CCLXIV. THE ACCUMULATION OF *l*-3:4-DIHYDROXYPHENYLALANINE IN THE TYROSINASE-TYROSINE REACTION

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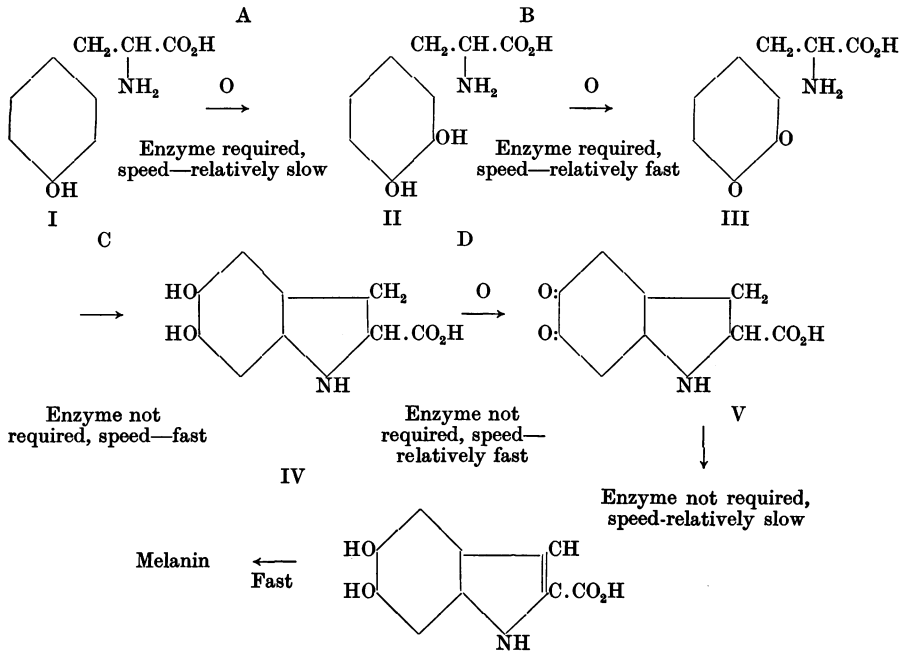
Although it is easily demonstrated that when tyrosinase acts upon tyrosine *l*-3:4-dihydroxyphenylalanine (dopa) is produced, it has hitherto not been clear why this is possible. Tyrosinase acts far more rapidly on dopa than on tyrosine, and dopa would therefore not be expected to accumulate in the reaction.

However, from a mealworm tyrosinase-tyrosine reaction, which has proceeded 2–5 hr., dopa can be isolated in yields varying from 10 to 20% of the actual tyrosine oxidized. Although, as shown in the preceding paper, the presence of peroxidase and other catechol oxidases explains satisfactorily why dopa accumulates to a smaller extent in the case of the plant tyrosinase preparation, it does not explain its accumulation in the animal tyrosinase-tyrosine reaction, in spite of the rate at which tyrosinase oxidizes dopa.

Indeed, it seems to be generally true that tyrosinases from all sources oxidize *o*-dihydric phenols at a much faster rate than monohydric phenols. Pugh [1930] and Graubard & Nelson [1935] have investigated the actions of tyrosinase preparations subjected to different methods of purification on catechol



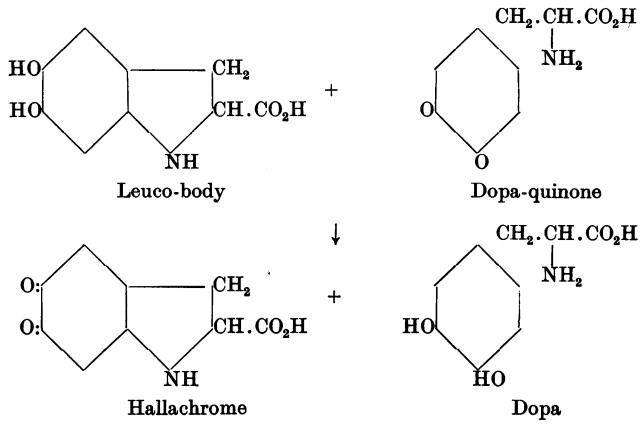
and *p*-cresol respectively. Their results show that the same enzyme is responsible for the catalytic oxidation of both mono- and *o*-di-hydric phenols. As long as the enzyme can act on *p*-cresol it can also act on catechol, although inactivation of the enzyme is brought about much more quickly by catechol than by *p*-cresol. When the enzyme is inactive towards catechol, it is also inactive towards *p*-cresol, and conversely.

