XLIV. OXIDISING ENZYMES. II. THE NATURE OF THE ENZYMES ASSOCIATED WITH CERTAIN DIRECT OXIDISING SYSTEMS IN PLANTS.

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THE present and the following communications are a continuation of work undertaken upon the oxidising enzymes of various fruits for the Food Investigation Board of the Department of Scientific and Industrial Research.

In a previous paper [Onslow, 1919] it has been stated that the tissues and tissue extracts of those plants which give a blue colour with guaiacum tincture contain some substance with the catechol grouping in addition to a peroxidase. In the case of other plants the blueing of guaiacum is only brought about after addition of hydrogen peroxide, and from these plants substances with the catechol grouping are absent. It was shown, moreover, that in the first type of plants, the tissues could be freed from the "catechol" substance by thoroughly pounding and extracting with cold alcohol. A water extract of the tissue residue will then only blue guaiacum on addition of hydrogen peroxide, that is, it apparently only contains a peroxidase. Also when this extract is added to solutions of substances containing the catechol grouping, *e.g.* catechol, protocatechuic acid or the compound from the plant itself, oxidation of these takes place with a brown coloration and the production of a peroxide (probably hydrogen peroxide from decomposition in aqueous solution of a peroxide of the catechol compound). The resultant combination, peroxide and peroxidase, will then blue guaiacum. It was suggested that the enzyme present, which could only be spoken of as a peroxidase since it did not blue guaiacum without hydrogen peroxide, catalysed the oxidation of the catechol compound with the production of peroxide. Enzyme extracts prepared in a similar way from plants without catechol substances were without effect on catechol, protocatechuic acid, etc. After heating, the enzyme extract loses its power to oxidise catechol as well as the power to blue guaiacum in the presence of hydrogen peroxide. Two reactions are thus catalysed by the enzyme extract in the first of the above-mentioned classes of plants. One is the oxidation of the catechol substance by means of molecular oxygen with the formation of a peroxide: the other, the decomposition of the peroxide

with the setting free of "active" oxygen. The catalysis of the second type of reaction has always been defined as the sole function of peroxidases. If a peroxidase only is present in the enzyme extract of "catechol" plants, it must be allowed that the peroxidases in the two classes of plants are very different, in that the enzyme of one class appears to catalyse rapidly the oxidation of the catechol substance in addition to the usual function, whereas that of the other class is without effect. Since autoxidation is undoubtedly a complex process, it is not impossible that one enzyme may in some way catalyse both reactions, though the hypothesis is not satisfactory. As an alternative, it is now suggested that a second enzyme, in addition to the peroxidase, is present in the first class of plants. The function of this additional enzyme is to catalyse the oxidation of the catechol substance with the formation of a peroxide, which, when formed, is acted on by the peroxidase in the usual way. It is proposed to call this second enzyme an oxygenase¹, a term originally used by Chodat and Bach for the portion of an oxidase which can be replaced by hydrogen peroxide. Chodat [1910] speaks of oxygenases thus: "...es sind fermentartige Körper, die sich mit dem Sauerstoff der Luft zu einem Peroxyd verbinden können." Thus these authors make no clear differentiation between the organic compound from which the peroxide is formed and the enzyme which forms the peroxide, as a result, in all probability, of not having effected in their experiments a separation of the catechol substance, or similar compound, from the enzymes involved. In recent years the term oxygenase has fallen into disuse in favour of the simpler explanation of oxidase action, *i.e.* as being due to a peroxide formed by autoxidation only, and a peroxidase.

In the present communication, the following additional evidence is offered in connection with these problems.

Spontaneous autoxidation of solutions of various phenols and aromatic acids.

Solutions of the following substances were exposed to air for 6-7 days: adrenaline, caffeic acid (crude extract only), carvacrol, catechol, cresol (ortho- and para-), gallic acid, guaiacol, orcinol, phenol, phloroglucinol, protocatechuic acid, pyrogallol, quinol, resorcinol, tannic acid and vanillin. One per cent. solutions were used, except in the cases of adrenaline and caffeic acid, of which the strength of the solutions was unknown. In the cases of caffeic acid, gallic acid, phenol, protocatechuic acid, pyrogallol and tannic acid, before exposure to air, dilute caustic potash was added to the solutions until they just failed to change the yellow colour of bromocresol purple, *i.e.* the P_H ranged between 5.2-6.8. After the long exposure to air, a few cc. were then tested for peroxide with a mixture of 5 cc. of 2 % potassium iodide, 2 cc. of 1 % acetic acid and

¹ The term oxygenase is not very satisfactory, since the function of the enzyme is not oxygen-splitting. It has been adopted for the present, however, to avoid further confusion of nomenclature. Should the enzyme be definitely isolated at some future date, the desirability of giving it a more suitable name might be considered.

