XIV. THE TYROSINASE-TYROSINE REACTION.

VI. PRODUCTION FROM TYROSINE OF 5:6-DIHYDROXY-INDOLE AND 5:6-DIHYDROXYINDOLE-2-CARBOXYLIC ACID—THE PRECURSORS OF MELANIN.

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IN the last communication of this series [Raper, 1926] it was shown that if a solution of the red pigment which is produced from tyrosine by tyrosinase is allowed to decolorise under suitable conditions, then 3:4-dihydroxyphenylalanine may be isolated from the solution. It was pointed out also that in the solution obtained by this method, the immediate precursor of melanin is present and that it possesses a structure in which the nitrogen is no longer in the amino form.

In the present paper an account is given of the identification of this precursor of melanin and the light it throws on the chemical changes involved in its production from tyrosine. It has not been possible to isolate the precursor itself because it is too easily oxidised, but its methyl derivative has been isolated in crystalline form and satisfactorily identified.

When the red solution obtained from tyrosine by the action of tyrosinase, as described previously [Raper, 1926], is allowed to decolorise *in vacuo* or in presence of sulphurous acid and then, after concentration in an atmosphere of CO_2 , is methylated by means of methyl sulphate in an atmosphere of hydrogen, from the reaction product two crystalline methylated products can be obtained. One of them is a feeble base and the other an acid. The former is obtained principally when the red substance has been allowed to decolorise *in vacuo* and the latter when decolorisation takes place in the presence of sulphurous acid. Both products give colour reactions characteristic of indole derivatives.

It was postulated in the paper already mentioned that the 3:4-quinone of phenylalanine (I) is formed prior to the production of the red substance in the tyrosinase-tyrosine reaction; and in attempting to determine the structure of the indole derivatives to which the red substance gives rise, it had to be considered whether in the formation of the indole ring the N of the amino group of the phenylalanine quinone attached itself in the 2 or 6 position in the benzene nucleus. Union in the 6 position would produce

I

II

III

the 5 : 6-quinone of dihydroindole-2-carboxylic acid (II) and in the 2 position, the 6 : 7-quinone of dihydroindole-2-carboxylic acid (III). Further oxidation of II and III would result in the formation of the quinones of the corresponding indole carboxylic acids.

From the work of Jones and Robinson [1917] on the orientation of substituted catechol ethers it appeared to be most probable that the 5 : 6-quinone of indole-2-carboxylic acid would be formed, and it was suspected therefore that the methylated indole derivative with acid properties which was isolated from the products of oxidation was 5 : 6-dimethoxyindole-2-carboxylic acid, the quinone having undergone reduction to the corresponding dihydroxy-derivative in presence of sulphurous acid. It was also apparent that by loss of CO_2 from this acid or from the quinone with subsequent reduction, 5 : 6-dihydroxyindole would be obtained and that the feeble base isolated after methylation was therefore likely to be 5 : 6-dimethoxyindole. Since 5 : 6-dimethoxyindole and its carboxylic acid had not been previously prepared they were therefore synthesised¹ and found to be identical in all respects with the two indole derivatives isolated from the oxidation products. This being established it is of interest to discuss the probable formula of the red substance, its mode of formation and the changes it undergoes when it becomes decolorised. The provisional formula for the red substance given previously [Raper, 1926] must now be considered to be very unlikely in view of the fact that in the presence of sulphurous acid it gives rise to 5 : 6-dihydroxyindole-2-carboxylic acid (V).

One way of explaining this transformation is to assume that the red substance is the 5 : 6-quinone of indole-2-carboxylic acid (IV) and that on reduction by sulphurous acid it yields the corresponding dihydroxy-compound. If this be the case then the decolorisation of the red substance which takes place in the absence of oxygen must be accompanied by a reduction as well as a loss of CO_2 since the main product obtained is 5 : 6-dihydroxyindole.

This process is slower than the decolorisation brought about by sulphurous acid, and since unchanged tyrosine is present in the solution it could be explained as being brought about at the expense of the tyrosine, which would be oxidised.

