I. OXIDISING ENZYMES. I. THE NATURE OF THE "PEROXIDE" NATURALLY ASSOCIATED WITH CERTAIN DIRECT OXIDISING SYSTEMS IN PLANTS.

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THE following account records some experiments which throw additional light on the nature of the oxidising enzymes of plants. The dual nature of an oxidase, *i.e.* peroxide-peroxidase, and the fact that one component, the peroxidase, is an enzyme, has been established by previous workers. The resolution of the system into its component parts has also been effected to some extent.

The present work shows that the peroxide may arise by oxidation of an aromatic compound of a particular type of structure, the oxidation being activated by the peroxidase itself. It is also shown that the aromatic compound can be separated from the peroxidase by a purely chemical method, and that the two components can afterwards be reunited again. The peroxide once formed cooperates, in the usual way, with the peroxidase in carrying out further oxidation reactions such as are characteristic of "oxidases."

DIRECT AND INDIRECT OXIDATION AND THE GENERALLY ACCEPTED INTERPRETATION.

It has been known for a long time that the tissues of many plants discolour or turn brown on injury, such as bruising, *e.g.* Potato tuber, Apple fruit¹. The same phenomenon may be brought about in many cases by exposure of the tissues to chloroform vapour. A certain number of plants, on the other hand, do not show discoloration under similar treatment.

Further, it has been shown that the expressed juices, or water extracts, of plants which exhibit discoloration on injury, when added to guaiacum tincture immediately produce a blue colour. The juices, or water extracts, of plants which do not discolour, do not give a blue colour with guaiacum tincture until hydrogen peroxide has been added.

¹ The discoloration on injury has been found to be characteristic of many or all members of certain Natural Orders, notably the Umbelliferae, Labiatae, Compositae and others. In other cases, only a fraction of the genera of an order apparently exhibit the phenomenon. Finally, in some orders, *e.g.* Cruciferae, the phenomenon is rare or possibly entirely absent [Wheldale, 1911]

It has been assumed, on the evidence of many investigators, that the phenomenon of discoloration of tissues on injury is connected with the action of oxidising enzymes.

The hypothesis generally accepted in regard to oxidising enzymes is one which classifies these substances as direct and indirect. When either the juice, or water extract, of a tissue gives immediately a blue colour, *i.e.* an oxidation product, with guaiacum solution, the tissue is said to contain a direct oxidising enzyme, or oxidase. If, however, the blue colour only appears after addition of hydrogen peroxide, the tissue is said to contain an indirect oxidising enzyme, or peroxidase. It has been postulated that, in general, a system which turns guaiacum blue consists of a peroxide and a peroxidase: the peroxidase acts upon the peroxide and transfers oxygen in an active state to readily oxidisable substances, such as guaiacum. In the case of plant tissues which give the direct reaction, it has been suggested that some organic substance in the plant acts as a peroxide, while in tissues giving the indirect reaction, the peroxidase only is present, and the peroxide may be supplied artificially in the form of hydrogen peroxide.

The original conception of the hypothesis of oxidising enzymes, which we owe to Chodat and Bach, was rather more complex than that outlined in the foregoing paragraph. These authors regarded an oxidase as consisting of two components, a peroxidase (as described above) and an oxygenase. "Als Oxygenasen bezeichnen Chodat und Bach den supponierten eiweisshaltigen oder organischen Anteil der bisherigen Oxydasen, der als Peroxyd bildender Körper sich mit dem Luftsauerstoff addierend, sich mit ihm zu einem Körper der allgemeinen Formel $F \begin{smallmatrix} \diagup O \\ \diagdown \end{smallmatrix}$ verbindet. Mit anderen Worten, es sind fer-

mentartige Körper, die sich mit dem Sauerstoff der Luft zu einem Peroxyd verbinden können. Sie werden, wie andere fermentartige Körper, durch Hitze zerstört, durch starken Alkohol gefällt, können vergiftet und geschädigt werden. Sie unterscheiden sich von gewöhnlichen Peroxyden nur dadurch, dass sie wahrscheinlich hochmolekulare Körper sind." [Chodat, 1910.]

