CXLIV. OXIDISING ENZYMES.

IX. ON THE MECHANISM OF PLANT OXIDASES.

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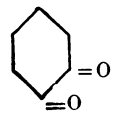
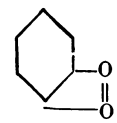
On a basis of the original conception of Bach and Chodat, the authors have considered the mechanism of the oxidase of the higher plants to be as follows: first, the formation of an organic peroxide from the dihydroxy-group characteristic of catechol, this reaction being catalysed by an oxygenase; second, the decomposition of this peroxide (or of hydrogen peroxide derived from it by the action of water) with the production of active oxygen, this reaction being catalysed by a peroxidase. The active oxygen so formed will oxidise guaiacum and other acceptors. The actual form of peroxide derived from the dihydroxy-group has not been postulated.

Recently Szent-Györgyi [1925] has made the enlightening suggestion, supported, to some extent, by experimental evidence, that an orthoquinone is the product of the action of the oxygenase upon catechol. This orthoquinone, moreover, will blue guaiacum in the absence of peroxidase. Hence, Szent-Györgyi has concluded that peroxidase is not a component of the type of oxidase under consideration. Orthoquinones, of two types:

(i)   (ii)

have been prepared by the oxidation of catechol with silver oxide by Willstätter and Müller [1908]. Owing to its far greater stability, Szent-Györgyi has suggested type (i) as the probable product formed by the oxygenase. Such a product prepared artificially will, he has shown, blue guaiacum.

In addition to the above modification of the conception of the oxidase mechanism, Szent-Györgyi is also of the opinion that peroxidases are merely attenuated oxidases and do not exist as specific enzymes. The absence of oxidases from, and the presence of authenticated peroxidases in, a certain percentage of the higher plants is due, in his opinion, to the absence of "catechol" compounds, the place of which can be taken by hydrogen peroxide. With this point of view we do not agree. Not only do the non-oxidase plants

contain no catechol compounds, but they contain no oxygenase which is capable of acting upon the catechol group, even when supplied.

The experimental evidence, offered by Szent-Györgyi in support of the view that the blueing of guaiacum in the oxidase reaction is due solely to orthoquinone, is as follows. An enzyme preparation, from the potato tuber, was allowed to act upon catechol in potassium dihydrogen phosphate solution. After a certain interval, excess of methyl alcohol was added for the precipitation of enzymes. The oxidised product (presumed to be orthoquinone), however, remained in solution, and, after the careful removal of alcohol by evaporation at low temperature and reduced pressure, caused the blueing of guaiacum. The above is no evidence, of course, for the formation of an orthoquinone, but demonstrates only that an oxidation product has been produced which will blue guaiacum in the absence of any enzyme.

Szent-Györgyi has stated that two factors have a considerable influence on the progress of the reaction, the concentration of catechol and the hydrogen ion concentration. From experimental evidence he has shown the optimum concentration of catechol to be about 0·005 %. The intensity of the guaiacum reaction decreased with higher concentrations of catechol. This, he has suggested, may be due to a reaction between the quinone and the unchanged catechol to form less active compounds.

With regard to hydrogen ion concentration, he found that at p_H 7·4 the oxidation of catechol was relatively rapid, but the guaiacum blue tended to fade. With increasing acidity, the oxidation of catechol takes place more slowly, but the blue colour is more stable. This, he has suggested, may be explained on the grounds that the above-mentioned combination of oxidised and unaltered catechol takes place more readily with increasing alkalinity. For a 10-minutes' period of reaction between enzyme and catechol, the optimum p_H appeared to be 6·4, which he also found to be that of potato juice.

The authors have repeated, exactly, as far as they can judge, the experimental procedure of Szent-Györgyi. In addition, a control experiment was performed using enzyme without catechol. In the latter, after removal of enzyme and alcohol, a test for peroxidase was carried out with hydrogen peroxide and guaiacum. Whereas the actual experiments were positive, it was found that this so-called control, as often as not, gave a peroxidase reaction; that is, the conditions of the experiment did not necessarily ensure complete precipitation of the peroxidase. Hence it formed no control, nor can one be sure, when a blue colour was given in the actual experiment with catechol, that it was not due to traces of peroxidase unprecipitated by alcohol.

In the above experiments a 0·2 *M* solution of potassium dihydrogen phosphate (p_H 4·6) was always employed. Subsequently, the concentration of alcohol was increased four times without necessarily giving a negative result in the so-called control.

Hence, by means of Szent-Györgyi's technique as employed by us, we were unable to ensure the absence of peroxidase.

