and carbon dioxide were collected in two micro-analytical absorption tubes. Calibration of the apparatus using potassium hydrogen carbonate in phosphate buffer showed that no water was getting through the drying tube. 78% of the carbon dioxide was collected in less than 5 hours and after 20 hours 99·4%.

3,4-Dihydroxyphenylalanine, in neutral phosphate buffer was oxidized in a rapid stream of oxygen by tyrosinase in the presence of catalase. 0·4 moles of carbon dioxide were collected during the first 10 hours, then the rate dropped to about 0·1 moles/day. This slow, long term, evolution of carbon dioxide must be due to slow degradation of the melanin. After 125 hours oxidation 88% w/w melanin was recovered. In another experiment 1 mole of carbon dioxide was collected after 150 hours and oxidation for

1000 hours gave 1·35 moles of carbon dioxide. After 7890 hours oxidation 94% w/w melanin was isolated.

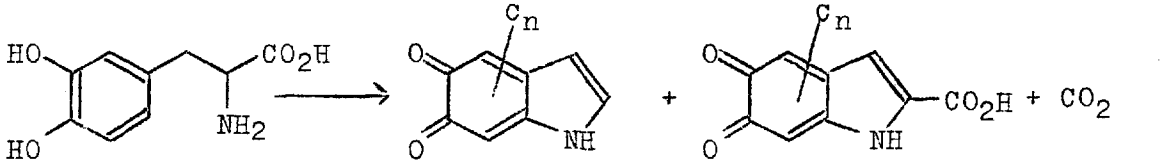


Since the oxidation of 3,4-dihydroxyphenylalanine to melanin was usually carried out for 8 hours, a reaction was stopped after this time and the products estimated:

Melanin	60% w/w
Carbon dioxide	7·1% w/w
Unreacted dopa[239]	31%

Allowing for the unreacted 3,4-dihydroxyphenylalanine, 0·46 moles of carbon dioxide were evolved. This suggested that about

half the molecules were decarboxylated. This would give an average molecular weight per indole unit of 169 which agreed closely with the observed yield of melanin of 170 mg. per mole of 3,4-dihydroxyphenylalanine.



M.W. 197 E.W. 145 E.W. 189 M.W. 44

46% 54% 46%

Ave. 169

These results may be taken to indicate that melanin is approximately a co-polymer of indole-5,6-quinone and indole-5,6-quinone-2-carboxylic acid units. The carbon dioxide need not be derived entirely from the carboxyl group and some ring fission may still occur.

Oxidation of Melanin to Pyrrole acids

The oxidation of melanin with alkaline permanganate or hydrogen peroxide gave low yields of a number of pyrrole acids (see review page 30). The principal product was pyrrole-2,3,5-tricarboxylic acid which could be isolated in 1-2% yield from the permanganate oxidation.[91] Since the yields were so low, it is doubtful whether the results are of much significance. In order to find out the true yield, pyrrole-2,3,5-tricarboxylic acid was estimated by radiochemical dilution by a method similar to that used to estimate pyrrole-2,3-dicarboxylic acid.[119]

D,L-3,4-dihydroxyphenyl[α-^{14}C]alanine was oxidized enzymically and the resulting [^{14}C]melanin completely oxidized with cold alkaline potassium permanganate in the presence of pyrrole-2,3,5-tricarboxylic acid*. The acid was reisolated as the trimethyl ester and rigorously purified. Its activity indicated a maximum yield of pyrrole-2,3,5-tricarboxylic acid of only 1·2%. Although this figure was considerably higher than that obtained with pyrrole-2,3-dicarboxylic acid (0·2-0·3%), the formation of pyrrole acids cannot give more than the slightest indication of the structure of melanin.

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* The author is indebted to Professor R. A. Nicolaus for his generous gift of triethyl pyrrole-2,3,5-tricarboxylate.

Estimation of Absorbed 3,4-Dihydroxyphenylalanine in Melanin

If a 5,6-dihydroxyindole were an intermediate in melanogenesis the oxidation of 3,4-dihydroxy[6-^{3}H]phenylalanine to melanin should result in complete loss of tritium. The retention of 13% could be due to uncyclized units absorbed or bonded in the melanin, or to indole units which were cyclized to the 2-position.[135] 1·4% of 3,4-dihydroxyphenylalanine was recovered, by radiochemical dilution, from an acid extract of [^{14}C]melanin. In order to check this result a sample of [^{14}C]melanin was extracted with acid in the presence of inactive 3,4-dihydroxyphenylalanine. After 4 hours refluxing with acid the extract was decanted and the melanin re-extracted for a further 24 hours with fresh reagents. 3,4-Dihydroxyphenylalanine was recovered from the extracts and rigorously purified. The residual activities indicated that 5·0% unreacted precursor was desorbed after 4 hours, and a further 0·55% after prolonged extraction. The total yield (5·6%) was significantly less than the 13% retention of the 6-tritium and proved that this retention of activity could not be entirely due to absorbed 3,4-dihydroxy[6-^{3}H]phenylalanine.

Experimental

The following instruments were used to record physical data:-

Melting points (m.p.)-uncorrected	Kofler block
Infra-red spectra (i.r.)	Unicam SP 200
Ultra-violet spectra (u.v.)	Unicam SP 700 or SP 800
Nuclear magnetic resonance spectra (n.m.r.)	Varian A 60
Mass spectra (m.s.)	A.E.I. MS 9.

N.m.r. spectra were determined by Mrs. I.A.Boston, mass spectra by Mr. P.N.Boshoff and micro-analyses by the staff of the Imperial College micro-analytical laboratories.

Mushroom tyrosinase	Koch-Light Laboratories Ltd.
Beef liver catalase	Koch-Light Laboratories Ltd.
Deuterium oxide (99·7%)	Norsk Hydro-elektrisk Kvaelstofaktie-selskab.
Tritiated water (0·2 C/ml.)	U.K.A.E.A.

Radiochemical compounds were assayed in liquid scintillator (1·2 ml.) (Nuclear Enterprises Ltd. NE 213), or if insoluble in a mixture of dimethylformamide (0·1 ml.) and liquid scintillator (1·1 ml.)

Petrol refers to petroleum ether (b.p. 60-80°).

Extracts from aqueous solutions were dried with sodium sulphate.

Thin-layer chromatography (t.l.c.) was carried out on silica gel or alumina GF_{254}.

Relative activities of radioactive compounds are shown in square brackets [ ]. Where necessary the activities are corrected for quenching.

Preparation and reactions of Pummerer's ketone

Pummerer's ketone[195]

A solution of p-cresol (1·08g., 10mmole) in ice-cold water containing sodium carbonate (2·12 g., 20mmole) was oxidized with aqueous potassium ferricyanide (4·94 g., 15 mmole). The mixture was stirred for 2 hours, filtered through cellite and the sticky precipitate washed well with water. The precipitate was extracted with ether, and the extract dried, evaporated and chromatographed on alumina grade III (35g.). Elution with benzene gave Pummerer's ketone (247 mg., 23%) which was recrystallized from benzene-petrol, m.p. 125° [lit.[195] 124·5°],

i.r. (CCl_4) ν 1690 cm^{-1}
u.v. (ethanol) λ 220 mμ (ε 7000), 300 (ε 3600)

Addition of methanol to Pummerer's ketone

Pummerer's ketone (243mg.) in methanol (50 ml.) was treated with 4N sodium hydroxide (0·3 ml.). After 3 hours at room temperature the solution was neutralized and evaporated, and the residue extracted with ether. Extraction of the residue from the ether with petrol, concentrated an oil (70 mg.) which was chromatographed on alumina grade III (35 g.) in benzene. A single fraction (64·5 mg.) was eluted and shown by t.l.c. to be a mixture of two compounds.

Rechromatography on silica gel also gave a single fraction (59·1 mg.). N.m.r. showed it to be a 1:1 mixture of Pummerer's ketone and the 1,2-methanol adduct.

Mixture	Pummerer's ketone
I.r. (CCl_4) ν 1723, 1690 cm^{-1}	1690 cm^{-1}
u.v. (EtOH) λ 205, 300 mμ*	
n.m.r. (CCl_4) τ 8·53 s. CMe	
8·47 s. CMe	8·48 CMe
7·71 s. 2ArMe	7·71 ArMe
7·29 m. $3CH_2$	7·22 CH_2
6·67 s. OMe	
6·55 m. CHOMe	
5·39 m. 2CHOAr	5·40 CHOAr
4·20 d. CH=	4·21 CH=
3·65 dd. CH=	3·62 CH=
3·0-3·5 m. 6ArH	3·0-3·4 3ArH

* Purified by t.l.c.

Oxidation of p-cresol with horseradish peroxidase[201]

A solution of p-cresol (0·50 g.) in 0·1M phosphate buffer (50 ml.) was oxidized with peroxidase (2·56 mg.) and hydrogen peroxide (0·3%, 50 ml.) over a period of 30 min. Pummerer's ketone was isolated by chromatography. Yield 24%, p-cresol (28%) was recovered from the aqueous residue.

[2,6-$^2H_2$]-p-Cresol

A mixture of p-cresol(1·19 g., 11 mmole), triethylamine (1·5 ml., 11 mmole) and deuterium oxide (3·0 ml., 140 mmole)

were refluxed for 5 hours (101°). The mixture was acidified with 2N hydrochloric acid (5·5 ml.) and extracted with ether (4 x 30 ml.) to give [2,6-$^2H_2$]-p-cresol. Yield c.a. 100%.

n.m.r. (CCl_4) τ 7·79 s. ArMe
4·08 bd.s. OH
3·43, 3·28, 3·10 ArH-2·18 H, 91% D

Oxidation of [2,6-$^2H_2$]-p-cresol with alkaline ferricyanide

The oxidation of [2,6-$^2H_2$]-p-cresol (1·07 g., 10 mmole) was carried out by the method described for p-cresol. Pummerer's ketone, yield 208 mg. (20%), m.p. 122·5°,

n.m.r. (CCl_4) τ 8·47 s. CMe
7·70 s. ArMe
7·22 m. CH_2 1·83 H, 0·17 D
5·39 m. OCH
4·20 d. =CHCO 0·85 H, 0·15 D
3·64 dd. CH=
3·08 s. ArH 1·95 H, 1·0 D.

[2,4,4-$^2H_3$]Pummerer's ketone

Pummerer's ketone (115 mg., 0·54 mmole) in dry dimethylformamide (0·3 ml.) containing triethylamine (0·05 ml., 0·36 mmole) and deuterium oxide (0·5 ml., 28 mmole) was refluxed for 15 minutes.

N.m.r. ($CDCl_3$) τ 8·44 s. CMe
7·70 s. ArMe
5·32 d. OCH $J_{1,5}$ 1·8 c/s
3·56 bd.s. CH=
2·9-3·3 m. 3ArH

Chromatography of [2,4,4-$^2H_2$]-p-cresol on alumina grade III in benzene gave Pummerer's ketone with 17% deuterium in the 2- and 4-positions.

[2-^{3}H]-p-cresol

A mixture of p-cresol (325mg., 3 mmole), triethylamine (150 mg., 1·5 mmole) and tritiated water (100 mg., 5 mmole) was heated in a Carius tube at 100° for 19 hours. The [2-^{3}H]-p-cresol was extracted with ether, diluted with inactive p-cresol and distilled.

Activity, 4·37 x 10^5 c/s/mmole (99% exchange)[as benzoate]

[2-^{3}H]-p-cresol benzoate

Benzoyl chloride (c.a. 50 mg.) was added to a solution of [2-^{3}]-p-cresol (c.a. 10 mg.) in 2N sodium hydroxide (0·5 ml.) at 5°. After shaking for 5 min. ice/water (5 ml.) was added and the precipitate filtered off, washed with water and recrystallized from petrol. M.p. 70·5-71°

A sample of [2-^{3}H]-p-cresol (c.a. 10 m.g.) was dissolved in 2N sodium hydroxide (0·5 ml.) at 5° and kept for 5 hours before adding benzoyl chloride to give the benzoate.

Activity 4·50 x 10^5 c/s/mmole

2,6-dibromo-p-cresol

A solution of [2-^{3}H]-p-cresol (100 mg.) in ethanol was treated with bromine in ethanol until the colour persisted. Evaporation of the solvent gave a pale yellow oil which was refluxed with petrol to remove the excess bromine. The crystalline residue was extracted with petrol (residue of 2-bromo-p-cresol) to give 2,6-dibromo-p-cresol which was recrystallized from petrol.

Activity, 308 c/s/mmole (0·07%)

Oxidation of [2-^{3}H]-p-cresol with alkaline ferricyanide

Oxidation of [2-^{3}H]-p-cresol (200 mg.) by the same procedure as p-cresol gave Pummerer's ketone (20%).

Extraction of the acidified aqueous residue gave p-cresol.

		relative
Activities,	Original p-cresol $4·46 \times 10^5$ c/s/mmole	[2·00]
	recovered p-cresol $3·89 \times 10^5$	[1·78]
	Pummerer's ketone $2·16 \times 10^5$	[0·99]
	(rechromatograph) $2·04 \times 10^5$	[0·91]

In order to check for exchange, a solution of [2-^{3}H]-p-cresol (20 mg.) in aqueous sodium carbonate (40 mg.) was stirred at 5° for 2½ hours, and the p-cresol reisolated.

Activity, recovered p-cresol $3·86 \times 10^5$ c/s/mmole [1·73]

Oxidation of [2-^{3}H]-p-cresol by peroxidase

A solution of [2-^{3}H]-p-cresol (187 mg.) was oxidized in neutral phosphate buffer with peroxidase and hydrogen peroxide by the same procedure as p-cresol to give Pummerer's ketone (20%).

			relative
Activities,	starting p-cresol	$4{\cdot}58 \times 10^5$ c/s/mmole	[2·00]
	recovered p-cresol	$4{\cdot}65 \times 10^5$	[2·03]
	Pummerer's ketone	$5{\cdot}02 \times 10^5$	[2·19]
	(rechromatograph)	$2{\cdot}51 \times 10^5$	[1·09]

The oxidation of [2-^{3}H]-p-cresol (900 mg.) was repeated. The crude oxidation product was filtered off, washed with water and dissolved in ether (100 ml.). Washing with 5% sodium hydroxide (2 x 50 ml.) and water (2 x 50 ml.) removed the phenols. The residue was chromatographed rapidly on alumina grade V in benzene to give Pummerer's ketone (23%).

Rotation, $[\alpha]_D^{27}$ 0·38° (c 3·5 in $CHCl_3$).

			relative
Activities,	starting p-cresol	$1{\cdot}49 \times 10^5$ c/s/mmole	[2·00]
	Pummerer's ketone	$2{\cdot}59 \times 10^5$	[3·00]
	(rechromatograph)	$0{\cdot}84 \times 10^5$	[0·99]
	(rechromatograph)	$0{\cdot}77 \times 10^5$	[0·90]

[2,4,4-$^3H_3$]Pummerer's ketone

Pummerer's ketone (208 mg., 0·97 mmole) in dry dioxan (2 ml.) containing triethylamine (0·1 ml., 0·7 mmole) and tritiated water (c.a. 25 mg.) was refluxed for 1 hour. The solvent

was evaporated and the residue recrystallized from benzene-petrol.

	relative
Activity 6·73 x 10^6 c/s/mmole.	[1·00]

[2,4,4-$^3H_3$]Pummerer's ketone (50 mg.) was dissolved in ether (50 ml.) and shaken for 1 minute alternately with cold 5% sodium hydroxide (50 ml.) and water (50 ml.). After 6 washings water (50 ml.) was added and the solution kept at 0° for 44 hours. The recovered Pummerer's ketone was recrystallized from benzene-petrol.

Activity 6·21 x 10^6 c/s/mmole.	[0·92]

2-(1'-Methyl-4'-oxocyclohexyl)-4-methylphenol[198]

Pummerer's ketone (4·28 g., 20 mmole) was hydrogenated in sodium ethoxide in ethanol over 5% palladium on charcoal.

Yield 4·36 g. (100%), m.p. 175° from ethanol-water (lit.[198] 175-176°).

I.r. (CCl_4) ν 3600, 3400 bd., 1710, 1611 cm^{-1}
u.v. (EtOH) λ 205·5, 219, 224, 282 mμ
n.m.r.($CDCl_3$) τ 8·58 s. CMe
8·1 m. $2CH_2$
7·72 . ArMe
7·5 m. $2CH_2CO$
4·5 bd. OH
2·6-3·5 m. 3ArH

Bromination of 2-(1'-methyl-4'-oxocyclohexyl)-4-methylphenol

T.l.c. showed a complex mixture of products from the following reactions:-

a) Bromine (1 mole) in acetic acid-sodium acetate.

b) Bromine (1 mole) in carbon tetrachloride.

c) Bromine (1 mole) in ethanol.

d) Bromine (1 mole) in ethanol-sodium ethoxide.

Excess of these reagents also gave complex products.

2-(1'-Methyl-4',4'-ethylenedioxycyclohexyl)-4-methylphenol

2-(1'-methyl-4'-oxocyclohexyl)-4-methylphenol (101 mg., 0·47 mmole) in dry benzene (30 ml) containing 1,2-dihydroxyethane (93 mg. 3·2 mmole) and p-toluenesulphonic acid (c.a. 5 mg.) was refluxed until the i.r. spectrum showed no ketone absorption ($\frac{1}{2}$ hr.).

