CLXVII. OXIDISING ENZYMES.

X. THE RELATIONSHIP OF OXYGENASE TO TYROSINASE.

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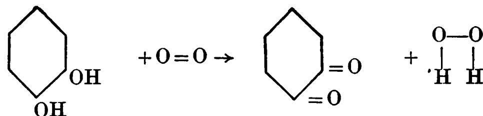
In a recent communication [Pugh and Raper, 1927], the following statement has been made: "tyrosinase has all the properties ascribed by Onslow to oxygenase and the system she describes is really that of tyrosinase with a catechol derivative....There is no evidence, therefore, that this is a new enzyme, and it seems unnecessary to retain the term oxygenase in the sense used by Onslow." The present communication records some observations made in connection with the above statement.

It is known that a preparation from the potato and many other Higher Plants giving the "direct oxidase" reaction made according to Onslow [1920], that is, by thorough extraction of the tissues with cold alcohol without undue oxidation, and subsequent extraction of the enzymes with water from the residue, will rapidly convert catechol and other *o*-dihydroxy-substances into *o*-quinones [Szent-Györgyi, 1925; Pugh and Raper,[1] 1927] and the mono-phenols, phenol, *p*-cresol, *m*-cresol and tyrosol, and *p*-hydroxybenzoic acid [Onslow and Robinson, 1925; Uys-Smith, 1926; Pugh and Raper,[1] 1927] and tyrosine [Onslow, 1923; Raper,[2] 1926] into the corresponding *o*-dihydroxy-compounds, with the subsequent formation of *o*-quinones. Such a preparation also contains peroxidase.

It is known [Onslow, 1920] that if an aqueous solution of catechol is exposed to air, it autoxidises with the formation of a peroxide, since it reacts with peroxidase and guaiacum. As far as qualitative tests are valid, the peroxide appears to be hydrogen peroxide on account of the reaction given with titanium sulphate [Onslow and Robinson, 1926; Platt and Wormall, 1927]. It is possible that *o*-quinone and hydrogen peroxide are formed according to the equation below:



[1] The *o*-quinone was identified by Raper using tyrosinase from the meal-worm, and catechol, phenol, *p*-cresol and *m*-cresol as substrates.

[2] 3 : 4-Dihydroxyphenylalanine was also identified by Raper in this reaction, again using tyrosinase from the meal-worm.

The *o*-quinone here, however, cannot be detected by guaiacum. It has been suggested [Szent-Györgyi, 1925] that it may condense under certain conditions with unchanged catechol.

Such a reaction now appears to represent that carried out by the enzyme, oxygenase, of the Higher Plants, the *o*-quinone having recently been isolated by Pugh and Raper (as stated above), who have, moreover, shown that the same product is formed from catechol by the action of peroxidase (horse-radish) and hydrogen peroxide.

Since such oxygenase plants as have been examined also contain a catechol derivative [Onslow, 1921], it is reasonable to assume that the above is the reaction which takes place, with simultaneous browning, on injury to the cell.

Now, the preparation as described by Onslow contains oxygenase and peroxidase, both of which can carry out the reaction denoted above. It is obvious, moreover, that such a preparation may always contain traces of *o*-quinone, though not sufficient to blue guaiacum, and possibly of catechol. The oxidation of catechol in the presence of such a preparation is therefore due to both oxygenase and to peroxidase and hydrogen peroxide; in addition, the oxygenase itself might be regarded essentially as the *o*-quinone, perhaps stabilised by association with a colloidal surface. The question also naturally arises as to whether the oxidation of the monophenols is brought about by the same enzyme as that which catalyses the oxidation of *o*-dihydroxy-compounds or by another catalyst; or whether it is a secondary oxidation in which *o*-quinone, formed from the diphenols originally present, takes part. Bearing in mind, therefore, that the *o*-quinone may be an intrinsic part of the oxidising system, we have tried experimentally to obtain some evidence on this point.

On this assumption, a plant tissue which has been allowed to oxidise should yield a more active enzyme preparation than one which has not received such treatment, because, in the former, more *o*-quinone will presumably be present. We therefore took two small portions (about 20 g. each) from the same potato. One portion was coarsely pounded in a mortar and allowed to oxidise for about 10 minutes; it was then thoroughly ground and extracted five times with alcohol and air-dried. The other portion was very rapidly sliced under alcohol with maximum care to avoid contact with air, and immediately ground and extracted with alcohol five times and air-dried. Equal portions of both preparations were then treated with equal volumes of freshly-prepared 0·1 % aqueous catechol, 0·1 % *p*-cresol in phosphate buffer (p_H 7·3), and a saturated solution of tyrosine in the same buffer. The oxidised preparation acted more rapidly on all three substances, the differences in rate being small but definite for catechol, and greater for *p*-cresol and tyrosine. We have so far been unable to maintain this difference on reprecipitation of the two enzyme preparations from aqueous solution by alcohol.

In addition, we have tried the effect of treatment of the enzyme preparation with charcoal, with the object of removing the traces of *o*-quinone present. About 75 g. of potatoes were pounded, extracted four times with alcohol and

air-dried. The residue was then extracted with 50 cc. of water, and filtered, about 40 cc. of extract being obtained. Preliminary tests with freshly prepared aqueous catechol, and with *p*-cresol and tyrosine, both in buffer solution, were made; the solution was then shaken up five times with Merck's charcoal (0·1 g. per 5 cc.), portions being tested between each adsorption. The results obtained are set forth in the following table.