Alternatively, the red substance may be considered to be the 5 : 6-quinone of dihydroindole-2-carboxylic acid (II) and to undergo slow autoreduction with the loss of CO_2 , thus producing 5 : 6-dihydroxyindole. This however

¹ The synthesis will be described elsewhere.

would render the decolorisation of the red substance in presence of sulphurous acid more difficult to understand, since on reduction it would yield 5 : 6-dihydroxydihydroindole-2-carboxylic acid, whereas the compound isolated after methylation is not a dihydroindole derivative. That a dihydroindole derivative is actually produced but subsequently oxidises in the air in the process of extraction subsequent to methylation is possible but unlikely. On the other hand the sulphurous acid may act simply as a catalyst and accelerate the autoreduction, which, proceeding more rapidly, would be less likely to result in the loss of CO_2 and would thus give rise to the production of the dihydroxyindole carboxylic acid. When the autoreduction is not catalysed by SO_2 and consequently proceeds relatively slowly there is more opportunity for the loss of CO_2 and dihydroxyindole is produced. Another alternative formula for the red compound which gives it a *para*- instead of an *ortho*-quinonoid structure is the following:

This offers the same difficulty as II, namely, that it does not explain the production of an indole derivative rather than a dihydroindole derivative in presence of sulphurous acid. On the other hand, as in the case of (II) the difficulty disappears if it be assumed that the action of the sulphurous acid is catalytic and favours autoreduction. It might also be expected to have a yellow rather than a red colour which is more characteristic of the *ortho*-quinonoid grouping.

From these considerations it appears most probable that the red compound is the 5 : 6-quinone of dihydroindole-2-carboxylic acid and that this substance undergoes autoreduction and loss of CO_2 slowly at the ordinary temperature and more rapidly on heating with production of 5 : 6-dihydroxyindole. In the presence of sulphurous acid, however, the autoreduction proceeds more rapidly and consequently the loss of CO_2 is much less marked. This results in the production mainly, therefore, of 5 : 6-dihydroxyindole-2-carboxylic acid. The experimental results show that in presence of sulphurous acid this is almost the only product produced, only traces of the base being obtained. In the absence of sulphurous acid, however, the base forms almost the entire product which can be isolated, only traces of the acid being obtained. If these views be correct, then the formation of the red substance from the 3 : 4-quinone of phenylalanine may be represented as follows:

The first stage may be considered as analogous to the formation of an anilino-quinone, the second is brought about by the oxidation of the dihydroxydihydroindole carboxylic acid by the enzyme. This is probably rapid since it is analogous to the oxidation of 3:4-dihydroxyphenylalanine to the corresponding quinone which occurs in an earlier stage of the tyrosinase reaction and is known to take place rapidly.

The presence of dihydroxyphenylalanine in small amount along with the indole derivatives is accounted for partly by its being an intermediate between tyrosine and the quinone of phenylalanine, and partly by the reduction of the latter by sulphurous acid when this is used to accelerate the decolorisation of the red substance. It has already been suggested by the author that 3:4-dihydroxyphenylalanine is the first product produced from tyrosine when it is oxidised by tyrosinase and its isolation and identification as a product of the reaction, together with other evidence put forward in the last communication of this series, rendered this very probable. But it seemed to be important, in order to place the matter beyond doubt, to demonstrate that the red substance produced when tyrosinase acts on 3:4-dihydroxyphenylalanine will undergo transformation to yield the same indole derivative that tyrosine produces under the same conditions. This has now been done. When the red substance, obtained from 3:4-dihydroxyphenylalanine by the action of the enzyme, was allowed to decolorise *in vacuo*, it was possible to isolate 5:6-dimethoxyindole from the resulting products by the same procedure that was used in the case of tyrosine.

These results place on a more secure basis the hypothesis first advanced by Happold and Raper [1925] that tyrosinase produces *ortho*-quinones from those phenols which bring about deamination when acted upon by the enzyme in presence of an amino-acid. This view has been extended to oxidases in general which will act upon substances containing the catechol grouping by Szent-Györgyi [1925], who has also made it the basis of a theory as to the nature of oxidases. Experiments in progress in the author's laboratory indicate very clearly that tyrosinase acting on phenol and *p*-cresol produces *o*-quinones, for it has been found that when the enzyme is allowed to act on either of these phenols in the presence of aniline, considerable amounts of the anilino-quinones are obtained. The complete results of these experiments will be published shortly.

It is presumed from the results described in this paper that melanin production occurs by the oxidation in the air of 5:6-dihydroxyindole and to a lesser extent of 5:6-dihydroxyindole-2-carboxylic acid. These substances have not been synthesised, but there is no doubt that they would be extremely easily oxidised in the air and especially in slightly alkaline solution—the optimum condition for melanin production from tyrosine. Attempts to remove the methyl groups from dimethoxyindole or its carboxylic acid have been fruitless as might be expected, since this can only be done in acid solution, under which conditions both the indole and its carboxylic acid yield tarry

products [*vide* Perkin and Rubenstein, 1926]. Under slightly alkaline conditions the oxidation of the traces of dihydroxyphenylalanine which are found amongst the products of action of the enzyme would contribute to melanin formation also [Bloch and Schaaf, 1925].

