ENZYMES IN ONTOGENESIS (ORTHOPTERA)

XIII. Activation of Protyrosinase and the Oxidation of Ascorbic Acid*

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INTRODUCTION

A number of reports showing that quinonoid compounds can act as carriers in the oxidation of ascorbic acid by oxygen have been reviewed by King (1, 2). Since phenol oxidases bring about the production of quinones, the association of ascorbic acid oxidation with these oxidases seems well established. A study of such an oxidation in conjunction with the tyramine-tyrosinase and tyrosine-tyrosinase reactions should be of especial significance in view of the fact that an inactive tyrosinase (protyrosinase) can be obtained from the egg of the grasshopper, *Melanoplus differentialis* (3).

EXPERIMENTAL

Preparation of Protyrosinase.—The procedure for extracting protyrosinase from the grasshopper egg has been described (3). A number of eggs in the diapause stage were ground up and centrifuged in a 0.9 per cent NaCl solution. The fatty layer, which contains an activator of protyrosinase, was removed and the supernatant fluid decanted into a graduated cylinder. To this portion a ½ volume of M/15 KH_2PO_4 solution was added. After standing for several hours at 0°C. this fluid was centrifuged. The clear supernatant liquid, designated B_1 was removed and diluted with a volume of M/15 Na_2HPO_4 solution equal to the amount of KH_2PO_4 solution previously added. The B_1 was next dialyzed at 0°C. against a 0.9 per cent NaCl solution by placing 40.0 ml. of B_1 in a cellophane tube and suspending the tube in 10 to 12 volumes of the saline solution. The latter solution was renewed at the end of each 24 hours. After 3 days the contents of the cellophane tube (volume = 40.7 ml.) were removed and stored at 0°C.

Composition (and Volume) of Reaction Solutions.—The center wells of Warburg manometer vessels contained 0.1 ml. of a 10 per cent KOH solution and a small roll of filter paper. The reaction fluid volume was 3.0 ml. The side bulbs contained 0.5 ml. of the B_1 preparations. 2 ml. of Sorensen's M/15 phosphate buffer solution of a pH = 6.2 were placed in the reaction chamber. In the case of tyrosine this 2.0 ml. portion

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contained a known amount of tyrosine (Coleman and Bell). The composition of the remaining 0.5 ml. of aqueous solution was varied as to the amount of tyramine hydrochloride (Eastman), the presence of ascorbic acid (Eastman), and an excess of sodium oleate (Merck) or the commercial detergent, Aerosol (American Cyanamid).[1]

RESULTS AND DISCUSSION

The existence of an inactive tyrosinase, which upon the addition of an excess of various activators changes into a tyrosinase, has already been considered (4). It has become simpler to refer to the inactive form as protyrosinase and to the active form as tyrosinase. Throughout this discussion, the behavior of protyrosinase (without activation by sodium oleate or Aerosol) is contrasted to that of tyrosinase (with this activation).

In the presence of undialyzed tyrosinase, tyramine is 0.93 oxidized to melanin in 160 minutes (tyramine, tyrosinase; Fig. 1). A very distinct red color appears within 30 seconds after the commencement of the latter reaction. This color, due to an indole quinone, indicates that the fifth intermediary product of the oxidation of tyramine to melanin is accumulating (5). The uppermost curve (tyramine, ascorbic acid, and tyrosinase) shows an initial rapid uptake of oxygen succeeded by completion of the oxidation of tyramine to melanin. During the first 115 c.mm. oxygen uptake, although the oxidation of tyramine to melanin has started, the time of appearance of the indole quinone red color is delayed for some 8 minutes until the ascorbic acid is oxidized to dehydroascorbic acid. Therefore, the apparent inhibition of tyrosinase, if one views the rate of color formation as a measure of enzyme activity, is probably concerned not with a primary effect upon the enzyme but rather with an alteration in the velocity of formation of intermediary products (6). Judging from both the lack of a red color during these first 8 minutes and from the rapidity of oxygen uptake it seems that the oxidation of ascorbic acid involves the reduction of the quinone of 3:4 dihydroxyphenylethylamine, the third intermediary product in the oxidation of tyramine to melanin (5). In contrast to these two systems the following experiments, also with undialyzed extracts resulted in no observable oxygen uptake: protyrosinase alone; tyrosinase alone; protyrosinase and ascorbic acid; protyrosinase and tyramine; protyrosinase, ascorbic acid, and tyramine (Fig. 1).

