LXXII. A COMPARISON OF CERTAIN OXIDISING ENZYMES OF THE HIGHER AND LOWER PLANTS.

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PEROXIDASE, the enzyme which induces the oxidation of guaiacum and other oxidisable substances by hydrogen peroxide, is known to be present in nearly all living plant cells. Many plants, moreover, are able to cause the blueing of guaiacum without the addition of hydrogen peroxide; these have been considered to contain a direct oxidase, phenolase or laccase. Bach and Chodat consider such an enzyme system to be composed of oxygenase in addition to peroxidase, oxygenase being described as an enzyme-like substance, which can unite with the oxygen of the air to form a peroxide, the system being analogous to that formed by hydrogen peroxide and peroxidase [Chodat, 1910]. The two components, oxygenase and peroxidase, have been separated to a certain extent by fractional precipitation with alcohol [Bach and Chodat, 1903], by dialysis, and by ultra filtration [Bach, 1916]. Most of the work of Bach and Chodat on oxidases has been carried out on the basidiomycetes, but oxygenase has been mentioned in connection with various phanerogams, *e.g.* *Rhus vernicifera*, the potato, the apple [Chodat, 1910], wheat and the sunflower [Bach and Oparin, 1923]. Onslow, M. W. [1920] has postulated that, for the higher plants, three components are present in what has been termed an oxidase or laccase, namely: an aromatic substance with two hydroxyl groups in the *ortho*-position, or "catechol" substance, from which a peroxide can be formed, and two enzymes; an oxygenase which catalyses the formation of peroxide from the "catechol" substance, and a peroxidase, which decomposes the peroxide with the production of active oxygen. The catechol substance is soluble in alcohol, therefore when an enzyme preparation is made by the usual method of alcoholic precipitation, it does not cause the direct blueing of guaiacum unless a substance containing the *ortho*-dihydroxy grouping (catechol, protocatechuic acid or aldehyde, caffeic acid, etc.) is added. The action of the oxygenase is apparent in the rapid darkening of the *ortho*-dihydroxy substance. It is, moreover, postulated that the discoloration on injury of plants containing a direct oxidase system is due to the action of the oxygenase on the catechol compound.

An enzyme solution prepared as described by Onslow [1920] oxidises *p*-cresol and causes quinol to darken, the products of the latter reaction, however, do not appear to blue guaiacum. Resorcinol and phloroglucinol are unattacked. Gallagher [1923] has denied that peroxide formation in the plant is due to enzyme action, and has stated that the so-called oxygenase, in the case of the potato, is in reality an autoxidisable lecithin-like substance which, on extraction from the plant, shows peroxide-forming properties. The ferric chloride reaction (catechol reaction) in the case of the mangold is affirmed to be caused by the presence of tannin, while the darkening of aqueous extracts of such plants as the potato and mangold is attributed to the action of tyrosinase on tyrosine, and not to the oxidation of a catechol derivative under the influence of oxygenase.

The experiments described by Onslow [1920] were repeated in the cases of the potato, apple, pear and banana, for plants showing the direct oxidase reaction, and the horse-radish and water melon, as peroxidase plants, the results described by the above investigator being corroborated. In addition to these, the artichoke and the groundsel, as oxidase plants, and the turnip, as a plant giving only the peroxidase reaction, were examined. In all cases where a direct oxidase reaction was present evidence was obtained for the existence of the two enzymes, oxygenase and peroxidase, and the presence of the "catechol" substance was indicated by the characteristic action of *o*-dihydroxy aromatic substances, namely a green reaction with ferric chloride in neutral solution, followed by a purple or red coloration on addition of sodium carbonate; moreover, the complete oxidase system could be synthesised by adding an extract of the aromatic constituents of the plant [Onslow, 1920] to an enzyme extract, prepared by alcohol precipitation, containing oxygenase and peroxidase, but no catechol substrate. In the case of the peroxidase plants there was no evidence for the presence of oxygenase, *i.e.* an enzyme extract did not catalyse the darkening of catechol derivatives with ensuing direct blueing of guaiacum, while no trace of the ferric chloride and sodium carbonate reaction was manifest.

In the case of the mangold, the oxygenase and peroxidase could again be demonstrated, while the presence of the catechol derivative, though indubitable, was sometimes difficult to show. Addition of an extract of the aromatic constituents of the plant to an enzyme extract prepared by alcohol precipitation caused, as in the cases cited above, the synthesis of the oxidase system, shown by the direct blueing of guaiacum.