Solutions tested with 0·5 cc. enzyme extract		Original extract	Extract after 1st adsorption with charcoal	After 2nd adsorption	After 3rd adsorption	After 4th adsorption	After 5th adsorption
0·1 % catechol	1 cc.	Instantaneous	Instantaneous	Instantaneous	Instantaneous	1 min.	3 min.
0·1 % *p*-cresol Water	1 cc. 1 cc.	3 min.	42 min.	55 min.	85 min.	Negative after 2 days	Negative after 2 days
0·1 % *p*-cresol 0·0005 % catechol	1 cc. 1 cc.	—	4 min.	5 min.	8 min.	23 min.	>1 hour colour fainter
Sat. solution tyrosine Water	1 cc. 1 cc.	50 min.	Negative after 2 days	Negative	Negative	Negative	Negative
Sat. solution tyrosine 0·0005 % catechol	1 cc. 1 cc.	35 min.	6 min.	10 min.	15 min.	>1 hour	Negative

The times recorded refer in each case to the first appearance of a definite colour.

It will be seen from the table that the onset of oxidation of catechol was not obviously retarded until after the fourth adsorption, although the intensity of the reaction, as indicated by the colour, gradually decreased. The oxidation of *p*-cresol, on the other hand, was diminished very considerably after the first adsorption and eventually ceased, whereas it was restored to practically the original value by the addition of about 0·005 mg. of catechol. The extract was unable to bring about the oxidation of tyrosine after the first adsorption; this capacity was restored to a small extent by the addition of traces of catechol; unlike, however, the case of *p*-cresol, oxidation does not proceed normally, a very small amount of the tyrosine present being oxidised.

From the observations recorded above, we conclude that the activity of the colloidal constituent is at first almost unimpaired by charcoal, as shown by the continued rapid reaction with catechol. On the other hand, the oxidation of *p*-cresol is greatly retarded by the first adsorption with charcoal, and gradually ceases with successive treatments; it is, moreover, restored to almost its initial strength by the addition of a trace of catechol. It may be assumed, therefore, that it is a secondary oxidation depending on the presence of *o*-quinone, which has been largely removed by the first, and probably completely so, by the fifth treatment. It seems as if the addition of a trace of catechol may result in the production of *o*-quinone, which initiates the autocatalysis following on the induced oxidation with production of homocatechol from *p*-cresol.

In the case of tyrosine, induced oxidation seems to take place to a certain

extent, after adsorption, on the addition of traces of catechol, as the solutions turn pale pink and darken slightly; but, as the reaction never increases in intensity, autocatalysis apparently does not proceed far under these conditions. It is known from the work of Raper [1927], that further oxidations take place in the formation of melanin from tyrosine, and the reaction is obviously more complicated than in the case of *p*-cresol.

Peroxidase has always been detected in our enzyme extracts after the last adsorption. The presence of peroxidase has a certain significance in the experiment, for, as stated earlier in the paper, a solution of catechol slowly autoxidises in air, producing, in all probability, hydrogen peroxide. In the presence of the latter and peroxidase, *o*-quinone is formed from catechol. The difference in effect between a freshly prepared catechol solution and one which has stood for 24 hours or more could be demonstrated, not only with the original enzyme extract, but also after each successive adsorption, the colour appearing slightly earlier with the autoxidised catechol.

It appears to us to be certain that there is an enzyme, present in plants giving the "direct oxidase reaction," which catalyses the autoxidation of *o*-dihydroxy-compounds with the probable production of hydrogen peroxide and *o*-quinone; the plants which give the "peroxidase reaction" only are incapable of originating such a catalysis. We see no reason for discarding the term oxygenase for this enzyme, or for substituting the term tyrosinase for an enzyme which primarily catalyses the oxidation of substances with the *o*-dihydroxy-grouping. From the evidence given in this paper, it seems highly probable that the *o*-quinones produced from such substances will bring about, as a secondary oxidation, the formation of dihydroxy-compounds from monophenols, such as *p*-cresol. To a slight extent the *o*-quinone can initiate the formation of melanin from tyrosine, but the fact that the oxidation is not greater in extent may indicate that some additional factor is concerned in the normal reaction. It seems, nevertheless, that the term, tyrosinase, may, when the conditions are definitely realised, ultimately come to be interpreted as oxygenase plus an *o*-dihydroxy-compound or its derived quinone. If, in addition, it should be proved that the oxygenase itself is really an *o*-quinone in conjunction with a colloidal surface, then the original conception of Bach and Chodat [Chodat, 1910] of the oxygenase as "an enzyme-like peroxide" would be vindicated, though re-expressed in more modern terminology. Throughout the present paper, our remarks only apply to enzymes of the Higher Plants. Further, we have been unable to demonstrate a tyrosinase reaction in any plant which did not also contain the catechol-oxygenase system. In regard to the polyphenolase and laccase oxidising systems of the Higher Plants, our view is that these terms are synonymous with the oxygenase-catechol system. It is true that certain laccases of the Higher Plants [Bertrand, 1896] have been stated to have no action on tyrosine. We also have found a few cases of oxygenase-catechol plants not acting on tyrosine; but, as the conditions of the reaction are not completely understood, they cannot yet be

established as definite exceptions. We have retained the name, oxygenase, on account of the classical terminology of Bach and Chodat. Until it should be known definitely that oxygen is not activated in the reaction, we see no reason for substituting another term.

Concerning the classification of aerobic oxidases proposed by Pugh and Raper [1927], we would like to point out, in connection with system (1), namely peroxidase plus an autoxidisable substance [Gallagher, 1923], that a criticism of the interpretation of this author's observations has been published by one of us [Robinson, 1924]. The work of Euler and Bolin [1908], which concerns the proposed system (3), has been criticised by Bunzel [1915]. System (4), tyrosinase, we prefer to interpret on the lines indicated above, where we have expressed our views as to the connection of this enzyme with the catechol-oxygenase, the polyphenolase and the laccase systems.

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