DEVELOPMENT OF THE ABOVE HYPOTHESIS SUGGESTED BY THE PRESENT WORK.

The following modification of the hypothesis of oxidising enzymes, or, at any rate, of a certain *class* of these substances, is suggested from experimental evidence to be described later.

In plants, which brown on injury and give the direct oxidase reaction, there is present a peroxidase and, in addition, some aromatic compound containing the dihydroxy grouping characteristic of catechol. On injury or autolysis, the peroxidase itself activates the oxidation of the aromatic compound, and the oxidised product constitutes a peroxide (of Chodat and Bach). The peroxide-peroxidase system so formed will then blue guaiacum tincture.

It would appear, moreover, that in the above-mentioned plants the peroxidase is always associated with compounds containing the "catechol" grouping.

It has been found possible to prevent the formation of the peroxide-peroxidase system by extracting the aromatic compound with alcohol, leaving the peroxidase in the tissue residue. The peroxidase can then only give the indirect reaction. On adding the extract of the aromatic substance to the peroxidase, the system can now be synthesised and will give the direct reaction.

The above provides an extension of the system of Chodat and Bach, in that the peroxidase activates the formation of its own peroxide.

In plants, on the other hand, which give the indirect reaction and do not brown on injury, it appears that the peroxidase is not associated with compounds containing the "catechol" grouping, nor will these enzymes activate the oxidation of such compounds.

Aromatic compounds, such as those suggested, containing the dihydroxy grouping of the phenol catechol

are widely distributed in plants [corroborated by Wolff, 1917 and Wolff and Rouchelman, 1917]. Such substances may be, in some cases, protocatechuic acid, protocatechuic aldehyde, caffeic acid or derivatives of these:

There is evidence (based on the characteristic ferric chloride reaction) that plants, which brown on injury and give the direct reaction, do contain substances of this nature and that they are absent from plants giving only the indirect reaction.

It has been shown that the peroxidases of plants which give the direct reaction when added to solutions of catechol, protocatechuic and caffeic acids (on neutralisation), give brown oxidation products which are in effect peroxides. On addition of guaiacum to the mixture of the oxidised product and peroxidase, a blueing takes place. The same result is also obtained using the aromatic substance extracted from the plant itself.

The peroxidases of plants which give only the indirect reaction, on the other hand, do not act upon catechol, protocatechuic acid, etc., and no system blueing guaiacum is formed in their presence.

Hence it is to be pictured that, on injury or autolysis of tissues which discolour, the peroxidase comes into contact with a "catechol-like" substance whose oxidation it activates. Extracts or juices of the tissues will then blue guaiacum.

The conception of peroxidases, which oxidise polyphenols in the absence of hydrogen peroxide or other peroxide, differs from that formulated by Chodat and Bach. It might be maintained that the peroxidase used in the present account had traces of peroxide as impurity: even if this were so, the enzyme must still be defined as a peroxidase, since it is without action on guaiacum except in the presence of added hydrogen peroxide.

PREPARATION OF THE PEROXIDASE.

The employment of either expressed juices or aqueous extracts of tissues is not recommended for use in experiments on oxidising enzymes, since the presence of numerous other substances (sugars, tannins) largely interferes with the action of the enzymes. Moreover, in such liquids, on standing, reactions of many kinds may take place including those of reduction. Hence the following method of procedure has been employed, and either the fruit of the Pear or the tuber of the Potato may be used as material, though the method has been applied to other plants. The tissues are very rapidly pounded and extracted with cold alcohol, whereby the enzymes are precipitated and retained in the cell residue, while other substances, such as sugars and aromatics, are more or less completely removed.

