XCV. THE TYROSINASE-TYROSINE REACTION.

V. PRODUCTION OF *l*-3·4-DIHYDROXY-PHENYLALANINE FROM TYROSINE.

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It has been shown by Raper and Wormall [1923] and Raper and Speakman [1926] that when tyrosine is oxidised by tyrosinase, the first visible change is the production of a red pigment. This red pigment is very unstable and changes spontaneously to a colourless substance which in turn undergoes oxidation in the air to produce melanin.

The exact nature of the chemical reactions accompanying these various changes has not yet been elucidated but it has been shown by Raper and Wormall [1925] that deamination of the tyrosine does not take place. It has also been shown by Happold and Raper [1925] that when tyrosinase acts on certain phenols it is probable that ortho-quinones are formed as intermediate products of the reaction and, since tyrosine contains a phenolic hydroxyl group, it would be expected that it also might yield an ortho-quinone under the influence of tyrosinase.

In the present paper it is shown that *l*-3·4-dihydroxyphenylalanine is produced when tyrosine is oxidised by tyrosinase, this substance having been isolated in small amount as one of the products of the reaction. It has been obtained from the solution which results when the red pigment has been converted into the colourless substance, either on standing *in vacuo*, or by boiling, or by standing at the ordinary temperature in the presence of sulphurous acid. This does not enable it to be decided definitely whether the dihydroxyphenylalanine thus isolated is a product of the decomposition of the red substance or is produced continuously from tyrosine by the action of the enzyme, but it provides satisfactory evidence that one point of attack in the tyrosine molecule, when it is oxidised by tyrosinase, is the ortho-position to the phenolic hydroxyl group. It also provides further evidence, if it were needed, that deamination of the side chain does not take place.

It is easily demonstrated that tyrosinase oxidises 3·4-dihydroxyphenylalanine more rapidly than it oxidises tyrosine. Both substances when oxidised by the enzyme give rise to unstable red pigments which, to judge by their properties, are identical. This fact coupled with the isolation of 3·4-dihydroxyphenylalanine as a product of the action of the enzyme on tyrosine suggests very strongly that the initial product of the oxidation of tyrosine is 3·4-di-

hydroxyphenylalanine and that this is converted by further oxidation into the red substance. The observations of Happold and Raper [1925] indicate that the dihydroxyphenylalanine would yield an ortho-quinone and it is probable therefore that the red substance is produced from this. The red substance is not a quinone as it does not give the guaiacum reaction, liberate iodine from potassium iodide or react with aniline. It is suggested therefore that the following are the stages of the reaction up to the production of the red pigment:

The quinonoid configuration for the red substance suggested in this scheme would be in conformity with its colour and instability, and its change into the colourless precursor of melanin is probably brought about by an intramolecular change in which a more stable cyclic compound is formed which no longer possesses the quinonoid structure. In favour of this view is the fact that melanin contains almost the same percentage of nitrogen as tyrosine [Raper and Wormald, 1925] and, as is shown in the experimental part, when the colourless substance has been produced from the red pigment, a greater part of the nitrogen which the solution contains is present in the non-amino-form. The question cannot be finally settled until the constitution of the colourless substance has been determined².

It has been suggested by Onslow [1923] that one component of tyrosinase is an enzyme (oxygenase), which catalyses the oxidation of ortho-dihydroxy-compounds to peroxides, the latter then serving to yield active oxygen under the influence of a peroxidase, which is another component of the enzyme. 3,4-Dihydroxyphenylalanine would serve as a substrate for this purpose and Onslow and Robinson [1925] have recently advanced the view that it is the initial product of the oxidation of tyrosine and that its continuous production is essential to the further progress of the reaction. If this be true, then its addition to a tyrosinase-tyrosine system should lead to an initial increase in the velocity of oxidation of the tyrosine. The effect would only be noticed in the early stages, for, on the assumption as to the various stages of the reaction put forward above, once the reaction has started, dihydroxyphenylalanine

¹ Alternatively the N may attach itself in the *para*-position.

² [Note added July 30th.] That the colourless substance is an indole derivative has been established, but its constitution is at present uncertain.

would be continuously present in the solution. The experiment has been tried and an initial increase in the velocity of the oxidation of tyrosine has been observed.

Incidentally, it may be pointed out that the observations in the present paper disprove the contention of McCance [1925] that *o*-dihydroxy-compounds are not formed in the tyrosinase reaction. McCance states that since catechol compounds combine with boric acid, to yield strongly acid substances, a borate buffer ought to inhibit the oxidation of tyrosine if 3·4-dihydroxyphenylalanine were an essential intermediate product of the reaction, whereas it does not. This view does not take cognisance of the fact that only traces of 3·4-dihydroxyphenylalanine would be present at any one time in the tyrosinase reaction and therefore any change in the p_H of the solution would be small, also that it is probable that the boric acid compound is slightly dissociated in solution and therefore free dihydroxyphenylalanine would be present. Actual experiment shows that 3·4-dihydroxyphenylalanine is readily oxidised by tyrosinase when it is added to a borate buffer with a reaction of p_H 8.