It appears to us very unlikely that a selective inactivation of tyrosinase towards dopa and not towards tyrosine should occur during the course of the enzyme action. It is more probable that the accumulation of dopa when using the animal tyrosinases is to be explained by the presence of some reducing agent or system, which reduces the dopa-quinone (III), the second product of the enzyme action.

Considering the scheme (p. 2162) which has been put forward to explain the mechanism of the tyrosinase-tyrosine reaction [Raper, 1927], it will be observed that there are two oxidation-reduction systems involved, namely: (i) dopa $\rightleftharpoons$ dopa-quinone (III), $E_0 = +0{\cdot}511$ V./*p*H 4·6 [Ball & Chen, 1933]; (ii) 5:6-dihydroxydihydroindole-α-carboxylic acid (IV) (leuco-compound) $\rightleftharpoons$ 5:6-quinone (V), $E_0 = +0{\cdot}170$ V./*p*H 4·6 [Friedheim, 1933].
(hallachrome)

The presence of reducing enzyme systems in the mealworm tyrosinase preparation which could reduce the dopa-quinone (III) or hallachrome (V) has been sought for without success. The mealworm enzyme, in virtue of the method of preparation, is unlikely to contain any substrate which could enter into a dehydrogenase system and utilize dopa-quinone (III) or hallachrome (V) as hydrogen acceptors. It was found, however, that hallachrome will act as a perfect hydrogen acceptor in xanthine oxidase and succinic oxidase systems, and satisfactorily replaces methylene blue. By analogy, although dopa-quinone has never been isolated, it could also act as a hydrogen acceptor in such systems.

The failure to find an enzyme system that could account for the reducing action led to the consideration of the possibility that some product of the reaction itself acted as a reducing agent. Of such products the most likely appeared to be 5:6-dihydroxydihydroindole-α-carboxylic acid. This is the product which results from the intramolecular rearrangement of dopa-quinone. It easily takes up oxygen from the air, and is converted into its corresponding quinone [Heard & Raper, 1932] and might therefore reduce dopa-quinone back to dopa, and itself be oxidized to its corresponding quinone (the red pigment, hallachrome, of the tyrosinase reaction), thus:

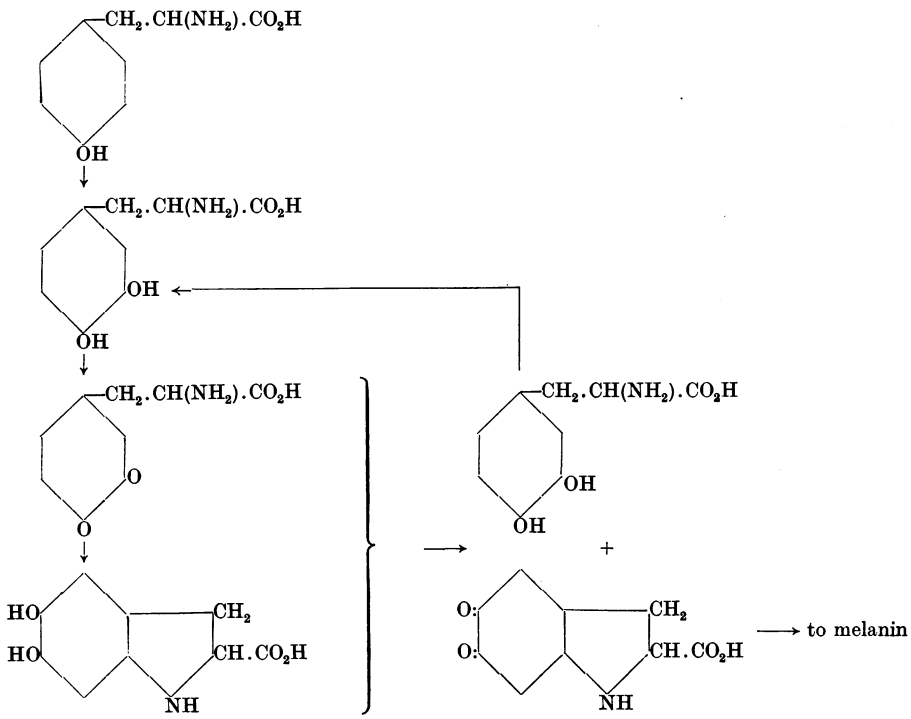


This is possible from a consideration of the oxidation-reduction potentials of the systems concerned, so far as they have been investigated.

