362. *Formation of Tyrosine Melanin. Part II.*

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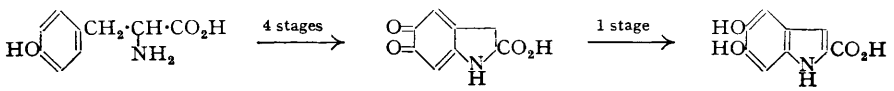
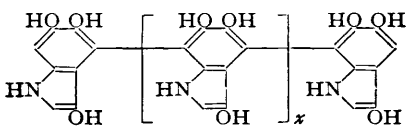
Derivatives of tyrosine and β-phenylalanine have been prepared and their reactions with tyrosinase investigated. The visible and ultra-violet spectra of the resulting pigments have been compared and the results discussed.

Although the formation of melanin from tyrosine has been extensively studied, the reactions involved and the structure of the pigment itself remain unsolved problems. Furthermore, there is still controversy about the relation between natural and synthetic melanins (Mason, *New York Acad Sci.*, 1948, **4**, 399; Ginsburg, *Genetics*, 1944, **29**, 176). The view that melanin formation from tyrosine (I) involves the five stages needed to convert it into (III) *via* the red pigment (II) was advanced by Raper (*Biochem. J.*, 1927, **21**, 89) and has received confirmation from the spectroscopic work of Mason (*J. Biol. Chem.*, 1948, **172**, 83) and the synthetic work of Robertson and his co-workers (*J.*, 1948, 2223; 1949, 2061).

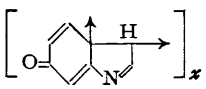


(I.) (II.) (III.)

Various suggestions have been advanced to account for the formation of melanin from (III), but none has been proved. Clemo and Weiss (*J.*, 1945, 702) suggested that the 5 : 6-dihydroxyindole couples in position 2 and is oxidised at position 3, and that the resulting indigoid structure then undergoes further polymerisation, but this view has been rendered unlikely by Harley-Mason (*J.*, 1948, 1244), who prepared 5 : 6 : 5′ : 6′-tetrahydroxyindigo and found that it did not yield melanin. Burton (*Chem. and Ind.*, 1948, **67**, 313) suggested 4 : 7-coupling of the dihydroxyindole and oxidation at position 3 to give a polymer of the type (IV).



(IV.)



(V.)

On the assumption that (III) is an essential intermediate, it is probable either that melanin results from the polymerised coupling at certain positions of the dihydroxyindole system, or that the catechol nucleus first undergoes oxidative fission as postulated by Woodward for the biogenesis of strychnine (*Nature*, 1947, **162**, 155) and by Robinson for that of emetine (*ibid.*, 1948, **162**, 524). If the latter mechanism were involved, (III) would be converted into a reactive ketonic substance and to test this view we added hydroxylamine to the tyrosine enzyme system. The further reaction was not inhibited and we conclude therefore that such an oxidative fission is improbable.

The oxidation of 6-hydroxy-3-phenylindole-2-carboxylic acid to a black pigment led Morton and Slaunwhite (*J. Biol. Chem.*, 1948, **179**, 259) to postulate the quinomethane system (V) for their pigment, which does not need a 5-hydroxyl group.