The production of 3·4-dihydroxyphenylalanine from tyrosine by a biological method of oxidation overcomes an obstacle which previously existed in accepting the view that tyrosine is the parent substance of adrenaline. The presence of an enzyme in the suprarenal gland which oxidises tyrosine has not yet been demonstrated but it is probable that 3·4-dihydroxyphenylalanine may be produced elsewhere in the body. Bloch [1917] and Bloch and Schaaf [1925] have presented evidence that it is the only substrate which is effective for the production of melanin in epithelial pigment cells by "dopa" oxydase. This enzyme does not attack tyrosine but its mere existence and its apparent specificity render the production of 3·4-dihydroxyphenylalanine in some situation in the body very probable.

EXPERIMENTAL.

Preparation of the enzyme.

The enzyme used in the experiments described in this paper was prepared from mealworms (*Tenebrio molitor*) by a modification of Gortner's process [1910]. About 100 g. mealworms are killed with chloroform water, washed and pounded in a mortar with successive portions of chloroform water until only the hulls remain. The resulting fluid is strained through coarse muslin to remove remnants of the hulls and at this stage measures about 400 cc. This is filtered and the residue on the filter paper washed with chloroform water containing a trace of acetic acid until the filtrate is colourless and gives only a slight turbidity with lead acetate and ammonia. This process usually occupies about 10 days because filtration is slow. The residue on the filter paper is now scraped off, ground up in a mortar with small amounts of chloroform water¹ and the whole transferred to a wide-mouthed stoppered bottle. A few cc. of

¹ In all about 400 cc.

chloroform are added and then ammonia until the reaction is slightly alkaline to litmus. With occasional shaking the enzyme gradually becomes very finely dispersed and is an extremely active preparation of tyrosinase. It retains its activity in this condition for many weeks. If the solution be acidified slightly with acetic acid, the enzyme is completely precipitated in an easily filterable condition.

Isolation of 1-3-4-dihydroxyphenylalanine.

1 g. of tyrosine is dissolved by boiling in 500 cc. of water and the solution diluted to 2 litres. By cooling, the temperature is adjusted to about 27° and 100 cc. of the enzyme preparation described above are added. By addition of very dilute acetic acid the reaction is adjusted to between p_H 6 and 6.5. The solution is placed in a 2½ litre bottle and oxygen bubbled through until the air above the liquid has been largely displaced by oxygen. The bottle is now stoppered and well shaken. The shaking is repeated frequently and about every hour the oxygen is renewed by a repetition of the initial bubbling. The liquid quickly becomes red and usually after 2-4 hours, depending on the temperature and the activity of the enzyme, the enzyme has separated out as a flocculent precipitate. This generally happens when about 50-60 % of the tyrosine has been oxidised and is apparently due to a reaction between the enzyme and some product of its action on tyrosine. To ensure complete precipitation, 10 cc. of 1 % acetic acid are added, and the solution filtered rapidly, using several large funnels. The filtrate is clear and has a fine red colour resembling diluted, laked blood. The red substance may now be rapidly converted into the colourless melanin-producing substance by boiling in an inert atmosphere and rapidly cooling out of contact with air, or more slowly by keeping the solution *in vacuo* for a few days or by adding 30 cc. of saturated sulphurous acid and allowing it to stand for 48 hours in a stoppered bottle. The last method has been the one most frequently used because of its simplicity. When the transformation of the red substance is complete the liquid has a pale brownish colour. If sulphurous acid has been added it must be removed at this stage by boiling and then cooling rapidly. The next step is the removal of the colourless precursor of melanin. 40 cc. of 20 % lead acetate are added and the solution freely exposed to air or shaken periodically in a large bottle. A whitish precipitate, consisting partly of lead sulphate, but also containing some of the melanin-producing substance, is formed when the lead acetate is added and this gradually darkens in the air. The solution is allowed to stand with occasional shaking for 48 hours to allow for complete precipitation of melanin and is then filtered. To the colourless filtrate 1 % ammonia is added until the liquid is faintly but distinctly alkaline to litmus. An excess of ammonia must be avoided as this throws down tyrosine, which is still present, as well as 3-4-dihydroxyphenylalanine. The liquid is allowed to stand and the precipitated lead compound of dihydroxyphenylalanine washed once by decantation. It is then redissolved by adding dilute acetic acid and the