The tissue of the Pear fruit constitutes a simpler case than that of the Potato tuber, since the latter contains, in addition to a peroxidase, a tyrosinase and a substance which it oxidises, presumably tyrosine. It is to the latter reaction that most of the darkening of expressed Potato juice is due. Tyrosinase, however, has no action on guaiacum [Chodat, 1910]. If Pear fruit is employed, it is advisable to select a variety which browns rapidly on injury, as the aromatic content, and possibly the activity of the peroxidase, may vary in different varieties.

For preparation of the peroxidase, a pear (or potato) is peeled, and from it are cut thin slices which are rapidly pounded in a mortar, after adding sufficient 96 % alcohol to prevent, as far as possible, any exposure of the tissue to air. The alcohol is then quickly filtered off on a filter pump. The residue can then be ground with more alcohol and filtered. The process should be repeated two or three times, and the grinding should be very thorough. After the final filtering, a white powder is left containing the insoluble residue of the cells including the peroxidase.

If a cold water extract of the residue be made and filtered, the following points can be demonstrated:

- (a) It does not darken on standing in air. (A very slight darkening may take place after standing a number of hours.)
- (b) Added to guaiacum tincture, it produces no blue colour within the time usually allowed for a positive reaction.
- (c) Added to guaiacum tincture and hydrogen peroxide, a deep blue colour is obtained.

From the above results it appears that the alcohol has removed some part of the system responsible for darkening and for the direct reaction. This point will be considered again later (see section on extraction of aromatic substances).

Another point to be noted is that if the grinding is not sufficiently rapid, the residue may be discoloured, in which case the water extract of the residue may give some direct reaction. This will also be considered later (see section on laccase).

Similar results to the above were obtained with the tissues of fruits of the Apple and Greengage.

ACTION OF THE PEROXIDASE ON SOLUTIONS OF VARIOUS PHENOLS AND AROMATIC ACIDS.

If an aqueous solution of the Pear (or Potato) peroxidase (as prepared in the previous section) is added to a dilute solution of pure catechol, a yellowish tint is rapidly developed which eventually deepens to a yellowish-brown. It was also shown by performing the experiment in a closed vessel attached to a gas burette that there was an absorption of oxygen during the action of the peroxidase on catechol. If some of this oxidised solution, also containing peroxidase, is added to guaiacum tincture, the latter is immediately turned blue.

With a solution of protocatechuic acid, instead of catechol, the same result is obtained, but only if the protocatechuic acid is first neutralised.

Solutions of the peroxidase, together with guaiacum tincture, were also added to solutions of other phenols, *i.e.* phenol, quinol, resorcinol, pyrogallol and phloroglucinol, and to various acids, *i.e.* gallic, tannic, benzoic and salicylic, each acid being neutralised with sodium carbonate before the addition of the enzyme. The solution of pyrogallol turned brown, and there was some darkening with quinol, but when guaiacum was subsequently added, there was no appreciable blueing in any case.

It would thus appear that the Pear and Potato peroxidases are able to activate the oxidation of compounds containing the catechol nucleus, and that the oxidation products can act as peroxides as regards the oxidation of guaiacum.

The action of the Pear and Potato enzymes on a crude solution of caffeic acid was examined in the following way. Coffee berries contain caffeatannic acid which is said to be a glucoside of caffeic acid. The ground berries were extracted with hot water, filtered, and the filtrate precipitated with lead acetate. A yellow precipitate of the lead salt of caffeatannic acid separated out. This was decomposed with sulphuric acid, filtered from lead sulphate and neutralised. When the Pear peroxidase was added to this neutralised extract, followed by guaiacum tincture, a blue colour was obtained. It is to be supposed that the berry contains some free caffeic acid, since the caffeatannic acid has both hydroxyls replaced by sugar.

A similar browning of catechol solution, followed by blueing when guaiacum tincture is added, was obtained with enzymes from the following tissues (prepared in the way above described by treating with cold alcohol, and extracting the tissue residue with water): fruit of Apple and Greengage: leaf of Pear: flowers of Horse Chestnut (*Æsculus Hippocastanum*), and of a white variety of Foxglove (*Digitalis*). There is reason to believe, that the enzymes of all plants which brown on injury and give the direct oxidase reaction (such as those above mentioned) will behave in the same way towards catechol and subsequently guaiacum.

