# The Oxidizing Enzymes of Sugarcane: Tyrosinase (Polyphenol oxidase)

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INTRODUCTION

When, in 1931, Sommer (57)[2] published the first convincing evidence of copper essentiality for higher plants, there was no inkling that this metal took part in distinct cellular functions, or that it could be a constituent of oxidizing enzymes. Indeed, for more than a century biologists had viewed the presence of copper in plant tissues as a kind of natural accident, of little or no consequence (8,24). Perhaps no experiments have so succinctly demonstrated the physiological potential of micronutrients than those dealing with the copper proteins. Foremost among this group is the oxidizing enzyme tyrosinase.

Catalyzing the type reaction

$$AH_2 + \tfrac{1}{2} O_2 \xrightarrow{\text{oxidase}} A + H_2O,$$
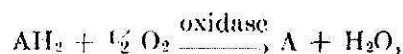
the copper-containing enzymes stimulate direct oxidation of their respective substrates by atmospheric oxygen. The reaction does not take place anaerobically. Dyes such as methylene blue are not altered by the copper enzymes, and no hydrogen peroxide formation results from their activity. Each enzyme is sensitive to cyanide.

Tyrosinase was discovered by Bertrand and Bourquelot (12,15,13), who demonstrated that darkening of mushroom tissues was due to the enzymatic oxidation of tyrosine, a phenolic amino acid.[3] Kubowitz (36,37) has proposed a sequence of copper-requiring reactions to account for the oxidation of a polyphenol, such as catechol, to its corresponding quinone. Thus the cupric state of the enzyme is reduced to the cuprous form, which, in turn, is reoxidized by oxygen:
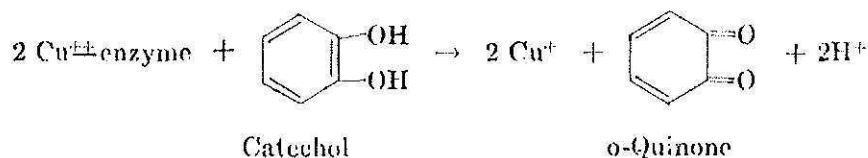
$$2\,Cu^{++}\text{enzyme} + \text{Catechol} \rightarrow 2\,Cu^{+} + \text{o-Quinone} + 2H^{+}$$

Copper apparently functions as a prosthetic group attached through a nonporphyrin linkage (27, p. 367). The darkening which accompanies tyrosinase action (i.e., the browning of cut or bruised tissues of fruits and vegetables) is believed due to a subsequent series of nonenzymatic reactions whereby quinones are converted to dark, melanin-like pigments (60, p. 241, 53,44,46,45).

The marked capacity of tyrosinase to catalyze electron transfer from metabolites to oxygen has led workers to suspect that this enzyme might be a key constituent of terminal oxidation. Unfortunately, a prolonged controversy over this point has left tyrosinase hanging, its true roles as yet unresolved.[4] Boswell and Whiting (16) felt that a catechol-like compound might be the natural hydrogen carrier operating adjacent to the terminal oxidase of potato. They were able to increase the oxygen uptake of potato slices by adding catechol, although later the reaction product apparently inhibited the oxidase. Baker and Nelson (9) used a substituted catechol, 4-t-butyl catechol, to show that decline of oxygen uptake did not result from oxidase inhibition. They further strengthened the case for tyrosinase as a respiratory enzyme by obtaining a prolonged oxygen uptake increase, with a concurrent increase of carbon dioxide evolution, thus maintaining the respiration quotient close to unity. Sreerangachar (58,59) reported evidence that tyrosinase serves as a terminal oxidase in tea leaves and stems. No participation of cytochrome oxidase, another suspected catalyst of terminal oxidation, was detected (58). Studies by Bonner and Wildman (14) led to similar conclusions regarding tyrosinase of spinach leaves, since more than 90 percent of the respiration was inhibited by p-nitrophenol. This agent inhibits tyrosinase but has no effect on the cytochrome enzymes.

Other workers have been skeptical of tyrosinase in respiration, favoring instead the iron-containing enzyme cytochrome oxidase.[5] Levy and coworkers (39) observed that potato respiration is inhibited by carbon monoxide, and that this inhibition is reversed by light. Brown and Goddard (17) recorded similar light effects while working with wheat embryos, although Allen and Goddard (7) failed to detect either carbon monoxide or light effects upon the respiration of mesophyll cells in mildewed wheat.

[4] For a more thorough review of the respiratory roles of tyrosinase, the reader is referred to the papers by Dawson and Tarpley (24), Nelson (48), Arnon (8), and by Goddard and Meeuse (28).

[5] Szent-Gyorgyi (63) has discounted outright the role of polyphenol oxidase (tyrosinase) in plant respiration, assigning the enzyme a dormant role in normal tissues. According to this view, only when tissues are damaged, as by bruising or pathogenic invasion, is the enzyme mobilized and required to produce a quinone which is toxic to the invader.

