CONTRIBUTION TO THE KNOWLEDGE OF PLANT PHENOLASES[1]

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(WITH ONE FIGURE)

Introduction

Respiratory chromogens are thought by PALLADIN and his coworkers (**16, 17**) to play an important rôle in plant respiration. Dihydroxy and trihydroxy phenols are found among these chromogens. They are oxidized to quinones by molecular oxygen in alkaline solution. In acid solution, a similar oxidation occurs in the presence of oxygen and plant phenolases. The possible rôle of phenolase in plant respiration has been questioned, because attempts to correlate activity with rate of respiration have failed; furthermore, phenolases could not be found in a large number of plants. It should, however, be born in mind that a correlation between phenolase activity and rate of respiration can be expected only if the enzyme is the limiting factor in the system. That this is not the case can be seen from the fact that the addition of phenolic compounds to a plant extract known to contain phenolase is followed by the development of colored products. In other words, the amount of enzyme present is larger than that required for the oxidation of a plant chromogen normally present and the chromogen appears to be the limiting factor. The fact that phenolases have not so far been demonstrated in a number of plants may be due to the fact that the possibility of specificity among phenolases such as is known to occur among other enzymes has been ignored. Thus the reagents used for the determination of their presence may have contained reactive groupings that are attacked by some phenolases but not by others. That such a condition prevails will be shown in this paper, in which observations are reported which appear to indicate the existence of three phenolases, specific for the ortho-, meta- and para-hydroxy grouping respectively.

Methods

The plant extracts were prepared from frozen material by extraction with 1 per cent. sodium bicarbonate solution. The extracts were immediately neutralized with glacial acetic acid and saturated with toluol. Phenolase activity was measured by observing manometrically the rate of oxygen absorption by the extract-substrate mixture in a Barcroft-Warburg respirometer. The reaction took place under oxygen at 25° C. in a

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medium buffered with an acetate mixture. None of the extracts showed a measurable oxygen absorption in the absence of a suitable poly-phenol. The details of the method have been discussed elsewhere (**19**).

Experimentation

The catalyzing power of different plant extracts was tested on various phenolic substrates at varying concentrations and pH. The extracts from fruits of apricot (var. Royal) and avocado (var. Spinx) oxidized catechol very rapidly and pyrogallol only slowly. The ratio for avocado was about 6 : 1[2]. Avocado extract oxidized phloroglucinol at an exceedingly slow rate, while apricot extract caused observable effect. Neither avocado nor apricot extracts oxidized phenol, resorcinol, quinol, or tryosine, although potato extracts oxidized tryosine at a rapid rate. The avocado and apricot extracts were used with resorcinol between 0.1 and 1 M concentration from pH 3.9 to 7.1, and with quinol at 0.15 and 0.3 M concentration between pH 3.9 and 7.1. The tyrosine was used only at pH 6.5.

An extract of lemon leaves (var. Eureka) was found to oxidize phloroglucinol very rapidly, but it oxidized resorcinol very slowly (pH 6.2, conc. 0.5 M). Catechol and pyrogallol were oxidized at a considerably slower rate than phloroglucinol (table I). No oxidation of phenol or tyrosine was obtained. The ratio of the rate of phloroglucinol oxidation to that of catechol was about 11 : 1. A rather interesting phenomenon observed was the action of catechol upon the phloroglucinol-oxidase system. Catechol inhibited the oxidation of phloroglucinol (table I).