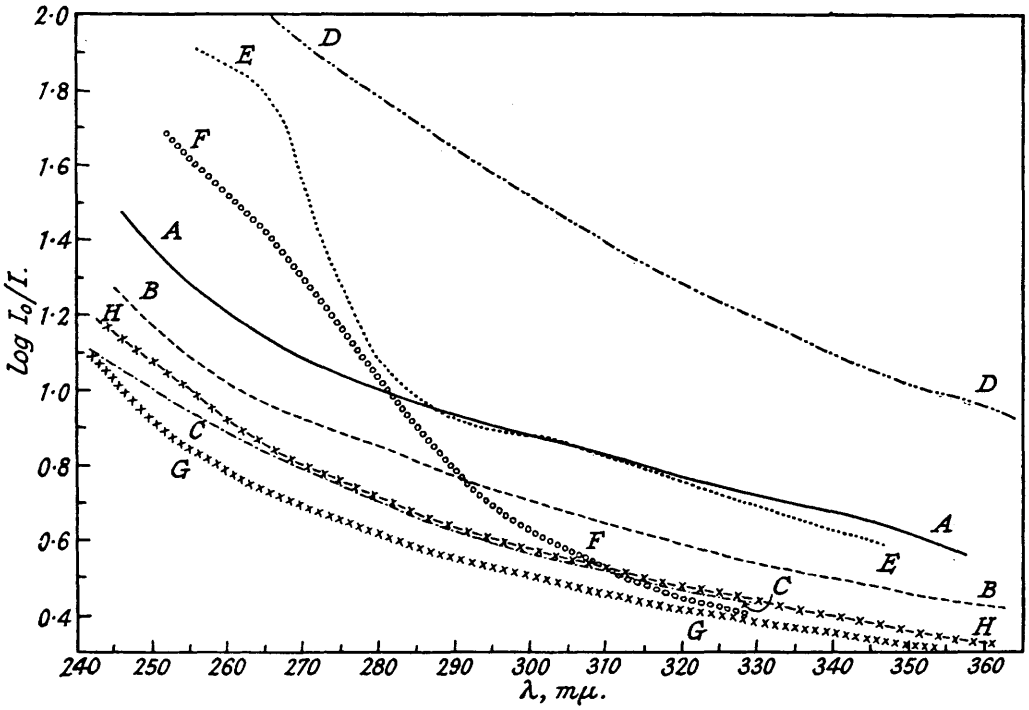
We have therefore based our work on the assumption that the indole nucleus undergoes oxidative coupling at some of the many available centres, and inhibition of melanin formation would be expected if the hydrogen atoms at the active centres were replaced by other groups. Accordingly, substituted tyrosines have been prepared and treated under the standard conditions for production of melanin from tyrosine by tyrosinase. Thus, 2- (Schmalfus and Peschke, *Ber.*, 1929, **62**, 2591) and 3-methyl-, and 3-methoxy-tyrosine yield no melanic pigment, although in all cases position 5 is free for the introduction of the hydroxyl group. The same is true for 2 : 4-dihydroxyphenylalanine (Hirai, *Biochem. Z.*, 1926, **177**, 449), for which the entry of a hydroxyl group at position 5 would be expected to be particularly easy; in this case, however, the enzyme is poisoned, but not in the three cases mentioned above. Although the enzyme is to some extent specific in this reaction the absence of the carboxyl group is stated to produce no difference in melanin formation since tyrosine and *p*-hydroxyphenylethylamine take up similar amounts of oxygen (Dulière and Raper, *Biochem. J.*, 1930, **24**, 259). The effect of blocking position 2 of the indole nucleus has been examined also by using tyrosine ethyl ester (E. Fischer,

Ber., 1901, **34**, 433). This forms a brown pigment insoluble in aqueous sodium hydrogen carbonate, but readily soluble in warm 10% aqueous sodium hydroxide. The solution yields on acidification a brown solid easily soluble in aqueous sodium hydrogen carbonate with the evolution of carbon dioxide. The ester group has thus been retained.

The ester-containing pigment, unlike tyrosine-melanin, cannot be formed by the autoxidation procedure, which suggests that the dihydroxyindole-2-carboxylic ester is not autoxidised in alkaline solution. The reaction may, therefore, proceed differently from that involved in melanin formation, although Robertson *et al.* (*loc. cit.*) and Burton have shown that a 2-methyl group in the case of 5 : 6-dihydroxy-2-methylindole does not prevent melanin formation.

FIG. 1.

Ultra-violet absorption spectra. Cell length = 1 cm. Concentrations are in mg./ml.



A, *Tyrosine-melanin* (0·066).
B, 2 : 3-*Dihydroxyphenylalanine-melanin* (0·033).
C, *Carboxyl-containing pigment after further enzyme action* (0·050).
D, *Carboxyl-containing pigment* (0·020).
E, N-*Methylmelanin* (0·016).
F, *Adrenalin-melanin* (<0·001).
G, *Tyrosine-melanin* (0·034), *blocked with hydroxylamine.*
H, *Tyrosine-melanin* (0·037), *blank for hydroxylamine test.*

The carboxyl-containing pigment when treated with tyrosinase is converted into a brownish-black pigment initially sparingly soluble, but after reprecipitation insoluble, in aqueous sodium hydrogen carbonate. This slight solubility suggests decarboxylation and the analytical figures suggest that a further reaction has occurred, but since the melanin pigments cannot be purified by recrystallisation—ethylene chlorohydrin, claimed as a suitable solvent by Lea (*Nature*, 1945, **156**, 478), has not proved useful in our hands—the usual analyses cannot be as accurate as for organic compounds generally.