Light reversal of CO inhibition is a characteristic of iron-porphyrin oxidase systems, most notably that of cytochrome C.

A perhaps more reasonable stand favors both copper and iron oxidases in plant respiration, one acting in the absence of the other, or both together. Guzman-Barron and coworkers (33) reported that 8-hydroxyquinoline completely inhibited potato polyphenol oxidase (tyrosinase), while having no effect on cytochrome oxidase. Since potato respiration was only partly inhibited by this agent, it was concluded that both enzymes take part in potato respiration. It is also important that, while carbon monoxide inhibition may be reversed by light for one tissue, carrot root, it may not be reversed for another, carrot leaf, of the same plant (43). Warburg (64) has shown that, in the presence of polyphenol oxidase (tyrosinase), there is a coupled oxidation-reduction between reduced pyridine nucleotides and polyphenols. During the discussion period following a paper by Nelson (48) on phenol oxidases in plant respiration, the following pertinent comments on the copper and cytochrome oxidases were contributed by Dr. S. Granick:[6]

The possibility of a copper enzyme coming into play in the cytochrome oxidase picture need not be dismissed summarily. Since one has to account for a transfer of 4 electrons from cytochrome C to D_2 to form $2H_2O$ and since such activity is yet known only in the heterogeneous mitochondrial material, it might be that a copper enzyme could cooperate with cytochrome oxidase in the process of oxidation with oxygen. Furthermore, if the energy released in cytochrome C oxidation is not to be wasted, it is necessary to postulate other enzyme interactions with this oxidation. The process of cytochrome oxidase reaction is, therefore, probably not to be regarded as a simple one-enzyme activity.

It is interesting to note that Keilin and Hartree (35), Graubard (30), and Eichel et al. (26) have all suggested that cytochrome oxidase itself may be a copper-containing enzyme.

Copper enzymes, like those of iron, have been studied as possible indicators of nutritional disorders in agricultural plants (10,18,19,20,51,2). Previous work in Puerto Rico has shown that tyrosinase is present in sugarcane-leaf tissues (1). Until now, however, the enzyme has not received the close attention given to those more directly related to sugar formation and breakdown.

Tyrosinase is important in cane to the extent that it participates in respiration,[7] including the metabolism of sucrose accumulations and of the

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[7] Goddard and Meeuse (28) define respiration as ". . . the oxidation of organic compounds with molecular oxygen serving as the ultimate electron acceptor; the oxidation may be complete with water and carbon dioxide as the final products, or it may be incomplete, with organic acids as the end products." In their report an arbitrary distinction was drawn between respiration and glycolysis, although in

hexose and phosphorylated metabolites which otherwise would have taken part in sucrose formation. In view of present concepts of terminal oxidation, either tyrosinase or cytochrome oxidase, separately or together, must serve as most critical factors during carbohydrate breakdown. This paper deals with the distribution and properties of cane tyrosinase as an additional step toward clarifying oxidase-sucrose relationships in sugarcane.

MATERIALS AND METHODS

Tyrosinase was obtained from meristem tissue of the sugarcane variety M. 336,[8] which had been frozen, lyophilized, and ground to pass a 60-mesh screen in accordance with procedures described earlier (1). Two grams of the dried material were extracted at laboratory temperature (19–21°C.) for 30 minutes in distilled water or 0.1 M[9] phosphate buffer, pH 7. Extraction was aided with a mechanical shaker operating at high speed. Extracts were clarified by expression through four layers of absorbent gauze, followed by centrifuging at 3,000 r.p.m.

After adjusting the pH to 7 with 0.25 N NaOH, sufficient solid ammonium sulfate was added to bring the solution to 80-percent saturation. This was accomplished over a 10-minute period with constant stirring of the solution. Precipitated protein was taken up in 4 ml. of distilled water, clarified by centrifuge, and refrigerated at 2°C. Appropriate dilutions were made just prior to running the tyrosinase assays.

Tyrosinase was assayed spectrophotometrically by measuring the optical density increase, at 390 mμ, of a mixture of catechol and enzyme in phosphate buffer. Techniques based upon the appearance of colored oxidation products have not been entirely satisfactory (62,52) because of a complex of factors influencing color formation. In particular, the orthoquinone which is produced from catechol apparently inactivates tyrosinase, although the oxidation rate is at first linear (29). Manometric techniques have been preferred by some workers (32,31,49,36), although Smith and Stotz (56) have published a more rapid colorimetric method which compares favorably with the manometric procedures. Probably the best tyrosinase assays available are the chronometric procedures of Dawson and Magee (23), and those of Miller et al. (47), in which orthoquinone is reduced back to catechol by ascorbic acid as soon as it is formed. The procedure adapted for cane tyrosinase is satisfactorily precise within a reaction period of 2

living tissues no reasonable line can be drawn to separate carbohydrate breakdown from respiratory pathways.