precipitation with lead acetate (10 cc.) and ammonia repeated. After again washing by decantation, the precipitate is filtered off by suction on a wad of paper pulp and washed several times with water. The pulp and adherent precipitate are transferred to a wide-mouthed, stoppered bottle, well shaken with 150 cc. water and decomposed with hydrogen sulphide. The pulp and lead sulphide are filtered off, washed, and the filtrate and washings concentrated to small bulk by distillation *in vacuo*. By cautious addition of ammonia, the solution is partially neutralised until it is acid to litmus but not to Congo-red. It is then clarified by boiling with charcoal, a trace of sulphurous acid is added and it is taken to dryness in a vacuum desiccator. The residue is extracted with a small amount of hot water and filtered to remove a small amount of insoluble inorganic matter. On again concentrating in a vacuum desiccator, dihydroxyphenylalanine crystallises out. At this stage it is best to work up the products from at least 2 g. of tyrosine because of the small yield. With 2 to 3 recrystallisations from water containing a trace of sulphurous acid the product is obtained pure. It crystallises, as observed by Guggenheim [1913], in thick prisms or fine needles according to conditions. From 2 g. of tyrosine 0.034 g. of the pure substance was obtained, m.p. 279° (corr.) with decomposition. Mixed with 3.4-dihydroxyphenylalanine from *Vicia faba*, the m.p. remained unchanged.

I am indebted to Dr F. R. Goss for a micro-analysis.

Found, C, 54.5 and 54.8 %; H, 5.8 and 5.7 %; N (Dumas), 7.1 %.

Calculated, C, 54.8 %; H, 5.6 %; N, 7.1 %.

The product gave a green colour with very dilute ferric chloride which was changed to purple by ammonia. It reduced ammoniacal silver nitrate at once in the cold. Treated with dilute sodium carbonate it gave a yellow solution which gradually became brown and then brownish black in the air. These properties and the M.P. are identical with those of the naturally occurring compound in *Vicia faba*. Since the naturally occurring tyrosine was used in the preparation, it was expected that the laevorotatory form of the compound would be obtained by the action of tyrosinase. The m.p. already indicated that this was so, inactive 3.4-dihydroxyphenylalanine having a m.p. of 263–272° [Funk, 1911]. To confirm this, 20 litres of a saturated tyrosine solution were oxidised by tyrosinase and the dihydroxyphenylalanine obtained by the method described above was examined polarimetrically. 0.155 g. in 9.94 cc. *N* HCl gave a rotation in a 1 dm. tube of -0.17° which gives $[\alpha]_D^{19} = -11.0^\circ$. Using more concentrated solutions, Guggenheim [1913] and Waser and Nevandowsky [1921] obtained values of -14.28° and -12.74° respectively, for the naturally occurring substance. The optical activity of the dihydroxyphenylalanine may be adduced as additional evidence, if it were required, that it is not formed from tyrosine by a deamination process with subsequent addition of ammonia.

Oxidation of 3·4-dihydroxyphenylalanine by tyrosinase.

The 3·4-dihydroxyphenylalanine used was prepared from *Vicia faba* by Guggenheim's method. 50 mg. were dissolved in 95 cc. of a phosphate buffer, p_H 6, 5 cc. tyrosinase were added and oxygen bubbled through the solution which was placed in a stoppered bottle. The solution was shaken frequently. It rapidly became red, and after an hour was very deeply coloured (under the same conditions it was judged that tyrosine would have taken at least ten times as long to produce the same depth of colour). The solution was filtered through a thick wad of paper pulp to remove the enzyme. 5 cc. of the filtrate were placed in a Thunberg tube and 1·03 cc. $N/5$ NaOH added to make the reaction approximately p_H 8. A control tube with 5 cc. filtrate and 1·03 cc. water was set up for comparison. Both tubes were immediately evacuated, placed in a bath at 37° and the colour changes observed. The tube at p_H 8 had lost its red tint after 15 minutes whereas that at p_H 6 was still a deep red. On admitting air to the tube at p_H 8 and shaking it blackened immediately. This behaviour is exactly like that described by Raper and Speakman [1926] for the red substance produced from tyrosine. The filtrate on boiling also decolorised rapidly like the red substance from tyrosine. It was not changed by adding aniline water. A small amount of dihydroxyphenylalanine was also oxidised by tyrosinase at p_H 6 in the absence of a phosphate buffer. When the red colour had developed, the solution was filtered and the filtrate boiled to decolorise it. On cooling and adding a drop of dilute ferric chloride the typical green colour given by dihydroxyphenylalanine was obtained but this then rapidly changed to a bluish black. This again is similar to the behaviour of the red substance prepared from tyrosine except that with the latter only a trace of green is obtained, owing to the very small amounts of dihydroxyphenylalanine present.

These various reactions of the red substance prepared from dihydroxyphenylalanine suggest that it is identical with the red substance obtained from tyrosine.

Nitrogen distribution in the solution after decoloration of the red substance.