1 cc. of 1 % starch solution. A blue colour appeared within half an hour in the cases of adrenaline, caffeic acid and catechol. No colour appeared in the other solutions on standing an hour. If 1 % sulphuric acid is substituted for 1 % acetic acid, in addition to the above three, the following also gave the peroxide test more or less rapidly: gallic acid, phenol, protocatechuic acid, pyrogallol, quinol and tannic acid. The remaining solutions gave no colour within the hour allowed. Controls of starch, iodide and acid also gave no colour on standing for the same length of time. It is evident, therefore, that a number of the above substances tend to form peroxides (or hydrogen peroxide by decomposition) on autoxidation, and those with the catechol grouping, on the whole, most readily. In no case did a freshly-made solution of any of the above substances give a reaction with iodide, starch and either acetic or sulphuric acid.

Behaviour, with peroxidase, of the above autoxidised solutions of various phenols and aromatic acids as peroxides.

The behaviour of the autoxidised solutions with a peroxidase (from horse-radish) and guaiacum was next investigated. The peroxidase solution was prepared by well pounding and extracting horse-radish root with 96 % alcohol, and then extracting the residue with water. On addition of the enzyme extract to the above solutions, followed by guaiacum tincture, a blue colour was obtained in the case of adrenaline, caffeic acid, catechol, protocatechuic acid and to some extent tannic acid. Hence such peroxides formed by autoxidation can function in the peroxide-peroxidase system, and, on the whole, those formed by compounds with the catechol grouping are most efficient. In no case was any result obtained when freshly prepared solutions were used.

Action on freshly-prepared solutions of the above phenols and aromatic acids of the crude enzyme extracts (oxygenase and peroxidase) from plants containing a "catechol" compound.

The potato tuber and pear fruit were used as sources of enzymes. The enzyme extracts were prepared in the usual way by rapidly pounding the tissues with cold alcohol, filtering on a pump, and repeating the process several times. A water extract of the colourless residue is used for the test. The results with extracts from both plants were as follows. A rapid browning of solutions of catechol and of caffeic and protocatechuic acids on neutralisation. On subsequent addition of guaiacum, a blue colour appeared in all three cases. A solution of adrenaline is rapidly turned red, but no blueing follows on addition of guaiacum (in contrast to the result in the last section). A solution of tyrosine is reddened (rapidly in the case of the potato, slowly with the pear) finally becoming black, but no blue colour appeared on addition of guaiacum at any stage. A solution of *p*-cresol is turned orange (rapidly with potato, slowly with pear), but unlike the case of tyrosine, a blue colour is

given on subsequent addition of guaiacum. No action was obtained with the remainder of the substances.

The reactions with tyrosine and *p*-cresol are presumably due to tyrosinase which has long been known to be present in the potato, though no previous reference can be discovered to its occurrence in the pear fruit. The reaction with adrenaline may be due, either to the presence of tyrosinase, which, according to some authors, acts on this substance, or to the presence of the oxidising enzyme which reacts with the catechol grouping, or to both. Points of interest are the non-blueing of guaiacum in the case of adrenaline and tyrosine, whereas it occurs in the case of *p*-cresol. Also the fact that when an autoxidised solution of adrenaline is used with a peroxidase, as in the last section, blueing occurs on subsequent addition of guaiacum.

Separation of two enzymes, oxygenase and peroxidase, from the enzyme extract of the potato.

In view of the suggestion that two enzymes, an oxygenase and a peroxidase are present in the pear and potato, an attempt was made to separate them by fractional precipitation. Such a method was originally employed by Chodat and Bach [1903: Bach, 1910] for separating their oxygenase component from the peroxidase in the case of an extract from the fungus, *Lactarius* (Basidiomycetes). Their extract was precipitated with 40 % alcohol which produced a precipitate rich in oxygenase, but poor in peroxidase. After continued fractional precipitation with alcohol, a final precipitate was obtained containing peroxidase only.