[8] Tyrosinase was also extracted from leaves −1 and 0, leaves +2 and +3, leaves +6 and +7, sheaths +6 and +7, 8 to 10 nodes, 8 to 10 internodes, nodes 1 to 3, and internodes 1 to 3.

[9] The letter M is used throughout this paper as an abbreviation for the word "molar."

minutes, and permits the handling of a very large number of sugarcane samples.

The standard tyrosinase assay was conducted at 20°C. according to the following procedure: 1.5 ml. of 0.1-M phosphate buffer, pH 7.2, was placed in duplicate 1-cm. pyrex cuvettes, followed by 0.5 ml. of enzyme preparation. About 2 or 3 units were added to each cuvette. One milliliter of distilled water was added to the reference vessel and the optical density reading for this solution was adjusted to zero. One milliliter of 0.05-M catechol solution was then added to the test cuvette, optical density of the test solution was recorded, and a stopwatch was started immediately. Readings were again taken for both reference and test solutions when 60 seconds had passed. One tyrosinase unit was arbitrarily defined as the amount of enzyme causing an optical-density increase of 0.10 under the prescribed conditions of the assay. Protein content of the enzyme preparations was determined colorimetrically by the method of Sutherland et al. (61), and tyrosinase action was expressed as specific activity in units per milligram of protein.

RESULTS AND DISCUSSION

EXTRACTION, FRACTIONATION, AND DISTRIBUTION

Preliminary extraction trials for powdered, freeze-dried leaf tissues were conducted with 0.1-M acetate buffer (pH 4.6 and 5.5), distilled water, 0.1-M phosphate buffer (pH 7.0 and 7.5), and with 0.1-M tris buffer (trishydroxymethylamino methane) of pH 8.0 and 8.5. Acetate buffer (pH 5.5) and phosphate buffer (pH 7) were superior to distilled water with regard both to total protein and tyrosinase obtained. Phosphate buffer (pH 7) was used throughout the remainder of the study.

Fractionation of the cane-leaf extracts revealed that tyrosinase was heavily precipitated over an unusually broad saturation range, from 25 to 70 percent (fig. 1). Only Q enzyme (4), starch phosphorylase (5), and an α-amylase (4) have previously been precipitated so readily at low-salt concentrations, and none of these was obtained over such a broad saturation range. Since protein is salted out when the protein-protein interaction begins to exceed the protein-water interaction, a tremendous variation must exist among cane proteins having tyrosinase activity, particularly with regard to —NH3+ and —COO− groups. It seems probable that the protein requirement of tyrosinase is far less specific than that of the copper cofactor.

Distribution of tyrosinase within the plant was ascertained with a series of tissue samples taken from 10-month old cane of the variety Uba Marot (table 1). By far the greatest specific activity was recorded with meristem preparations, with relatively strong activity also present in leaves —1 and

0. On a basis of activity in units per gram of dried tissue, meristem completely overshadowed all other tissues tested. This is not surprising in view of the rapid darkening of cane meristematic tissue observed whenever
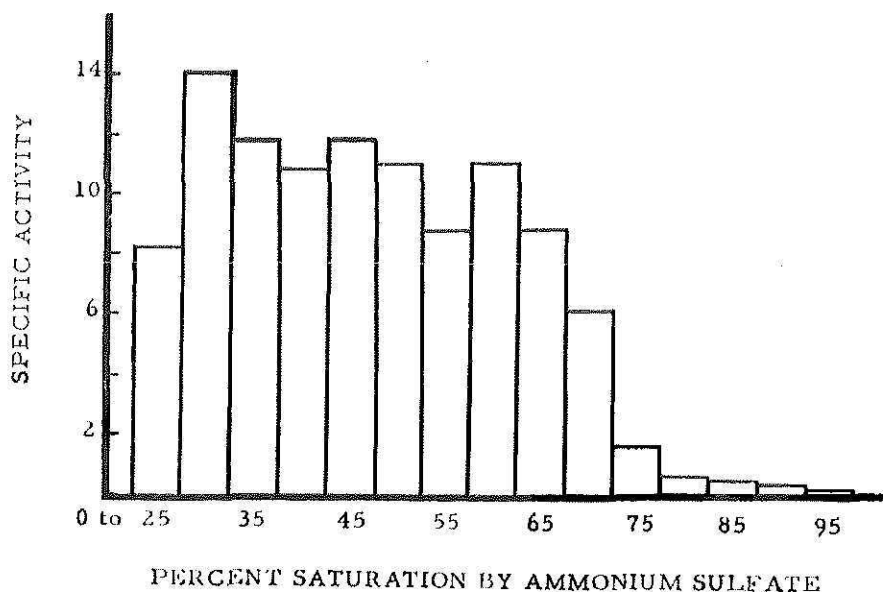

FIG. 1.—Tyrosinase activity among protein increments precipitated from extracts of sugarcane meristem.