The peroxidase from the fruit of Apple behaves like that of the Pear and Potato towards protocathechuic acid and crude caffeic acid, and also subsequently towards guaiacum.

On the other hand enzymes (by alcohol) from leaves of Yellow Alyssum, flowers of Mallow (*Malva moschata* var. *alba*), white Stock (*Matthiola*) and white Arabis were without action on catechol, and there was no blueing on subsequent addition of guaiacum tincture. The tissues of these plants give no browning on injury and no direct guaiacum reaction.

EXTRACTION OF AROMATIC SUBSTANCES.

This is carried out by cutting pears (or potatoes) into thin slices as rapidly as possible, and dropping the slices immediately into boiling 96 % alcohol. After boiling for a time, the hot alcohol is filtered off. The filtrate is then distilled *in vacuo* until all the alcohol is removed. The residue consists of a comparatively small bulk of a turbid aqueous solution of those substances which have been extracted by alcohol, the water being largely derived from the tissue of the fruit or tuber. To this extract, after filtering, a concentrated solution of lead acetate is added. A pale yellow precipitate is formed, and the acetate is added until no more precipitate is produced. The latter is filtered off and washed. It is then decomposed with the minimum amount of sulphuric acid, and the lead sulphate filtered off.

A portion of this filtrate is nearly neutralised (to litmus) with caustic potash solution, and with it the following tests are made:

(a) On addition of a few drops of 4 % ferric chloride solution, a green colour is produced. On further addition of a little dilute sodium carbonate, the green colour changes to bluish-purple and finally to purplish-red.

(b) An aqueous extract of the Pear (or Potato) peroxidase (prepared by alcohol) is added. The solution of aromatics, which has only a yellowish-brown tint, rapidly darkens to a deeper brown on standing about an hour.

(c) On addition of guaiacum tincture to the solution in (b) at any time within an hour after the addition of the peroxidase, a blue colour is obtained.

On addition of guaiacum tincture to the solution of aromatics which has been allowed to stand for some time without the presence of peroxidase, no blue colour is obtained within the time usually allowed for its appearance.

[The result in (a) is also given by extracts of the Apple and Greengage fruits and leaves of Pear, all of which brown on injury. There is no such result with extract of Alyssum leaves, which do not brown on injury.]

The remainder of the filtrate is then extracted with ether several times and the ether distilled off. The residue is taken up in water and the same test with ferric chloride repeated. It will be found to give the same reaction. It is not advisable to use the ether extract for testing with the oxidising enzyme, since ether itself may contain peroxides which complete the system.

From the above observations it is clear that hot alcohol will extract from the fruit (or tuber) a substance which is precipitated by lead acetate and is also soluble in ether. It gives a reaction with ferric chloride and sodium carbonate which is characteristic of catechol, protocatechuic acid, protocatechuic aldehyde and caffeic acid, all of which substances contain the ortho-dihydroxy grouping. This reaction will be referred to as the "catechol" reaction. It is also clear that the oxidation of the substance extracted in this way can be activated by the peroxidase, and the product of oxidation can function as a peroxide thus completing the system which oxidises guaiacum.

If the solution is neutralised before extraction with ether, the latter does not appear to extract the aromatic substance.

The precipitation by lead acetate, and the solubility in alcohol and ether, point to a resemblance between the aromatic substance of the Pear (or Potato) and both catechol and protocatechuic acid.