The result of dialyzing a B_1 preparation against the sodium chloride solution is graphically illustrated in Figs. 1 and 2. There is no significant change in the velocity of oxidation of 2.3×10^{-3} mM of tyramine in the presence of tyrosinase. Neither does dialysis affect the velocity of oxygen

[1] The authors wish to express their appreciation to the American Cyanamid Company for supplying the Aerosol used in these experiments.

uptake by this amount of tyramine and 0.01 mM of ascorbic acid (equivalent to 112 c.mm. of oxygen) in the presence of tyrosinase. It was also found that no measurable oxygen uptake occurred in the following experiments on dialyzed material; protyrosinase; tyrosinase; protyrosinase and tyramine; protyrosinase and ascorbic acid; protyrosinase, ascorbic acid, and tyramine. However, there is a marked difference in the effect of an undialyzed and a dialyzed tyrosinase upon the oxidation of ascorbic acid (Fig. 1). This particular oxidation of ascorbic acid in the presence of an undialyzed extract is probably due to a coupled reaction with some oxidation product of a naturally occurring substrate. After this substrate has diffused away, the protyrosinase can be activated and the resulting failure to oxidize ascorbic acid indicates the absence of a substance which can act as a carrier between oxygen and ascorbic acid (7). Since the oxygen uptake of dialyzed tyrosinase solutions is not perceptible, it seems that only a minute amount of the natural substrate is needed for the oxidation of ascorbic acid. This is borne out in experiments on dialyzed tyrosinase preparations in which less than 2.3×10^{-3} mM of tyramine still furnishes

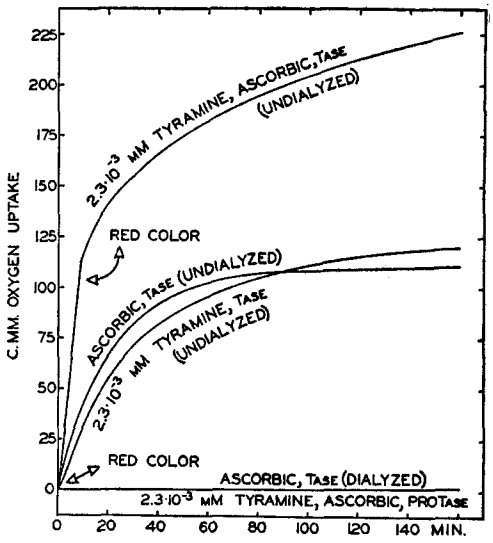

FIG. 1. The effect of dialyzed and undialyzed tyrosinase and protyrosinase preparations upon the oxidation of ascorbic acid. 0.01 mM ascorbic acid; concentration of activator, 0.07 per cent sodium oleate; pH = 6.1; T. = 25.0°C.

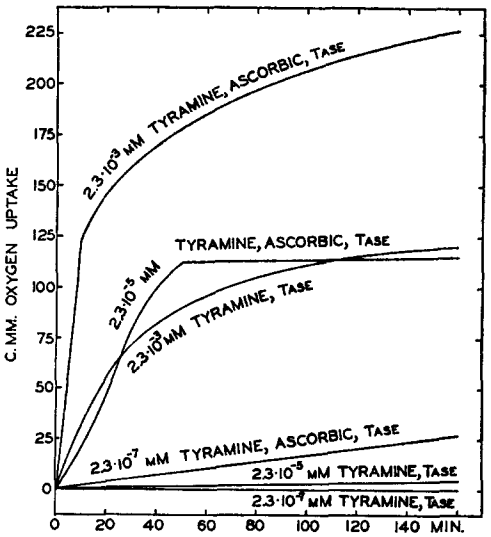

FIG. 2. The effect of dialyzed tyrosinase upon the oxidation of ascorbic acid in the presence of various amounts of tyramine. 0.01 mM ascorbic acid; activator, 0.07 per cent sodium oleate; pH = 6.0; T. = 25.0°C.

enough quinone to catalyze the oxidation of 0.01 mM of ascorbic acid to dehydroascorbic acid (Fig. 2). Obviously the tyramine-tyrosinase reaction with smaller amounts of tyramine oxidizes ascorbic acid at slower rates (Fig. 2).