TABLE 1.—*Activity of tyrosinase extracted from different tissues of 12-month-old sugarcane*[1]

Data classification	Data for indicated tissue source —								
	Meri-stem	Leaf (−1 & 0)	Leaf (+2 & +3)	Leaf (+6 & +7)	Sheath (+6 & +7)	Node (8-10)	Internode (8-10)	Node (1-3)	Internode (1-3)
Specific activity	148.2	29.4	12.5	10.5	9.6	2.2	1.9	1.8	3.6
Tyrosinase units per gram of tissue	2075.0	122.0	61.3	49.4	32.2	3.5	Trace	Trace	Trace

[1] All reactions were run for 2 minutes at 20°C. in phosphate buffer (pH 7.3). Catechol concentration was 0.1 M.

it is cut or damaged. Even freeze-dried meristem samples, initially white, totally dry and stored at −10°C., slowly turn brown with the passage of a few weeks. This is undoubtedly the work of tyrosinase acting upon endogenous phenolic compounds, uninspiring as these conditions must be for the catalyst.

PROPERTIES OF SUGARCANE TYROSINASE

Stability

Unlike cane peroxidase (6), tyrosinase showed no recuperative capacity after having been inactivated by boiling. Dialysis of the tyrosinase preparation against distilled water caused a 23-percent activity decline within 2 hours (table 2), although activity gradually increased to its former level by 32 hours. The dialysis effect is difficult to explain, although obviously

TABLE 2.—*Specific activity of cane tyrosinase after varying periods of dialysis against distilled water*[1]

Data classification	Data for indicated dialysis time (hours)							
	0	½	2	4	8	16	24	32
Specific activity	15.1	12.9	11.6	11.9	12.7	13.9	14.5	15.2

[1] Enzyme samples were dialyzed at 2°C. for the indicated period of time. Afterward they were stored at 2°C. until the 32-hour treatment had been completed. Each sample was then assayed according to the standard procedure.

TABLE 3.—*Specific activity decline of cane tyrosinase stored at room, laboratory, and refrigerator temperatures*[1]

Storage temperature	Data for indicated days following preparation					
	0	1	2	4	8	12
Room (28–29°C.)	15.1	3.4	1.1	0.5	0.3	0.2
Laboratory (19–21°C.)	15.1	5.6	2.5	1.1	.8	.6
Refrigerator (2°C.)	15.1	11.9	11.1	9.2	5.7	4.6

[1] Standard assay procedures were employed. Tyrosinase preparations were stored under toluene.

no essential cofactor was involved. Apparently both a potential activator and an inhibitor were present in the crude preparation, the former being more quickly removed by dialysis than the latter.

Stability of the tyrosinase preparation was tested at several storage temperatures, including room (27–29°C.), laboratory (19–21°C.), and refrigerator (2°C.). All samples were stored under toluene. As indicated in table 3, the preparations were extremely sensitive to room and laboratory temperatures, losing about 77 and 63 percent of their initial activity, respectively, within 24 hours of extraction. Samples stored at 2°C. deteriorated more slowly and tyrosinase was easily measurable after 12 days. This

response to cool temperature contrasts markedly with sugarcane peroxidase, the latter enzyme declining more rapidly in the refrigerator than when standing in the laboratory.

Optimum Temperature and pH

Cane tyrosinase was tested at temperatures ranging from 16° to 52°C. Since no satisfactory method is available for regulating temperatures of test solutions within the spectrophotometer employed, the following procedure was adapted: Duplicate test tubes containing all components of the standard digest except substrate were equilibrated for 5 minutes in a water bath adjusted to the desired temperature. Catechol solution was equili-
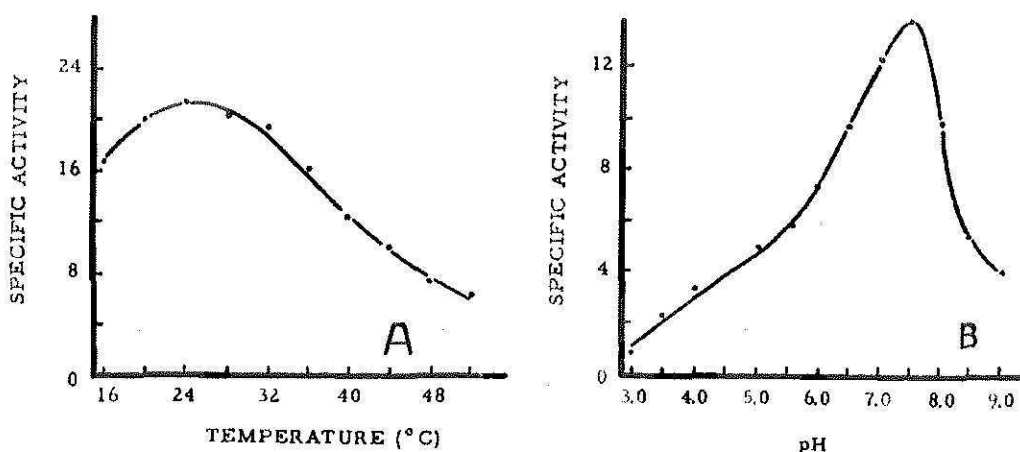