I.r. (CCl_4) ν 3610, 3400 bd., 1616, 1603 cm^{-1}.

Without isolating the ketal, bromine (75 mg., 0·47 mmole) in benzene was added and the solution refluxed for 3 min. The residue (64 mg.) after evaporating the benzene was chromatographed on alumina grade III in benzene.

Benzene eluate- 29 mg. (45%)- probably the 6-bromo-derivative

i.r. (CCl_4) ν 3510, 1604 cm^{-1}
u.v. (EtOH) λ 206, 284 mμ.

Ethyl acetate eluate- 26 mg. (40%).

i.r. (CCl_4) ν 3600, 3420 bd., 1618, 1603 cm^{-1}
u.v. (EtOH) λ 226, 282 mμ.

2-(1'-Methyl-4'-oxocyclohexyl)-4-methyl[6-^{3}H]phenol and exchange.

[6-^{3}H]Pummerer's ketone (10 mg.)[from the oxidation of [6-^{3}H]-p-cresol - page 116, second experiment] was hydrogenated in ethanol (5 ml.) containing sodium (11·5 mg.) over 5% palladium on charcoal (2 mg.).

Yield 9·3 mg.

This keto-phenol (7·9 mg. 36 μmole) was heated with water (1·0 ml., 50 mmole) and triethylamine (1·8 mg., 18 μmole) at 100° for 20 hours in a Carius tube. The product was extracted with dichloromethane (5 x 5 ml.) and purified by t.l.c. on silica gel (2 x 20^{2} cm.^{2} plates), eluting with 15% ethyl acetate-benzene. The keto-phenol fraction was scraped off and eluted with methylene chloride.

Yield 3·7 mg., m.p. 172-174°

		relative
Activity, Pummerer's ketone	0·65 x 10^{5} c/s/mmole	[1·00]
Keto-phenol	0·56 x 10^{5}	[0·86]

The exchange was repeated at 118° for 3 days using keto-phenol (1·4 mg., 6·5 μmole), triethylamine (0·50 mg., 5 μmole), water (0·5 ml. 25 mmole) and dimethylformamide (0·1 ml.)

Yield 0·4 mg., after t.l.c. on silica gel.

Activity, Keto-phenol	0·022 x 10^{5} c/s/mmole	[0·03]

Preparation and Reactions of Indolyl-quinones.

o-Benzoquinone

Silver oxide oxidation of catechol in anhydrous ether containing sodium sulphate[93,241] proved unsuccessful, so periodate oxidation was used.[207]

Catechol (300 mg., 2·7 mmole) in water (15 ml.) at 0° was oxidized with a solution of sodium metaperiodate (1·05 g., 4·9 mmole) in water (7·5 ml.). The red solution was immediately extracted with dichloromethane (3 x 10 ml.), and the extract dried and evaporated at 0° to 2 ml. Addition of petrol precipitated o-benzoquinone as a red powder.

Yield, 290 mg. (95%)

4-Methyl-o-benzoquinone[93]

Vanillin (7·60 g., 50 mmole) in absolute alcohol (50 ml.) containing 6N hydrochloric acid (0·3 ml.) was hydrogenolyzed over 10% palladium on charcoal (100 mg.). Hydrogen (100 mmole) was rapidly absorbed. The solution was filtered and evaporated to give 2-methoxy-4-methylphenol (creosol).

Yield, 6·60g. (96%), n^{25} 1·5361 (lit.[242] n_D^{25} 1·5354)

n.m.r. (CCl_4) τ 7·78 s. ArMe
6·24 s. OMe
4·65 bd.s. OH
3·48 d. 5-H $J_{5,6}$ 8·1 c/s
3·45 s. 3-H
3·31 d. 6-H

Creosol (414 mg., 3 mmole) in dry ether (5 ml.) was oxidized with sodium metaperiodate and the quinone extracted with dichloromethane. Precipitation with petrol gave a pale yellow solid, 4-methyl-o-benzoquinone.

Yield, 350 mg. (95%), m.p. 62-67° (lit.[207] 65-67°)
i.r. ($CHCl_3$) ν 1737, 1720, 1692, 1665, 1636 cm^{-1}
u.v. (EtOH) λ 210, 254 sh., 390 mμ.

4-(2'-Methylindol-3'-yl)-o-benzoquinone

Addition of o-benzoquinone in 1:1 ethanol-water to 2-methylindole in 1:1 ethanol-water gave an almost black precipitate of the indolyl-quinone.[93]

An alternative method gave a purer product, A solution of catechol (0·70 g., 6·4 mmole) and 2-methylindole (0·66 g., 5·1 mmole) in dioxan (25 ml.) was oxidized by sodium metaperiodate (2·20 g., 10·3 mmole) in water (25 ml.). The precipitate of indolyl-quinone was filtered off, washed with hot water and ether, and dried.

U.v. (EtOH) λ 222, 280, 286, 394, 581 mμ
(Dioxan) λ 224, 281, 288, 360, 510 mμ.

4-(Indol-3'-yl)-o-benzoquinone

Indole reacted with o-benzoquinone in aqueous ethanol to give the indolyl-quinone.[93]

U.v. (EtOH) λ 218, 281, 288, 351 sh., 519 mμ.

4-(Indol-3'-yl)-5-methyl-o-benzoquinone

Indole reacted with 4-methyl-o-benzoquinone in aqueous ethanol to give the indolyl-quinone.[93]

Oxidation of a solution of indole (1·17 g., 10 mmole) and creosol (1·38 g., 10 mmole) in dioxan (25 ml.) with sodium metaperiodate (4·28 g., 20 mmole) in water (70 ml.) at 0° gave a blue-black precipitate of the indolyl-quinone after dilution with water (200 ml.). This was filtered off, washed with hot water, and extracted with ether until the filtrate was colourless.

Yield 0·97 g. (41%)

i.r. (Nujol) ν 3240, 1668 w., 1616, 1610 cm^{-1},

u.v. (EtOH) λ c.a.200, 284, 290, 390, 553 mμ.

3-(3',4'-Dihydroxyphenyl)-2-methylindole

A solution of 4-(2'-methylindol-3'-yl)-o-benzoquinone (50 mg.) in dioxane (5 ml.) was reduced by sodium dithionite (100 mg.) in water (5 ml.). The intense purple colour of the indolyl-quinone was rapidly discharged to leave a pale yellow solution which was concentrated *in vacuo* and extracted with chloroform (3 x 10 ml.). The extract was dried, evaporated to dryness, and the residue vacuum sublimed (140°, 5 x 10^{-5} mm. Hg)

Yield 20 mg. (40%), m.p. 169°

i.r. ($CHCl_3$) ν 3540, 3470, 3300 bd., 1718 w., 1605 cm^{-1}
u.v. (EtOH) λ 228, 263, c.a. 290 mμ

n.m.r. (Pyridine) τ 7·43 s. ArMe
(D.M.S.O.) τ 2·3-3·3 m. 7ArH
1·2 bd.s. 2OH (exchanged by D_2O)
-0·95 bd.s. NH (exchanged by D_2O)

3-(3',4'-Dihydroxy-6'-methylphenyl)indole

4-(Indol-3'-yl)-5-methyl-o-benzoquinone (100 mg.) was reduced with sodium dithionite in aqueous dioxan or aqueous acetone and the product vacuum sublimed (150°, 5 x 10^{-5} mm. Hg).

Yield, 86 mg. (85%),

i.r. ($CHCl_3$) ν 3550, 3480, 3320 bd., 1716 w., 1606 cm^{-1}
u.v. (EtOAc) λ 254, 282 mμ
n.m.r. ($CDCl_3$) τ 7·87 s. ArMe
4·9 bd.s. 2OH (exchanged by D_2O)
3·23 s. 5'-H
3·17 s. 2'-H
2·5-3·1 m. 5ArH
2·0 bd.s. NH (exchanged by D_2O)

[6-^{2}H]Creosol

Creosol (0·62 g., 4·5 mmole) was refluxed with deuterium oxide (1·0 g., 50 mmole) containing triethylamine (0·5 g., 5 mmole) for 7 hours under nitrogen (96°). The mixture was acidified with 2N hydrochloric acid (3 ml.) and extracted with ether (3 x 5 ml.). N.m.r. of the product (c.a. 100%) showed that it contained only 0·50 deuterium.

The deuteration was repeated with refluxing for 60 hours.

N.m.r. (CCl_4) τ 7·78 s. ArMe
6·21 s. OMe
4·8 bd.s. OH (exchanged by D_2O)
3·48 s. 3,5-H_2, 1·82 H 1·0 D
i.r. (liq.) 3560 (OH), 2650 (OD), 1608 cm^{-1}.

4-(Indol-3'-yl)-5-methyl[3-^{2}H]-o-benzoquinone

Indole and [6-^{2}H]creosol (166 mg., 1·2 mmole) were oxidized by sodium meta-periodate in aqueous dioxan.

Yield 185 mg. (65%).

3-(3',4'-Dihydroxy-6'-methyl[2'-^{2}H]phenyl)indole

4-(Indol-3'-yl)-5-methyl[3-^{2}H]-o-benzoquinone (170 mg.) in acetone (12 g.) was reduced by sodium dithionite (400 mg.) in water (16 ml.) at 0°. The acetone was evaporated and the solution extracted with chloroform (3 x 10 ml.). The extract was dried, evaporated and the residue vacuum sublimed (160°, 10^{-4} mm. Hg).

N.m.r. ($CDCl_3$) τ 7·88 s. ArMe
5·0 bd.s. OH, 1·7 H, 0·3 D
3·22 s. 5'-H, 1·04 H
3·16 s. 2'-H, 0·62 H, 0·38 D
2·4-3·1 m. 5ArH
2·0 bd.s. NH 0·66 H, 0·34 D.

[O,O,N-$^2H_3$]-3-(3',4'-Dihydroxy-6'-methylphenyl)indole

3-(3',4'-Dihydroxy-6'-methylphenyl)indole (96 mg., 0·4 mmole) in dry dioxan containing deuterium oxide (0·3 ml.) was warmed for 10 min. then evaporated. The trideutero-derivative was vacuum sublimed (150°, 10^{-4} mm. Hg).

N.m.r. ($CDCl_3$) τ 7·91 s. ArMe
5·0 bd.s. OH 0·71 H, 1·29 D,
2·4-3·4 m. ArH 6·1 H, 0·9 D,
2·0 bd.s. NH 0·47 H, 0·53 D.

Methylation of 3-(3',4'-dihydroxy-6'-methylphenyl)indole

3-(3',4'-Dihydroxy-6'-methylphenyl)indole (96 mg., 0·4 mmole) in dry dimethylformamide (5 ml.) was treated under nitrogen with a 53% dispersion of sodium hydride in mineral oil (72 mg., 1·6 mmole) and methyl iodide (142 mg. 1·2 mmole) in dry dimethylformamide (5 ml.). After 30 min. water (50 ml.) was added and the solution neutralized with 6N hydrochloric acid. The precipitate was filtered off, washed with water, dried, dissolved in benzene and chromatographed on alumina grade I.

Benzene eluate- 13 mg. (12%)- 3-(3',4'-dimethoxy-6'-methyl-phenyl)-1-methylindole. M.p. 105-111°, vacuum sublimed (100°, 7 x 10^{-5} mm. Hg) m.p. 113-114°.

i.r. (CCl_4) ν 1719 w., 1610 cm^{-1}
u.v. (EtOH) λ 224, 259 sh., 284, 291 sh. mμ

n.m.r. (CCl_4) τ 7·82 s. ArMe
6·27 s. } 2OMe
6·24 s. }
6·22 s. } NMe
2·6-3·4 m. 7ArH.

Analysis, found: C, 77·1; H, 6·9; N, 5·0.
$C_{18}H_{19}NO_2$ required: C, 76·8; H, 6·8; N, 5·0%.

15% Ethyl acetate-benzene eluate- 35 mg. (32%)-
3-(3',4'-dimethoxy-6'-methylphenyl)indole.
M.p. 156-160°, vacuum sublimed (120°, 6 x 10^{-5} mm. Hg)
m.p. 163-164°,

i.r. (CCl_4) ν 3490, 1720 w., 1614 cm^{-1}
u.v. (EtOH) λ 225, 259 sh., 292 mμ
n.m.r. ($CDCl_3$) τ 7·73 s. ArMe
6·13 s. OMe
6·07 s. OMe
2·3-3·2 s. 7ArH
1·65 bd.s. NH.

Analysis, found: C, 76·6; H, 6·4; N, 5·3.
$C_{17}H_{17}NO_2$ required: C, 76·4; H, 6·4; N, 5·2%.

Alternative procedure.

3-(3',4'-Dihydroxy-6'-methylphenyl)indole (50 mg.) in ether was treated with excess ethereal diazomethane. After 20 hr. the solvent was evaporated and the residue chromatographed on alumina grade III (5 g.).

Benzene eluate- 7·5 mg. (13%)- 3-(3',4'-dimethoxy-6'-methyl-phenyl)-indole.

I.r. (CCl_4) ν 3480, 1727 w., 1610 cm^{-1}.

20% ethyl acetate-benzene eluate- 17 mg. (32%)-
3-(3'-hydroxy-4'-methoxy-6'-methylphenyl)indole or
3-(4'-hydroxy-3'-methoxy-6'-methylphenyl)indole.
I.r. (CCl_4) ν 3540, 3470, 1727 w., 1610 cm^{-1}.

[5-^{3}H]Vanillin[214]

Alkaline exchange of vanillin in the presence of tritiated water gave [5-^{3}H]vanillin.

Relative

Activity, 1·08 x 10^5 c/s/mmole. [1·00]

[6-^{3}H]Creosol

[5-^{3}H]Vanillin (921 mg., 6 mmole) was hydrogenolyzed in ethanol (20 ml.), containing a trace of 6N hydrochloric acid, over 5% palladium on charcoal (50 mg.).

Yield, 773 mg. (96%).

Activity, 1·10 x 10^5 c/s/mmole. [1·01]

4-(Indol-3'-yl)-5-methyl[3-^{3}H]-o-benzoquinone

[6-^{3}H]Creosol (276 mg., 2 mmole) was oxidized by sodium metaperiodate in the presence of indole in aqueous dioxan at 0°. The precipitate was extracted with ether for 2 hr.

Yield, 149 mg. (31%).

3-(3',4'-Dihydroxy-6'-methyl[2'-^{3}H]phenyl)indole

4-(Indol-3'-yl)-5-methyl[3-^{3}H]-o-benzoquinone (119 mg., 0·5 mmole) in acetone (9·5 ml.) was reduced by sodium dithionite (300 mg., 1·7 mmole) in water (10 ml.) at 0°. The acetone was evaporated at room temperature and the aqueous solution extracted with chloroform (3 x 20 ml.). The extract was dried and evaporated in the absence of oxygen.

Methylation of 3-(3',4'-dihydroxy-6'-methyl[2'-^{3}H]phenyl)indole

The crude indolyl-catechol from the preceding reaction was methylated with methyl iodide and sodium hydride in dry dimethylformamide. The products were separated by chromatography and purified by vacuum sublimation.

3-(3',4'-Dimethoxy-6'-methyl[2'-^{3}H]phenyl)-1-methylindole

Yield, 63 mg. (50%)

Activity, (crystallized) 0·96 x 10^{5} c/s/mmole
(vacuum sublimed) 0·94 x 10^{5} c/s/mmole. [0·86]

3-(3',4'-Dimethoxy-6'-methyl[2'-^{3}H]phenyl)indole

Yield, 33 mg. (28%)

Activity, (crystallized) 0·89 x 10^{5} c/s/mmole
(vacuum sublimed) 0·95 x 10^{5} c/s/mmole. [0·88]

5-Bromo[6-^{3}H]creosol

[6-^{3}H]Creosol was brominated at room temperature with excess bromine in carbon tetrachloride.

M.p. 67° (from petrol).

Activity, 0·97 x 10^5 c/s/mmole. [0·87]

5-Bromovanillin

[5-^{3}H]Vanillin was brominated at room temperature with bromine (1 mole) in acetic acid-sodium acetate.

Activity, 340 c/s/mmole [0·0034]

7b,14b-Dimethyl-5a,7b,12a,14b-tetrahydrobisindolo[2,3-b;2',3'-b']-benzo[1,2-d;4,5-d']difuran[93]

3-Methylindole (1·31 g., 10 mmole) reacted with p-benzoquinone (1·08 g., 10 mmole) in glacial acetic acid (10 ml.) to give a pale pink precipitate.

Yield, 0·62 g. (30%).
M.p., 250° (nitrobenzene) (lit., 250-252°[93], 255-256°[213]),
i.r. (nujol) ν 3420, 1610 cm^{-1}
u.v. (EtOH) λ 206 mμ (ε 16,200)
225infl (ε 6800)
318 (ε 4150)
n.m.r. (CF_3CO_2H) τ 8·10 s. CMe
7·92 s. CMe
5·90 s. CH
2·2-2·5, 2·90, 3·13 m. 10 ArH.

Acetate, m.p. 385-387° (decomp.) (lit.,[213] 414-416°)

i.r. (nujol) ν 1679, 1602 cm^{-1}

u.v. (EtOH) λ 235, 308 mμ.