The several mangold plants examined seemed to contain very different amounts (which were never very large) of this substance, so that in some cases the oxidase reaction was weak and an aromatic extract gave only a slight reaction with ferric chloride. The mangold appears to contain considerable quantities of a substance which is probably of pectic nature, being insoluble in strong alcohol, and precipitated by lead acetate. It seems possible that the catechol derivative is in combination with this substance. Pectic acid

itself, on hydrolysis with 1 % oxalic acid, under pressure, yields an insoluble acid which is probably galacturonic acid, analogous to glycuronic acid; this latter substance is known to combine with phenolic compounds. Attempts to hydrolyse the supposed pectic substance of the mangold with 1 % sulphuric acid, and to extract the catechol derivative with ether were unsuccessful.

It may be remarked, moreover, that the tannin reaction of an alcohol-ether extract of the mangold (*i.e.* a dark blue coloration with ferric chloride) obtained by Gallagher, was not always present.

The small content of the catechol derivative in the mangold may be attributed to the fact that this plant has a number of cultivated varieties, the chemical composition of which may well vary from that of the original type. Similar variations are shown in the potato; the variety King Edward, for example, gives a slight oxidase reaction and contains comparatively little of the catechol derivative.

The experiments of Gallagher [1923] in connection with an extractable lecithin-like substance were then repeated¹. The phosphatide, after preparation and purification as described by the above author, was dissolved in alcohol, and, after standing for 19 days in an uncorked vessel, the alcoholic solution gave evidence of peroxide formation, on testing with horse-radish peroxidase and guaiacum. It is considered, however, that a reaction which requires so long a period to develop is hardly comparable with the instantaneous phenomena evident in the plant; there is, moreover, evidence that alcohol itself slowly undergoes peroxide formation on standing [Neuhaus, 1905; De Stoecklin, 1907]. Again, lecithin substances are present in peroxidase, as well as in oxidase plants, a phosphatide preparation from the turnip as an example from the former class, made exactly as that described in the case of the potato, showed similar peroxide forming properties.

The oxidase system of the fungus *Lactarius vellereus*, studied by Bach and Chodat, and others, was then investigated. Preparations of a crude enzyme extract were made according to the method described by Onslow [1920] which had been used for the phanerogams, namely, the plant tissue was rapidly and thoroughly pounded with 97 % alcohol and was repeatedly filtered to dryness on a Buchner funnel; the residue was then extracted with water for about 15 minutes, and filtered, the filtrate containing a crude solution of the enzymes present. Unlike a similar enzyme preparation from the potato or other phanerogam, this water extract contains a complete direct oxidase system, that is, it is able to blue guaiacum without the addition of H_2O_2 . If, therefore, any substrate comparable to the catechol derivative of the higher plants exists, this substance does not seem to be soluble in alcohol. Thorough extraction of the plant tissue with acetone, ethyl acetate, benzene

¹ An alcoholic extract of potatoes, prepared as described, was concentrated *in vacuo*; the residue was then divided into two portions, one half being extracted with ether, and the other with chloroform, the latter procedure providing a control for the more rapid peroxide-forming properties of the ether itself.

and ether failed to disintegrate the oxidase system. The oxidases of the basidiomycetes seem thus to be fundamentally different from those of the higher plants. The behaviour of the phenolases of the fungi *Lepiota Prossera*, *Agaricus campestris* and *Russula fragilis* appeared to be similar to that of *Lactarius vellereus*.

Further examination of *Lactarius* showed that an enzyme extract prepared as above oxidises tyrosine and *p*-cresol and causes the phenols, quinol, resorcinol and phloroglucinol, to darken. The action on catechol is extremely rapid, suggesting the existence of some system analogous to the oxygenase described by Onslow for the phanerogams.

The latex of *Lactarius vellereus* does not darken on injury of the plant; moreover, extraction with hot alcohol, as described by Onslow [1920], of both this fungus and *Agaricus campestris*, failed to reveal any evidence of the presence of an *o*-dihydroxy aromatic compound. These differences between the oxidase systems of the higher and lower plants may explain the existence of the different conceptions of the nature of the oxygenase postulated by Bach and Chodat and by Onslow. Fraenckel [1922], in accordance with the theory of the former authors, explains the disintegration of the oxidase system of the higher plants by assuming that, in this case, the oxygenases are much more susceptible to the toxic action of alcohol than those of the basidiomycetes.

Similarly, the various conflicting theories on the nature of tyrosinase may be explicable if this enzyme shows corresponding differences in the phanerogams and the cryptogams. The observations which have been made on this subject are difficult to correlate and to interpret.