A series of experiments was next performed identical with the above, except that distilled water was employed instead of phosphate solution. The results were, with one doubtful exception, negative; namely, no blueing of guaiacum was obtained. The control experiments, after addition of hydrogen peroxide, were almost always positive. From the above results, the conclusion may be drawn that, at approximately p_H 7, the "orthoquinone" is not stable, but is removed by condensation or otherwise. The positive results with the control may possibly be explained by the fact that in water the enzymes were less completely precipitated by alcohol than in phosphate solution.

A modification of the experiment was made by adding an extract of horseradish peroxidase to a solution of the "orthoquinone" after removal of alcohol. On subsequent addition of guaiacum, no intensification of blueing was observed, as compared with a control without peroxidase. From this crude experiment, it does not appear that peroxidase catalyses the oxidation of guaiacum by the "orthoquinone."

On the assumption that the alcohol technique may not be valid, we tried to solve the problem by substituting other enzyme precipitants in Szent-Györgyi's method. Those selected were colloidal ferric hydroxide, barium chloride, tannic acid, mercuric chloride and Esbach's reagent (1 g. picric acid and 1 g. citric acid per 100 cc. of water).

Colloidal ferric hydroxide. Three times the quantities given by Szent-Györgyi were used, namely, 90 mg. of enzyme, 3 cc. of phosphate and 3 drops of 0·1 % catechol solution. These were incubated at 37°, 1·5 cc. of colloidal ferric hydroxide was added, the solution filtered from the precipitate, and tested with guaiacum. The results were always positive, the controls without catechol negative. Though, naturally, the use of colloidal ferric hydroxide is open to criticism in connection with guaiacum, not only did the ferric hydroxide precipitates fail to give any positive reaction with this reagent, but control experiments with catechol and phosphate, incubated and subsequently precipitated with colloidal ferric hydroxide, were also negative.

Barium chloride and mercuric chloride. The precipitation of the enzyme was not complete. Of other mercury salts, mercuric nitrate itself was found to blue guaiacum.

Tannic acid. This reagent completely inhibited the blueing of guaiacum by the "orthoquinone."

Esbach's reagent. This was successful as in the case of colloidal ferric hydroxide. For 90 mg. of enzyme, etc., 0·5 cc. of the reagent was used. In spite of the colour of the picric acid, the blueing of guaiacum could be distinctly seen.

Colloidal ferric hydroxide was also employed with an aqueous catechol solution without phosphate. It was, however, unsuccessful. If sufficient iron were used to ensure complete precipitation of the enzyme, the ferric hydroxide itself was not precipitated. The use of Esbach's reagent with aqueous catechol

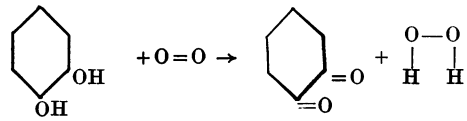
was successful in removing the enzyme, and the results were negative as determined by the guaiacum reaction.

From the above, we conclude that, within a certain range of hydrogen ion concentration, an oxidation product (possibly orthoquinone) is formed from catechol as a result of catalysis by oxygenase, and this product is capable of blueing guaiacum in the absence of any enzyme.

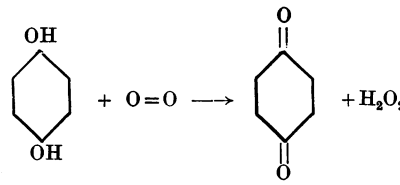
In a previous communication [Onslow and Robinson, 1925], the authors have shown that when a potato enzyme preparation is allowed to act on *p*-cresol, a dihydroxy-compound, presumably 3·4-dihydroxytoluene, is first formed, and is subsequently oxidised by the oxygenase. This statement is also supported by the observations of Uys-Smith [1926], and if correct, it should be possible to detect the formation of an "orthoquinone," as it is in the case of catechol.

Experiments were therefore made using *p*-cresol, instead of catechol, in phosphate solution with colloidal ferric hydroxide. A positive reaction was obtained with guaiacum, showing that an analogous oxidation product has been formed from *p*-cresol.

A further aspect of the problem will now be considered. If the view be accepted that orthoquinone is the probable oxidation product of catechol in the oxidase system, then a simple expression of the reaction would be as follows:



In connection with this question of the nature of the products formed on oxidation of catechol, some recent work of Wieland and Fischer [1926] is of importance. In these researches, the applicability of Wieland's conception of oxidation, by means of hydrogen activation and transport to molecular oxygen, has been tested in the case of oxidation of various phenols by a Basidiomycete catalyst, namely that of *Lactarius vellereus*. From this fungus, preparations were made which contained a system (whether equivalent to an oxygenase we cannot say) which was eventually obtained almost entirely free from peroxidase and catalase by fractional precipitation. The action of such a system on quinol was accompanied by a production of hydrogen peroxide in amount approaching the theoretical value required for the reaction:



Hydrogen peroxide was also obtained in considerable quantity from the action of the system upon catechol, pyrogallol and guaiacol. Wieland and

Fischer were, however, unable to obtain dehydrogenation anaerobically when molecular oxygen was replaced by methylene blue and various other hydrogen acceptors.