2,5-di(3'-Methylindol-2'-yl)hydroquinone

Vacuum sublimation (200-250°, 1 x 10^{-4} mm. Hg) of the dimethyltetrahydrobisindolobenzodifuran gave a pale yellow solid.

M.p. 270-292° (decomp.) (lit.,[213] 281-285°)

i.r. (nujol) ν 3480, 1567 cm^{-1}

u.v. (EtOH) λ 227·5 mμ (ε 46,700)
251infl. (ε 19,500)
307 (ε 16,000)
353 (ε 26,000)

n.m.r. (D.M.S.O.) τ 2·3-3·1 m. 11·4ArH*
0·75 s. 2OH (exchanged by D_2O)
-0·80 s. 2NH (exchanged by D_2O)

* Inaccurate integral.

Acetate, m.p. 266-267°

i.r. ($CHCl_3$) 3400, 1742, 1580 w., 1567w. cm^{-1}.

5a,10b-Dihydro-2-hydroxy-10b-methylindolo[2,3-b]benzo[d]furan

3-Methylindole (0·65 g., 5 mmole) and p-benzoquinone (0·54 g., 5 mmole) were dissolved in acetone and treated with acetic acid (0·1 ml.). After 12 hr. at room temperature no reaction had occurred so 6N hydrochloric acid (0·2 ml.)

was added. After 2 hr. the acetone was evaporated and the products extracted into chloroform and chromatographed on alumina grade III in benzene. The main fraction (chloroform eluate) was rechromatographed on silica gel (Hopkin and Williams, M.F.C.) in benzene. The product contained a trace of impurity which was removed by rechromatography on alumina grade III. The product was an unstable oil which failed to crystallize.

I.r. ($CHCl_3$) ν 3630, 3430 bd., 1782, 1609 cm^{-1}

u.v. (EtOH) λ 226, 316 mμ

n.m.r. ($CDCl_3$) τ 8·55 s. CMe
4·97 bd.s., OH, NH (exchanged by D_2O)
4·12 s. OCHN
2·9-3·7 m. 7ArH.

2-(3'-Methylindol-2'-yl)hydroquinone

Dihydrohydroxymethylindolobenzofuran isomerized slowly at room temperature. Vacuum sublimation (135°, 1 x 10^{-4} mm. Hg) also gave pale yellow crystals of the isomer.

M.p. 173·5-174°

i.r. (nujol) ν 3510, 3400, 1617 cm^{-1}

u.v. (EtOH) λ 226 mμ (ε 37,000)
244infl.(ε 16,000)
304infl.(ε 19,500)
321 (ε 10,200)

n.m.r. (D.M.S.O.) τ 7·82 s. CMe
2·4-3·3 m. 7ArH
1·18,1·27 s. 2OH
-0·61 s. NH

Acetate,

i.r. ($CHCl_3$) ν 1757 cm^{-1}

u.v. (EtOH) λ 222 mμ (ε 15,700)
238infl. (ε 10,700)
300 (ε 6740).

Quenching

The efficiency of the tritium standard was determined in the presence of inactive Pummerer's ketone.

Pummerer's ketone 0 mg.	Efficiency 20·68%
0·36	20·10
0·57	19·59
1·05	18·60
1·22	18·18
2·34	16·02

Hence Q = 0·0491

Other values of Q are tabulated on page 73.

3,4-Dihydroxyphenyl[β-$^2H_2$]alanine - 1

3,4-Dimethoxyphenylmorpholineacetonitrile

Veratraldehyde (1·66 g., 10 mmole) and morpholine perchlorate (prepared from morpholine and 60% perchloric acid in ether) (2·06 g., 11 mmole) were dissolved in morpholine (10 ml.). After 1 hr. a solution of sodium cyanide (0·53 g., 11 mmole) in water (1·5 ml.) was added and the reaction mixture warmed to 80° for 2 hr., then cooled and poured into ice/water (200 ml.). The precipitate was filtered off (2·04 g., 78%) and the aqueous solution extracted with chloroform to give more product (0·47 g., 18%). The morpholino-nitrile was recrystallized from iso-propyl ether-petrol.

Yield 2·36 g. (90%), m.p. 96-96·5° (lit.,[242] 105-107°)
i.r. ($CHCl_3$) ν 1597 cm^{-1}
u.v. (EtOH) λ 234, 279, 284 infl. mμ
n.m.r. ($CDCl_3$) 7·35-7·55 m. $2NCH_2$
6·2-6·4 m. $2OCH_2$
6·10 s. 2OMe
5·24 s. α-CH
2·9-3·2 m. 3ArH.

3,4-Dimethoxyphenylmorpholine[α-^2H]acetonitrile

3,4-Dimethoxyphenylmorpholineacetonitrile (5·24 g., 20 mmole) was dissolved in dry dimethylformamide (distilled with benzene and stored over calcium hydride) (25 ml.) under dry oxygen-free nitrogen and treated with a 53% suspension of sodium

hydride in mineral oil (2·72 g., 60 mmole). After 4 hr. at room temperature deuterium oxide (2·0 ml., 110 mmole) was added and after 1 hr. ice/water (400 ml.) and extracted with chloroform (3 x 100 ml.). The extract was washed with water and evaporated to dryness. The residue was recrystallized from iso-propyl ether-petrol.

Yield 4·92 g. (94%)

i.r. (CCl_4) ν 1597 cm^{-1}

n.m.r. ($CDCl_3$) 99·0% D.

Oxidation of 3,4-dimethoxyphenylmorpholineacetonitrile

3,4-Dimethoxyphenylmorpholineacetonitrile (262 mg., 1 mmole) was treated with 53% sodium hydride (270 mg., 6 mmole) in dry dimethylformamide (10 ml.), under nitrogen, air or oxygen. After 2 hr. the reaction mixture was diluted with water and the products separated on alumina grade V. Two main fractions were obtained:

A. Benzene eluate- 3,4-dimethoxyphenylmorpholineacetonitrile
B. Ethyl acetate eluate- 3,4-dimethoxybenzoyl morpholine

1. Under nitrogen	A. 86%	B. 14%
2. under air	69%	31%
3. under oxygen	0	95%

The structure of the oxidation product was determined from its physical and chemical properties.

M.p. c.a. 0°, n^{23} 1·5658

i.r. (liq.) 3500 bd. 1630 cm^{-1}, (CCl_4) 1642 cm^{-1}

u.v. (EtOH) λ 251, 282 mμ

n.m.r. ($CDCl_3$) τ 6·35 s. $2OCH_2$ + $2NCH_2$
6·13 s. 2OMe
2·9-3·1 m. 3ArH.

Analysis, found: C, 59·9; H, 7·2; N, 5·3; M, 266.

$C_{13}H_{17}NO_4$ required: C, 61·7; H, 6·8; N, 5·6%; M, 251.

Hydrolysis with aqueous ethanolic hydrochloric acid gave ethyl veratrate (chromatographed on grade V alumina in benzene).

i.r. (liq) ν 1709 cm^{-1}

u.v. (EtOH) λ 220, 262, 282 mμ

n.m.r. ($CDCl_3$) τ 8·62 t. $C\underline{H}_3CH_2$ J 7·1 c/s
6·09 s. 2OMe
5·67 q. $MeC\underline{H}_2$ J 7·1 c/s
3·13 d. 5-ArH
2·47 d. 2-ArH } $J_{5,6}$ 8·2 c/s
2·32 d,d. 6-ArH } $J_{2,6}$ 1·8 c/s

Hydrolysis with 4N sodium hydroxide in ethanol gave veratric acid (m.p. and mixed m.p. 185°).

3,4-Dimethoxybenzoyl morpholine (c.f. benzoyl morpholine[243])

Veratric acid (200 mg.) was chlorinated with thionyl chloride (3 ml.). Veratroyl chloride (200 mg., 1 mmole), triethylamine (0·25 ml., 2·5 mmole), morpholine (110 mg., 1·3 mmole), and dry benzene (3 ml.) were refluxed for 2 hr. The solution was filtered and evaporated to dryness to give a clear oil. I.r., u.v. and t.l.c. were similar to those observed for the oxidation product of 3,4-dimethoxyphenylmorpholineacetonitrile. $n^{20·5}$ 1·5530.

[α-^{2}H]Veratraldehyde

3,4-Dimethoxyphenylmorpholineacetonitrile (2·63 g., 10 mmole) was hydrolyzed in refluxing 2N hydrochloric acid (50 ml.) for 1 hr.

Yield, 1·67 g. (100%), recrystallizedfrom iso-propyl ether petrol.

I.r. (CCl_4) ν 2105, 2090, 2050, 1683 cm^{-1} (Perkin-Elmer 257)

n.m.r. (CCl_4) τ 6·06 s. OMe
6·08 s. OMe
3·10 d. 5-ArH
2·66 d. 2-ArH } $J_{5,6}$ 8·7 c/s
2·60 d,d. 6-ArH } $J_{2,6}$ 1·7 c/s
0·20 s. CHO (99·0% D)

m.s. (hot-box 75°) [on a different sample, 95% D (n.m.r.)]

m/e				m/e	
169	1·1%			97	17%
168	8·2			96	27
167	100	$ArCDO^+$	94% D	83	10
166	9·7	$ArCHO^+$		81	14
165	35	$ArCO^+$		79	14
152	20			78	13
111	27			77	12
101	13			69	39

Hydantoin[244]

Hydantoin was prepared from glycine (15·2 g., 0·2 mole) and potassium cyanate (20 g., 0·25 mole).

Yield 13·9 g. (69%), m.p. 220-221° (lit.,[244] 220°).

I.r. (nujol) ν 3300, 3160, 3070, 1696 bd., 1669 cm^{-1}

u.v.(EtOH) λ c a. 203 mμ
(0·1 N NaOEt) λ 222 mμ (ε 4950).

Veratralhydantoin[196,197]

Veratraldehyde (2·00 g., 12 mmole), hydantoin (1·8 g., 18 mmole) and anhydrous sodium acetate (1·80 g., 22 mmole) were refluxed in acetic anhydride (18 ml., 320 mmole) for 1½ hr. (c a. 140°) under dry nitrogen. The hot solution was poured into hot water (120 ml.) and warmed for 10 min., then allowed to stand for 2 hr.

Yield 2·11 g. (71%), m.p. 227-236°, 233-236° (from ethanol) (lit. 204-206°[196], 176-186°[197]).

I.r. ($CHCl_3$) ν 3250, 1790, 1739, 1709 cm^{-1}
(nujol) ν 3420, 3190, 1775, 1711 cm^{-1}
u.v. (EtOH) λ 206, 243 infl., 345 mμ.

Veratralhydantoin- alternative method (c.f. benzalhydantoin[215])

Veratraldehyde (0·83 g., 5 mmole), hydantoin (0·5 g., 5 mmole), pyridine (0·8 ml., 10 mmole) and diethylamine (0·5 ml., 5 mmole) were refluxed (c.a. 100°) for 8 hr. under nitrogen. Water (5 ml.) was added and the precipitate filtered, washed with water and dried.

Yield 0·30 g. (24%), m.p. 274-276°.

Veratrylhydantoin

a) Veratralhydantoin (312 mg., 1·25 mmole) in dioxan (20 ml.) and water (0·5 ml.) was reduced with 3% sodium amalgam[245] (10 g., 13 mmole) over 1 hr. The solution was decanted from the mercury, diluted with water (30 ml.) and neutralized with 6N hydrochloric acid. After decolorizing with activated charcoal the solution was evaporated to dryness. The residue was extracted with hot ethanol (3 x 20 ml.). The residue from this extraction was re-extracted with hot ethyl acetate.

Yield, 255 mg. (81%)

b) Veratralhydantoin (312 mg.) was dissolved in acetic acid (2·0 g., 33 mmole) and dioxan (10 ml.) and reduced with sodium amalgam. The reaction was very slow and little was reduced after 1 week at room temperature.

c) Reduction of veratralhydantoin (312 mg.) was also slow in acetic acid (2·0 g.) and acetic anhydride (10 ml.)

d) Veratralhydantoin (312 mg.) was reduced in water (1·0 ml, 54 mmole) and dioxan (5 ml.) at 75° with sodium amalgam (10 g.). The reduction was complete in 2 hrs. and the product was isolated as in (a).

Yield 282 mg. (90%), m.p. 205-210° (lit., 164°[196], 148-153°[197]).

I.r. ($CHCl_3$) ν 3400 bd., 1722, 1602 cm^{-1}

(nujol) ν 3280 bd., 1772, 1705 cm^{-1}

u.v. (EtOH) λ 280, 231 mμ

n.m.r. (CF_3CO_2H) τ 6·7 bd,m. CH_2
6·07 s. 2OMe
5·4 bd,m. CH
2·95-3·15 m. 3ArH.

3,4-Dimethoxyphenylalanine[196,197]

Veratrylhydantoin (280 mg., 1·1 mmole) was refluxed for 9 hr. with a solution of barium hydroxide (5·0 g.) in water (10 ml.). Water (10 ml.) was added and the hot solution exactly neutralized with 6N sulphuric acid. The precipitate was filtered off and washed with hot water. The filtrate was evaporated to dryness.

Yield, 298 mg.

I.r. (nujol) ν 3380, 3190, 1591 bd. cm^{-1}
u.v. (EtOH) λ 278 mμ.

3,4-Dihydroxyphenylalanine[197]

3,4-Dimethoxyphenylalanine was refluxed for 3 hr. under nitrogen with 55% hydriodic acid (4 ml.) containing red phosphorous (10 mg.). The solution was filtered and evaporated and the residue dissolved in water (5 ml.) and re-evaporated. The residue, in water (2 ml.), was neutralized with concentrated aqueous ammonia and evaporated until crystallization started. After standing at 0° overnight the product was filtered off, washed with cold water (0·5 ml.) and acetone (5 ml.).

Yield, 85 mg. (39%, based on veratrylhydantoin)

3,4-Dihydroxyphenylalanine ethyl ester

3,4-Dihydroxyphenylalanine (5 mg.) was refluxed with ethanolic hydrochloric acid for 4 hr., then evaporated to dryness and neutralized with ammonia. Extraction with chloroform gave the ester.

M.s. (probe 200°)

m/e				m/e		
225	8%	M^+		103	42%	
152	60	$ArCH_2CH=NH_2^+$		102	57	$H_2N=CHCO_2Et^+$
124	38			79	22	
123	100	$ArCH_2^+$		78	23	
107	29			77	54	
106	14			74	75	$H_2N=CHCO_2H^+$ (m* 102-74).

[α-^{2}H]Veratryl[5-^{2}H]hydantoin

Veratralhydantoin (248 mg., 1 mmole) in dioxan (10 ml) containing deuterium oxide (0·2 ml., 12 mmole) was heated to exchange the OH and NH groups, then evaporated to dryness.

The residue was dissolved in dioxan (4 ml.) and deuterium oxide (0·85 ml., 47 mmole) at 80° and treated with 3% sodium amalgam (5 g., 6·5 mmole) over 1 hr. allowing the temperature to drop to 50°. The solution was stirred at 45° for 1 hr, then cooled, decanted and neutralized as described above.

Yield, 181 mg. (73%)

i.r. (nujol) 3280 bd., 1719 cm^{-1}.

3,4-Dimethoxyphenyl[β-^{2}H]alanine

[α-^{2}H]Veratryl[5-^{2}H]hydantoin (170 mg., 0·7 mmole) was hydrolyzed with barium hydroxide as described above.

Yield, 172 mg.

i.r. (nujol) ν 3400 bd., 1591 bd. cm^{-1}

u.v. (EtOH) λ 199, 229, 278 mμ

n.m.r. (D_2O/DCl) τ 6·83 t. CHD J 6·2 c/s (i.e. $J_{\alpha,\beta} \approx J_{H,D}$)
6·15 s. OMe
6·13 s. OMe
5·65 d. CH J 6·3 c/s
3·10 bd. 3ArH.

3,4-Dihydroxyphenyl[β-^{2}H]alanine ethyl ester

3,4-Dihydroxyphenyl[β-^{2}H]alanine (144 mg.) was hydrolyzed with 55% hydriodic acid as described above.

The product was refluxed with ethanolic hydrochloric acid to give the ethyl ester.

Yield, 106 mg. (80%, based on [α-^{2}H]veratryl[5-^{2}H]hydantoin)

m.s. (probe 110°)

m/e				m/e		
227	5·9%	M^+D_2	β-96% D_1 α- 9% D_1	125	53%	
226	27	M^+D_1		124	100	$ArCHD^+$
225	1·2	M^+D_0		108	13	
154	10			103	94	
153	70	$ArCHDCH{=}NH_2^+$		102	98	$H_2N{=}CHCO_2Et^+$
152	11			74	88	$H_2N{=}CHCO_2H^+$ (m*103-74, 102-74)
137	17			61	33.	

Condensation of [α-^{2}H]veratraldehyde with hydantoin

[α-^{2}H]Veratraldehyde (167 mg., 1 mmole), hydantoin (150 mg., 1·5 mmole) and anhydrous sodium acetate (166 mg., 2 mmole) were refluxed in acetic anhydride (3 ml.) for 2 hr. and the product isolated as described above.