A solution of the red substance free from enzyme was obtained from tyrosine as described earlier in this paper. 100 cc. were kept *in vacuo* until the red colour had disappeared and it was then distilled *in vacuo* until about one-third of the solution remained. With portions of this, estimations of total nitrogen, amino-nitrogen and ammonia (Folin) were made. The figures obtained per 100 cc. were: total N, 20·16 mg., amino-N, 9·71 mg., ammonia-N, 2·88 mg. The non-amino-N not accounted for by ammonia was thus 7·57 mg. The ammonia present was due to that contained in the enzyme solution, so that the non-amino-N accounted for 44 % of the total N minus ammonia-N. In a similar experiment in which the red substance was allowed to decolorise in

presence of sulphurous acid, a figure of 41 % non-amino-N was obtained. It was proved (by the bromination method) that in these experiments 57 % of the tyrosine had been oxidised, so that of the amino-N originally present in the tyrosine which had been oxidised, $\frac{44}{57} \times 100$, or 77 % was now present in the non-amino-form. This shows that in the colourless substance which is produced from the red pigment, the nitrogen is probably part of a cyclic structure.

Velocity of oxidation of tyrosine in the presence of dihydroxyphenylalanine.

The method used was that described by Raper and Wormall [1923], the tyrosine being estimated by bromination. A preliminary experiment showed that any dihydroxyphenylalanine present in the samples of the oxidation mixtures withdrawn for analysis was destroyed by the subsequent treatment with alkali which was adopted by Raper and Wormall prior to the bromination. This ensured therefore that tyrosine alone was being estimated in those mixtures to which 3·4-dihydroxyphenylalanine had been added.

The determination of the effect of dihydroxyphenylalanine on the amount of tyrosine oxidised was carried out as follows.

About 0·15 g. tyrosine was dissolved in a phosphate buffer, p_H 6 (solution A), and a solution of dihydroxyphenylalanine was made containing 12·8 mg. in 15 cc. water (solution B).

Flask 1. 135 cc. solution A + 10 cc. water + 5 cc. enzyme.

Flask 2. 135 cc. solution A + 10 cc. solution B + 5 cc. enzyme.

Flask 3. 135 cc. buffer solution + 10 cc. water + 5 cc. enzyme.

Temperature 17·5°. Immediately after making up each mixture, duplicate 20 cc. samples were withdrawn for tyrosine estimation. The air current was then started and samples were withdrawn after 65, 125 and 205 minutes. The samples as withdrawn were run into flasks containing 0·5 cc. 10 % acetic acid and the estimation carried out exactly as described by Raper and Wormall.

The following figures were obtained:

Time (minutes)	Tyrosine in 20 cc. (mg.)		Tyrosine oxidised per 20 cc. (mg.)	
	Flask 1	Flask 2	Flask 1	Flask 2
0	8·06	7·89	0	0
65	6·91	6·40	1·15	1·49
125	5·62	5·35	2·44	2·54
205	4·52	4·42	3·54	3·47

These results show a 30 % increase in oxidation of tyrosine in the first 65 minutes when dihydroxyphenylalanine is present. After 125 minutes the increase has diminished to 4 %, which is almost within the experimental error of the method, and after 205 minutes no increase is observed.

Oxidation of 3·4-dihydroxyphenylalanine in a borate buffer.

This experiment was designed to test the validity of the conclusions of McCance [1925]. A low concentration of dihydroxyphenylalanine was used purposely, since if the hypothesis which has been advanced in the introduction to the present paper is correct, only a very small concentration of this substance could be present at any one moment owing to its ease of oxidation by tyrosinase. 11 mg. dihydroxyphenylalanine were dissolved in 50 cc. of a borate buffer, p_H 8; 2 cc. of enzyme were added and oxygen bubbled through the solution for a short time. The bottle containing the solution was stoppered and shaken frequently. After 8 minutes the development of a red colour was clearly visible and after 23 minutes the solution was a deep reddish crimson. The colour deepened as the oxidation proceeded and became finally a dark reddish brown. This experiment proves therefore that McCance's conclusions were based on false assumptions.

SUMMARY.

1. *l*-3·4-Dihydroxyphenylalanine has been isolated and identified as a product of the action of tyrosinase on *l*-tyrosine. The yield of the pure substance is not more than 3 % of the tyrosine oxidised.

2. It is suggested that 3·4-dihydroxyphenylalanine is the first product produced in the tyrosinase-tyrosine reaction and that this on further oxidation produces the red pigment which is characteristic of the reaction. A probable formula for the red pigment is given.

3. In the presence of a small amount of 3·4-dihydroxyphenylalanine the rate of oxidation of tyrosine by tyrosinase is initially increased. This supports the views of Onslow on the mechanism of action of tyrosinase.

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