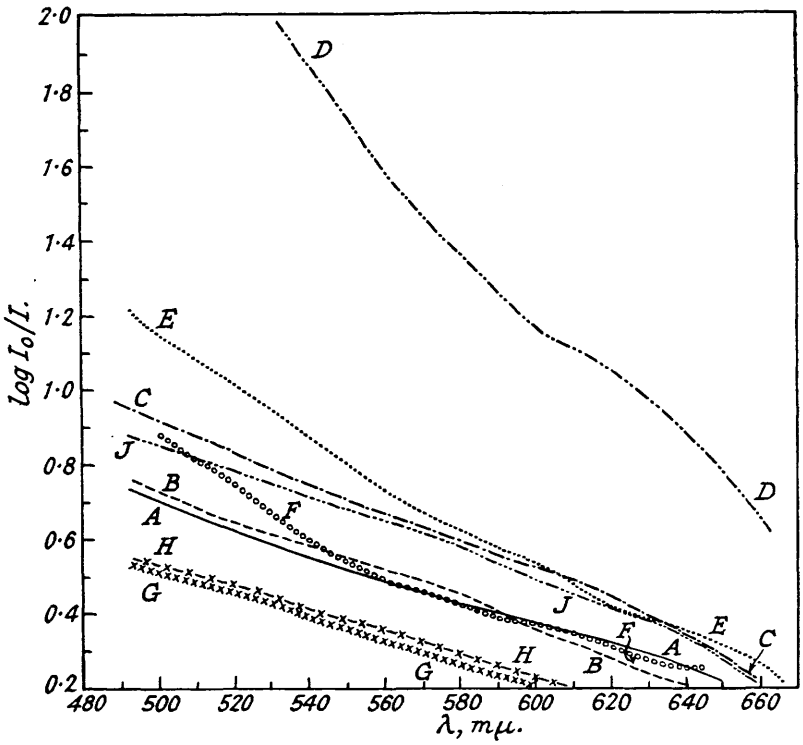
Raper infers that *N*-methyltyrosine (Corti, *Helv. Chim. Acta*, 1949, **32**, 681) forms *N*-methylmelanin which indicates that the blocking of position 1 of the indole nucleus does not inhibit pigment formation and we have found that the ethyl ester of *N*-methyltyrosine, which is blocked in both the 1 and the 2 position yields no pigment. Tyrosine-melanin is only very slightly soluble in aqueous sodium hydroxide after being heated on the water-bath, whereas *N*-methylmelanin as well as the above carboxyl-containing pigment both readily dissolve and

these solutions are thus very suitable for the determination of their visible and ultra-violet spectra (Figs. 1 and 2).

2 : 3-Dihydroxyphenylalanine has also been prepared and found to yield a black pigment easily soluble in N-potassium hydroxide, but not in sodium hydrogen carbonate solution, thus indicating that the 4-hydroxyl group of the indole nucleus is probably free. This pigment is spectroscopically identical with tyrosine-melanin, and this suggests that position 4 may not be needed for polymer coupling as required by Burton's formula.

FIG. 2.

Visible-light absorption spectra. All concentrations are 0·095 *mg./ml., except where otherwise stated. Cell length* = 2·5 *cm.*



A, *Tyrosine-melanin.*
B, 2 : 3-*Dihydroxyphenylalanine-melanin.*
C, *Carboxyl-containing pigment after further enzyme action* (0·107).
D, *Carboxyl-containing pigment.*
E, N-*Methylmelanin.*
F, *Adrenalin-melanin* (<0·005).
G, *Tyrosine-melanin* (*blocked with hydroxylamine*).
H, *Tyrosine-melanin* (*blank for hydroxylamine test*).
J, 2 : 3-*Dihydroxyphenylalanine-melanin* (*substituted indole allowed to autoxidise*).

Burton also suggests the introduction of a hydroxyl group at position 3, but we find that adrenalin which has a potential hydroxyl group at position 3 is converted by tyrosinase into a brown pigment spectroscopically different from *N*-methyltyrosine-melanin.