M.s. (probe 200°)

m/e				
291	18%	$M+Ac^+D_1$		
290	10	$M+Ac^+D_0$		
251	2·2			
250	16			
249	100	M^+D_1	(m* 291-249)	53% D i.e. 66% retention
248	77	M^+D_0	(m* 290-248)	53% D i.e. 66% retention
234	11	$M-Me^+D_1$	(m* 249-234)	
233	8	$M-Me^+D_0$	(m* 248-233)	
178	12	$M-CONHCO^+D_1$	(m* 249-178)	
177	13	$M-CONHCO^+D_0$	(m* 248-177)	
163	15	$M-Me-CONHCO^+D_1$	(m* 178-163, 234-163)	
162	11	$M-Me-CONHCO^+D_0$	(m* 177-162, 233-162)	
132	5			
131	5			
91	5			
90	5			
43	60			
42	5			

3,4-Dimethoxyphenylmorpholine[α-^{3}H]acetonitrile

Prepared from 3,4-dimethoxyphenylmorpholineacetonitrile (262 mg.) using sodium hydride and tritiated water by a method similar to that described above.

Activity, 1·57 x 10^7 c/s/mmole (97% incorporation)

[α-^{3}H]Veratraldehyde

3,4-Dimethoxyphenylmorpholine[α-^{3}H]acetonitrile (26·2 mg., 100 μmole) was hydrolyzed in aqueous ethanolic hydrochloric acid and the [α-^{3}H]veratraldehyde diluted with veratraldehyde (403 mg.) and recrystallized from iso-propyl ether-petrol(40-60).

Activity, 4·10 x 10^5 c/s/mmole, Relative activity [1·00]

Condensation of [α-^{3}H]veratraldehyde with hydantoin

[α-^{3}H]Veratraldehyde (166 mg., 1 mmole) was condensed with hydantoin in refluxing acetic anhydride containing sodium acetate and samples were taken at intervals and the veratral-hydantoin isolated. The products were recrystallized from acetone-water, acetone, and methanol.

Activities,	10 min.	4·1 x 10^5 c/s/mmole	[1·00]
	20	3·9 x 10^5	[0·95]
	40	3·6 x 10^5	[0·88]
	65	3·7 x 10^5	[0·90]
	115	3·0 x 10^5	[0·73]
	160	2·3 x 10^5	[0·56]
	240	2·5 x 10^5	[0·61]
	300	2·0 x 10^5	[0·49]

Condensation of [α-^{3}H]veratraldehyde with hydantoin- 2

[α-^{3}H]Veratraldehyde (83 mg., 0·5 mmole), hydantoin (50 mg., 0·5 mmole), pyridine (0·10 ml.) and diethylamine

(0·06 ml.) were heated in a Carius tube at 100° for 22 hr.

Yield, 5 mg. (4%)

Activity, 2·48 x 10^5 c/s/mmole [0·61]

[1,3,5,5-$^2H_4$]Hydantoin

Hydantoin (2·25 g., 22·5 mmole) in dry dimethylformamide (10 ml.) at 100° was treated with small portions of deuterium oxide (12 x 0·1 ml., 66 mmole) in dimethylformamide (5 ml.) acidified with thionyl chloride (100 mg.). Each fraction was equilibrated for 5 min., then the reaction mixture was heated to distil off the water, before adding more deuterium oxide. When the deuteration was complete the solution was evaporated to dryness.

N.m.r. (D.M.S.O.) [tetrachlorobenzene as reference for integral]

τ 6·52 m. CHD c.a. 88% D

0·1 bd. NH c.a. 95% D

[α-^{2}H]Veratralhydantoin

[α-^{2}H]Veratraldehyde (2·078 g., 12·5 mmole) and [$^2H_4$]hydantoin (2·00 g., 20 mmole) were refluxed in acetic anhydride (dried by refluxing with magnesium) (10 ml.) containing anhydrous sodium acetate (2·05 g., 25 mmole) and deuterium oxide (0·4 ml.). After 1 hr. the reaction mixture was poured into water (100 ml.) and the product isolated.

Yield, 2·561 g. (83%), m.p. 271-273°.

I.r. (nujol) ν 3200 bd; 1774, 1739, 1705, 1648.cm^{-1}

m.s. 57% D.

[α-$^2H_2$]Veratryl[5-^2H]hydantoin

[α-^2H]Veratralhydantoin (2·49 g., 10 mmole) was reduced with sodium amalgam in aqueous dioxan as described above.

Yield, 1·875 g. (74%), m.p. 164-167°.

I.r. (nujol) ν 3280, 3180, 3060, 1745 sh., 1706 cm^{-1}.

3,4-Dimethoxyphenyl[β $^2H_2$]alanine

[α-$^2H_2$]Veratryl[5-^2H]hydantoin (1·86 g., 7·3 mmole) was hydrolyzed with aqueous barium hydroxide as described above.

Yield, 1·542 g. (93%).

I.r. (nujol) ν 3380, 3200, 1622 sh., 1608, 1590 cm^{-1}

u.v. (H_2O) λ 198 mμ (ε c.a. 30,000)
228 (ε 6500)
278 (ε 2560) (i.e. 94% pure)
281 sh.(ε 2330)

n.m.r. (D_2O) τ 6·81 d,d. CHD $J_{\alpha,\beta}$ 6·0 c/s, $J_{H,D}$ 5·6 c/s
6·28 s. OMe
6·21 s. OMe
5·61 s. C<u>H</u>-CD_2
d. C<u>H</u>-CHD $J_{\alpha,\beta}$ 6·0 c/s
3·1-3·3 m. 3ArH.

3,4-Dihydroxyphenyl[β-$^2H_2$]alanine

3,4-Dimethoxyphenyl[β-$^2H_2$]alanine (1·32 g., 5·4 mmole) was hydrolyzed with hydriodic acid and the product isolated as described previously.

Yield, 0·806 g. (54%), m.p. 271-2° decomp. (sealed tube)
D,L-dopa m.p. 271-2° decomp.

I.r. (nujol) ν 3420, 3220, 3080, 1703 w., 1652 cm^{-1}

u.v. (H_2O) λ 199 mμ (ε c.a. 27,400)
220 (ε 6380)
280 (ε 2730) (i.e. 100% pure).

3,4-Dihydroxyphenyl[β-$^2H_2$]alanine ethyl ester

3,4-Dihydroxyphenyl[β-$^2H_2$]alanine (5 mg.) was esterified in ethanolic hydrochloric acid.

M.s. (probe 110°)

m/e				m/e		
228	3·0%	M^+D_3	β-96% D	125	37%	$ArCD_2^+$
227	14	M^+D_2		124	100	$ArCHD^+$
226	19	M^+D_1	β-59% D	123	60	$ArCH_2^+$
225	0·3	M^+D_0	α- 9% D	109	11	
155	10			108	15	
154	57	$ArCD_2CH{=}NH_2^+$		103	86	
153	43	$ArCHDCH{=}NH_2^+$		102	81	$H_2N{=}CHCO_2Et^+$
152	10	$ArCH_2CH{=}NH_2^+$		79	18	
				78	17	
				74	85	$H_2N{=}CHCO_2H^+$ (m*103-74, 102-74).

3,4-Dihydroxyphenyl[β-$^2H_2$]alanine - 2

Veratryl alcohol[217,218]

Veratraldehyde (1·61 g., 1 mmole) was dissolved in a mixture of ethanol (1 ml.) and water (2 ml.) containing potassium hydroxide (110 mg., 2 mmole). T.l.c. showed little reaction after 20 hr. at room temperature, but disproportionation was complete after 2 hr. at 90°. The product was extracted with dichloromethane after evaporating the ethanol.

Yield, 0·894 g. (100%).
I.r. (liq.) ν 3600 bd., 1609, 1596 cm^{-1}
n.m.r. ($CDCl_3$) τ 4·41 s. OMe
4·39 s. OMe
5·88 s. OH (exchanged by D_2O)
5·67 s. CH_2
3·3-3·4 m. 3ArH
(0·47 s. 3% residual aldehyde).

Acidification of the aqueous residue with 6N sulphuric acid gave veratric acid.

Yield, 0·893 g. (98%).

Veratryl chloride[112]

Veratryl alcohol (0·849 g., 5 mmole) was chlorinated at room temperature for ½ hr. with thionyl chloride (1 ml.) in ether (10 ml.).

Yield, 0·884 g. (94%).
i.r. (liq.) ν 1605, 1591 cm^{-1}.

Diethyl acetamidoveratrylmalonate

Diethyl acetamidomalonate (1·03 g., 0·474 mmole) in dry dimethylformamide was treated with 50% sodium hydride (215 mg., 0·474 mmole). After ½ hr. at room temperature veratryl chloride (0·884 g., 0·474 mmole) in dry dimethylformamide was added and the solution stirred at 40-50° for 5 hr. The reaction mixture was cooled and diluted with ice/water (100 ml.), then extracted with dichloromethane (3 x 50 ml.). The extract was washed with water (50 ml.) and evaporated. The crystalline residue was extracted with petrol to remove unreacted diethyl acetamidomalonate.

Yield, 1·614 g. (93%), m.p. 105° (lit.,[112] 106-107°).
I.r. ($CHCl_3$) ν 3427, 1735, 1674, 1608, 1592 cm^{-1}
n.m.r. ($CDCl_3$) 8·70 t. Me J 7·0 c/s
7·98 s. NAc
6·40 s. $ArCH_2$
6·19 s. 2OMe
5·72 q. OCH_2
3·1-3·6 m. 3ArH.

[α-$^2H_2$]Veratryl alcohol

[α-^2H]Veratraldehyde (3·94 g., 23·6 mmole) was refluxed with sodium hydroxide (2·0 g., 50 mmole) in a mixture of ethanol (4 ml.) and water (4 ml.) at 90° for 5 hr.

N.m.r. showed 1·70 D in the α-position (85% D) indicating a 12% loss from the starting aldehyde (97·7% D).

Recovered [α-^2H]veratraldehyde (2%) contained 98·2% D.

The reaction of [α-^{2}H]veratraldehyde (172 mg.) with sodium hydroxide (80 mg.) in a mixture of ethanol (0·2 ml) and water (0·2 ml.) at 40° took 120 hr. and gave a product containing 1·85 D (94% retention).

The reaction of veratraldehyde with aqueous sodium hydroxide

a) 20% sodium hydroxide in water refluxed at 105°,
4 hr., 80%; 8 hr., 90% reacted.

b) 20% sodium hydroxide in aqueous dioxan refluxed at 105°,
5 hr., 10% reacted.

c) 50% sodium hydroxide in water heated to 155° in a sealed tube,
½ hr., 80% reacted.

d) 50% sodium hydroxide in water refluxed at 120°,
20 min., 85%; 1½ hr., 100% reacted.

e) As (d) with added hydrogen peroxide (0·2%),
20 min., 85% reacted.

f) Saturated sodium hydroxide in water refluxed at 173°,
5 min., 97%; 15 min., 100% reacted.

[α-$^2H_2$]Veratryl alcohol - 2

[α-^{2}H]Veratraldehyde (5·00 g., 30 mmole) was refluxed with 50% aqueous sodium hydroxide (7·5 ml.) for 2½ hr. at 120°.

Yield, 2·55 g. (100%), n^{17} 1·5500 (lit.,[246] n_D^{17} 1·555).

I.r. (liq.) 3450 bd., 2165, 2130, 2070, 1611, 1593 cm^{-1} (Perkin-Elmer 257).

N.m.r. indicated 1·954 D in the α-position (97·7% D).

[α-^{2}H]Veratraldehyde contained 0·970 D.

[α-$^2H_2$]Veratryl alcohol

[α-$^2H_2$]Veratryl alcohol (2·50 g., 15 mmole) was chlorinated with thionyl chloride in ether for 1 hr. at room temperature.

Yield, 2·77 g. (100%).

Diethyl acetamido[α-$^2H_2$]veratrylmalonate

[α-$^2H_2$]Veratryl chloride (1·89 g., 10 mmole) was condensed with diethyl acetamidomalonate as described previously.

Yield, 3·32 g. (90%).

3,4-Dihydroxyphenyl[β-$^2H_2$]alanine

Diethyl acetamido[α-$^2H_2$]veratrylmalonate (3·109 g., 8·5 mmole) was refluxed with a mixture of 55% hydriodic acid (micro-analytical reagent) (6 ml.) and 45% hydrobromic acid (5 ml.) for 7 hr. under nitrogen in the presence of red phosphorous (10 mg.). The product was isolated by the same method as described previously.

Yield, 1·320 g. (79%), 94% pure (u.v.)
0·883 g. (53%), 100% pure (recrystallized from water).

M.p. 271-272° decomp. (sealed tube).

I.r. (nujol) ν 3400, 3210, 3060, 1652 cm^{-1}
u.v. (H_2O) λ 218 sh. mμ (ε 6700)
281 (ε 2830).

3,4-Dihydroxyphenyl[β-$^2H_2$]alanine ethyl ester

3,4-Dihydroxyphenyl[β-$^2H_2$]alanine (5 mg.) was esterified in ethanolic hydrochloric acid.

M.s. (probe 140°)

m/e				m/e		
228	3·3%			109	13%	
227	19	M^+D_2	≥95% D	108	12	
226	1·6	M^+D_1		103	85	
225	0·5	M^+D_0		102	82	$H_2N{=}CHCO_2Et^+$
154	62	$ArCD_2CH{=}NH_2^+$		79	20	
153	12	$ArCHDCH{=}NH_2^+$		77	12	
126	50			74	95	$H_2N{=}CHCO_2H^+$ (m*103-74, 102-74)
125	100	$ArCD_2^+$		69	14	
124	11	$ArCHD^+$.				

Oxidation of 3,4-dihydroxyphenyl[β-$^2H_2$]alanine to melanin[119]

3,4-Dihydroxyphenyl[β-$^2H_2$]alanine (400 mg.) in M/15 phosphate buffer at pH 6·80 (80 ml.) was oxidized by oxygen in the presence of mushroom tyrosinase (14 mg.) and beef-liver catalase (55 mg.). After 8 hr. the melanin was precipitated by the addition of ammonium nitrate (25 g.) and centrifuged off. The product was washed with water (4 x 100 ml.) and acetone (3 x 100 ml.) and dried in vacuo over phosphorous pentoxide.

Yield, 249 mg. (67% w/w).

Analysis, found; C, 52·7; H, 4·1; N, 8·6; Ash, 0%.

Preparation of melanin

3,4-Dihydroxyphenylalanine (50 mg.) in M/15 phosphate buffer (5 ml.) was oxidized with oxygen in the presence of tyrosinase (1·8 mg.) and catalase (7·5 mg.).

Precipitation:

a) 8N Hydrochloric acid- rapid precipitate, yield 50%.

b) Saturated sodium chloride, pH 7 - precipitated in ½ hr.

c) Saturated sodium sulphate, pH 7 - precipitated in ½ hr.

d) Saturated ammonium sulphate, pH 5- precipitated in 5 min.

e) Saturated ammonium acetate, pH 8 - precipitated in ½ hr.

f) Saturated sodium bicarbonate,pH 10- clear solution, no precipitate.

g) Precipitated after standing for 2 weeks.

Ammonium nitrate was used to precipitate melanin in all preparations from specifically labelled 3,4-dihydroxyphenylalanine.

Deuteration and Tritiation of Aldehydes

The experiments performed jointly with Dr. D. J. Bennett are indicated with an asterisk (*).

Benzaldehyde

Phenylmorpholineacetonitrile*

Benzaldehyde (1·06 g., 10 mmole) and morpholine perchlorate (2·06 g., 11 mmole) were dissolved in morpholine (10 ml.) and treated with sodium cyanide (0·53 g., 11 mmole) dissolved in a minimum of water. The solution was warmed to 85° for 1 hr., then cooled. Addition of ice/water (100 ml.) gave a precipitate which was filtered off, washed with water and dried (1·768 g., 88%). The product was recrystallized from petrol.

Yield, 1·634 g. (81%), m.p. 69-70° (lit.[233] 68-70°).

I.r. (CCl_4) ν 1604 cm^{-1}

n.m.r. ($CDCl_3$) τ 7·3-7·5 m. $2NCH_2$
6·2-6·4 m. $2OCH_2$
5·17 s. CH
2·4-2·7 m. 5ArH.

Phenylmorpholine[α-^{2}H]acetonitrile

a) Sodium hydride method*

Phenylmorpholineacetonitrile (202 mg., 1 mmole) was dissolved in dry dimethylformamide (20 ml.) under nitrogen

and treated with 53% sodium hydride in mineral oil (96 mg., 2 mmole). After 1 hr. the yellow solution was treated with deuterium oxide (0·2 ml., 11 mmole) and, after 20 min., acidified with thionyl chloride. Water (20 ml.) was added and the solution extracted with chloroform (3 x 30 ml.). The extract was washed with water (2 x 40 ml.), dried and evaporated. The residue was recrystallized from petrol.

Yield, 172 mg. (85%).

N.m.r. 92% D.

b) Direct exchange

Phenylmorpholineacetonitrile (101 mg., 0·5 mmole), deuterium oxide (0·2 ml., 11 mmole) and dimethylformamide (0·4 ml.) were heated in a Carius tube under nitrogen for 2 hr. at 100°. The tube was cooled, opened and the solution evaporated to dryness. The residue was recrystallized from petrol.

Yield, 101 mg. (100%).

N.m.r. 87% D, 89% exchange.

After heating to 100° for 64 hr. with deuterium oxide phenylmorpholineacetonitrile was completely deuterated.