To determine whether, in the higher plants, hydrogen peroxide is formed simultaneously with the quinone is a difficult problem. If we assume the above equation to be a correct representation, then in a mixture of an enzyme (potato) preparation and an aqueous solution of catechol, the following are present: oxygenase, peroxidase, catalase (probably) and catechol: orthoquinone and hydrogen peroxide are produced. The former tends to disappear, we may suppose, by condensation. It would seem, moreover, that under these conditions, if any hydrogen peroxide were formed, it would tend to be decomposed either by (*a*) peroxidase giving active oxygen which can be accepted by unchanged catechol, or (*b*) catalase, giving molecular oxygen.

On addition of guaiacum to such a system, it would be oxidised by the orthoquinone. It might also be oxidised by the hydrogen-peroxide-peroxidase system, though we do not know how the hydrogen peroxide may be distributed in the reactions in which catalase, peroxidase, guaiacum and catechol are involved.

Nevertheless, an attempt was made to see if it were possible to detect the presence of hydrogen peroxide from the oxidation of catechol. When an aqueous solution of catechol is left in air, it slowly oxidises and becomes brown. Such an autoxidised solution will not blue guaiacum alone, but will do so on addition of peroxidase (from horse-radish, etc.). Hence we must conclude that a peroxide is present. Titanium sulphate appears to be the only reagent which discriminates between organic peroxides and hydrogen peroxide. We therefore proceeded as follows: a 5 % aqueous solution of catechol was kept at 37° for 12 hours. Since we found that catechol itself gives a yellow colour with titanium sulphate, the autoxidised solution was extracted with specially purified ether until all catechol was removed (as detected by the ferric chloride reaction). After removal of the catechol, titanium sulphate solution was added. A definite yellow colour appeared. As a control we tested a freshly prepared catechol solution which had been extracted with the same sample of ether. This form of control we found especially necessary, since the washings from ordinary laboratory ether were found to give very strong reactions with titanium sulphate owing to the presence of peroxide. Hence, for the above extraction of catechol, we employed purified ether which had been washed with water and distilled from concentrated sulphuric acid and afterwards from sodium. As an additional confirmation of the above test with titanium and the autoxidised catechol, it was repeated with the following modification: the solution, after ether extraction, was divided into two portions, and to one-half we added liver catalase which had been freed from peroxidase by filtration through a Chamberland candle. On the addition of titanium sulphate no yellow colour appeared in the half to which catalase had been added, though the control gave a positive reaction. From the above experiments

we assume that, in the autoxidation of catechol, hydrogen peroxide is most probably formed.

The detection of hydrogen peroxide when the oxidation is catalysed by an oxygenase was also attempted. The potato enzyme was allowed to act on a 2·5 % solution of catechol in distilled water at 37° for about 30 minutes. The mixture was then extracted with peroxide-free ether until free from catechol. A distinctly positive reaction was given with titanium sulphate; this reaction, moreover, did not appear after the addition of catalase. As far as these qualitative tests are valid, we draw the conclusion that hydrogen peroxide is probably formed when the oxidation of catechol is catalysed by the oxygenase. It is apparent, however, that the difficulty of demonstrating the presence or absence of hydrogen peroxide in the higher plants may be largely due to the fact that a separation of oxygenase from peroxidase and catalase has not yet been achieved.

The facts so far described lead to the possibility of considering the oxygenase of the higher plants as a dehydrogenase. In this case it is of some importance to find out whether catechol can be oxidised anaerobically, molecular oxygen being replaced by other hydrogen acceptors.

Attempts to oxidise catechol anaerobically in the presence of oxygenase, using methylene blue as a hydrogen acceptor, have so far been unsuccessful. In view of this result, advantage was taken of a new series of indicators introduced by Mansfield Clark [Needham and Needham, 1926]. We are indebted to Dr J. Needham for samples of these, and to Dr M. Dixon for assistance in making up the necessary solutions.

The indicators used were *o*-cresol-2·6-dichloroindophenol, *o*-cresolindophenol, 2·6-dibromophenolindophenol and *o*-chlorophenolindophenol. All are reduced more readily than methylene blue and are arranged above in order of increasing reducibility.

The experiments were conducted in Thunberg tubes carefully evacuated and tested during the course of the experiments, the tubes being kept in a bath at 37°.