TABLE I

EFFECT OF CATECHOL UPON THE RATE OF OXYGEN ABSORPTION BY LEMON LEAF EXTRACT IN THE PRESENCE OF PHLOROGLUCINOL AT pH 6.2 AFTER 20 MINUTES (IN MG. × 10^3)

SUBSTRATE	0.0125 M PHLOROGLUCINOL	0.0125 M PHLOROGLUCINOL 0.05 M CATECHOL	0.05 M CATECHOL
Enzyme and substrate	315	166	138
Substrate blank	0	90	110
Enzyme and substrate corrected for blank	315	76	28

After exposure to oxygen without the enzyme the phloroglucinol remained almost colorless although having a greenish-yellow tinge; the catechol had browned; but a mixture of the two became green. When the enzyme was

[2] Unless stated otherwise, this ratio refers to absorption of oxygen under optimum substrate concentration.

present the mixture of the two substrates turned greenish black. The mixture of catechol and phloroglucinol prepared in a test-tube prior to the determination remained almost colorless in absence of the buffer. When auto-oxidation was hastened by raising the pH the dark green pigment developed. The amounts of absorption by the substrate blanks (table I) seem to indicate that the presence of phloroglucinol only slightly inhibited the auto-oxidation of catechol. From this we may infer that the reactive OH groups of the catechol were not involved in the reaction causing the formation of the green pigment associated with the inhibition of enzyme action of phloroglucinol in the presence of catechol. A possible explanation of the inhibition of phloroglucinol oxidation by catechol would seem to lie in a reaction of the phloroglucinol with the oxidation product of the catechol.

The extracts from young pear leaf (var. Bartlett) very rapidly oxidized quinol. Catechol was oxidized at about one-fourth the rate of quinol (table II), and pyrogallol still more slowly. Resorcinol, phloroglucinol, phenol, and tyrosine were not oxidized to a measurable extent.

The various extracts had different properties in respect to their power of oxidizing different phenolic groupings. Apricot and avocado extracts oxidize most rapidly the ortho-hydroxy-phenol, lemon extract the meta-hydroxy-grouping, and pear the para-compounds. More than one such group was attacked by most extracts. On the assumption that more than one enzyme was present, attempts were made to separate them. The enzymes were fractionally precipitated by alcohol, filtered, dried, and redissolved in water. The relative activity of the precipitate for different substrates was tested. However, no significant differences could be obtained because of the great differences in the rate of catalysis of any one plant extract toward any two substrates. This necessitated correspondingly large dilution of the stronger one, thus increasing the error considerably. The enzymes in avocado, apricot, and lemon could be precipitated with 75 per cent. alcohol while the enzymes in the pear could even be precipitated with 45 per cent. alcohol.

It has been shown (**21**) that the catalytic power of apricot extract is destroyed by exposure for only 1 hour at 70° C. Extracts of pear leaf and lemon leaf (without buffer) were brought to boiling and then immersed in boiling water for one-half hour. Table II shows the effect produced by boiling. While the power to oxidize catechol was lost by the lemon leaf extract, it was still able to slowly oxidize phloroglucinol. The power to catalyze the oxidation of quinol had been completely retained by the pear leaf extract while it had lost most of its action on catechol. Thus in the same extract one catalytic power could be destroyed, while the other remained unharmed, which lends strong support to the theory that we are dealing with more than one enzyme.

TABLE II

EFFECT UPON PHENOLASE ACTIVITY OF HEATING FOR ONE-HALF HOUR AT 100° C.

SOURCE OF EXTRACT	SUBSTRATE	pH*	$O_2 \times 10^3$ MG. ABSORBED† AFTER 10 MINUTES	
			UNHEATED	HEATED
Lemon	0.5 M catechol	5.6	97	0
	0.0125 M phloroglucinol	6.3	2670	47
Pear	0.5 M catechol	5.6	56	8
	0.05 M quinol	6.0	236	237

* pH at which oxygen absorption was determined (buffer added after heat axposure).
† All values corrected for auto-oxidation.