FIG. 2.—A, Effects of temperature on cane tyrosinase activity; B, effects of variable pH. Buffers (0.1 M) were employed as follows: Acetate, pH 3.5-5.5; phosphate, pH 6.0-7.5; and tris (trishydroxymethylamino methane), pH 8.0-9.0. The standard assay was used throughout in conjunction with the indicated variables.

brated separately. One milliliter of distilled water was placed in the control tube and the reaction was then initiated by transferring 1 ml. of catechol solution to the test vessel. Two minutes were allowed for the reaction to proceed. Contents of the test tubes were rapidly transferred to 1-cm. cuvettes where optical-density differences between reference and test solutions were measured as usual. About 2 minutes and 15 seconds elapsed for each reaction. Although some error was unavoidable, the results were nevertheless relative, and indicated that optimum temperature for cane tyrosinase is about 24°C. (fig. 2,A). Thus, quite by accident, the laboratory temperature we ordinarily employed was very near optimum. Dawson and Magee (23) recommended 25°C. for both "cresolase" and "catecholase" assays, whereas Smith and Stotz (56) suggested 30°C. for colorimetric measurement of general plant tyrosinase. Peroxidase of sugarcane was

more than twice as active at 36°C. than at the 20°C. temperature where it was usually measured.

Optimum pH for cane tyrosinase was about 7.5 (fig. 2,B). This corresponds closely with the optimum pH for cane peroxidase (7.4), yet was higher than that recommended by Dawson and Magee for "cresolase" (7.0) and "catecholase" (5.1), or by Miller *et al.* for general tyrosinase (5.5). Smith and Stotz found "catechol oxidase" (potato) to be considerably less active at pH 4.5 than at higher pH values, but observed little difference between pH 6.0 and 7.5.
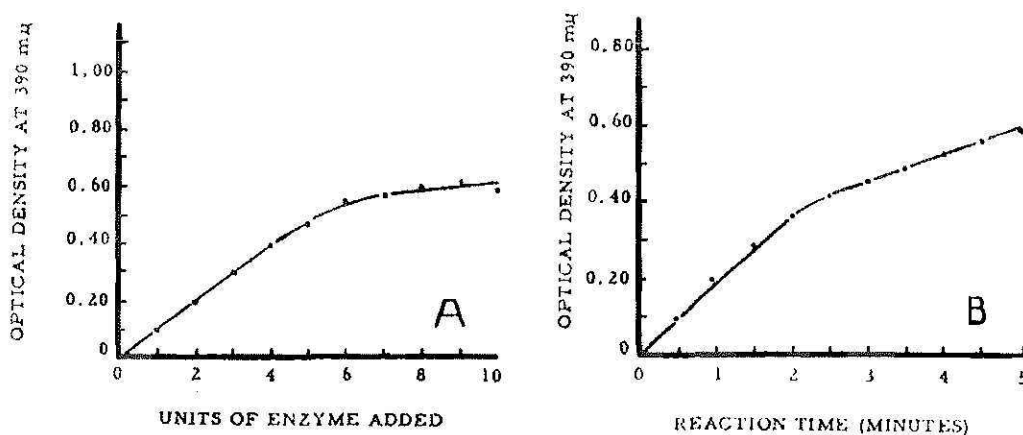

FIG. 3.—A, Proportionality of tyrosinase activity with concentration; B, proportionality of activity with time. There were 2.5 units employed for the time experiment, otherwise the standard assay was used throughout in conjunction with the indicated variables.

Reaction Velocity vs. *Enzyme Concentration and Time*

A linear relationship exists between reaction velocity and tyrosinase content to a concentration of 5 units per digest (fig. 3,A). Ordinarily, about 2 to 3 units of enzyme were employed for the standard tyrosinase assay.

Orthoquinone is generally regarded as an inhibitor of tyrosinase when the enzyme acts upon catechol (*60*, p. 242, *36,49,23*). In some instances it has even been found necessary to add ascorbic acid to the extracting media in order to reduce quinones formed from endogenous phenolic compounds, and thereby to prevent tyrosinase inhibition while the enzyme is being prepared (*56*). A major concern with cane tyrosinase was the development of rapid procedures whereby large numbers of samples could be prepared and assayed in a single day. Consequently, it was necessary to determine for how long the direct oxidation of catechol could be regarded as a reliable measure of tyrosinase action (*i.e.*, until quinone began to inhibit). As indicated in figure 3,B, the enzyme obeyed a linear reaction for only 2 min-

utes, which, nevertheless, sufficed for establishing a standard assay based upon a 60-second reaction period.

Substrate Concentration, Km

Cane tyrosinase was tested with catechol concentrations ranging from 1 to 50 μmols/ml. of digest (fig. 4). Maximum velocity was recorded with 8 μmols of catechol. Km is therefore about 4.0×10^{-3} mols of catechol per liter.