[α-^{2}H]Benzaldehyde

Phenylmorpholineacetonitrile (150 mg.) was hydrolyzed

for 1 hr. with dilute hydrochloric acid (10 ml.) and the product extracted with dichloromethane.

Yield, 71 mg. (97%).
N.m.r. 89% D; 3% loss of deuterium,
i.r. (liq.) ν 2140, 2100, 1685 cm^{-1}.

p-Nitrobenzaldehyde

p-Nitrophenylmorpholineacetonitrile*

p-Nitrobenzaldehyde (0·75 g., 5 mmole) was reacted with morpholine perchlorate and sodium cyanide as described above.

Yield, 0·728 g. (60%) crude,
0·600 g. (48%) crystallized from benzene-petrol-iso-propyl ether. M.p. 146·5-148·5°.

Analysis, found: C, 58·2; H, 5·2; N, 17·2.
$C_{12}H_{13}N_3O_3$ requires: C, 58·3; H, 5·3; N, 17·0%.

N.m.r. ($CDCl_3$) τ 7·3-7·5 m. $2NCH_2$
6·15-6·35 m. $2OCH_2$
5·03 s. CH
2·18 d. 2,6-ArH } J 8·5 c/s.
1·71 d. 3,5-ArH }

p-Nitrophenylmorpholine[α-^{2}H]acetonitrile

Sodium hydride method*

p-Nitrophenylmorpholineacetonitrile (246 mg., 1 mmole) was treated with sodium hydride in dimethylformamide and the deep purple anion (λ 520 mμ) quenched with deuterium oxide. The product was isolated as described above and purified by

chromatography on alumina grade III in benzene.

Yield, 190 mg. (78%).
N.m.r. c.a. 0% D.

p-Nitro[α-^{2}H]benzaldehyde

Direct exchange and in situ hydrolysis

p-Nitrophenylmorpholineacetonitrile (103 mg., 0·42 mmole), deuterium oxide (0·2 ml., 11 mmole) and sulpholan [redistilled, m.p. c.a. 30° (lit.[247] 20-21°)] (2·0 ml.) were sealed in a Carius tube under nitrogen together with a brake-seal phial of deutero-acid [prepared *in situ* from sulphuryl chloride (85 mg., 0·63 mmole) and deuterium oxide (50 mg.)]. The Carius tube was heated to 100° for 1 hr. then cooled and shaken to brake the phial of acid. Heating to 100° for 1 hr. hydrolyzed the morpholino-nitrile. The tube was cooled, opened and the product extracted with chloroform.

Yield, 30 mg. (48%).
N.m.r. c.a. 100% D,
i.r. (CCl_4) ν 2140, 2100, 1690 cm^{-1}.

m-Nitrobenzaldehyde

m-Nitrophenylmorpholineacetonitrile*

m-Nitrobenzaldehyde (1·50 g., 10 mmole) and morpholine perchlorate (2·06 g., 11 mmole) were dissolved in morpholine (10 ml.) and stirred at 60° for 1 hr. The solution was cooled and sodium cyanide (0·53 g., 11 mmole) in water was added

then warmed to 90° for 1 hr. The solution was cooled and diluted with ice/water (100 ml.). The precipitate was dissolved in chloroform, dried and evaporated. The residue was chromatographed on alumina grade V in benzene, then recrystallized from iso-propyl ether.

Yield, 1·89 g. (77%), m.p. 87·5-88·5°.

Analysis, found: C, 58·0; H, 5·3; N, 17·0.

$C_{12}H_{13}N_3O_3$ required: C, 58·3; H, 5·3; N, 17·0%.

N.m.r. ($CDCl_3$) τ 7·25-7·5 m. $2NCH_2$
6·1-6·4 m. $2OCH_2$
5·03 s. CH
2·37 t. 5-ArH
2·07 d,t. 6-ArH
1·76 d,t. 4-ArH
1·59 t. 2-ArH

$J_{4,5} \approx J_{5,6} = 7·8$ c/s
$J_{2,4} \approx J_{2,6} \approx J_{4,6} = 1·6$ c/s

m-Nitrophenylmorpholine[α-*H]acetonitrile

a) Sodium hydride method

m-Nitrophenylmorpholineacetonitrile (246 mg., 1 mmole) was treated with sodium hydride in dimethylformamide and the turquoise-green anion quenched with deuterium oxide after 2 hr. The product was isolated as described above and purified by chromatography on alumina grade III in benzene.

Yield, 188 mg. (89%).

N.m.r. 75% D.

b) Direct exchange

m-Nitrophenylmorpholineacetonitrile (123 mg., 0·5 mmole), tritiated water (2 mC/ml) (0·2 ml.) and dimethylformamide (0·5 ml.) were exchanged at 100° for 15 hr. The product was extracted into chloroform and chromatographed on alumina grade V in benzene.

Yield, 25 mg. (20%).

Activity, $1 \cdot 41 \times 10^5$ c/s/mmole (84% incorporation)

m-Nitro[α-*H]benzaldehyde

a) Hydrolysis of m-Nitrophenylmorpholine[α-^{3}H]acetonitrile

m-Nitrophenylmorpholine[α-^{3}H]acetonitrile (51·7 mg.) was hydrolyzed for 2 hr. with aqueous ethanolic hydrochloric acid (3:1:1 ethanol, water, concentrated hydrochloric acid) (5 ml.). The alcohol was evaporated and the product extracted with chloroform.

Yield, 32 mg. (100%)

Activity, $0 \cdot 43 \times 10^5$ c/s/mmole (30% retention)

b) Direct exchange and in situ hydrolysis

m-Nitrophenylmorpholineacetonitrile (123 mg., 0·5 mmole), deuterium oxide (0·4 ml.) and sulpholan (0·5 ml.) were heated to 100° for 2 hr as described above. The reaction mixture was acidified with sulphuryl chloride (68 mg., 0·5 mmole) and

hydrolyzed for 1 hr. at 100°. The product was extracted with chloroform and crystallized.

Yield, 57 mg. (76%).
N.m.r. 98·6% D, 100% exchange,
i.r. (CCl_4) ν 2160, 2100, 1689 cm^{-1}.

m-Nitrobenzoic acid

m-Nitro[α-^{3}H]benzaldehyde was oxidized with alkaline permanganate and the product recrystallized from carbon tetrachloride, chloroform and then vacuum sublimed (100°, 5 x 10^{-5} mm. Hg).

Activity, 96 c/s/mmole (0·07% of morpholino-nitrile)

p-Cyanobenzaldehyde

p-Cyanophenylmorpholineacetonitrile*

p-Cyanobenzaldehyde (1·31 g., 10 mmole) was reacted with morpholine perchlorate and sodium cyanide as described for benzaldehyde.

Yield, 2·136 g. (88%) crude,
1·764 g. (78%) recrystallized from carbon tetrachloride-petrol, m.p. 128-128·5.
Analysis, found: C, 68·7; H, 5·8; N, 18·3.
$C_{13}H_{13}N_3O$ requires: C, 68·7; H, 5·8; N, 18·5%.
N.m.r. ($CDCl_3$) τ 7·3-7·5 m. $2NCH_2$
6·15-6·35 m. $2OCH_2$
5·08 s. CH
2·78 s. 4ArH.

p-Cyanophenylmorpholine[α-*H]acetonitrile

a) Sodium hydride method

p-Cyanophenylmorpholineacetonitrile (227 mg., 1 mmole) was treated with sodium hydride in dimethylformamide and the deep red anion quenched with deuterium oxide. The product was purified as above and crystallized from carbon tetrachloride-petrol.

Yield, 208 mg. (91%).

N.m.r. >95% D.

b) Direct exchange

p-Cyanophenylmorpholineacetonitrile (114 mg., 0·5 mmole) was exchanged with tritiated water as described above. After 15 hr. the product was isolated by diluting the reaction mixture with water and filtering off the crystals.

Yield, 107 mg. (95%).

Activity, 1·63 x 10^{5} c/s/mmole (99% incorporation).

p-Cyano[α-*H]benzaldehyde

a) Hydrolysis of p-cyanophenylmorpholine[α-*H]acetonitrile

p-Cyanophenylmorpholine[α-^{2}H]acetonitrile (114 mg.) was hydrolyzed in aqueous ethanolic hydrochloric acid (5 ml.) for ½ hr. and the product chromatographed on alumina grade V.

Yield, 94 mg. (100%).

N.m.r. 68% D (72% retention).

p-Cyanophenylmorpholine[α-^{3}H]acetonitrile (61 mg.) was hydrolyzed in aqueous ethanolic hydrochloric acid (5 ml.) for 2 hr. and the product extracted with chloroform.

Yield, 20·4 mg. (58%).

Activity, 0·16 x 10^{5} c/s/mmole (10% retention).

b) Direct exchange and in situ hydrolysis

p-Cyanophenylmorpholineacetonitrile (114 mg., 0·5 mmole), deuterium oxide (0·2 ml.) and sulpholan (0·4 ml.) were reacted at 100° for 2 hr. The product was acidified with sulphuryl chloride (68 mg., 0·5 mmole) and heated at 100° for 2 hr. The product was extracted with chloroform and crystallized.

Yield, 57 mg. (86%).

N.m.r. 93% D, 96% exchange,

i.r. ($CHCl_3$) ν 2240, 2140, 2100, 1693, 1683 cm^{-1}.

p-Cyanobenzoic acid

p-Cyano[α-^{3}H]benzaldehyde (10 mg.) was oxidized with alkaline permanganate and the product recrystallized from iso-propyl ether–petrol, carbon tetrachloride and vacuum sublimed (130°, 5 x 10^{-5} mm. Hg).

Activity, 880 c/s/mmole (0·5% retention of morpholino-nitrile)

p-Diethylaminobenzaldehyde

p-Diethylaminophenylmorpholineacetonitrile*

p-Diethylaminobenzaldehyde (1·77 g., 10 mmole) was reacted with morpholine perchlorate and sodium cyanide as described for benzaldehyde.

Yield, 1·305 g. (96%), oil.

N.m.r. ($CDCl_3$) τ 8·83 t. $2CH_3$ J 7·0 c/s
7·3-7·55 m. $2NCH_2CH_2O$
6·66 q. $2NCH_2Me$
6·2-6·4 m. $2OCH_2CH_2N$
5·28 s. CH
3·15 d. 3,5-ArH } J 8·9 c/s
2·60 d. 2,6-ArH }

p-Dimethylaminobenzaldehyde

p-Dimethylaminophenylmorpholineacetonitrile

p-Dimethylaminobenzaldehyde (1·49 g., 10 mmole) was reacted with morpholine perchlorate and sodium cyanide as described for m-nitrobenzaldehyde. The product precipitated on diluting the reaction mixture with ice/water and did not require purification.

Yield 2·40 g. (98%), m.p. 138·5°.

Analysis, found: C, 68·7; H, 7·8; N, 17·3.
$C_{14}H_{19}N_3O$ requires: C, 68·5; H, 7·8; N, 17·1%.

N.m.r. ($CDCl_3$) τ 7·3-7·55 m. $2NCH_2$
7·03 s. $2NCH_3$
6·2-6·4 m. $2OCH_2$
5·30 s. CH
3·29 d. 3,5-ArH } J 8·8 c/s
2·67 d. 2,6-ArH.

p-Dimethylaminophenylmorpholine[α-^{2}H]acetonitrile

Sodium hydride method

p-Dimethylaminophenylmorpholineacetonitrile (245 mg., 1 mmole) was treated with sodium hydride in dimethylformamide and the yellow anion quenched with deuterium oxide. The product was purified as above and recrystallized from carbon tetrachloride-ether.

Yield, 196 mg. (80%).

N.m.r. 94% D.

p-Dimethylamino[α-^{2}H]benzaldehyde

p-Dimethylaminophenylmorpholine[α-^{2}H]acetonitrile (123 mg., 0·5 mmole) was hydrolyzed in 2N hydrochloric acid (3 ml.) for ½ hr. The reaction mixture was neutralized and the product extracted with dichloromethane and purified by t.l.c. on silica gel (3 x 20^2 cm^2), eluting with 15% ethyl acetate-benzene.

Yield, 44 mg. (60%) (n.m.r. showed the presence of some cyanohydrin in the product).
N.m.r. 72% D (77% retention),
i.r. ($CHCl_3$) ν 2140, 2100, 1676 cm^{-1}.

Hydrolysis of p-Dimethylaminophenylmorpholineacetonitrile

Hydrolysis in aqueous ethanolic hydrochloric acid was rapid, but the principal product was p-dimethylaminophenyl-α-ethoxyacetonitrile.

Yield, 89%, purified by t.l.c. on silica gel.

I.r. (CCl_4) ν 1614 cm^{-1}

u.v. (EtOH) λ 198, 269 mμ

n.m.r. (CCl_4) τ 8·78 t. $C\underline{H}_3CH_2$ J 7·0 c/s
7·05 s. $2NCH_3$
6·45, 6·41 2q. $OC\underline{H}_2Me$ J 7·0 c/s
4·98 s. CH
3·38 d. 3,5-ArH } J 8·9 c/s
2·78 d. 2,6-ArH }

A trace of a compound, which gave an intense green spot on the t.l.c. plate on exposure to air or iodine, was also formed and could be isolated by preparative-scale t.l.c.

p-Dimethylaminophenylmorpholineacetonitrile (123 mg., 0·5 mmole) was hydrolyzed in 2N hydrochloric acid and the products separated by t.l.c. The compound which gave the green spot was isolated in 3% yield and identified as bis-p-dimethylaminophenylacetonitrile.

Treatment of the morpholino-nitrile with 1 equivalent of aqueous acid gave a better yield (44%).

M.p. 122-123° (lit.[248] 124°)

i.r. ($CHCl_3$) ν 2252, 1613 cm^{-1}

u.v. (EtOH) λ 205, 270, 300 sh. mμ

n.m.r. ($CDCl_3$) τ 7·11 s. $4NCH_3$
5·02 s. CH
3·29 d. 3,5,3',5'-ArH } J 8·7 c/s
2·80 d. 2,6,2',6'-ArH }

m.s. (probe, 120°)

m/e			m/e		
280	22%		279/2	23%	M^{++}
279	100	M^+	277/2	30	
278	31	$M-H^+$	278/3	0·9	$M-H^{+++}$
253	9	$M-CN^+$	277/3	0·2	
235	31	$M-NMe_2^+$ (m* 279-235)	254/3	0·5	
159	41	$M-C_6H_4NMe_2^+$			
158	46	$M-H-C_6H_4NMe_2^+$			
157	10				
144	16				
120	19				
118	11				
77	10.				

Analysis, found: C, 77·4; H, 7·5; N, 14·9.

$C_{18}H_{21}N_3$ requires: C, 77·4; H, 7·5; N, 15·0%.

α-Naphthaldehyde

1-Naphthylmorpholineacetonitrile *

1-Naphthaldehyde (1·56 g., 10 mmole) was reacted with morpholine perchlorate and sodium cyanide as described for m-nitrobenzaldehyde. The product was purified by chromatography on alumina grade V in benzene.

Yield, 2·194 g. (87%) crude,
2·062 g. (82%) recrystallized from petrol, m.p. 103-104°.

Analysis, found: C, 76·1; H, 6·4; N, 11·1.
$C_{16}H_{16}N_2O$ requires: C, 76·2; H, 6·4; N, 11·1%.
N.m.r. ($CDCl_3$) τ 7·25-7·5 m. $2NCH_2$
6·2-6·45 m. $2OCH_2$
4·59 s. CH
2·9-3·2 m. 7ArH.

1-Naphthylmorpholine[α-^{2}H]acetonitrile

Sodium hydride method

1-Naphthylmorpholineacetonitrile (253 mg., 1 mmole) was treated with sodium hydride in dimethylformamide and the resulting orange anion quenched with deuterium oxide. The product was isolated as above.

Using a reaction time of 2 hr. at room temperature only 79% deuteration was achieved, so the reaction was carried out at 40° for 2 hr.

Yield, 224 mg. (89%).

N.m.r. 97% D.

1-[α-^{2}H]Naphthaldehyde

1-Naphthylmorpholine[α-^{2}H]acetonitrile (132 mg.) was hydrolyzed in aqueous ethanolic hydrochloric acid.

Yield, 82 mg. (100%).
N.m.r. 79% D (100% retention),
i.r. (liq.) ν 2140, 2100, 1676 cm^{-1}.

Cinnamaldehyde

Styrylmorpholineacetonitrile*

Cinnamaldehyde (1·32 g., 10 mmole) was reacted with morpholine perchlorate and sodium cyanide as described for benzaldehyde. The crude product was chromatographed on silica gel (100 g.) in chloroform.

1st fraction- 0·377 g. (17%)- probably 2-benzyl-1-morpholino-acrylonitrile.

I.r. (CCl_4) ν 2220, 1673, 1615 cm^{-1} (1673 cm^{-1}- cinnamaldehyde)
u.v. (EtOH) λ 262 sh., 266·5, 271 mμ
n.m.r. ($CDCl_3$) τ 7·0-7·2 m. $2NCH_2$
6·15-6·35 m. $2OCH_2$
6·35 d. $C\underline{H}_2CH$ } J 8·0 c/s
4·61 t. $CHCH_2$ }
2·74 s. 5ArH

2nd fraction- 28 mg. (1%)- styrylmorpholineacetonitrile.