These extracts were purified by precipitation and reprecipitation with three times their volume of alcohol, and their pH-activity curves (fig. 1) and Michaelis constant were determined. The activities, as influenced by pH, are plotted here on different scales in order to be able to show the curves on one graph. In each case, however, the intersection of the ordinate on the abscissa is used as the zero point. While the apricot-catechol and the lemon-phloroglucinol curves show definite optima in the acid range, no optima occur in the pH range studied for the pear-quinol and the avocado-pyrogallol systems. No measurements were taken above pH 6.2 because of the greatly increased auto-oxidation of the substrate. It has been pointed out (20) that the catechol system, when obtained from different sources, shows an optimum pH, which is presumably due to the accompanying specific colloids as it differs for different plants. Further work would probably establish a similar relative pH relationship between the various enzyme-substrate systems in different plants.

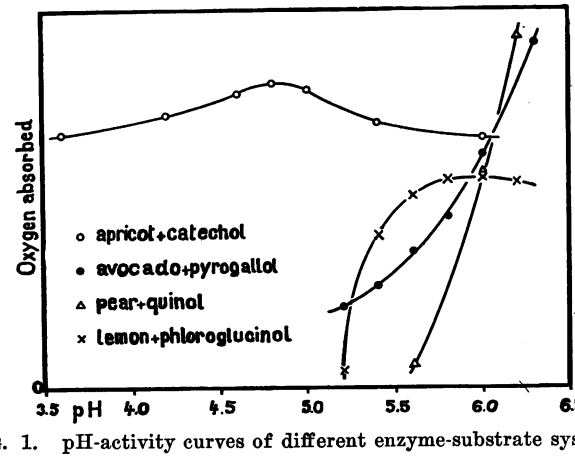

FIG. 1. pH-activity curves of different enzyme-substrate systems.

The Michaelis constant was determined by plotting substrate-concentration-activity curves and finding by this graphic method the concentration at which 50 per cent. activity was obtained. The results are shown in table IV.

TABLE IV

MICHAELIS CONSTANT OF DIFFERENT PHENOLASES

SUBSTRATE	CATECHOL	PYROGALLOL	PHLOROGLUCINOL	QUINOL
Source of extract	Avocado Apricot	Avocado	Lemon	Pear
Michaelis constant	7×10^{-2}	$2\text{–}3 \times 10^{-2}$	6×10^{-3}	$1\text{–}2 \times 10^{-2}$

These values, ranging between 6×10^{-3} and 1×10^{-2}, differ in their order of magnitude from those reported in the literature. Thus WARBURG (**24**) found oxygenase of yeast to have a K_M of 0.5×10^{-7} M oxygen, and WILLSTÄTTER and WEBER (**26**) reported a $K_M = 6 \times 10^{-6}$ M leucomalachite green for peroxidase of horse-radish. These and similar values for other oxidases caused HALDANE (**8**) to suggest that the low constant of oxidases may prove to be a fundamental distinction from hydrolytic enzymes. EULER and LAURIN (**5**) have shown that purification does not influence the Michaelis constant. The different values which they obtained for the Michaelis constant of saccharase from various races of yeast seem to be due to the presence of inactive enzymes which have retained their affinity for the substrate but do not dissociate from it. Since this would tend to produce lower values rather than higher ones, this factor does not seem to enter into the divergence which is reported here from the results of other workers.

Discussion

The observations reported in this paper tend to indicate that phenolases from different sources may be of a different nature. This expresses itself primarily in their specificity towards definite groupings. BERTRAND (**1**) compared the relative rate of oxidation of phenols in the presence of lactase and found the rate of oxidation was in the order: quinol > catechol > resorcinol. More recently BUNZEL (**2**), working with various organs of the potato plant, came to the conclusion that the order of oxidation by oxidase was: para > ortho > meta. On the other hand, he found later (**3**) that this relation may not hold true in other plants and that the relative order of ease of oxidation of different phenols may vary in different plants. In no case was the meta position found to be easily oxidized.

The writer's plant material has the advantage that in all cases tyrosinase was absent, and that it was not measurable by the test-methods employed. This enzyme, which OKUYAMA (**14**) has shown to be essentially

a dehydrogenase, may be responsible for the high rate of oxidation of the para grouping reported in some of the data. In each of the three types of material under discussion a di- or trihydroxy phenol with a different relative position of the hydroxy groups in the phenol ring was preferentially oxidized by the enzyme. We prefer to speak of relative rates rather than of absence of the oxidation of certain groupings because the oxidation may have taken place at such a slow rate that it remained unnoticed under the conditions of the experiment.