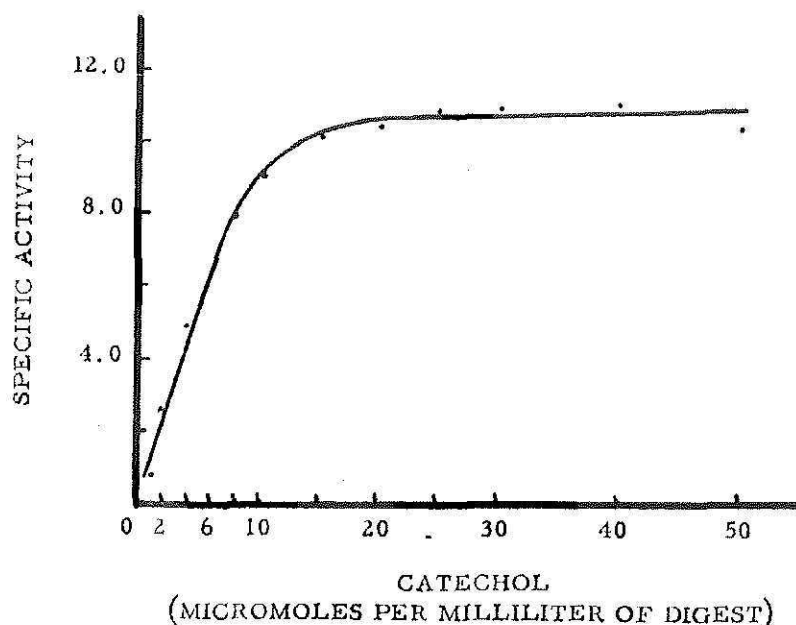FIG. 4.—Effects of variable substrate concentration on the activity of tyrosinase from sugarcane meristem.

Substrate Specificity

Tyrosinase will conceivably be found accompanied by such enzymes as peroxidases, ascorbic acid oxidase, and other phenol oxidases, including laccase. Such enzymes might alter or modify tyrosinase action, particularly so when tyrosinase has been precipitated over an unusually broad salt-saturation range. Tyrosinase can be distinguished from laccase in view of its capacity to catalyze two distinct oxidations, the addition of a hydroxyl group into a monohydric phenol (ortho to the one already present), and oxidation of orthodihydric phenols to the corresponding orthoquinones. Laccase can bring about the oxidation of both ortho- and paradihydric phenols, but does not act upon monohydric phenols.

Cane tyrosinase acted upon tyrosine, catechol, DOPA (3,4-dihydroxy-

phenylalanine), pyrogallol, guaiacol, resorcinol, hydroquinone, and para-cresol. No reaction was detected against phenol or metacresol. The possibility cannot be ruled out that laccase (a true polyphenol oxidase) composes part of the cane tyrosinase preparation.

Cushing (22) has studied the effects of tyrosinase on a series of substituted phenols. Oxidation of complicated, high molecular-weight substances by tyrosinase has also been reported (55).

Activation and Inhibition

A series of cations and anions were tested for possible regulatory effects upon tyrosinase (table 4). Also tested were glucose, cysteine, thiourea, hydroxylamine, and ascorbic acid. Thiourea and hydroxylamine markedly

TABLE 4.—*Effects of additives on tyrosinase from sugarcane meristem*[1]

Additive	Specific activity	Additive	Specific activity	Additive	Specific activity
Control (H₂O)	11.3	I	12.1	Glucose	10.8
Ca	10.8	Cu	12.5	Nitrate	11.3
Mg	11.5	As	10.4	Cysteine-HCl	0
B	9.0	Zn	10.0	Thiourea	6.1
F	10.1	CN	.9	Hydroxylamine	5.0
Br	10.8	β-GP	11.3	Ascorbic acid	0

[1] Each element or compound was used at the rate of 1 μmol/ml. of digest, in accordance with the standard assay. The following sources were employed: Calcium chloride, magnesium chloride, boric acid, sodium fluoride, sodium bromide, potassium iodide, cupric sulfate, sodium arsenate, zinc sulfate, potassium cyanide, sodium β-glycerophosphate, and sodium nitrate.

inhibited the enzyme at the rate of 1 μmol/ml. of digest. Inhibition was virtually complete at 10 times this concentration. Cysteine and ascorbic acid permitted no measurable activity, and cyanide, also at the rate of 1.0 μmol/ml. of digest, allowed only a trace of tyrosinase activity. Separate experiments showed the enzyme to be completely inhibited by carbon monoxide.

The inhibitory effects of thiourea, cyanide, and cysteine were anticipated from the review by Sumner and Somers (60, p. 242), although no mention was made of hydroxylamine as a tyrosinase inhibitor. Curiously, hydroxylamine did inhibit cane peroxidase (6), which prompted us to test it on tyrosinase, and Sumner and Somers do list it as an inhibitor of horseradish peroxidase (60, p. 223).