The determining factors in the accumulation of dopa in the mealworm tyrosinase-tyrosine reaction would then be the relative velocities of the enzymic oxidation process and of the internal oxidation-reduction reactions.

If this is the correct explanation, then it would be anticipated that 5:6-dihydroxydihydroindole-α-carboxylic acid, when added to a tyrosinase-tyrosine system, would increase the dopa accumulation by increasing the efficiency of the reducing mechanism. The ratio dopa produced/tyrosine oxidized should therefore be increased. Experiment has shown that this is the case. The leuco-compound, obtained by large scale preparation from the tyrosinase-tyrosine reaction, and also from the worm *Halla parthenopoea* by reduction of hallachrome with palladium-norite and hydrogen, when added continuously in small quantities to a proceeding tyrosinase-tyrosine reaction, increased the ratio dopa produced/tyrosine oxidized from 0·123 without its addition (control), to 0·193 with its addition.

The following scheme is therefore advanced as representing an interplay of chemical reactions in the tyrosinase-tyrosine system.



Although not concerned in the accumulation of dopa in the tyrosinase-tyrosine system, an attempt was made to study quantitatively the effects of other biological reducing systems on the tyrosinase-tyrosine system as regards dopa accumulation. Preliminary observation showed that hallachrome acted as a hydrogen acceptor in the xanthine oxidase and succinic oxidase systems. On attempting quantitative experiments in systems in which tyrosinase and xanthine oxidase were present with their respective substrates, it was found to be

impossible to estimate unchanged tyrosine owing to the presence of interfering substances. Hence, although an increased production of dopa was observed as compared with that found when tyrosinase acted alone, the result must be regarded as inconclusive, since the dopa produced could not be equated with the tyrosine oxidized. Similar experiments in presence of succinic oxidase and succinate did not reveal any significant change in the ratio dopa produced/tyrosine oxidized. Lastly, the action of ascorbic acid in the tyrosinase-tyrosine reaction was investigated. It is well known that vitamin C stabilizes adrenaline [Heard & Raper, 1932] and from analogy it would be expected also to stabilize dopa. This is quite in accord with the oxidation/reduction potentials of the systems concerned, for ascorbic acid $E_0' = -0{\cdot}045$ V. at *p*H 7 and 30° [Green, 1934]; and dopa $\rightleftharpoons$ dopa-quinone $E_0' = +0{\cdot}345$ V. at *p*H 7 [Ball & Chen, 1933].

Test-tube experiments showed that ascorbic acid, added in small amounts to the tyrosinase-tyrosine system inhibited pigment formation for some time. This may not mean that it inhibits the formation of dopa from tyrosine by the enzyme, since if it keeps the dopa in the reduced state, no pigmentation will be visible. The results so far show that there is an increase in the ratio dopa produced/tyrosine oxidized when ascorbic acid is introduced into the system. More work on the effect of ascorbic acid on the accumulation of dopa in the tyrosinase system is necessary before the mechanism is fully understood, and it is hoped to investigate this point, together with the effects of the addition of glutathione to the tyrosinase system.

Experimental

(I) *Preparation of 5:6-dihydroxydihydroindole-α-carboxylic acid*

This substance can be obtained in aqueous solution from two sources: (*a*) isolation of the naturally occurring 5:6-quinone of dihydroindole-α-carboxylic acid from the polichaete worm *Halla parthenopoea* Costa [Mazza & Stolfi, 1931] and reduction with palladium and hydrogen; (*b*) isolation and reduction of the 5:6-quinone from the tyrosinase-tyrosine reaction, or reduction of the concentrated 5:6-dihydroxyindole-α-carboxylic acid catalytically to 5:6-dihydroxydihydroindole-α-carboxylic acid. Both methods have been used.