The formation of melanin from dihydroxyindole and its carboxylic acid is of considerable interest from the point of view of the normal production of melanin in pigment cells and in melanotic tumours. From time to time observations have been made which indicated that melanin on destructive decomposition yielded cyclic compounds containing nitrogen which give the pine-shaving reaction. This has been taken by some to mean that melanins were possibly formed from blood pigment. Evidence against this view has been collected by von Fürth [1912] and it is now not generally accepted. On the other hand it has led to the view (von Fürth) that tryptophan might be the mother-substance of melanin in pigment-producing cells. The isolation from the urine in a case of melanotic sarcoma of a substance which gave indole reactions has also given support to this view [Eppinger, 1910] and especially so since administration of tryptophan increased the amount of this substance excreted, whereas tyrosine did not. On the other hand, Bloch and Schaaf [1925] have advanced the view that melanin is formed from 3:4-dihydroxyphenylalanine by a specific enzyme "dopa oxydase," and a structure for the pigment is given in which its formation is based on the production of the 3:4-quinone of phenylalanine, two molecules of which are represented as condensing with one molecule of the 3:4-quinone of phenylacetaldehyde.

It would appear from the observations recorded in this paper that the cyclic group containing nitrogen is already present in the precursors of melanin and that it is likely to persist when melanin has been formed by oxidation of these precursors in the air or by means of tyrosinase. Whether, however, the melanin obtained from tyrosine by oxidation with tyrosinase is identical with that found in pigment cells cannot be yet considered as proven. It is on the whole unlikely, since Abderhalden and Guggenheim [1907, 1908] have shown that peptides containing tyrosine will also produce melanin when oxidised by the enzyme and it is likely that the peptide linkage will persist in the final product. It does however seem probable that the reactions leading to the formation of the indole nucleus from tyrosine, or peptides containing it, as described in this paper, are an essential preliminary in the natural production of melanin.

EXPERIMENTAL.

The enzyme used in the following experiments was prepared from the mealworm (*Tenebrio molitor*) as described previously by the author [Raper, 1926].

I. *Production of 5:6-dimethoxyindole from tyrosine.*

1 g. of tyrosine is dissolved in 500 cc. hot water and the solution diluted to 2 l. in a large stoppered bottle.

The temperature is adjusted to 26–30° by cooling or warming as required and 100 cc. of the enzyme preparation are added. The p_H is now adjusted to between 6 and 6.5 by the cautious addition of 1 % acetic acid. Oxygen is bubbled through the solution for 1–2 minutes and the bottle is stoppered and well shaken. About every half-hour oxygen is bubbled through the liquid and it is frequently shaken. The solution quickly becomes red and in 2 to 4 hours most of the enzyme has been precipitated. To ensure complete precipitation 10 cc. of 1 % acetic acid are added and after standing for a few minutes the solution is filtered quickly, using several large funnels. The deep red filtrate is placed in a large filter flask and the flask evacuated. The liquid is well shaken to remove as much dissolved oxygen as possible and hydrogen is then admitted. It is now again evacuated and re-filled with hydrogen. The red liquid is allowed to stand at the ordinary temperature under hydrogen until the red tint has been lost and replaced by a pale brown. 10 cc. of a saturated solution of SO_2 are now added to minimise oxidation in the succeeding operations. The solution is now filtered to remove a small amount of brown deposit and placed in a flask from which it can be displaced gradually and continuously to a large distillation flask while at the same time being kept under hydrogen. It is then concentrated by vacuum distillation in a stream of CO_2 until it measures about 20 cc. The reaction products in this solution together with unchanged tyrosine are now methylated in an atmosphere of hydrogen. The flask is detached and fitted with a reflux condenser, inlet and outlet tubes for hydrogen and a dropping funnel. The flask is evacuated and filled with hydrogen, this process being repeated once to ensure complete absence of oxygen.

With a slow stream of hydrogen passing through the flask, the methylation is carried out by first adding 10 cc. of 20 % NaOH, shaking gently till the contents of the flask are all in solution, and then adding 3.5 cc. dimethyl sulphate in two portions (2.5 and 1 cc.). After each addition the flask is vigorously shaken. It is now heated on the water-bath for an hour, cooled and the brown alkaline solution extracted three times with ether. The ether extract is washed with a small amount of water. On removal of the ether by distillation, from 100 to 150 mg. of the crystalline dimethoxyindole have usually been obtained. It was recrystallised from a small volume of alcohol and was pure after two crystallisations, M.P. 154–5°. Mixed with synthetic 5 : 6-dimethoxyindole the M.P. remained unchanged.

Analysis. Found: C, 67.6 %; H, 6.2 %; N, 8.3 %.

Calculated: C, 67.8 %; H, 6.2 %; N, 7.9 %.