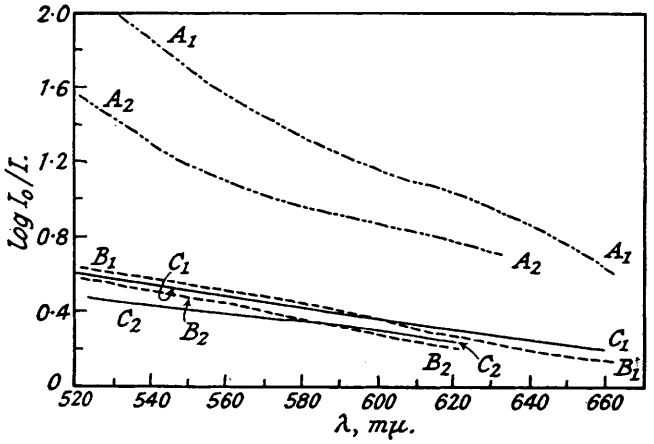
The absorption spectra of the pigments (Figs. 1 and 2) have characteristic gradients. When the solutions are kept in alkaline solution the absorption generally decreases (Fig. 3), no deposit is observed, and the gradient remains constant.

This result is in agreement with Dulière and Raper's observation (*loc. cit.*) that more oxygen is taken up in solutions of pH greater than 8, thus indicating further oxidative coupling whereby the molecular concentration decreases so that absorption becomes less intense. The analytical results obtained for the above-described pigments (dried to constant weight over sulphuric acid in a vacuum) show consistently higher hydrogen values than those quoted previously for melanin and indicate that in all cases formation of the pigment is accompanied by an oxygen uptake.

3-Methoxytyrosine was prepared by a route alternative to that employed by Johnson and Bengis (*J. Amer. Chem. Soc.*, 1913, **35**, 1606). We found the melting point of one of the intermediates, α-oximino-β-(4-hydroxy-3-methoxyphenyl)pyruvic acid, to differ from that recorded by Fischer and Hibbert (*J. Amer. Chem. Soc.*, 1947, **69**, 1208).

FIG. 3.

Visible-light absorption spectra. Decreases in absorption when substances are kept standing in alkaline soln. Concns. as in Fig. 2.



A_1, *Carboxyl pigment.*
A_2, A_1 *after 7 days.*
B_1, 2 : 3-*Dihydroxyphenylalanine-melanin.*
B_2, B_1 *after 24 hours.*
C_1, *Tyrosine-melanin.*
C_2, C_1 *after 24 hours.*

Our results show that the pigments from *N*-methyltyrosine and tyrosine ethyl ester differ from tyrosine-melanin. Further, the evidence indicates that positions 1 and 2, but not 4, are involved in the oxidative polymerisation. The work is being continued.

EXPERIMENTAL.

(M. p.s are uncorrected.)

3-*Methyltyrosine*.—Diketopiperazine (2·0 g.), 4-hydroxy-3-methylbenzaldehyde (4·8 g.), freshly fused sodium acetate (9 g.), and distilled acetic anhydride (9 ml.) were heated for 7 hours at 145—150°. The mixture gradually solidified to a dark brown mass. After addition of water (40 ml.), the cooled product was warmed, filtered, washed with hot water, and recrystallised from acetic acid, 2 : 5-*diketo*-3 : 6-*di*-(4-*acetoxy*-3-*methylbenzylidene*)*piperazine* being obtained as golden-yellow plates (3·9 g., 51%), m. p. 280° (Found : C, 66·5; H, 5·5. $C_{24}H_{22}O_6N_2$ requires C, 66·4; H, 5·1%). The benzylidenepiperazine (1·4 g.), hydriodic acid (22 ml.), and red phosphorus (2·0 g.) were refluxed for 10 hours. The cooled deep-red solution was diluted with water (20 ml.) and filtered, the filtrate evaporated to dryness, water (10 ml.) added, and the yellow solution titrated with aqueous ammonia (*d* 0·986) until the colour just disappeared (pH 5). The free amino-acid was filtered and recrystallised twice from water (charcoal), 3-methyltyrosine being obtained as colourless prisms (0·7 g., 54%), m. p. 276° (Found : C, 61·3; H, 6·7. Calc. for $C_{10}H_{13}O_3N$: C, 61·5; H, 6·7%).