From the worms (obtained from the Naples Biological Station) the procedure of Mazza & Stolfi was followed. The alcoholic extract of the worms, coloured deep red with a bluish fluorescence, was concentrated in a stream of hydrogen (under reduced pressure) at 25°. The residue was treated with a small volume of glacial acetic acid, the solution kept for some time and then filtered, the filtrate being coloured a good red-violet. Anhydrous acetone (4 vol.) was added and the solution left in the ice-chest for some time, the small brown precipitate which was deposited being filtered off. The filtrate was evaporated to dryness at 30° *in vacuo*, taken up again in a small volume of anhydrous acetone, filtered and evaporated to dryness over H_2SO_4. A rose-brown residue was left, easily soluble in water giving a brilliant reddish purple solution and possessing all the properties of hallachrome. A concentrated solution was easily reduced by the aid of palladium-norite (0·1 %) and hydrogen to the colourless leuco-body. This colourless solution, on shaking with air, absorbed oxygen again, with the production of the 5:6-quinone.

In the present work a good supply of the leuco-body was necessary. Since it is not easy to get a good supply of *Halla*, it was thought possible to try and isolate the hallachrome from the products of the tyrosinase-tyrosine reaction. Hitherto, it had been believed that the red quinone was too unstable to withstand isolation.

As hallachrome is easily destroyed by boiling, it is important that the evaporation of large quantities should be accomplished at a low temperature and preferably in an atmosphere of hydrogen. Tyrosine in saturated solution (1 g./2 l.) was oxidized, using a purified potato tyrosinase (acetone-ether precipitate from potato juice), and when a good red colour had been obtained, the enzyme was precipitated by the cautious addition of acetic acid. Solid CO_2 was added to the filtered, port wine-coloured solution to saturate with CO_2 and cool, and the solution evaporated at 25–30° at low pressure using a high-vacuum pump. The two receivers were cooled in freezing mixtures, and traps of conc. H_2SO_4 and $CaCl_2$ were used. At no stage in the evaporation was air let in, the apparatus being thoroughly washed out with hydrogen at the start.

The residue from large scale preparations of many litres of hallachrome solution, after evaporating to dryness, was taken up in anhydrous acetone in absence of air and kept in the ice-chest. It was coloured a deep reddish brown, and was quickly filtered and evaporated again under hydrogen *in vacuo*. In this way, a dark reddish brown solid was obtained, soluble in water giving a deep reddish violet colour in concentrated solution. It behaved in every way like the 5:6-quinone of dihydroindole-α-carboxylic acid and gave the Erhlich reaction on boiling. The yield was, however, very small and at the stage where it was taken up in anhydrous acetone, a considerable brown residue did not go into solution; presumably owing to decomposition in the process of preparation.

The substance finally obtained was dissolved in water and this concentrated solution was reduced catalytically by means of palladium-norite and hydrogen.

(II) *Action of 5:6-dihydroxydihydroindole-α-carboxylic acid on the mealworm tyrosinase-tyrosine reaction*

The solution of reduced quinone (leuco-body) was electrometrically titrated in a stream of hydrogen and the *p*H adjusted to 7 by dropping small amounts of ammonia from a microburette fitted into the rubber bung. The bottom of the titration vessel was connected with a 25 ml. burette whose outlet at the top was connected by a three-way tap to a reservoir of hydrogen or the suction pump. In this way the neutralized, reduced quinone (leuco-body) was kept under hydrogen until the experiment proper.

Exp. 1. Solutions: (*a*) Tyrosine (250 ml. 1/2000); mealworm enzyme (12·5 ml.); water (25 ml.); adjusted to *p*H 7. (*b*) Tyrosine + enzyme (same amounts) + leuco-body (25 ml.); adjusted to *p*H 7. (*c*) Control of (*a*) using water (250 ml.) instead of substrate + enzyme (12·5 ml.). (*d*) Control of (*b*) using water (250 ml.) + leuco-body (25 ml.) + enzyme (12·5 ml.). Temperature of bath 25°. Time of aeration, 2·5 hr. (oxygen). Toluene and capryl alcohol were added and the dopa and tyrosine at the end of the enzyme action assayed. The results obtained are shown in Table I.