The method adopted for the potato was as follows. The tuber was pounded and extracted with cold alcohol as previously described. The residue was then extracted for an hour with about three times its weight of water, and the extract squeezed out through muslin. The extract was then centrifuged, the opalescent solution decanted and absolute alcohol added until the concentration reached 40 %. The precipitate taken up with water gave in several experiments a stronger oxygenase reaction (blueing with catechol solution and guaiacum in the presence of peroxidase) than peroxidase reaction (blueing with hydrogen peroxide and guaiacum). Reprecipitation of the first fraction failed to remove the peroxidase, nor was the result more successful by making the concentration of alcohol 20 or 30 % for the first precipitation. By further precipitation of the first filtrate four or five times with additional volumes of alcohol equal to that first added, a precipitate was eventually obtained which ceased to give the oxygenase reaction though it still gave a good peroxidase reaction. By adding saturated ammonium sulphate (20 cc. to 80 cc. of extract) instead of alcohol for the first precipitation, in one trial a first fraction was obtained which gave only a slight peroxidase, but a strong oxygenase reaction. The experiment, however, could not be repeated with any certainty.

It therefore appears that the method of fractional precipitation is not wholly satisfactory for the separation of the two enzymes. More recent experiments of Bach [1916] would seem to confirm this result since he has tried to separate Chodat and Bach's two components of *Lactarius* oxidase by ultrafiltration. Of the two, he found the peroxidase to be much the more rapidly adsorbed by ultrafiltration. The oxygenase remains on the ultrafilter, and its oxidising power can be regenerated by addition of horse-radish peroxidase.

The above results furnish evidence in favour of the existence of two enzymes in plants which contain a catechol substance, turn brown on injury and blue guaiacum without addition of hydrogen peroxide. In plant tissues, in cases so far investigated, we conclude then that three factors are necessary for the blueing of guaiacum: an aromatic substance with a dihydroxy-grouping in the ortho-position; an enzyme, oxygenase, which catalyses the oxidation of such substances with the production of an organic peroxide (or hydrogen peroxide by decomposition); and, finally, a peroxidase which transfers "active" oxygen to readily oxidisable compounds, *e.g.* guaiacum. Artificially, the place of the oxygenase and aromatic compound can be taken either by hydrogen peroxide or by peroxides formed by the autoxidation of certain substances. If the oxidase (synonymous with laccase, phenoloxidase and phenolase) of the pear and potato is to be defined as an enzyme blueing guaiacum, then all three components are essential to its constitution, for the mixture of enzymes alone, oxygenase and peroxidase, is unable to blue guaiacum unless contaminated with the catechol substance.

It is probable that the oxidases (laccases, phenoloxidases, phenolases) obtained by previous workers by precipitation of plant juices with alcohol, are in many cases mixtures of oxygenase and peroxidase with adsorbed catechol compound. Such mixtures would blue guaiacum, and would transfer oxygen to many other substances, such as phenols and aromatic acids, to a greater or less degree. The enzyme mixture, oxygenase and peroxidase (which needs care to be prepared free from catechol compound), has, by virtue of the oxygenase, so far been found only to act on the dihydroxy-grouping in the ortho-position. It can be readily seen that the combination, catechol compound and oxygenase, behaves in every way like Chodat and Bach's organic substance of an enzyme-like nature endowed with the power of forming peroxides, which they call oxygenase.

CONCLUSIONS.

1. Solutions of catechol, caffeic acid, protocatechuic acid and adrenaline, compounds which have the dihydroxy-grouping in the ortho-position, tend to autoxidise slowly when left in air with the formation of peroxides.
2. When a solution of a peroxidase is added to any of the above peroxides, the combination constitutes an oxidase system which will blue guaiacum.

3. The autoxidation, with production of peroxides, of catechol, proto-catechuic acid and caffeic acid is accelerated by enzyme extracts of plants which turn brown on injury, and of which the juices blue guaiacum without addition of hydrogen peroxide.

4. Such plants have been found to contain a compound giving the "catechol" reaction. It is suggested that they also contain, in addition to a peroxidase, a second enzyme, which will be termed an oxygenase. The function of the latter is to catalyse the autoxidation of the "catechol" compound with the formation of a peroxide.

5. Three components therefore, in the cases investigated, are present in what has been termed an oxidase (laccase, phenoloxidase or phenolase): a "catechol" compound from which a peroxide can be formed, and two enzymes, an oxygenase which catalyses the production of peroxide, and a peroxidase which decomposes the peroxide with the formation of "active" oxygen. All three components are essential for the blueing of guaiacum.

6. The peroxidase can be separated from the oxygenase by fractional precipitation with alcohol, though the converse has not been accomplished.

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