3-*Methoxytyrosine*.—Sodium (1·7 g.) in absolute ethanol (70 ml.) was added to a warm solution of hydroxylamine hydrochloride (4·8 g.) in the minimum amount of water. After removal of sodium chloride, α-thio-β-(4-hydroxy-3-methoxyphenyl)pyruvic acid (5 g.) was added, the mixture was refluxed for 45 minutes, cooled, and evaporated to dryness in a vacuum. The pale yellow product was dissolved in a minimum amount of aqueous sodium hydroxide (5%), filtered from sulphur, and cooled to 0°. Hydrochloric acid (10%) was added until the solution became pale yellow. α-Oximino-β-(4-hydroxy-3-methoxyphenyl)pyruvic acid slowly crystallised out and was recrystallised from water (yield, 3 g., 60%; m. p. 156°) (Found : C, 53·7; H, 5·2. Calc. for $C_{10}H_{11}O_5N$: C, 53·3; H, 4·9%). To the oximino-acid (4 g.) in aqueous sodium hydroxide (8%; 30 ml.) sodium amalgam (4%; 80 g.) was added during 30 minutes. The solution was evaporated to 30 ml., treated with charcoal, and cooled. The amino-acid was filtered off, dried at 100°, and recrystallised from water. 3-Methoxytyrosine was obtained as colourless prisms (3·1 g., 80%), m. p. 257° (Found : C, 48·4; H, 6·9. Calc. for $C_{10}H_{13}O_4N,2H_2O$: C, 48·6; H, 6·9%. Found, after drying in a vacuum at 160° : C, 56·8; H, 6·6. Calc. for $C_{10}H_{13}O_4N$: C, 56·9; H, 6·2%).

Ethyl Ester of L-N-*Methyltyrosine*.—L-*N*-Methyltyrosine (3 g.) was dissolved in ethanol (20 ml.), the cooled solution saturated with hydrogen chloride, ethanol (42 ml.) added, the whole refluxed for 7 hours and then evaporated to dryness in a vacuum, and the residue dissolved in water (20 ml.). The solution was neutralised (potassium carbonate) and extracted with ethyl acetate, the solvent removed, and the *ethyl* ester distilled (b. p. 158—162°/2 mm.) as a colourless oil which solidified and recrystallised from ethyl acetate in colourless prisms, (2·0 g., 59%), m. p. 122° (Found : C, 64·7; H, 7·3. $C_{12}H_{17}O_3N$ requires C, 64·6; H, 7·6%).

α-Amino-β-(2 : 3-dihydroxyphenyl)propionic Acid.—*o*-Vanillin (5 g.), hippuric acid (6·2 g.), anhydrous sodium acetate (3·0 g.), and acetic anhydride (10·3 ml.) were heated on a water-bath for 30 minutes. The mixture gradually solidified. Water (25 ml.) was added and the yellow product warmed, filtered off, washed with hot water, and recrystallised from absolute ethanol. The *azlactone* of α-benzamido-β-(2-acetoxy-3-methoxyphenyl)cinnamic acid was obtained as yellow needles, (9 g., 82%), m. p. 156—157° (Found : C, 67·7; H, 4·7. $C_{19}H_{15}O_5N$ requires C, 67·7; H, 4·4%).

A solution of the azlactone (5 g.) and sodium hydroxide (1·8 g.) in 50% ethanol (75 ml.) was warmed for 15 minutes, water (150 ml.) added to the deep-red solution, and hydrochloric acid (20%) added dropwise with stirring until the acid was precipitated and the solution assumed a pale yellow colour. The *α-benzamido-β-(2-hydroxy-3-methoxyphenyl)cinnamic acid* recrystallised from 75% ethanol in colourless plates (3·2 g., 74%), m. p. 226° (Found : C, 65·7; H, 4·8. $C_{17}H_{15}O_5N$ requires C, 65·2; H, 4·8%). The recrystallised cinnamic acid (2·5 g.), hydriodic acid (15 ml.; *d* 1·7), acetic anhydride (15 ml.), and red phosphorus (1·5 g.) were refluxed under hydrogen for 2½ hours. Water (30 ml.) was added, the phosphorus and some benzoic acid were filtered off, the yellow filtrate was extracted 3 times with ether, and the aqueous layer evaporated under hydrogen. The clear brown residual oil was dissolved in water (20 ml.) containing a little sulphur dioxide and treated with charcoal, and the resulting pale yellow solution was treated dropwise with aqueous ammonia (*d* 0·986) till it became green and commenced to darken. The α-amino-β-(2 : 3-dihydroxyphenyl)propionic acid was filtered off and twice recrystallised from aqueous sulphur dioxide (charcoal), forming colourless prisms (1·2 g., 71%), m. p. 265° (Found : C, 55·0; H, 5·8. $C_9H_{11}O_4N$ requires C, 54·8; H, 5·6%).