If the alkaline solution resulting from the methylation after extraction with ether is acidified to Congo red and again shaken out several times with ether, traces of an acid are obtained which on crystallisation from benzene containing a small amount of acetone melts at 202–3° with evolution of gas. This acid is therefore 5 : 6-dimethoxyindole-2-carboxylic acid (see next section).

A trace of the base dissolved in alcohol and mixed with a few drops of *p*-dimethylaminobenzaldehyde in alcohol gave a violet colour on adding a drop of 20 % HCl. Addition of a trace of sodium nitrite changed the colour to a deep crimson. The substance gave a reddish violet colour with sodium nitroprusside and caustic soda which was changed to a pure blue on adding acetic acid. Mixed with glyoxylic acid and run on to concentrated sulphuric acid the substance gave a red colour at the junction of the two liquids.

II. *Production of 5 : 6-dimethoxyindole-2-carboxylic acid from tyrosine.*

The procedure to be carried out up to the stage at which the solution of the red pigment is obtained is the same as described in the previous section. Instead of keeping the red solution under hydrogen, it is mixed with 50–60 cc. of a saturated solution of SO₂ and allowed to stand for 1 to 2 days in a stoppered bottle. The pale yellow solution is now concentrated *in vacuo* in a large distilling flask and when reduced to about 20 cc. is methylated in an atmosphere of hydrogen exactly as described above. The alkaline solution obtained after methylation is extracted with ether and usually yields a trace of crystalline substance which gives indole reactions and is presumably 5 : 6-dimethoxyindole. On acidifying and shaking out several times with ether a substance is obtained which is left as a vitreous residue after evaporation of the ether. It crystallises on allowing it to stand with water for a few hours and is crude 5 : 6-dimethoxyindole-2-carboxylic acid. The yield has usually been about 0.2 g. from each gram of tyrosine initially taken. It was purified by dissolving in the minimal volume of acetone and adding 15 volumes of hot benzene, filtering at once and allowing to stand. It crystallised in flattened, rhombic prisms, m.p. 202–3° with evolution of gas. Mixed with synthetic 5 : 6-dimethoxyindole-2-carboxylic acid the m.p. remained unchanged.

Analysis. Found: C, 59.8 %; H, 5.1 %; N, 6.6 %.

Calculated: C, 59.7 %; H, 5.0 %; N, 6.3 %.

In the *p*-dimethylaminobenzaldehyde reaction it gives a less intense violet colour than 5 : 6-dimethoxyindole. If it be heated above its m.p. until CO₂ evolution ceases and the residue then tested, the indole reaction is more marked and similar in all respects to that given by pure 5 : 6-dimethoxyindole. The acid or base dissolved in glacial acetic acid gives a yellow colour when one drop of nitric acid is added (brucine test).

III. *Production of 5 : 6-dimethoxyindole from 3 : 4-dihydroxyphenylalanine.*

1 g. dihydroxyphenylalanine prepared from *Vicia faba* according to the directions of Guggenheim [1913] was dissolved in 2 l. water, cooled to 27° and 70 cc. of the enzyme solution added. The *p_H* was adjusted to 6.5 and the solution saturated with oxygen. The solution rapidly became a deep red and in 10 minutes the enzyme had precipitated out. After half-an-hour 10 cc. of 1 % acetic acid were added and the solution was filtered. The red filtrate was

dealt with exactly as described in the first section. After methylation 136 mg. of the crude base were obtained. This was recrystallised twice from alcohol and had m.p. 154–5°. Mixed with synthetic 5:6-dimethoxyindole the m.p. remained the same. It gave the typical indole reaction with *p*-dimethylamino-benzaldehyde.

SUMMARY.

1. 5:6-Dihydroxyindole and 5:6-dihydroxyindole-2-carboxylic acid have been identified as products of the action of tyrosinase on tyrosine and are to be regarded as the immediate precursors of melanin.

2. 5:6-Dihydroxyindole is the chief product produced when the red pigment which is characteristic of the tyrosinase reaction undergoes intramolecular change. 5:6-Dihydroxyindole carboxylic acid is however produced principally when the red substance is allowed to decolorise in the presence of sulphurous acid.

3. 3:4-Dihydroxyphenylalanine also gives rise to 5:6-dihydroxyindole under the same conditions that result in its production from tyrosine. This confirms the previous suggestion that 3:4-dihydroxyphenylalanine is the first intermediate product in the tyrosinase-tyrosine reaction.

4. The formula previously given to the red pigment produced from tyrosine has been revised in view of the further evidence presented in this paper. It is probably the 5:6-quinone of dihydroindole-2-carboxylic acid.

5. The production of melanin is discussed.

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