3rd fraction- 0·632 g. (28%)- unknown, possibly HCN adduct.

I.r. (CCl_4) ν 2220, 1672 cm^{-1}
u.v. (EtOH) λ c.a. 206 mμ
n.m.r. ($CDCl_3$) τ 7·3-7·6 m. NCH_2 (2·3 H)
6·2-6·4 m. OCH_2 (2·8 H)
2·65-2·70 m. 5ArH

The morpholino-nitrile was prepared via the tertiary imminium salt. (c.f. pyrrolidinium salt[234])

Cinnamaldehyde (2·62 g., 20 mmole), morpholine perchlorate (4·12 g., 22 mmole) and triethylamine (0·1 ml.) were dissolved

in hot ethanol (200 ml.). On cooling yellow plates separated. The reaction mixture was diluted with ether (200 ml.) and the product filtered off and recrystallized from acetonitrile-ethanol.

Yield 5·0 g. (78%), m.p. 209-210·5°.

Analysis, found: C, 51·7; H, 5·4; N, 4·4.
$C_{13}H_{16}NO_5Cl$ requires: C, 51·7; H, 5·3; N, 4·6%.
I.r. (nujol) ν 1656, 1650, 1612, 1596 cm^{-1}
u.v. (EtOH) λ 337 mμ.

Cinnamaldehyde morpholinium perchlorate (3·02 g. 10 mmole) was added to aqueous sodium cyanide (0·54 g. 11 mmole). The precipitate was extracted with chloroform, dried and evaporated at room temperature.

Yield 1·69 g. (74%), m.p. 93-95°

Analysis, Found: C, 73·5; H, 7·0; N, 12·2.
$C_{14}H_{16}N_2O$ requires: C, 73·7; H, 7·1; N, 12·3%.
U.v. (EtOH) λ 251 mμ
N.m.r. ($CDCl_3$) τ 7·35-7·5 m. $2NCH_2$
6·15-6·4 m. $2OCH_2$
5·60 d,d. γ-CHCN — $J_{\alpha\beta}$ 16·2 c/s
3·87 d,d. α-CHPh — $J_{\beta\gamma}$ 4·8 c/s
3·09 d,d. β-CH= — $J_{\alpha\gamma}$ 1·7 c/s
2·61 s. 5ArH

Deuteration of styrylmorpholineacetonitrile

Styrylmorpholineacetonitrile (228 mg., 1 mmole) was treated with sodium hydride in dimethylformamide and the

resulting orange anion quenched with deuterium oxide. The product was chromatographed on silica gel in dichloromethane.

Yield, 70 mg. (53%)- cinnamaldehyde.

The product was converted to the semicarbazone and the mass spectrum taken:

Undeuterated- 12%, D_1- 36%, D_2-52%.

The deuteration was repeated and the principal product isolated by preparative-scale t.l.c. on silica gel.

I.r. (liq.) ν 2240, 1610 cm^{-1}

u.v. (EtOH) λ 255 sh., 259·5, 265, 269·5 sh. mμ

n.m.r. (CCl_4) τ 7·05-7·25 m. $2NCH_2$

6·15-6·35 m. $2OCH_2$

4·75 bd,s. β-CH=

2·82 s. 5ArH

This compound was probably [α-$^2H_2$]benzyl-1-morpholino-acrylonitrile.

n-Butyraldehyde

α-Morpholinevaleronitrile

n-Butyraldehyde (0·72 g., 10 mmole) was reacted with morpholine perchlorate and sodium cyanide by the method described for m-nitrobenzaldehyde.

Yield, 1·68 g. (100%), m.p. 31-33° (lit.[249] 32°).

N.m.r. ($CDCl_3$) τ 9·02 t, CH_3 J 6 c/s
8·25-8·7 m. $2CH_2$
7·3-7·6 m. $2NCH_2$
6·52 t. CH J 7 c/s
6·2-6·4 m. $2OCH_2$.

α-Morpholine[α-^{3}H]valeronitrile

α-Morpholinevaleronitrile (83 mg., 0·5 mmole), tritiated water (2 mC/ml) and dimethylformamide (0·25 ml.) were heated in a Carius tube at 155° for 3 hr.

Yield, 63 mg. (76%).

Activity, 1·93 x 10^5 c/s/mmole (≡ 1·3 ^{3}H).

Veratraldehyde

The preparation of [α-^{2}H]- and [α-^{3}H]veratraldehyde by the sodium hydride method is described above (pages 133-135, 142-143).

Veratrylmorpholine[α-*H]acetonitrile

b) Direct exchange

Veratrylmorpholine-acetonitrile (131 mg., 0·5 mmole), deuterium oxide (0·1 ml., 5 mmole) and sulpholan (0·25 ml.) were heated at 100° in an n.m.r. tube.

1 hr. - 23% D, 25% exchange.
2½ hr. - 63% D, 69% exchange.

Veratrylmorpholineacetonitrile (131 mg., 0·5 mmole), tritiated water (0·2 ml.) and dimethylformamide (0·5 ml.) were heated at 100° for 75 hr. The product was extracted with chloroform and recrystallized from iso-propyl ether-petrol.

Yield, 92 mg. (70%).

Activity, $1·58 \times 10^5$ c/s/mmole (103% exchange).

[α-^{3}H]Veratraldehyde

Veratrylmorpholine[α-^{3}H]acetonitrile was hydrolyzed in aqueous ethanolic hydrochloric acid.

Activity, $1·56 \times 10^5$ c/s/mmole (101% ^{3}H).

Veratric acid

[α-^{3}H]Veratraldehyde was oxidized with alkaline permanganate and the product recrystallized from ether and ethyl acetate-ether.

Activity, 10 c/s/mmole (0·006% ^{3}H).

Oxidation of 3,4-dihydroxyphenylalanine

Ferricyanide oxidations

The oxidation of D,L-3,4-dihydroxyphenylalanine was followed spectroscopically using 1 cm. cells and a SP800 spectrometer.

Sample cell:	M/1000	dopa	0·5 ml.
	M/500	ferricyanide	0·25-1·5 ml.
	M/10	sodium carbonate	0·1 ml.
		water	to 2·5 ml.
Reference cell:			
	M/500	ferrocyanide	0·25-1·5 ml.
	M/10	sodium carbonate	0·1 ml.
		water	to 2·5 ml.

Ratio Fe^{III} : Dopa	Spectra
1 1	λ 282mμ(ε3900), 301 (1680), 470 (930)
2 1	287mμ(ε4900), 304 (5300), 470 (1900)
4 1	306 (9500), 472 (3850)
6 1	306 (11,900), 470 (3900), 422 (5400).

The 4:1 ratio corresponds to the formation of dopachrome and these data were used for the enzyme reactions.

λ (0·0032M $NaHCO_3$) 306 mμ (ε 9500)
472 mμ (ε 3850).

Dopachrome

A solution of 3,4-dihydroxyphenylalanine (197 mg., 1 mmole) in water (50 ml.) was oxidized by potassium ferricyanide

(1·317 g., 4 mmole) in the presence of sodium carbonate (212 mg., 2 mmole). After 5 min. at 0° the ferrocyanide was precipitated by aqueous lead nitrate (2·650 mg., 8 mmole). The precipitate was centrifuged off and the red solution freeze dried.

U.v. (H_2O) λ 305, 470 mμ.
n.m.r. (D_2O) τ 6·5-7·2 m. CH, CH_2
3·15-3·45 bd,s. 2CH=.

Enzymic oxidation of 3,4-Dihydroxyphenylalanine

The factors influencing the rate of oxidation of 3,4-dihydroxyphenylalanine by oxygen, in the presence of mushroom tyrosinase, were investigated spectroscopically using 1 cm. cells of a SP700 spectrometer set at the absorption maximum of dopachrome (472 mμ) and recording absorbance as a function of time. Enzyme activities are given in international units (μmoles/min./mg. enzyme) and were measured at 30°. The activities are corrected, where necessary, for the decomposition of the tyrosinase solution (2%/day).

Standard solutions were prepared with water redistilled under nitrogen in an all glass apparatus.

3,4-Dihydroxyphenylalanine	M/1000	
Phosphate buffer, pH 6·8	M/15 or M	
Tyrosinase	0·270-0·290 mg./10 ml.	Stored at 0°
Catalase	1·451 mg./10 ml.	Stored at 0°

The cells were saturated with oxygen before adding the enzyme to start the reaction. The activity was calculated from the maximum rate of formation of dopachrome (ε 3850).

Dopa conc. M/10,000	Enz. conc. %	Buffer M	Other factors	Activity U/mg.
2	·111	0	Enzyme batch c.	1·41
2	·111	·026		1·21
2	·111	·1		1·26
2	·111	·1	0·44% catalase	1·41
4	0	·1	0·44% catalase	<0·02
2	·116	·026	D-Dopa	0·75
2	·116	·026	L-Dopa	1·66
2	·116	·026	D,L-Dopa	1·21
1	·111	·1		0·98
3	·111	·1		1·67
4	·111	·1		2·14
5	·111	·1		1·92
4	·087	·1		1·84
4	·058	·1		1·88
4	·029	·1		2·02
4	·110	·1	2 year old enzymes a.	1·38
4	·112	·1	b.	1·24
4	·115	·1	New enzymes c.	2·14
4	·108	·1	d.	2·13

Enzymic oxidation of L-tyrosine

Tyrosine was oxidized by a method similar to that described above.

L-tyrosine M/500

The reaction was preceded by an induction period during which little dopachrome was formed.

Tyr. conc. M/10,000	Enz. conc. %	Buffer M	Other factors	Activity U/mg.	Time lag min.
1	·108	·1	Enzyme batch d.	·204	2
2	·108	·1		·310	2
4	·108	·1		·430	4
5	·108	·1		·484	6
4	·054	·1		·700	7
4	·027	·1		·222	14
4	·110	·1	2 year old enzyme a.	·232	5
4	·112	·1	2 year old enzyme b.	·167	6
4	·115	·1	New enzyme c.	·373	4
4	·108	·1	New enzyme d.	·466	3
2	·111	·1	25°, L-tyr.	·145	5
2	·111	·1	25°, [3,5-$^2H_2$]tyr.	·119	11

Oxidation of 3,4-dihydroxyphenylalanine in presence of ascorbate

3,4-Dihydroxyphenylalanine was oxidized by oxygen, in the presence of mushroom tyrosinase, with added ascorbic acid to reduce the dopaquinone as it was formed. The rate of loss of ascorbate was measured directly by measuring the difference in absorbance between the sample and reference cells, both of which contained ascorbic acid. The activity of the enzyme was calculated from the maximum rate of loss of ascorbic acid (λ 266 mμ, ε 14,440).

Dopa conc. M/10,000	Enz. conc. %	Buffer M	Ascorbate M/10,000	Enz. batch	Activity U/mg.
2	·111	·1	·8	c.	2·16
4	·110	·1	1	a.	3·15
4	·112	·1	1	b.	3·10
4	·115	·1	1	c.	5·75
4	·108	·1	1	d.	6·07

Oxidation of tyrosine in the presence of ascorbate

L-Tyrosine was oxidized by a method similar to that described above. The results were calculated using the formula derived on page 103:

$$a_T = \frac{-1}{W_e{}^2 k} \cdot \frac{d^2[\text{Ascorbate}]}{dt^2}$$

a_T tyrosine hydroxylase
W_e weight enzyme
k $d[a_D]/d[\text{Dopa}]$.

Tyr. conc. M/10,000	Enz. conc. %	Buffer M	Ascorbate M/10,000	Other factors	a_T U/mg.
2	·111	·1	·8		0·42
2	·111	·1	·8	[3,5-$^2H_2$]Tyr.	0·30

Oxidation of [2-^{3}H]-p-cresol with mushroom tyrosinase

A solution of [2-^{3}H]-p-cresol (55 mg., 0·5 mmole) in M/15 phosphate buffer at pH 6·80 (20 ml.) was oxidized with oxygen in the presence of mushroom tyrosinase (3·5 mg.) and beef liver catalase (15 mg.) for 16 hr. 4N Sodium hydroxide (5 ml.) and Pummerer's ketone (54·8 mg.) were added and the

solution extracted with chloroform. The extract was dried and evaporated, and the residue chromatographed on alumina grade III in benzene. The resulting Pummerer's ketone (45 mg.) was repeatedly recrystallized from benzene-petrol.

Activity, p-cresol $3{\cdot}32 \times 10^5$ c/s/mmole
recovered Pummerer's ketone 18·2 c/s/mmole.

Radiochemical yield, 0·022%.

Estimation of carbon dioxide in melanogenesis

Carbon dioxide evolved during the oxidation of 3,4-dihydroxyphenylalanine was absorbed from the oxygen in soda-asbestos. Pure oxygen was passed through the following vessels:

1. bubbler- 5% barium hydroxide
2. absorption tower- potassium hydroxide pellets
3. reaction vessel equipped with tap funnel
4. trap
5. U-tube containing anhydrone-pumice
6. micro-analytical tube- anhydrone, 14-22 mesh (M.A.R.)
7. micro-analytical tube- soda-asbestos, 14-22 mesh (M.A.R.)
8. trap
9. U-tube - calcium chloride
10. bubbler- 5% barium hydroxide.

The vessels were connected by glass tubing with short rubber links. The rubber tubing on the inlet side of the reaction vessel was cured in 45% potassium hydroxide at 60° for 1 hr. and in steam for 2 hr. and the rubber tubing on

the outlet side was impregnated with paraffin wax and cleaned with glycerol.[250] Permanent joints were sealed with beeswax and moveable ones with Apiezon T.

Calibration:

Potassium hydrogen carbonate (22·88 mg.) was added to M/15 phosphate buffer at pH 6·8 (10 ml.) in the reaction vessel, and a slow stream of oxygen passed through.

Time 1.20 hr.	3·35 mg. CO_2 (33%)
4.10	7·85 (78%)
20.25	10·02 (100%)
25·25	10·11 (101%)

During this time 0·18 mg. of water were collected.

Oxidation of 3,4-dihydroxyphenylalanine

3,4-Dihydroxyphenylalanine (100 mg.) was oxidized in M/15 phosphate buffer (20 ml.) in the presence of mushroom tyrosinase (3·54 mg.) and beef liver catalase (14 mg.). Some of the data are given below, and the results shown graphically on page 106. The figures are corrected for a slow gain in weight of the soda-asbestos tube of 13 μg/hr.

Time 0.30hr.	CO_2 0·65mg.	0·029mmolc	24hr.	CO_2 9·83mg.	0·440mmole
1.00	1·54	0·068	30	10·54	0·472
2.10	4·22	0·189	100	17·3	0·775
4·10	6·55	0·293	152	21·9	0·983
6.00	7·48	0·335	200	24·4	1·08
8.30	8·42	0·377	337	27·2	1·22
12.25	9·15	0·410	480	28·1	1·26

After 7890 hr. oxidation the reaction was worked up and melanin (94·0 mg.) isolated.

In a second oxidation melanin (88·4 mg.) was isolated after 125 hr. oxidation.

Oxidation of Melanin to Pyrrole acids

[^{14}C]Melanin

3,4-Dihydroxyphenyl[α-^{14}C]alanine (197 mg., 1 mmole, 10 μC) was oxidized by oxygen in the presence of mushroom tyrosinase (7·0 mg.) and catalase (28 mg.) in M/15 phosphate buffer at pH 6·80 (20 ml.). The carbon dioxide evolved was estimated.

Time $1\frac{1}{2}$ hr.	CO_2 7·22 mg.
5	11·55
8	13·98

The melanin was isolated after 8 hr. by precipitation with ammonium nitrate (page 151).

Yield, 117·4 mg.

Residual 3,4-dihydroxyphenylalanine,[239] 61·2 mg.

Pyrrole-2,3,5-tricarboxylic acid

Triethyl pyrrole-2,3,5-tricarboxylate* (20·2 mg.) was refluxed in 5% potassium hydroxide (1·0 ml) under nitrogen

* The author is indebted to Prof. R. A. Nicolaus for his generous gift of triethyl pyrrole-2,3,5-tricarboxylate.

for 6 hr. The solution was acidified with acetic acid and the product allowed to crystallize at 0° (1 week).

Yield, 8·3 mg. (54%).

Oxidation of [^{14}C]Melanin with Potassium Permanganate

[^{14}C]Melanin (21·7 mg.) and pyrrole-2,3,5-tricarboxylic acid (7·4 mg.) in water (5 ml.) containing 2N potassium carbonate (0·4 ml.) were oxidized by M potassium permanganate (10 ml.) for 2 hr. The manganese dioxide was reduced with sulphur dioxide and the solution evaporated to dryness. The residue was extracted with hot acetone for 2 hr. Evaporation of the acetone gave a residue (123 mg.) which was methylated with diazomethane in methanol (20 ml.). Trimethyl pyrrole-2,3,5-tricarboxylate was isolated by preparative-scale t.l.c. on silica gel, eluting with ethyl acetate. (R_F 0·70).

Yield, 3·8 mg. crude,
1·1 mg. crystallized from benzene-petrol, m.p. 119-121°.

Activities

3,4-dihydroxyphenyl[α-^{14}C]alanine	2·71 x 10^5 c/s/mmole
Recovered pyrrole ester	8·56 x 10^3 c/s/mmole.

Radiochemical yield, 1·2 %.