The enzyme extracts were made by a method, previously described [Onslow, 1920], of extracting the residue (prepared by treating the tissues with alcohol) with water and filtering; or, in some cases, by precipitation of the water extract with alcohol and again taking up in water. For control experiments, the enzyme solution was well boiled, filtered and well cooled in air. Phosphate buffers were employed ranging from p_H 5·8–7·0. The concentration of catechol solution was 0·1 %.

Definite amounts of buffer, enzyme extract and indicator were introduced into the Thunberg tube. The catechol solution was always measured into a very small test-tube placed within the Thunberg tube. By this means any oxidation, before complete evacuation, was avoided. After evacuation, the liquids were mixed by inverting the tube. A control, using boiled enzyme, was always set up, and, in some cases, controls of enzyme and indicator alone,

and catechol and indicator alone. The time for complete decoloration was noted.

The above indicators were reduced by catechol alone, given a sufficient period of time. The object of the research was to ascertain whether, in plants containing the oxygenase-catechol system, the enzyme catalysed the reduction of these indicators. If so, the inference may be drawn that catechol is simultaneously oxidised as a result of hydrogen transference.

A number of oxidase and non-oxidase plants were investigated as follows:

	Time in minutes for decoloration		
	Unboiled	Boiled	Control
Oxidase plants			
1. Potato tuber	9	24	180
2. Artichoke tuber	25	45	—
3. „ leaf	10	30	195
4. Banana fruit	Boiled and unboiled almost simultaneously in 40 minutes		180
5. Lilac leaf	Both in 87 minutes		—
6. Apple fruit	Unboiled, slightly paler in 120 minutes		—
Peroxidase plants			
7. Horse-radish root ...	60	30	—
8. „ ...	Both in 30 minutes		—
9. Chickweed (*Stellaria*) plant	Both simultaneously		—
10. Cucumber fruit	Both in 230 minutes		—
11. „	100	130	—
12. Turnip root	70	90	—
13. „	70	100	—
14. „	55	100	—
15. „	60	80	—

In the case of the potato tuber, artichoke tuber and turnip root, both crude extract and solution of precipitated enzyme were used.

It should be noted that when several experiments were performed, different enzyme preparations were used. Owing to the impossibility of obtaining comparable strengths of enzyme when using crude extracts, concordance in the times of decoloration for separate experiments cannot be expected. When enzyme preparations were made from leaves, difficulty was experienced in extracting the enzymes from the tissue residue, especially when tannins are present, as is usually the case. These appear to render the enzymes precipitated *in situ* much less soluble.

In addition to the above, preparations were made from leaves of dead-nettle (*Lamium*) and goutweed (*Aegopodium*), both oxidase plants, and from iris rhizome and dried peas, both tissues of non-oxidase plants. In these cases the enzyme extracts reduced the indicators rapidly in air.

From the above data, we conclude there is very little, if any, evidence for an enzymic reduction of the indicators, the decrease in rate in the case of the boiled enzyme being possibly due to removal of proteins[1]. Since the results of using enzymes from both oxidase and peroxidase plants are, on the whole, similar, there is no evidence from these experiments for the point of view

[1] Pure pseudoglobulin, kindly prepared for us by Mr G. S. Adair, decolorised the indicator in 2 hours, either in the presence or absence of catechol, whereas boiled controls did not.

that the oxygenase, acting as a dehydrogenase, can bring about an anaerobic oxidation of catechol.

SUMMARY.

1. We have confirmed the observations of Szent-Györgyi, that an oxidation product, formed by the action of the oxygenase of the potato tuber on catechol, is capable of blueing guaiacum in the absence of peroxidase.

2. The procedure adopted by Szent-Györgyi for removal of peroxidase by precipitation with methyl alcohol was not successful when applied by us. When colloidal ferric hydroxide or picric acid was substituted for alcohol, the removal of peroxidase appeared to be complete.

3. The suggestion offered by Szent-Györgyi, that the above-mentioned oxidation product may be orthoquinone, is favourable to the hypothesis of oxidation of catechol, by dehydrogenation, with the formation of hydrogen peroxide with molecular oxygen. The oxygenase in this case would be a dehydrogenase.

4. As far as could be detected qualitatively with titanium sulphate, hydrogen peroxide appears to be formed during the autoxidation of catechol: it is probably also produced when the oxidation is catalysed by oxygenase.

5. Experiments were carried out to ascertain whether the oxygenase, assuming it to be a dehydrogenase, is capable of oxidising catechol anaerobically in the presence of a hydrogen acceptor. Methylene blue and the other more readily reducible indicators of Mansfield Clark were employed. No evidence was obtained from these experiments that oxygenase can act in this capacity.

In conclusion we are much indebted to Sir F. G. Hopkins for many suggestions and criticisms.

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