Chodat [1910] considers tyrosinase to be a single enzyme which oxidises tyrosine, *p*-cresol, and certain other substances when in the presence of the latter compound. He has prepared a tyrosinase free from both phenolase and peroxidase from the fungi *Lactarius vellereus* and *Russula foetens*. Chodat and Staub [1907] prepared an oxidase-free tyrosinase from potato peelings¹; they found that this enzyme brings about the deamination of amino-acids, resulting in the formation of aldehydes. Schweizer [1917] suggests that deamination caused by tyrosinase is due to its activity as an oxidising enzyme, as deaminases, although their existence has been long assumed, have never been isolated. He remarks, moreover, that laccase is an improbable constituent of tyrosinase, as the two enzymes can work in different reaction media.

Bertrand [1886] prepared a laccase-free tyrosinase from *Russula*, and Bertrand and Muttermilch [1907] made from wheat bran a tyrosinase which gave none of the reactions of laccase.

Bach [1914] on the contrary, has been unable to prepare tyrosinase which does not contain both phenolase and peroxidase either from the fungi or from

¹ Tyrosinase prepared by alcohol precipitation from the phanerogams does not give the guaiacum reaction for laccase (*i.e.* blueing of guaiacum without the addition of H_2O_2) as the "catechol" substrate for the oxygenase is removed by alcohol.

the potato. He attributes the inactivity towards oxidase reagents of the enzymes obtained by Chodat and his pupils to an alteration of salt content. He considers, moreover, that in the preparation from wheat bran obtained by Bertrand and Muttermilch the oxygenase was partly destroyed (see footnote, p. 546) and found that acceleration of the oxidation of tyrosine was brought about by addition of hydrogen peroxide. Bach therefore considers that tyrosinase is a mixture of two enzymes, a deaminase and a phenolase.

Onslow [1923] in studies of the phanerogams, using crude enzyme extracts, could find no plant without phenolase if tyrosinase were present. In certain plants, however, which contained a well-marked oxidase, no tyrosinase could be demonstrated (*Urtica dioica*, *Nicotiana tabacum*, etc.).

Dodge [1919] considers that fungus tyrosinase has no deaminase action, as ammonia estimations made during the course of the reaction with tyrosinase bore no relationship to the disappearance of amino nitrogen, which was investigated concurrently. He attributes the ammonia found by Chodat and Schweizer to the activity of a deaminase precipitated with tyrosinase from the plant extracts used. This author cites the work of Beyerinck [1913] in support of the theory that tyrosinase contains two component enzymes.

The possibilities as to the nature of tyrosinase seem therefore to be as follows:

1. Laccase and tyrosinase may be two separate unassociated enzymes, as found by Chodat. As Bach and Onslow have found, however, that tyrosinase is always accompanied by laccase, the latter enzyme may always be a component of the former.

2. The two classes of reactions may be due to the same enzyme—to which special names have been applied when the reaction substrates and products are different. The inactivity towards tyrosine of certain oxidase preparations and of hydrogen peroxide and peroxidase may be due to the inadequate salt content of the medium.

It has been mentioned above that Gallagher [1923] attributes the darkening of the mangold to tyrosinase rather than to the action of oxygenase on a catechol derivative. This may seem possible for such a plant as the mangold where the amount of the catechol substance is small and the oxidase itself is feeble, but such an interpretation appears to be inadequate in a case such as that of the apple, where the presence of tyrosinase cannot invariably be demonstrated, possibly owing to the high concentration of acid.

The oxidation of *p*-cresol, with the production of a deep orange colour, is held by Chodat [1907] to be a specific reaction for tyrosinase, the oxidase or peroxide-peroxidase system giving a colourless substance insoluble in water, with this reagent. Onslow [1923] from work on the higher plants has found, however, that enzyme extracts from all the oxidase plants examined oxidise *p*-cresol, the products of the reaction being able to blue guaiacum. *p*-Cresol in such cases acts analogously to a catechol derivative, that is, as a substrate for oxygenase, rather than as a reagent for "active" oxygen. Onslow has

suggested that an *o*-dihydroxy-derivative may be formed from *p*-cresol by "active" oxygen or that traces of such a dihydroxy-derivative may be present as an impurity in the substance itself. As, however, the characteristic orange colour does not always appear in the absence of tyrosinase, it seems possible that a specific oxidising enzyme may be necessary to act on *para*-hydroxy-compounds. Further work on the action of oxidases on such substances might give indications of the relationship of laccase with the oxidising constituent of tyrosinase.

SUMMARY.

1. A fundamental difference appears to exist between the oxidases of the higher plants and those of the basidiomycetes, in that those of the former which have been examined consist of three components, a catechol derivative and two enzymes, oxygenase and peroxidase, while the oxidase of the basidiomycetes has so far been separated into two constituents only, an enzyme-like peroxide and peroxidase.

2. The experiments of Onslow [1920] on certain phanerogam oxidases have been repeated and the results confirmed. The work of Gallagher [1923] on extractable plant phosphatides has been repeated; its interpretation as regards the oxidase reaction is discussed in the text.

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