Table I

	Tyrosine oxidized mg.	Dopa produced mg.	$\frac{\text{Dopa}}{\text{Tyrosine}}$
Mealworm enzyme and tyrosine	71·0	8·1	0·114
Mealworm enzyme, tyrosine and leuco-body	65·5	10·3	0·157

As seen, the ratio dopa produced/tyrosine oxidized is greater on addition of the 5:6-dihydroxydihydroindole-α-carboxylic acid. It was thought that the introduction of the leuco-body, completely at the start of the enzyme action, did not simulate the continuous oxidation and reduction equilibrium set up in the

reaction mixture, when a definite concentration of red quinone is established. What is required, if possible, to favour dopa accumulation, is a continuous high preponderance of the leuco-body. Hence the addition of the leuco-body slowly over a period of time would more closely resemble what is required for dopa accumulation, and it was considered advisable to try this.

Exp. 2. Solutions: (*a*) Tyrosine (250 ml.); mealworm enzyme (12·5 ml.); adjusted to *p*H 7. (*b*) Tyrosine (250 ml.); enzyme (12·5 ml.); 25 ml. leuco-body added from burette under hydrogen, with its delivery tube fitting into the reaction vessel. The addition was adjusted so that about 5 ml. were added during 30 min. Time of aeration, 3 hr. (oxygen). Temperature of bath, 25°. (*c*) and (*d*) Controls of (*a*) and (*b*) as in Exp. 1, the leuco-body being added simultaneously as in (*b*). The dopa and tyrosine were estimated at the end. Table II shows the collected results.

Table II

	Tyrosine oxidized mg.	Dopa produced mg.	$\frac{\text{Dopa}}{\text{Tyrosine}}$
Mealworm enzyme and tyrosine	72	8·9	0·123
Mealworm enzyme, tyrosine and leuco-body	68	12·9	0·19

The results indicate that there is again a definite increase in dopa accumulation on addition to the enzymic reaction mixture of a solution of the reduced quinone (leuco-body). This is in accordance with the theory put forward to account for dopa accumulation in the mealworm enzyme tyrosinase-tyrosine system. There must be a limit to the concentration of dopa which can accumulate in the tyrosinase system; the yield of dopa in the second experiment was 18·9 % of the tyrosine oxidized, and it is quite probable that it represents very nearly a maximum.

(III) *Demonstration of the action of hallachrome as hydrogen acceptor in dehydrogenase systems*

By the use of the Thunberg tube technique, the presence of dehydrogenase systems, e.g. xanthine oxidase, succinic oxidase, glucose dehydrogenase, amino-acid dehydrogenase, were looked for, using methylene blue as hydrogen acceptor and with the addition of the appropriate substrate, but none was found to be present. It was quite easily demonstrable, however, that hallachrome could act as a perfect hydrogen acceptor for the xanthine oxidase and succinic oxidase systems.

Table III

Tubes	Hypoxanthine (0·1 %) ml.	Xanthine oxidase (2 %) ml.	Hallachrome in buffer *p*H 6·5 ml.	Methylene blue *M*/5000 ml.
A	1	1	3	—
B	—	1	3	—
C	—	—	3	—
D	1	—	3	—
E	1	1	—	1

As a source of xanthine oxidase the "whey preparation" of Dixon & Kodama [1926] *p*H 6·5 was used as a 2 % solution in phosphate buffer. The substrate (hypoxanthine) was placed in the small side arm of the Keilin tube, and when evacuation and filling with nitrogen was complete, it was mixed with the other constituents of the tube. Tubes A and E were rapidly decolorized in a few

seconds, B, C and D remaining unchanged for many hours. Admittance of air into A and E brought back the bright red and blue colours respectively, but on evacuation again both tubes were decolorized. Hence the reaction leuco-body $\rightleftharpoons$ hallachrome is reversible. When some of the hallachrome solution was boiled it immediately became colourless again (owing to the formation of 5:6-dihydroxy-indole-α-carboxylic acid). This colourless solution, as was expected, could not be reduced back to the leuco-body of hallachrome by the xanthine oxidase system.

It appears probable that the system dopa $\rightleftharpoons$ dopa-quinone would also be affected by the xanthine oxidase system and that dopa-quinone could thus act in a similar way as a hydrogen acceptor, since it has been found by Green [1934] that the normal potential of the xanthine oxidase system is very negative.

System	E_0 at *p*H 7/30°
Hypoxanthine-xanthine	−0·371 V.
Xanthine-uric acid	−0·361 V.