Melanin Formation.—The amino-acid (0·1 g.) was dissolved in water (200 ml.), the enzyme solution (10 ml.) (Raper, *loc. cit.*) and phosphate buffer (8 ml.; pH 8) were added, and air was bubbled through the solution for 24 hours. 3-Methoxy- and 3-methyl-tyrosine produced no pigment. 2 : 4-Dihydroxyphenylalanine poisoned the enzyme. The ethyl ester of L-*N*-methyltyrosine gave a yellow-green deposit. The ethyl ester of L-tyrosine produced a brown pigment different from the black one from L-*N*-methyltyrosine. 2 : 3-Dihydroxyphenylalanine gave a deep-maroon-coloured solution, which after 24 hours gradually deposited a black pigment.

In the cases where no pigment was formed, tyrosine (0·1 g.) in water (40 ml.) was subsequently added, to check the enzyme activity.

The autoxidation procedure employed by Raper (*Biochem. J.*, 1925, **19**, 90) was applied to L-tyrosine ethyl ester and 2 : 3-dihydroxyphenylalanine.

A solution of the amino-acid (or ester) (0·2 g.), water (400 ml.), and enzyme solution (20 ml.) was oxygenated until the red colour was a maximum : 2 hours for the ester, 4 hours for the acid. The solution was acidified (10% acetic acid; 8 ml.) and boiled. The precipitated products were filtered off and the colourless filtrate treated with aqueous sodium carbonate (10%; 10 ml.). The mixture was shaken with air for 24 hours.

2 : 3-Dihydroxyphenylalanine yielded a black pigment, similar to the above. Tyrosine ethyl ester yielded a reddish solution; this was treated with enzyme solution (20 ml.) and oxygenated; the ester-containing pigment was then deposited.

Carboxyl-containing Pigment.—The ethyl tyrosine ester-melanin was centrifuged with other biological impurities, and the brown solid treated with aqueous potassium hydroxide (20 ml.; 10%) and warmed on a water-bath for 30 minutes. The solution was centrifuged till no more deposit was observed and then acidified (10% hydrochloric acid). The free acid was centrifuged off, redissolved in 2% sodium hydrogen carbonate solution, filtered, centrifuged, and re-acidified. The process was repeated twice, and the pigment well washed with distilled water (Found : C, 51·9, 52·5; H, 5·2, 5·8; N, 9·3, 9·4%).

Alkaline hydrogen peroxide bleached the pigment and reduction with Raney nickel alloy in sodium hydroxide solution gave a pale brown solution similar to that obtained from melanin. On treatment with tyrosinase, the acid yields a brownish-black pigment sparingly soluble in 2% sodium hydrogen carbonate solution. After reprecipitation from the latter it is insoluble in aqueous sodium hydrogen carbonate, but soluble in sodium hydroxide (Found : C, 50·0, 49·8; H, 5·4, 5·6; N, 10·4, 10·8%).

2 : 3-Dihydroxyphenylalanine-melanin.—2 : 3-Dihydroxyphenylalanine on treatment with tyrosinase formed a black pigment. Its properties were similar to those of tyrosine-melanin, except that it was soluble in cold N-potassium hydroxide. Purification was effected by using the autoxidation procedure and then proceeding as described for the carboxyl-containing pigment. N-Potassium hydroxide was used instead of sodium hydrogen carbonate solution (Found : C, 59·7, 59·2; H, 4·9, 5·4; N, 8·1, 8·4%).

Adrenalin-melanin.—Adrenalin hydrochloride (20 mg.), water (40 ml.), enzyme solution (2 ml.), and buffer solution (1·5 ml.) were oxygenated for 24 hours. The brownish-black pigment deposited was centrifuged off and purified as above. It was soluble in cold N-potassium hydroxide. Purification was effected as above.

Melanin Formation in the Presence of Ketonic Reagents.—A mixture of tyrosine (0·1 g.) in water (200 ml.) and enzyme solution (10 ml.) was oxygenated until the deep-red coloration was observed (3—4 hours). The solution was acidified (10% acetic acid; 4 ml.) and boiled. The precipitated biological products were filtered off, and the colourless filtrate (stable in acid solution) was treated with a solution of hydroxylamine hydrochloride (0·5 g.) and sodium carbonate (1·2 g.) in the minimum amount of water.

Slight melanin formation was observed before the addition of alkali. 2 : 4-Dinitrophenylhydrazine (0·2 g.) and dimedone (0·1 g.) were added to separate portions of the acid solution. Pigment formation was still observed, although acid conditions are not favourable for melanin formation.

Determination of Spectra.—All the absorption spectra were observed by use of the Hilger Medium

Quartz Spectrograph and the Hilger Nutting Spectrophotometer. N-Potassium hydroxide was the solvent.

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