It is, however, of interest to note in connection with the predominant rôle which ONSLOW (**15**) places upon catechol compounds, that the ortho grouping was more or less oxidized by the extracts in all three cases. Furthermore the oxidation of pyrogallol was slower than that of catechol, which shows that a group in a different position than those directly attacked may influence the reaction.

These findings give rise to the following question: Do the preparations represent different enzymes, or is there fundamentally one enzyme with its activity modified by various plant colloids? BUNZEL (**3**), who did not have quite as striking a case with his material, thought that the modification in the relative rate of oxidation of several substrates by various plants was due only to differences in the colloidal substances accompanying the enzyme. He stated that this contention was supported by the fact that all organs of the same plant behaved alike, while if he had dealt with a mixture of enzymes, they would have been expected to differ in their behavior. Yet his data do not seem to bear out his conclusion inasmuch as various organs apparently show such differences.

Of the three types of extracts discussed in this paper, the pear-leaf extract can be readily singled out from the other two. Para-phenolase of pear leaves is heat stable while the accompanying ortho-phenolase is easily destroyed by heat. Indeed, the heat stability of the former would lead one to believe that it is an inorganic catalyst. Heat stable oxidases have been reported in alfalfa (**4**), tobacco (**6**), and mushroom (**25**). Furthermore a major part of this para-phenolase was precipitated by the addition of an equal volume of alcohol to the solution, while the other phenolases necessitated the use of a higher concentration of alcohol for precipitation.

The enzymes of the lemon-leaf extract could not be separated by fractional precipitation with alcohol. Indeed, the fact that catechol greatly retarded the oxidation of phloroglucinol by lemon-leaf extract may provide an explanation for the reported very weak oxidation of the meta grouping found in the case of avocado. Avocado reacts positively to the ferric chloride test used by ONSLOW (**15**) for the identification of catechol compounds. Citrus was classed by her among "peroxidase" plants for the lack of response to this test. This would seem to suggest that we may

be dealing with the same enzyme, the oxidation of the meta grouping in avocado and other "catechol plants" being inhibited by the presence of an ortho-phenol. However, it is difficult to see how such a small concentration of ortho-phenol, as naturally occurs in the plant, should completely inhibit meta oxidation as, *e.g.,* in apricot. While much higher concentrations used *in vitro* for lemon extract fail to stop it completely. The inhibition of meta oxidation by catechol cannot be explained on the basis of a greater combining power of the enzyme with catechol as compared with phloroglucinol because the Michaelis constant of the meta oxidation is only half that of the ortho reaction, showing that the meta grouping is more strongly attached to the enzyme.

Thus we found an oxidase able to oxidize ortho-phenols in apricot and avocado, plants containing tannins which gave a test for the catechol grouping. The concentrated tannin extracts of apricot (**20**) and solutions of commercial tannins were rapidly oxidized by the diluted oxidase of apricot and avocado. Lemon-leaf extract oxidized phloroglucinol preferentially, while in common with other citrus plants it is known to contain hesperidin, a glucoside of a meta-phenol. A para-phenolase was expected in pear leaves, because they are known to contain arbutin, a glucoside of quinol, and it was actually found to be present in large amounts. Apparently different specific oxidases and their proper substrates are present in different plants.