A similar and probably identical substance giving the "catechol" reaction was obtained by Reinke [1882] by hydrolysing Potato juice with hydrochloric acid and extracting with ether. It had been previously shown by Preusse [1879] that from a neutral or alkaline aqueous solution catechol can be extracted by ether, but not protocatechuic acid. Reinke obtained no substance giving the "catechol" reaction on ether extraction of the hydrolysed potato juice after neutralisation, and hence he concluded that the substance he described was not catechol. This conclusion appears to be confirmed by the experiments described in the present account. Reinke was also unable to identify his product with protocatechuic acid on account of its ready solubility in water, protocatechuic acid being only slightly soluble. Reinke suggests caffeic acid as the only alternative. The fact that the substance described in the present account is not extracted by ether from neutral aqueous solution indicates that it is probably an acid, but for complete identification it would be necessary to prepare it on a larger scale.

RELATIONSHIP BETWEEN THE PEROXIDASES OF THE PEAR AND POTATO AND LACCASE.

The direct oxidases of plants have generally been termed laccases by previous investigators [Chodat, 1910]. They are soluble in water, from which solution they can be precipitated by alcohol. Hence the method of preparation is usually by precipitation of the expressed plant juices by alcohol.

From results described in the present account it appears possible that many of the so-called laccases may be produced by the adsorption of a certain amount of the oxidised aromatic (produced during crushing and extraction) by the precipitate (by alcohol) containing the crude peroxidase. On redissolving in water, both peroxide and peroxidase will be present. It has been noted already that if the Pear (or Potato) tissue is either allowed to brown while pounding with alcohol, or is insufficiently extracted, the residue may be coloured, and in this case the water extract of the residue will give a direct guaiacum reaction.

It has been found that in the case of some tissues giving the direct reaction, it is practically impossible to prevent browning during maceration with alcohol, so that the residue is always somewhat coloured, and consequently the water extract gives the direct reaction.

INHIBITION OF OXIDISING ENZYMES.

It has been observed by several investigators that the action of oxidising enzymes may be inhibited by the presence of tannin, and, possibly, sugars. There is reason to believe that many plants may contain an enzyme of the type of that in the Pear fruit and Potato tuber, and also an aromatic with the catechol grouping, and yet, on injury, no browning takes place owing to inhibition by tannin present in the tissues.

CONCLUSIONS.

1. The tissues of many plants (*e.g.* fruit of Pear, tuber of Potato), on injury or exposure to chloroform vapour, turn brown: in other plants no browning occurs. Extracts of tissues which brown give the direct oxidase reaction with guaiacum: of those which do not brown, the indirect reaction only.

2. It would appear that the direct oxidase system in the Pear fruit and Potato tuber is due to the presence of a peroxidase (which blues guaiacum only on addition of hydrogen peroxide) and an aromatic substance giving the reaction characteristic of the catechol grouping. On injury, the peroxidase activates the oxidation of the aromatic with the formation of a peroxide. The peroxide-peroxidase system so formed will then blue guaiacum.

3. The aromatic substance giving rise to the peroxide can be extracted and separated from the peroxidase, thus preventing formation of the system. The system can be synthesised afterwards by combining the extracted aromatic and the enzyme.

4. There is evidence that in plants in general which brown on injury the peroxidase is associated with an aromatic substance giving the reaction characteristic of the catechol grouping. In such plants the peroxidases activate the oxidation of the aromatic substances (either obtained from their tissues or supplied from an artificial source) giving rise to peroxides, and the system

peroxide-peroxidase will then blue guaiacum. (In some plants however of this type the action may be masked or inhibited on injury by presence of tannins or other substances.)

5. Plants which do not brown on injury (and in which there is no apparent inhibition) do not contain a substance with the catechol grouping, and their enzymes do not catalyse the oxidation of substances with such a grouping.

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REFERENCES.

- Chodat (1910). *Handbuch der biochemischen Arbeitsmethoden*. E. Abderhalden, Berlin, **3** (1), 42.
Preusse (1879). *Zeitsch. physiol. Chem.* **2**, 324.
Reinke (1882). *Zeitsch. physiol. Chem.* **6**, 263.
Wheldale (1911). *Proc. Roy. Soc. B.* **84**, 121.
Wolff (1917). *Ann. Inst. Past.* **31**, 92.
Wolff and Rouchelman (1917). *Ann. Inst. Past.* **31**, 96.