Estimation of absorbed 3,4-Dihydroxyphenylalanine in Melanin

[^{14}C]Melanin (35·0 mg.) and 3,4-dihydroxyphenylalanine (35·0 mg.) were refluxed in N hydrochloric acid (1·0 ml.) for 4 hr. The solution was decanted and the residue washed with water (3 x 1 ml.). The total solution was evaporated to dryness and the residue, in water, neutralized exactly with aqueous ammonia. The solution was decolorized with activated charcoal and the 3,4-dihydroxyphenylalanine crystallized.

Activity, 1·38 x 10^4 c/s/mmole (5·01%)

The residual melanin was re-extracted with N hydrochloric acid for 24 hr. in the presence of 3,4-dihydroxyphenylalanine (35·0 mg.).

Activity, 0·150 x 10^4 c/s/mmole (0·55%).

References

1. Aristotle, 'History of Animals,' Book IV, Chap. 1, translated by D'Arcy Wentworth Thompsom.

2. Ann. N. Y. Acad. Sci., 1963, 100.
R.H.Thomson, 'Comparative Biochemistry', eds. M.Florkin and H.S.Mason, Academic Press, N.Y., 1962, 3, part A, p. 727.
R.H.Thomson, 'Recent Progress in the Chemistry of Natural and Synthetic Colouring Matters and Related Fields', eds. T.S.Gore, B.S.Joshi, S.V.Sunthanker, and B.D.Tilch, Academic Press, N.Y., 1962, p. 99.
H.S.Mason, 'Plant Cell Biology', ed. M.Gordon, Academic Press, N.Y., 1959, p. 563.
M.Thomas, 'Modern Methods of Plant Analysis', eds. K.Paech and M.V.Tracey, Springer, Berlin, 1955, p. 661.
A.B.Lerner and T.B.Fitzpatrick, Physiol. Rev., 1950, 30, 91.

3. D.Kertesz and R.Zeto, 'Oxygenases', ed. O.Hayaishi, Academic Press, N.Y., 1962, 307.
H.S.Mason, Adv. Enzymol., 1957, 19, 79; 1955, 16, 105.
A.B.Lerner, Adv. Enzymol., 1953, 14, 73.
J.M.Nelson and C.R.Dawson, Adv. Enzymol., 1946, 4, 99.

4. H.S.Raper, Biochem. J., 1927, 21, 89.

5. A.Bach, Biochem. Z., 1914, 60, 221.
R.Chodat and K.Schweizer, Arch. Sci. phys. nat., 1912, 33, 70; 1915, 35, 140.

6. J.Folpers, Biochem. Z., 1916, 78, 180.

7. F.C.Happold and H.S.Mason, Biochem. J. 1925, 19, 92.

8. H.S.Mason and A.Wormall, Biochem. J., 1925, 19, 84.

9. M.W.Onslow, Biochem. J., 1923, 17, 216.

10. J.Liebig, Annalen, 1846, 57, 127.

11. E.Erlenmeyer and A.Lipp, Ber., 1882, 15, 1544.
E.Erlenmeyer and J.T.Halsey, Annalen, 1899, 307, 138.
E.Fischer, Ber., 1899, 32, 3638.

12. C.Funk, J. Chem. Soc., 1911, 99, 554.

13. H.Stephen and C.Weizmann, J. Chem. Soc., 1914, 105, 1152.
K.Hirai, Biochem. Z., 1921, 114, 67.

14. T.Torquati, Chem. Zentr., 1913, II, 517, 518.

15. M.Guggenheim, Z. physiol. Chem., 1913, 88, 276.

16. E.Miller, J. Biol. Chem., 1920, 44, 481.

17. M.Damodaran and R.Ramaswamy, Biochem. J., 1937, 31, 2149.

18. H.S. Raper, Biochem. J. 1926, 20, 735.

19. W.C.Evans and H.S.Raper, Biochem. J. 1937, 31, 2155.

20. W.C.Evans and H.S.Raper, Biochem. J. 1937, 31, 2162.

21. K.Hempel, H.F.K.Männl, and M.Stehr, Biochim. Biophys. Acta, 1966, 124, 192.
S.H.Pomerantz and M.C.Warner, Biochem. Biophys. Res. Commun., 1966, 24, 25.

22. L.P.Kendal, Biochem. J. 1949, 44, 442.
K.Takenouchi and K.Aso, J. Vitaminol., 1964, 10, 123.

23. L.E.Arnow, J. Biol. Chem., 1937, 120, 151.

23a. R.Pitt-Rivers, Biochem. Soc. Symp., 1950, 5, 55.

24. G.R.Clemo and F.K.Duxbury, J. Chem. Soc., 1950, 1795.

25. M.Rolland and S.Lissitzky, Nature, 1962, 193, 881.

26. P.Larway and W.C.Evans, Biochem. J., 1962, 85, 22P

27. G.A.Swan, Ann. N. Y. Acad. Sci., 1963, 100, 1005.

28. S.Senoh, B.Witkop, C.R.Creveling and S.Undenfriend, J. Amer. Chem. Soc., 1959, 81, 1768.

29. S.Senoh, C.R.Creveling, S.Undenfriend, and B.Witkop, J. Amer. Chem. Soc., 1959, 81, 6236.

30. S.H.Pomerantz, Biochem. Biophys. Res. Commun., 1964, 16, 188.

31. S.H.Pomerantz, J. Biol. Chem., 1966, 241, 161.

32. H.S.Mason, W.L.Fowlks and E. Peterson, J. Amer. Chem. Soc., 1955, 77, 2914.

33. D.Kertesz, Bull. Soc. Chim. biol., 1953, 35, 993.

34. M.D.Hawley, S.V.Tatawawadi,S.Piekarski, and R.N.Adams, J. Amer. Chem. Soc., 1967, 89, 447.

35. S.Bouchilloux and A.M.Kodja, Bull. Soc. Chim. biol., 1960, 42, 1045.

36. S.Senoh and B.Witkop, J. Amer. Chem. Soc., 1959, 81, 6222.

37. H.S.Mason and E.W.Peterson, J. Biol. Chem., 1959, 234, 3291.

38. M.Rangier, Compt. rend., 1945, 220, 246,
J.Ruinz-Gijon, Nature, 1950, 166, 831.

39. J.D.Bu'Lock and J.Harley-Mason, J. Chem. Soc., 1951, 712.

40. W.Brackman and E.Havinga, Rec. Trav. chim., 1955, 74, 937, 1021, 1070.

41. S.Senoh and B.Witkop, J. Amer. Chem. Soc., 1959, 81, 6231.

42. O.T.Schmidt and W.Schönleben, Z. Naturforsch., 1957, 12b, 262.
H.Wyler and A.S.Dreiding, Helv. Chim. Acta, 1957, 40, 191.
H.Wyler, G.Vincenti, M.Mercier, G.Sassu and A.S.Dreiding, Helv. Chim. Acta, 1959, 42, 1696.

43. H.Wyler and A.S.Dreiding, Helv. Chim. Acta, 1959, 42, 1699; 1962, 45, 638.
T.S.Mabry, H.Wyler, G.Sassu, M.Mercier, and I.Parikk, Helv. Chim. Acta, 1962, 45. 640.

44. M.Piatteli, L.Minale and G.Porta, Ann. Chim., 1964, 54, 955. M.Piattelli and L.Minale, Phytochemistry, 1964, 3, 307.

45. M.E.Wilcox, H.Wyler, T.S.Mabry, and A.S.Dreiding, Helv. Chim. Acta, 1965, 48, 252.

46. H.Wyler, T.S.Mabry, and A.S.Dreiding, Helv. Chim. Acta, 1963, 46, 1745.

47. L.Hörhammer, H.Wagner and W.Fritzsche, Biochem. Z. 1964, 339, 398.
M.Piattelli and R.A.Nicolaus, Rend. Accad. Sci. Fis. Mat., 1965, 32.

48. G.Berti, A.D.Settimo, and D,Segnini, Ann. Chim. 1962, 52, 535.

49. H.S.Raper and A.Wormall, Biochem. J., 1923, 17, 454.

50. C.Mondier, J.N.Williams and H.A.Weisman, Arch. Biochem. Biophys., 1957, 72, 255.

51. F.P.Mazza and G.Stolfi, Arch. Sci. biol., 1931, 61, 183; Boll. Soc. ital. Biol. sper., 1930, 5, 74, 1121; Chem. Abs., 1932, 26, 216; 1930, 24, 4337; 1931, 25, 3402.

52. E.A.Friedheim, Biochem. Z. 1933, 259, 257.

53. J.D.Bu'Lock, J.Harley-Mason and H.S.Mason, Biochem J. 1950, 47, xxxii.

54. M.D.Sutherland and J.W.Wells, Aust J. Chem., 1967, 20, 515. V.H.Powell, M.D.Sutherland, and J.W.Wells, Aust. J. Chem., 1967, 20, 535. V.H.Powell and M.D.Sutherland, Aust. J. Chem., 1967, 20, 541.

55. H.S.Mason, J. Biol. Chem., 1948, 172, 83.

56. H.Sobotka and J.Austin, J. Amer. Chem. Soc., 1951, 73, 3077.

57. S.Ellis and R.N.Jones, J. Pharmacol., 1943, 79, 364.

58. F.Bergel and A.L.Morrison, J. Chem. Soc., 1943, 48.

R.A.Heacock and B.D.Scott, Can. J. Chem., 1960, 38, 508.

59. J.D.Bu'Lock and J.Harley-Mason, J. Chem. Soc., 1951, 2248.

60. J.Iwao and K.Tomio, J.Pharm. Soc. Japan, 1956, 76, 808; Chem. Abs., 1957, 51, 1177.

61. R.A.Heacock, O.Hutzinger, B.D.Scott, J.W.Daly, and B.Witkop, J. Amer. Chem. Soc., 1963, 85, 1825.

62. G.L.Mattok and D.L.Wilson, Can. J. Chem., 1967, 45, 327.

63. H.Burton, J. Chem. Soc., 1932, 546.

64. H.S.Mason and C.I.Wright, J. Biol. Chem., 1949, 180, 235.

65. J.D.Bu'Lock and J.Harley-Mason, Nature, 1950, 166, 1036.

66. D.Green and D.Richter, Biochem. J., 1937, 31, 596.

67. D.Buchnea, Ger. P., 860,065, 1952. Chem. Abs., 1959, 53, 1172. R.A.Heacock, C.Nerenberg and A.H.Payza, Can. J. Chem., 1958, 36, 853.

68. G.L.Mattok and R.A.Heacock, Can. J. Chem., 1964, 42, 484.

69. R.A.Heacock, Chem. Revs. 1959, 59, 181.

70. K.Wiesner, Biochem. Z. 1942, 313, 48.

71. J.Harley-Mason, Experimentia, 1948, 4, 307.

72. J.Harley-Mason, J. Chem. Soc., 1950, 1276.

73. J.D.Bu'Lock, J. Chem. Soc., 1961, 52.

74. P.Fischer, G.Durant, H.Lambert, and J.Lecompte, Bull. Soc. chim. belges., 1930, 59, 72.

75. A.E.Oxford and H.S.Raper, J.Chem. Soc., 1927, 417.

76. W.L.Dulière and H.S.Raper, Biochem. J. 1930, 24, 239.

77. W.H.Perkin and L.Rubenstein, J. Chem. Soc., 1926, 357.

78. S.Bouchilloux and A.Kodja, Compt. rend., 1958, 247, 2484.

M.Piattelli, E.Fattorusso, and S.Magno, Rend. Accad. Sci. Fis. Mat., 1961, 28, 168

79. R.I.T.Chromartie and J.Harley-Mason, J. Chem. Soc., 1953, 3525.

80. R.I.T.Chromartie and J.Harley-Mason, J. Chem. Soc., 1952, 2525.

81. J.Harley-Mason, J. Chem. Soc., 1953, 200.

82. E.J.Forbes, J. Chem. Soc., 1955, 3926; 1956, 513.

83. R.I.T.Chromartie and J.Harley-Mason, Chem.and Ind., 1953, 972.

84. R.I.T.Chromartie and J.Harley-Mason, Biochem. J., 1957, 66, 713.

85. M.Rangier, Compt. rend., 1952, 234, 2652.

86. R.J.S.Beer, K.Clarke, H.G.Khorana and A.Robertson, J. Chem. Soc., 1948, 2223.

87. M.Piattelli, E.Fattorusso, S.Magno and R.A.Nicolaus, Tetrahedron, 1963, 19, 2061.

88. J.D.Bu'Lock, Arch. Biochim. Biophys., 1960, 91, 189.

89. R.J.S.Beer, L.McGarth, A.Robertson and, A.B.Woodier, J. Chem. Soc., 1949, 2061.

90. H.Burton, Chem and Ind., 1947, 383.

91. M.Piattelli and R.A.Nicolaus, Tetrahedron, 1961, 15, 66.

92. M.Piattelli, E.Fattorusso, S.Magno and R.A.Nicolaus, Tetrahedron, 1962, 18, 941.

93. J.D.Bu'Lock and J.Harley-Mason, J. Chem. Soc., 1951, 703.

94. R.J.S.Beer, T.Broadhurst, and A.Robertson, J. Chem. Soc. 1954, 1947.

95. G.R.Clemo and J.Weiss, J. Chem. Soc., 1945, 702.

96. J.Harley-Mason, Nature, 1947, 159, 338.
J.Harley-Mason, J. Chem. Soc., 1948, 1244.

97. G.N.Cohen, Bull. Soc. Chim. biol., 1946, 28, 104, 107, 354.

98. J.Harley-Mason and J.D.Bu'Lock, Chem. and Ind., 1954, 377.

99. H.Burton, Chem. and Ind., 1948, 313.

100. L.Horner and W.Spietschka, Annalen, 1955, 591, 1.

101. A.Angeli, Rend.Accad. Sci. Fis. Mat., 1915, 24, 1; Gazzetta, 1918, 48, 67; Atti Accad. Lincei, 1918, 27, 417.

102. A.Angeli, Gazzetta, 1918, 48, 21; Atti Accad. Lincei, 1918, 27, 209.

103. A.Angeli, Atti Accad. Lincei, 1927, 6, 87.

104. J.Böeseken and G.Sloof, Proc. Acad. Sci. Amsterdam, 1929, 32, 1043; Chem. Abs., 1930, 24, 1085.

105. J.Ppspíšil and V.Ettel, Chem. průmysl, 1957, 7, 244; Chem. Abs., 1958, 52, 14561.

106. O.Hayaishi and K.Hashimoto, J. Biochem., 1950, 37, 371. W.C.Evans and B.S.W.Smith, Biochem. J., 1951, 49, x.

107. D.L.McDonald, R.Y.Stanier, and J.L.Ingraham, J. Biol. Chem., 1954, 210, 809.

108. G.R.Clemo and F.K.Duxbury, Chem. and Ind., 1953, 195.

109. Z.E.Jolles, Chem. and Ind., 1953, 845.

110. J.M.Bruce, J. Appl. Chem., 1954, 4, 469.

111. G.R.Clemo, F.K.Duxbury, and G.A.Swan, J. Chem. Soc., 1952, 3464.

112. N.C.Robson and G.A.Swan, 'Symposium on the Structure and Control of the melanocyte', 6th International Pigment Cell Conference of the International Union against Cancer, Springer-Verlag, Berlin, 1966, p. 155.

113. G.A.Swan and D.Wright, J. Chem. Soc., 1954, 381.

114. G.A.Swan and D.Wright, J. Chem. Soc., 1956, 1549.

115a. W.Brackman and E.Havinga, Rec. Trav. chim., 1955, 74, 1100.

116a. D.Keilin and E.F.Hartree, Biochem. J. 1945, 39, 293.
F.K.Duxbury, Chem. and Ind., 1953, 1364.

117a. F.H.J.Figge, Proc. Soc. Exp. Biol. Med., 1939, 41, 127; 1940, 44, 293.

118a. N.Millott, Experimentia, 1952, 8, 301.

119a. A.Angeli and C.Lutri, Atti Accad. Lincei., 1920, 29, 14; Gazzetta, 1920, 50, 128.

120a. A.Angeli and A,Pieroni, Atti Accad. Lincei, 1915, 24, 3.

115. L.Panizzi and R.A.Nicolaus, Gazzetta, 1952, 82, 435.
R.A.Nicolaus, Gazzetta, 1953, 83, 239; 1955, 85, 659.
R.A.Nicolaus and G.Oriente, Gazzetta, 1954, 84, 230.
R.A.Nicolaus and L.Mangori, Gazzetta, 1955, 85, 1397.
F.Binns and G.A.Swan, Chem. and Ind., 1957,396.
R.A.Nicolaus, A.Vitale, and M.Piattelli, Rend. Accad. Sci. Fis. Mat., 1958, 25, 220.
M.Piattelli, E.Fattorusso, and S.Magno, Tetrahedron Letters, 1961, 718.