Table IV

Tube	Na succinate (*M*/50) ml.	Succinic oxidase g.	Hallachrome in buffer *p*H 6·5 ml.	Methylene blue *M*/5000 ml.
A	1	0·5	5	—
B	—	0·5	5	—
C	1	—	5	—
D	1	0·5	—	1

The succinic oxidase was prepared from sheep's heart by Andersson's method [1927]. Tubes A and D became decolorized in a few minutes. Hence hallachrome can replace methylene blue and act as a hydrogen acceptor for this system as well.

Quantitative experiments on the effect of the xanthine oxidase system on the tyrosinase system

Solutions: (*a*) Tyrosine (1/200, 250 ml.); mealworm tyrosinase (20 ml.); water (50 ml.) adjusted to *p*H 7. (*b*) Tyrosine (250 ml.); hypoxanthine (0·2 g.); tyrosinase (20 ml.); xanthine oxidase (50 ml., 5 % solution "whey preparation"). (*c*) Control of (*a*) using water. (*d*) Control of (*b*) using 250 ml. water instead of the tyrosine; hypoxanthine (0·2 g.); xanthine oxidase (50 ml., 5 %); tyrosinase (20 ml.). Toluene and capryl alcohol were added. Temperature 25°. All solutions were adjusted to *p*H 7. Aeration for 2 hr. (oxygen).

At the end of this time acetic acid (10 %) was cautiously added to precipitate the enzymes, but great difficulty was experienced in precipitating all the protein. Finally, addition of excess picric acid and removal of the excess of the latter with nitron acetate, followed by the removal of the slight excess of nitron with potassium nitrate was tried [Greenwald, 1924; Busch & Blume, 1908]. This was effective in removing most of the interfering substances, and the dopa was estimated by the usual colorimetric method.

The filtrate was quite colourless, but on applying the usual methods of estimating tyrosine, it was found that the controls containing the "whey preparation" also gave Folin's Millon reaction. The bromination method of estimating the tyrosine could not be used either, since uric acid absorbs bromine under the conditions employed and it is present as a product of oxidation of the hypoxanthine by xanthine oxidase. After many trials, no accurate method of assaying the tyrosine was found. The dopa figures, however, are given in Table V.

Table V

Solutions	Dopa in mg.
Mealworm tyrosinase and tyrosine	5·4
Mealworm tyrosinase, tyrosine, xanthine oxidase and hypoxanthine	7·2

Since the tyrosine oxidized cannot be estimated, it cannot be definitely concluded from these experiments that the xanthine oxidase system favours the accumulation of dopa.

Quantitative effect of the succinic oxidase system on the tyrosinase reaction

The succinic oxidase of sheep's heart was used. The muscle was finely chopped and ground with 0·25% NaCl containing boric acid (1%) as antiseptic. The thoroughly washed tissue pulp was then used.

Solutions: (*a*) Tyrosine (250 ml. 1 g./2000); mealworm enzyme (20 ml.); adjusted to *p*H 7. (*b*) Same as (*a*) + succinic oxidase (2 g. tissue pulp); sodium succinate (0·5 g.). (*c*) Control of (*a*) using water (250 ml.) instead of the substrate. (*d*) A similar control of (*b*). (*e*) Tyrosine (250 ml.); mealworm enzyme (20 ml.); succinic oxidase (2 g.). Adjusted all solutions to *p*H 7. Temperature of bath, 25°. Aeration time, 3 hr.

The dopa and tyrosine were estimated by the molybdate method and by Folin's adaptation of Millon's reaction, respectively, as the bromination method could not be used, owing to the succinic acid-fumaric acid system. The results are shown in Table VI.

Table VI

Solutions	Tyrosine oxidized mg.	Dopa produced mg.	$\frac{\text{Dopa}}{\text{Tyrosine}}$
Tyrosinase and tyrosine	56·6	6·5	0·115
Tyrosinase-tyrosine system and succinic oxidase-succinate system	41·0	5·2	0·127
Tyrosinase-tyrosine system and succinic oxidase alone	37·0	4·8	0·13

As can be seen from the table, there is no significant accumulation of dopa due to the introduction of the succinic oxidase-succinate system.