The essentiality of copper as a cofactor of cane tyrosinase was determined by the method of Kubowitz (38), who originally demonstrated that

this type of enzyme is a copper-protein. Kubowitz could not remove copper by ordinary dialysis against water, but by inactivating tyrosinase with HCN, he was then able to separate copper from the enzyme by dialysis against running water. The oxidase protein, still inactive after dialysis, could be reactivated by adding copper. Other metals were not effective in restoring activity.

Cane tyrosinase which had been treated with KCN and dialyzed for 40 hours against distilled water lost all but a small fraction of the original activity (table 5). As little as 0.2 μmol of copper per milliliter of digest sufficed to activate the dialyzed enzyme to about 67 percent of control values, and 1 μmol of copper returned better than 100 percent of the enzyme's original activity. None of the other metals or ionic substances

TABLE 5.—*Stimulating effects of copper on cane tyrosinase following inactivation with potassium cyanide*[1]

| Data classification	Data for indicated treatment of tyrosinase preparations						
	Undialyzed (control)	Dialyzed enzyme + Cu at indicated concentrations (μmol/ml. of digest)—					
		0	0.2	0.4	0.6	0.8	1.0
Specific activity	23.7	1.9	15.8	20.2	22.2	24.8	25.9

[1] Whereas control tyrosinase was prepared as usual with water, test preparations were made up in 0.025 M KCN, which totally inactivated tyrosinase. The latter were dialyzed for 40 hours at 20°C., against 5 changes of distilled water to separate cyanide and copper from the free protein. Standard assays were then run with undialyzed tyrosinase in water solution, and with dialyzed enzyme in the presence of copper additives.

tested as possible activators (table 4) served to activate the KCN-treated enzyme. The slight activity remaining after the KCN and dialysis treatments might be due to an iron-bearing enzyme such as peroxidase, or a noncopper-requiring oxidase in the tyrosinase preparation.

SIGNIFICANCE OF TYROSINASE IN SUGARCANE

Having already mentioned the controversy as to whether tyrosinase or cytochrome oxidase is the responsible catalyst of terminal oxidation, it is obvious that one or the other must play an unusually significant role in sugarcane as a key system in the pathway of sugar breakdown. At the moment we feel that tyrosinase must assume a part or all of this role. The high incidence of tyrosinase in meristem tissues could hardly be an accident, and previous efforts to detect cytochrome oxidase in cane have proven unsuccessful (*1*).

Another possible function of tyrosinase in cane is that of an indirect oxidase for ascorbic acid. Enzymes which act upon phenols, including tyrosinase and peroxidase in the presence of hydrogen peroxide, form quinones which in turn may act upon ascorbic acid. Ascorbic acid might therefore serve as a reducing agent in the following tyrosinase-induced reaction sequence:
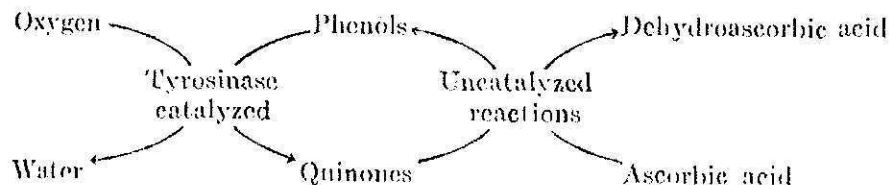
Some evidence indicates that the enzyme ascorbic acid oxidase, which oxidizes the acid directly, may catalyze electron transfer in respiration of higher plants (34,65). It is probable that a combination of tyrosinase and ascorbic acid would accomplish the same task in tissues lacking ascorbic acid oxidase.

A potentially critical function of tyrosinase might arise from the capacity of this enzyme to oxidize the tyrosyl groups of proteins, as evidenced by the work of Sizer (55). Tyrosinase did not affect the activity of the tyrosyl-bearing enzymes pepsin, trypsin, and chymotrypsin, presumably because only a small fraction of the tyrosine was oxidized. Also meriting consideration is the work of James et al. (34), which revealed a relationship between glycolysis and oxidation by the ascorbic acid system. Addition of ascorbic acid to barley preparations stimulated the loss of hexose diphosphate. Tyrosinase thus might affect sugar levels via its capacity to serve as an indirect ascorbic acid oxidase.