Lest it be thought that this dialyzable substance be a copper compound which is activated into directly catalyzing the oxidation of ascorbic acid, the following observations should be added. Although the copper-proteinate experiment of Stotz, Harrer, and King (8) could be duplicated, the addition of sodium oleate did not increase the activity of copper with respect to ascorbic acid oxidation in the presence of various amounts of egg albumin. If grasshopper egg tyrosinase is also a copper-proteinate (9–12), one might then conclude that this oxidase has different properties from ascorbic acid "oxidase." Such a conclusion seems to be in logical agreement with the distinctions which McCarthy, Green, and King (13) found to exist between ascorbic acid oxidases and catechol oxidase.

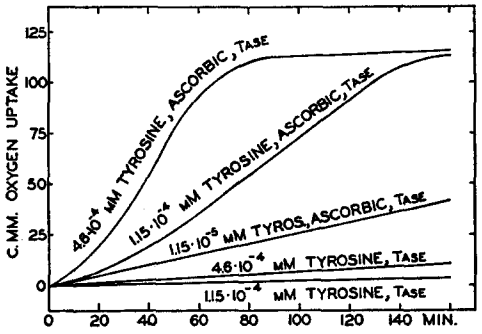

FIG. 3. The oxidation of ascorbic acid in the presence of various amounts of tyrosine and dialyzed tyrosinase. 0.01 mM ascorbic acid; activator, 0.017 per cent Aerosol; pH = 6.1; T. = 25.0°C.

Since the oxidation of small amounts of substrate can be expressed in terms of the readily observed coupled reaction with ascorbic acid, it is possible to use a solution of tyrosine as a substrate. The solubility of tyrosine limits the amount which can be added as a solution, but with concentrations of tyrosine from 4.6×10^{-4} to 1.15×10^{-5} mM (equivalent to 25.8 and 0.7 c.mm. oxygen) there is a coupled oxidation of ascorbic acid (Fig. 3). It was also observed that this reaction with tyrosine and ascorbic acid did not occur unless an excess of Aerosol or sodium oleate was present to function as an activator of the protyrosinase. Neither was there an oxidation of tyrosine in the presence of protyrosinase. Hence, under these conditions, with tyrosine as with tyramine, for a substrate there is still the distinction as to protyrosinase and tyrosinase.

SUMMARY AND CONCLUSIONS

1. Protyrosinase from the egg of the grasshopper, *Melanoplus differentialis*, can be activated by excess sodium oleate or Aerosol.

2. The 3:4 quinone products of the reaction of activated protyrosinase with tyramine or tyrosine will oxidize ascorbic acid to dehydroascorbic acid.

3. The velocity of this latter oxidation of ascorbic acid increases with the amount of tyramine or tyrosine.

4. The oxidation of ascorbic acid by the tyramine-tyrosinase reaction delays the time of appearance of a red color associated with an indole quinone intermediary product in the formation of melanin.

5. Protyrosinase, in itself, and in the presence of tyrosinase substrates does not bring about the oxidation of ascorbic acid.

6. A naturally occurring substrate in a preparation of protyrosinase, sufficient to cause the oxidation of ascorbic acid, can be removed by dialysis against a 0.9 per cent sodium chloride solution.

7. Dialysis against such a solution does not change the properties of protyrosinase; the inactive enzyme must still be activated before it will catalyze the oxidation of tyramine or tyrosine.

8. When the natural substrate, tyrosine, or tyramine is absent, activation of protyrosinase does not result in the oxidation of ascorbic acid.

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