Effect of ascorbic acid on dopa production in the tyrosinase-tyrosine reaction

It has been shown that vitamin C inhibits the Bloch pigmentation reaction of animal tissues [Schroeder, 1932]. Szent-Györgyi [1932] and Morawitz [1934] have also found that administration of ascorbic acid decreased pigmentation in Addison's disease. Heard & Raper [1932], in perfusion experiments with the suprarenal gland, showed that adrenaline was stabilized by ascorbic acid. These observations suggest that vitamin C stabilizes catechol derivatives and prevents their oxidation to melanin or melanin-like pigments. This is quite in accord with the oxidation-reduction potentials of the systems concerned. No evidence as yet, however, has been obtained indicating the effect of ascorbic acid on the production of catechol derivatives from monohydric phenols under the influence of tyrosinase and oxygen.

Test-tube experiments showed that the addition of ascorbic acid in small amounts to the tyrosinase system inhibited pigmentation for some time—presumably until the vitamin itself had been oxidized in the aerated solution.

This may not mean that it inhibits dopa formation, since if it keeps the dopa in the reduced state no pigmentation will be visible. It was decided to try a quantitative experiment as follows: (*a*) Tyrosine (250 ml., 1/2000); mealworm tyrosinase (10 ml.) adjusted to *p*H 7. (*b*) Same as (*a*) but with the addition of ascorbic acid (0·1 g.) + the calculated quantity of sodium carbonate to neutralize it, at intervals. (*c*) Control of (*a*) with water + enzyme. (*d*) Control of (*b*) with water + enzyme + neutralized ascorbic acid. Temperature of bath, 25°. Aeration time, 3 hr. When a red colour started to appear in the flask *b*, a further quantity of neutralized ascorbic acid was added.

The dopa and tyrosine were estimated by the Vulpian and Folin methods respectively. The molybdate colorimetric estimation for dopa cannot be applied since ascorbic acid interferes.

Table VII

Solution	Tyrosine oxidized mg.	Dopa produced mg.	Dopa / Tyrosine
Tyrosinase and tyrosine	47·2	7·5	0·16
Tyrosinase, tyrosine and ascorbic acid	32·4	12·9	0·39

The results in Table VII indicate that ascorbic acid does not prevent the oxidation of tyrosine to dopa by tyrosinase. No attempt was made to follow the disappearance of ascorbic acid from the solution, but it appears that it protects the dopa from enzymic oxidation so long as there is an appreciable concentration in solution.

Summary

1. The addition to the tyrosinase-tyrosine system of 5:6-dihydroxydihydroindole-α-carboxylic acid, an intermediate product in the tyrosinase-tyrosine reaction, increases the amount of dopa produced in the reaction. It is therefore concluded that dopa normally accumulates in the reaction because of the reduction of dopa-quinone by the dihydroindole derivative.

2. Evidence is also presented that addition of ascorbic acid to the tyrosinase-tyrosine system increases the accumulation of dopa by reduction of dopa-quinone.

3. No increase in dopa production was observed when the succinic oxidase-succinate system was present along with the tyrosinase-tyrosine system.

4. Attempts to determine the effect of the xanthine oxidase-xanthine system on the tyrosinase-tyrosine reaction were inconclusive, since no satisfactory method was found for the estimation of the tyrosine oxidized.

REFERENCES

Andersson (1927). *Skand. Arch. Physiol.* **52**, 187.
Ball & Chen (1933). *J. biol. Chem.* **102**, 691.
Busch & Blume (1908). *Z. angew. Chem.* **21**, 354.
Dixon & Kodama (1926). *Biochem. J.* **20**, 1104.
Friedheim (1933). *Biochem. Z.* **259**, 257.
Graubard & Nelson (1935). *J. biol. Chem.* **111**, 757.
Green (1934). *Biochem. J.* **28**, 1550.
Greenwald (1924). *J. biol. Chem.* **59**, 334.
Heard & Raper (1932). *Biochem. J.* **27**, 36.
Mazza & Stolfi (1931). *Arch. Sci. biol.* **16**, 182.
Morawitz (1934). *Klin. Wschr.* **13**, 324.
Pugh (1930). *Biochem. J.* **24**, 1442.
Raper (1927). *Biochem. J.* **21**, 89.
Schroeder (1932). *Klin. Wschr.* **11**, 553.
Szent-Györgyi (1932). *Dtsch. med. Wschr.* **852.**