The question need be raised, as to why the oxidation of these phenolic chromogens does not take place in so many plant tissues unless they are injured, in view of the fact that both substrate and enzymes are present in the vacuolar sap (**18**). This failure of pigment formation in the living cell, assuming the amount of the enzyme or any additional oxidative agent and the pH of the cells to be constant, may be due to one or more of the following mechanisms: (1) the rate of oxygen supply, (2) the amount and availability of the substrate present, or (3) the amount of an additional reducing substance present. The work of STEWARD (**21**) seems to indicate that oxidation and respiration of plant cells at cut surfaces may be limited by oxygen supply and it would seem that the influx of oxygen may play a major rôle in the darkening of cells beneath the cut surfaces of solid tissues. The fact that the substrate may be the limiting factor has been illustrated recently by KERTESZ (**11**). He described the oxidase system of the "Sunbeam" peach, and he believed that the enzyme is apparently not different from that of other stone fruits, but that tannin is almost absent. This variety of peach will not discolor upon injury. In addition to a difference in quantity of the substrate there may also be a modification of its availability. Thus LLOYD (**12**) reports that for various plants tannin may be held at different stages of growth within a gel of cellulose-like substance which may prevent its color reaction with iron salts. A complex

formation of hesperidin, in which the glucoside is surrounded by sugar molecules, has been mentioned by HALL (**10**). These colloidal formations may very well form systems which modify the rate of oxidation in the plant cell by limiting the available free substrate. The presence and activity of hydrolytic enzymes forms a very important mechanism to make the active grouping available for the oxidative system if it is bound, *e.g.*, as glucoside.

A third factor to be considered is the presence of a reducing substance providing for the reversibility of the system. SZENT-GYÖRGYI (**22**) showed that in the potato ascorbic acid could play this rôle. The writer observed the effect of ascorbic acid upon the systems discussed in this paper and found that with all substrates formation of a visible pigment was prevented in the presence of ascorbic acid, while the uptake of oxygen continued. The oxidation of ascorbic acid could be followed in all cases by means of iodine titration. Furthermore, if the pigment had been produced by oxidation of the phenol in alkaline solution, it could be reduced to the leucoform by ascorbic acid. Further evidence of this possible rôle of ascorbic acid in pigment formation is furnished by MORGAN, FIELD, and NICHOLS (**13**), who found that apricots, which had darkened, had lost vitamin C, and by JOSLYN, MARSH, and MORGAN (**10**) who showed that darkening of orange juice would take place only after the amount of ascorbic acid had sunk to a low level. Reducing substances, other than vitamin C, may possibly prevent the formation of the respiratory pigments in plant cells. An enzymatic oxidation of the products obtained by alkaline hydrolysis of sugar has been reported by GUTHRIE (**7**). It would seem probable that the unstable forms of sugar, as they occur in the plant, are able to reduce the quinones, which are formed by the action of oxidases on phenols, or that the oxidized ascorbic acid may, in turn, oxidize other reducing substances as suggested by SZENT-GYÖRGYI (**23**). Such a system may form an important part of the respiratory system in the plant.

Summary

1. The catalytic action of a number of plant extracts upon the oxidation of phenols was tested in a Barcroft-Warburg respirometer.

2. All extracts catalyzed the oxidation of more than one phenol, but preferential oxidation was shown by the various extracts for different phenols.

3. The ortho-phenolase from the fruits of avocado and apricot, which oxidized catechol rapidly and pyrogallol less rapidly, was inactivated by heating at relatively low temperatures. Meta-phenolase from lemon leaves, which oxidized phloroglucinol rapidly and resorcinol exceedingly slowly, was somewhat more heat resistant. Para-phenolase of pear leaf oxidized quinol and was heat stable.

4. The pH-activity curves and the Michaelis constants for these enzyme systems are reported.

5. Attention is called to the fact that glucosides, containing the phenols corresponding to the respective phenolases, are found in the plants examined.

6. The oxidized forms of these phenols could be reduced in each case by means of ascorbic acid, thus showing that the systems are reversible under natural conditions.

The writer wishes to express his indebtedness to Dr. J. P. BENNETT for the valuable aid and suggestive criticism offered throughout this work, to Dr. W. V. CRUESS for his help and the permission to use the facilities of his division, and to Dr. D. M. GREENBERG for valuable suggestions.

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