It is conceivable that direct use might eventually be made of information concerning tyrosinase or its associated oxidases in cane respiration. From time to time efforts have been made to regulate the sugar content of standing sugarcane, or to prevent periodic sugar losses by applying materials bearing strong capacity to alter the physiology of plants. Recent examples include the use of 2,4-dichlorophenoxyacetic acid (11,40,41), maleic hydrazide with sugarcane (42,3) and with sugar beet (54), and indole-3-acetic acid (3). Maleic hydrazide, as well as the chemicals CMU and Diquat, have been applied to foliage in order to evaluate the role of leaves in biochemical processes of cane flowering (21). Oils have been used as foliar sprays to prevent seasonal sucrose losses (50,25). The specific action of such materials is seldom known when they are applied, and usually it is hoped, without very much basis, that more desirable sugar or physiological objectives will somehow come to pass. In any event, the curtailment of respiratory mechanisms, of which the terminal oxidases are both critical and sensitive catalysts, appear as most likely areas for action of applied

chemicals. It is therefore probable that, when the properties of cane oxidases are thoroughly clarified, such objectives may be more readily achieved by use of enzyme regulators aimed specifically at catalysts such as tyrosinase.

SUMMARY

A study was made of the distribution and properties of tyrosinase (polyphenol oxidase) in sugarcane. The enzyme was extracted with phosphate buffer (pH 7) from freeze-dried tissues which had been ground to pass a 60-mesh screen. Tyrosinase was assayed spectrophotometrically at 390 mμ by measuring the optical density increase of a buffered mixture of catechol and enzyme.

Fractionation of cane extracts with ammonium sulfate showed that tyrosinase is precipitated readily from 20- to 70-percent saturation. The richest source of the enzyme was meristematic tissue. Considerable activity was also obtained from leaves —1 and 0, whereas only traces of the enzyme were present in both 8 to 10 and 1 to 3 nodes and internodes.

Tyrosinase preparations were heat-sensitive, losing most of their activity within 24 hours of extraction at room temperature (28–29°C.) and laboratory temperature (19–21°C.). In contrast to cane peroxidase, no recuperative capacity was evident after inactivation by boiling.

Substrates acted upon by tyrosinase included tyrosine, catechol, DOPA (3,4-dihydroxyphenylalanine), pyrogallol, guaicol, resorcinol, hydroquinone, and paracresol. No reaction was observed with phenol or metacresol. Optimum temperature was about 24°C., and optimum pH was 7.5. Apparent Km was 4.0×10^{-3} mols of catechol per liter. Thiourea and hydroxylamine markedly inhibited the enzyme at concentrations of 1 μmol/ml. Cysteine, ascorbic acid, and cyanide caused virtually complete inhibition at this concentration. Carbon monoxide likewise inhibited.

Tyrosinase which was inactivated by KCN was reactivated following prolonged dialysis against distilled water and addition of copper. No other metal tested (molybdenum, manganese, zinc, iron, magnesium) served to reactivate the catalyst.

Possible roles and significance of cane tyrosinase are discussed.

RESUMEN

Se hizo un estudio de la distribución y propiedades de la tirosinasa (oxidasa de polifenol) en la caña de azúcar. La enzima se extrajo con un amortiguador de fosfato (pH 7.0), de tejidos secados por congelación que se trituraron hasta poder pasarse por una criba de mallas, tamaño 60. La prueba se hizo en un espectrofotómetro a 390 mμ, midiéndose el aumento en densidad óptica de una mezcla amortiguada de catecol y enzima.

Al separarse por fraccionación los extractos de la caña con sulfato amónico, se precipitó la tirosinasa fácilmente entre una saturación de 20 a 70 por ciento. En el tejido meristemático fue que se encontró la mayor cantidad de la enzima. También se consiguió una actividad considerable en las hojas — 1 y 0, mientras que sólo se encontraron trazas de la enzima en los nudos y entrenudos del 8 al 10, y del 1 al 3.

Las preparaciones de tirosinasa se mostraron sensitivas a la acción del calor, inactivándose casi totalmente a las 24 horas después de haberse extraído a una temperatura ambiental de 28 a 29°C. y de 19 a 21°C. en el laboratorio. En contraste con lo que sucede con la peroxidasa de la caña, la tirosinasa no evidenció capacidad recuperativa después de la inactividad que le causara hervirla.

Los siguientes fueron los sustratos sobre los cuales actuó la tirosinasa: catecol, DOPA (3,4-dihidroxifenilalanina), pirogalol, guayacol, resorcinol, hidroquinona y para-cresol. No se observó reacción alguna con el fenol o con el metacresol. La temperatura óptima fue de alrededor de 24°C. y el pH óptimo 7.5. El Km aparente fue 4.0×10^{-3} moles de catecol por litro. La tiourea y la hidroxilamina inhibieron la enzima marcadamente a concentraciones de 1 μmol por mililitro de digesto. La cisteína, el ácido ascórbico y el cianuro causaron una inhibición casi completa a tal concentración. El monóxido de carbono actuó de igual manera.

La tirosinasa inactivada por KCN se reactivó tras una diálisis prolongada en presencia de agua destilada seguida por la adición de cobre. Ninguno de los otros metales que se probaron (molibdeno, manganeso, zinc, hierro, magnesio) sirvieron para reactivar el agente catalizador.

Se discute en este trabajo la posible función que pueda ejercer la tirosinasa y la importancia que pueda tener en la caña de azúcar.

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