116. R.A.Nicolaus, M.Piattelli, and G.Narni, Tetrahedron Letters, 1959, 21, 14

117. M.Piattelli, E.Fattorusso, S.Magno, and R.A.Nicolaus, Rend. Accad. Sci. Fis. Mat., 1961, 28, 337.

118. R.A.Nicolaus, M.Piattelli, and E.Fattorusso, Tetrahedron. 1964, 20, 1163.

119. L.Ogunkoya, Ph.D. Thesis, London, 1965.

120. R.A.Nicolaus and M.Piattelli, Rend. Accad. Sci. Fis. Mat., 1965, 32.

121. M.Piattelli, E.Fattorusso, S.Magno, and R.A.Nicolaus, Rend. Accad. Sci. Fis. Mat., 1961, 28, 165.

122. M.Piattelli, E.Fattorusso, R.A.Nicolaus, and S.Magno, Tetrahedron, 1965, 21, 3229.

123. L.A.Griffiths, Nature, 1959, 184, 58.

124. P.Correale and I.Cortese, Naturwiss., 1953, 40, 57. New Scientist, 1966, 30, 641.

125. H.S.Mason, H.Kahler, R.C.MacCardle, and A.J.Dalton, Proc. Soc. Exp. Biol. Med., 1947, 66, 421.

126. C.Steelink and G.Tollin, A.E.C. Accession No. 10463, 1964; Chem. Abs., 1966, 65, 14006.
G.Tollin and C.Steelink, Biochim. Biophys. Acta, 1966, 112, 377

127. H.S.Mason and A.Lada, Fed. Proc., 1952, 11, 254.

128. T.G.Bonner and A.Duncon, Nature, 1962, 194, 1078.

129. B.Commoner, J.Townsend, and G.E.Pake, Nature, 1954, 174, 689. A.B.Zahlan, J.E.Maling, and M.S.Blois, Photochem. Photobiol., 1966, 5, 269.

130. H.S.Mason, D.J.E.Ingram, and B.Allen, Arch. Biochem. Biophys., 1960, 86, 225.

131. H.C.Longuet-Higgins, Arch. Biochim. Biophys. 1960, 86, 231.

132. R.J.S.Beer, K.Clarke, H.G.Khorana, and A.Robertson, J. Chem. Soc., 1948, 1605.

133. J.L.Stoves, Chem. and Ind., 1954, 158.

134 R.J.S.Beer, J.P.Brown and A.Robertson, J. Chem. Soc., 1951, 2426.

135. G.W.Kirby and L.Ogunkoya, Chem. Commun., 1965, 546.

136. G.A.Swan and R.A.Nicolaus, Rend. Accad. Sci. Fis. Mat., 1964, 31, 212.

137. K.Hempel and W.Erb, Z. Zellforsch., 1962, 58, 125.
K.Hempel and M.Deimel, Arch. exp. Path. Pharmak., 1963, 246, 203; Chem. Abs., 1964, 60, 7254.

138. J.Renson, J.Daly, H.Weissbach, B.Witkop and S.Undenfriend, Biochem. Biophys. Res. Commun., 1966, 25, 504.

139. G.Guroff, M.Levitt, J.Daly, and S.Undenfriend, Biochem. Biophys. Res. Commun., 1966, 25, 253.

140. S.Undenfriend, P.Zaltzman-Nirenberg, J.Daly, G.Guroff, and B.Witkop, Reference from- J.Daly and B.Witkop, J. Amer. Chem. Soc., 1967, 89, 1032.

141. G.Guroff, K.Kondo and J.Daly, Biochem Biophys. Res. Commun., 1966, 25, 622.

142. H.Yoshida, J.Chem. Soc., 1883, 43, 472.

143. P.L.d'Incarville, Mém. Math. Phys. presenté Acad. Roy. Sci, Paris, 1760, 3, 117.

144. W.F.Symes and C.R.Dawson, J. Amer. Chem. Soc., 1953, 75, 4952; 1954, 76, 2959; Nature, 1953, 171, 841.
S.V.Sunthankar and C.R.Dawson, J. Amer. Chem. Soc., 1954, 76, 5070.
B.Loev and C.R.Dawson, J. Amer. Chem. Soc., 1956, 78. 1180.

145. G.Bertrand, Compt. rend., 1896, 122, 1215.

146. Report of the Commission on Enzymes of the International Union of Biochemistry, Oxford, Pergamon Press, 1961.

147. R.A.Gortner, J. Chem. Soc., 1910, 97, 110.

148. C.Gessard, Compt. rend., 1903, 136, 1086.

149. C.Neuberg, Biochem. Z., 1908, 8, 383.

150. C.Gessard, Compt. rend. Soc. Biol., 1902, 54, 1304.

151. S.Soloway, U.S.P. 3,251,742 (1966).

152. H.S.Raper and H.B.Speakman, Biochem. J., 1926, 20, 69.

153. H.Onslow, Proc. Roy. Soc., 1917, B89, 36.
D.Gaudin and J.H.Fellman, Biochim. Biophys. Acta, 1967, 141, 64

154. B.Bloch, Amer. J. Med. Sci., 1929, 177, 609.
G.F.Lindlow, Amer. J. Path., 1932, 8, 477.
G.H.Hogeboom and M.H.Adams, J. Biol. Chem., 1942, 145, 273.

155. F.Kubowitz, Biochem. Z. 1938, 299, 32.

156. M.F.Mallette and C.R.Dawson, Arch. Biochem. Biophys. 1949, 23, 29.

157. C.R.Dawson and R.J.Magee,'Methods in Enzymology', eds. S.P.Colowick and N.O.Kaplan, Academic Press, N. Y., 1955, 2, p. 817.
T.Matsuzawa, J. Expl. Med., 1960, 7, 143.

158. H.A.El Bayoumi and E.Frieden, J. Amer. Chem. Soc., 1957, 79, 4854.

159. K.Yasunobu, L.C.G.Thomsom and H.S.Mason, Fed. Proc., 1957, 16, 275.

160. M.F.Mallette, S.Lewis, S.R.Ames, J.M.Nelson, and C.R.Dawson, Arch. Biochem. Biophys., 1948, 16, 283.

161. J.L.Smith and R.C.Kreuger, J. Biol. Chem., 1962, 237, 1121.

162. D.Kertesz and R.Zito, Nature, 1957, 179, 1017.

163. R.C.Kreuger, Fed. Proc., 1959, 18, 267.

164. D.Kertesz and R.Zito, Biochim. Biophys. Acta, 1962, 64, 153.

165. D.Kertesz and R.Zito, Biochim. Biophys. Acta, 1965, 96, 447.

166. S.Bouchilloux, P.McMahill and H.S.Mason, J. Biol. Chem., 1963, 238, 1699

167. R.L.Jolley and H.S.Mason, J. Biol. Chem., 1965, 240, 1489.

168. M.Fling, N.H.Horowitz and S.F.Heinemann, J. Biol. Chem., 1963, 238, 2045.

169. A.S.Sussman, Arch. Biochem. Biophys., 1961, 95, 407.

170. F.C.Brown and D.N.Ward, Proc. Soc. Expl. Biol. Med., 1959, 100, 701; J. Amer. Chem. Soc., 1957, 79, 2647.

171. S.H.Pomerantz, J. Biol. Chem., 1963, 238, 2351.

172. A.Schweiger and P.Karlson, Z. Physiol. Chem., 1962, 329, 210. P.Karlson and C.E.Sekeris, Biochim. Biophys. Acta, 1962, 63, 489.

173. D.A.Robb, T.Swain and L.W.Mapson, Phytochemistry, 1966, 5, 665.

174. S.Patil and H.J.Evans, Nature, 1963, 200, 1322.

175. F.Kubowitz, Biochem. Z., 1937, 292, 221.

176. E.Frieden, G.Ezell, and Y.Karkhanis, Fed. Proc., 1957, 16, 183.

177. D.Kertesz, Nature, 1957, 180, 506.

178. R.G.R.Brown and H.A.O.Hill, Quart. Rev., 1965, 19, 95.

179. L.Malatesta, Gazzetta, 1941, 71, 467, 580. M.W.Lister, Canad. J. Chem., 1953, 31, 638.

180. K.Shimao, Biochim. Biophys. Acta, 1962, 62, 205. S.Osaki, Arch. Biochem. Biophys., 1963, 100, 378. E.A.Kean, Biochim. Biophys. Acta, 1964, 92, 602. M.Ikeda, L.A.Fahien, and S.Undenfriend, J. Biol. Chem., 1966, 241, 4452.

181. R.C.Behm and J.M.Nelson, J. Amer. Chem. Soc., 1944, 66, 711. L.P.Kendal, Biochem. J., 1949, 44, 442. A.B.Lerner, T.B.Fitzpatrick, E.Calkins, and W.H.Summerson, J. Biol. Chem., 1951, 191, 799.

182. D.Kertesz and D.Azzopardi, Biochim. Biophys. Acta, 1962, 64, 149.

183. D.Keilin and T.Mann, Proc. Roy. Soc., 1939, 125B, 187.

184. S.Friedman and S.Kaufman, J. Biol. Chem., 1966, 241, 2256.

185. W.Brackman and E.Havinga, Rec. Trav. chim., 1955, 74, 1107. E.Ochiai, Tetrahedron, 1964, 20, 1831.

186. L.V.Gorbunova, O.N.Efimov, V.V.Karpov, G.A.Razuvaev, V.B.Turchaninov, M.L.Khidekd, Mekhanizm i Kinetika Fermentativnogo Kataliza Akad. Nauk. S.S.S.R., 1964, 227; Chem. Abs., 1965, 62, 5518.

187. T.Nakamura, Biochim.Biophys. Acta, 1958, 30, 44.

188. T.Omura, J. Biochem., 1961, 50, 265; Chem. Abs., 1961, 56, 9080.

189. T.Nakamura, Biochim. Biophys. Acta, 1958, 30, 538, 640. T.Nakamura,and Y.Ogura, J. Biochem,, 1966, 59, 447; Chem. Abs., 1966, 65, 7550.

190. A.Nishimura, T.Omura, H.Endo, and A.Nagato, Repts. Liberal Arts Fac. Shizuoka Univ. Nat. Sci., 1956, 9, 35; Chem. Abs., 1958, 52, 3043.

191. R.Mosbach, Biochim. Biophys. Acta, 1963, 73, 204.

192. S.Shichiji, Y.Saeki, and T.Murakami, Kogyo Gijutsuin Hakko Kenkjusho Kenkyu Hokokou, 1965, 27, 1; Chem. Abs., 1966, 64, 5367.

193. L.Broman, B.G.Malmström, R.Aasa, and T.Vängård, J. Mol. Biol., 1962, 5, 1301.
A.Ehrenberg, B.G.Malmström, L.Broman, and R.Mosbach, J. Mol. Biol., 1962, 5, 450.

194. T.Omura, J. Biochem,, 1961, 30, 305.

195. R.Pummerer, D.Melamed, and H.Puttfarcken, Ber., 1922, 55, 3116.
R.Pummerer, H.Puttfarcken, and P.Schopflocher, Ber., 1925, 58, 1808.

196. V.Deulofeu and G.Mendivelzua, Z. Physiol. Chem., 1933, 219, 233.

197. J.C.Reid,'Isotopic Carbon', eds. M.Calvin, C.Heidelberger, J.C.Reid, B.M.Tolbert,and P.F.Yankwich, J.Wiley, N.Y.,1949,p.225.

198. D.H.R.Barton, A.M.Deflorin, and O.E.Edwards, J. Chem. Soc., 1956, 530.

199. T.Matsuura and K.Ogura, J. Amer. Chem. Soc., 1966, 88, 2602.

200. K.Bowden and C.H.Reece, J. Chem. Soc., 1950, 2249.

201. W.W.Westerfield and C.Lowe, J. Biol. Chem., 1942, 145, 463.

202. G.W.Kirby and L.Ogunkoya, J. Chem. Soc., 1965, 6914.

203. G.W.Kirby and H.P.Tiwari, J. Chem. Soc., 1964, 4655.

204. A.I.Scott, Quart. Revs., 1965, 19, 1.

A.J.Baker, T.A.Davidson, A.C.Day, M.B.Meyers, J.Nabney, and A.I.Scott, Bull. Nat. Inst. Sci. India, 1965, 31, 191.

205. J.M.Bruce, J. Chem. Soc., 1959, 2366.

206. J.M.Bruce, J. Chem. Soc., 1960, 360.

207. E.Adler and R.Magnusson, Acta. Chem. Scand., 1959, 13, 505.
E.Adler and S.Hernestam, Acta. Chem. Scand., 1955, 9, 319.

208. W.Otting and G.Stanger, Chem. Ber., 1955, 88, 828.

209. B.C.Challis and F.A.Long, J. Amer. Chem. Soc., 1963, 85, 2524.

210. R.L.Hinman and E.B.Whipple, J.Amer. Chem. Soc., 1962, 84, 2534.
A.H.Jackson and A.E.Smith, J. Chem. Soc, 1964, 5510.

211. E.Fischer and T.Schmidt, Ber., 1888, 21, 1811.
A.H.Jackson and A.E.Smith, Tetrahedron, 1965, 21, 989.
A.H.Jackson and P.Smith, Chem. Commun., 1967, 264.
N.P.Buu-Hoï and P. Jacquignon, Bull. Soc. chim. France, 1967, 1104.

212. F.J.Baude, Diss. Abs., 1965, 26, 82.

213. W.E.Noland and F.J.Baude, J. Org. Chem., 1966, 31, 3321.

214. D.J.Bennett, Ph.D. thesis, London, 1965.

215. W.J.Boyd and W.Robson, Biochem. J., 1935, 29, 542.

216. C.Kaiser and A.Burger, J. Amer. Chem. Soc., 1957, 79, 4365.

217. H.Decker and R.Pschorr, Ber., 1904, 37, 3396.

218. T.A.Geissman, Org. Reactions, 1944, 2, 94.

219. D.R.Lachowicz and R.J.Gritter, J. Org. Chem., 1963, 28, 106.

220. C.R.Hauser, P.J.Hamrick and A.T.Stewart, J. Org. Chem., 1956, 21, 260.

221. K.B.Wiberg, J. Amer. Chem. Soc., 1954, 76, 5371.

222. I.A.Pearl , J. Org. Chem., 1947, 12, 85.

223. Y.Urushibara and M.Takebayashi, Bull. Chem. Soc. Japan, 1937, 12, 328; Chem. Abs., 1937, 31, 7394.
M.S.Kharasch and M.Foy, J. Amer. Chem. Soc., 1935, 57, 1510.

224. J.E.Zanetti and D.V.Sickman., J. Amer. Chem. Soc., 1935, 57, 2735; 1936, 58, 2034.

225. A.F.Thompson and N.H.Cromwell, J. Amer. Chem. Soc., 1939, 61, 1374.

226. K.B.Wiberg, J. Amer. Chem. Soc., 1954, 76, 5371.
K.B.Wiberg and R.Stewart, J. Amer. Chem. Soc., 1955, 77, 1786.
W.M.Schubert and H.Burkett, J. Amer. Chem. Soc., 1956, 78, 64.

W.M.Schubert and P.C.Myhre, J. Amer. Chem. Soc., 1958, 80, 1755.

227. V.Franzen, Annalen, 1956, 600, 109.
A.Streitwieser and J.R.Wolfe, J. Amer. Chem. Soc., 1957, 79, 903.

228. T.Cohen and I.H.Song, J. Amer. Chem. Soc., 1965, 87, 3780.

229. D.Seebach, B.W.Erickson, and G.Singh, J. Org. Chem., 1966, 31, 4303.

230. D.J.Bennett, G.W.Kirby, and V.A.Moss, Chem. Commun., 1967, 218.

231. R.A.Henry and W.M.Dehn, J. Amer. Chem. Soc., 1950, 72, 2804.

232. H.Najer, P.Chabrier, R.Giudicelli and J.Sette, Bull. Soc. chim. France, 1958, 1189.

233. L.H.Goodson and H.Christopher, J. Amer. Chem. Soc., 1950, 72, 358.

234. N.J.Leonard and J.V.Paukstelis, J. Org. Chem., 1963, 28, 3021.

235. D.J.Blythin, PhD. thesis, London, 1967.

236. F.Reverdin, Ber. 1907, 40, 2442.
G.J.Esselen and L.Clarke, J. Amer. Chem. Soc., 1914, 36, 308.

237. C.Grundmann and M.B.Fulton, Chem. Ber., 1964, 97, 566.

238. E.Frieden and M.Ottesen, Biochim. Biophys. Acta, 1959, 34, 248.

239. L.E.Arnow, J.Biol. Chem., 1937, 118, 531.

240. V.Arkley, F.M.Dean, A.Robertson, and P.Sidisunthorn, J. Chem. Soc., 1956, 2322.
G.G.Haynes, A.H.Turner, and W.A.Waters, J. Chem. Soc., 1956, 2823.

241. L.Horner, Annalen, 1953, 579, 170.

242. J.Klosa, J. prakt. Chem, 1961, 12, 258.

243. Israel P., 13,383, 1960. Chem. Abs., 1961, 55, 7451.

244. E.C.Wagner and J.K.Simmons, J. Chem. Educ., 1936, 13, 203.

245. A.I.Vogel, 'Textbook of Practical Organic Chemistry,' 2nd Edn., Longmans, 1951, 190.

246. G.Vavon, Compte. rend., 1912, 154, 360.

247. H.J.Backer and C.C.Bolt, Rec. Trav. chim., 1935, 54, 538.

248. H.Weil, Ber., 1894, 40, 1403.

249. H.Böhme, H.Ellenberg, O.E.Herboth, and W.Lehners, Chem. Ber., 1959, 92, 1608.

250. F.Pregl,'Quantitative Organic Micro-analysis,' J.Churchill, London.