VI. MECHANISM OF OXIDATION IN THE PLANT. PART II.

AN INVESTIGATION OF SUBSTANCES WHICH ARE CAPABLE OF BEHAVING AS PEROXYDASES.

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THE question of peroxide formation in plant tissues has been considered in a previous paper [Gallagher, 1923]. There remains to be considered the mechanism by which the oxygen of peroxides may be rendered active for purposes of oxidation. This leads to the study of the enzyme peroxydase.

Peroxydase is characterised by its ability to oxidise guaiaconic acid and other oxydase reagents in presence of peroxide. Pure peroxydase preparations have no oxidising action on these reagents in the absence of peroxide; likewise, the peroxide is relatively inactive as an oxidising agent in the absence of peroxydase. It has been pointed out by Wolff [1908] that the behaviour of peroxydase in this respect is similar to that of certain salts of copper and of iron. Woker [1914] states that formaldehyde and acetaldehyde are capable of behaving as peroxydases and catalases. The very frequent presence of iron in peroxydase preparations has led to the belief that this metal in some form is the active constituent of the enzyme, a view which recalls the suggested connection of manganese with oxydases of the laccase type. Woker, as a result of her observations, suggests that peroxydases are substances of aldehyde nature.

The preparation of oxidising enzymes which have been shown to be free from manganese or iron [Rosenfeld, 1906; de Stoecklin, 1907; Bach and Tcherniak, 1908; Bach, 1910, Willstätter and Stoll, 1918 and Van der Haar, 1921] would appear to indicate that the mineral constituents of oxydase preparations are not the main active components of the enzyme. Rather does it appear likely that the chief active enzyme constituent is a substance of organic nature.

As a preliminary to the study of the chemical nature of naturally occurring peroxydase an examination was made of a variety of compounds which are capable of replacing peroxydase in the oxidation of guaiaconic acid by peroxide. The object of the study was to determine the nature of the reaction

by means of which the oxygen of the peroxide is set free in an active condition. It was also sought to determine whether there existed among compounds which behave as peroxydases a common peculiarity of structure to which their reactivity in this respect might reasonably be ascribed.

THE PEROXYDASE REACTION OF ALDEHYDES.

The peroxydase and catalase reactions of formaldehyde and acetaldehyde led Woker [1914] to the conclusion that naturally occurring peroxydases, catalases and reductases are due to the same agent which is a substance of aldehyde character. Benzaldehyde, it was stated, showed no activity as a peroxydase until after repeated rectification, and no catalase action was observed in the case of this aldehyde.

An investigation which was carried out in the present work on the behaviour of a number of aldehydes towards a mixture of guaiacum tincture and peroxidised turpentine led to the conclusion that the peroxydase reaction is generally associated with aliphatic aldehydes. No specimen of benzaldehyde examined appeared to have any action on a mixture of guaiacum and peroxide, but samples of propyl, *n*-butyl, *n*-valeric and aldehydes, as well as oenanthaldehyde, formaldehyde and acetaldehyde, were found to give distinct peroxydase reactions. These were the only aliphatic aldehydes examined. It was found, however, that different specimens of the same aldehyde frequently reacted to a markedly different degree with a mixture of guaiacum and turpentine. While some laboratory specimens of formaldehyde, for instance, gave a strong blue colour with a mixture of guaiacum solution and turpentine, others, of apparently similar concentration and purity, when tested under comparable conditions, gave but a faint coloration. These differences of behaviour admit of two explanations: either the specimens of low reactivity contain traces of a substance which inhibits the reaction with guaiacum, or else the peroxydase reaction is due to an impurity in the aldehyde and not to the aldehyde itself. The above-mentioned aldehydes were consequently rectified. In no instance did the fresh distillate show any reactivity towards a mixture of guaiacum and peroxide, but in each case the peroxydase reaction was found to reappear on storing the aldehyde in an unstoppered flask for several days. Specimens of the freshly distilled substances which were stored out of contact with air in sealed tubes failed to acquire a peroxydase reaction. It is thus evident that the peroxydase reaction commonly associated with aliphatic aldehydes is due to the presence of an impurity which is produced by the action of air on the aldehyde.

Nature of the substance to which the peroxydase reaction commonly given by aliphatic aldehydes is due.

The fact that by the action of air on many aliphatic aldehydes a substance is produced which is capable of oxidising guaiaconic acid in presence of peroxide, points to the likelihood that this reactive substance is an oxidation

product of the aldehyde. The aldehydes in question are known to be fairly easily oxidised in the air to the corresponding acids. It is necessary to remember that not alone are the acids incapable of inducing the oxidation of guaiacum but they tend to inhibit it. Clearly, therefore, the substance with which we are concerned must be an oxidation product intermediate between the aldehyde and the acid, or, which seems less likely, an oxidation product bearing no relation whatsoever to the acid. The facts of the case recall the problem of the autoxidation of benzaldehyde. In connection with this substance it has been fairly definitely established that a substance of peroxide nature is formed as an intermediate product in the oxidation to the acid. There is this essential difference, however; benzaldehyde appears to form a peroxide which is capable of oxidising guaiaconic acid in presence of peroxidase, while the substances derived from the aldehydes under consideration are only capable of replacing the peroxidase in the oxidation of guaiaconic acid by an added peroxide such as turpentine peroxide or ethyl hydroperoxide. The peroxide of benzaldehyde accordingly appears to be constituted similarly to hydrogen peroxide, while the substance formed from aliphatic aldehydes appears to be directly related in some way to peroxidase. Baeyer and Villiger [1900] have prepared a peroxide of benzaldehyde by treating the substance with hydrogen peroxide. Aliphatic aldehydes do not readily form peroxides with hydrogen peroxide [Langheld, Weyl, 1914].

The only known substances of peroxide nature derived from aliphatic aldehydes are the so-called monoperoxides or ozonides described by Harries and Langheld [1906]. The aldehyde monoperoxides are formed when the aldehyde is treated with ozone. One atom of oxygen is added on per molecule of aldehyde, and the formula at present accepted for these substances is $R \cdot CH = O = O$. That the peroxidase reaction of the aldehydes under consideration is very probably due to a substance of this type is shown by the following experiment.

Exp. A. One gram of pure freshly rectified oenanthaldehyde was placed in a tube immersed in a good freezing-mixture and a current of ozonised oxygen from a single Siemen's induction tube was passed through the liquid at the rate of about one bubble per second for half an hour. The generation of ozone was then stopped and the current of oxygen was continued at a somewhat faster rate for about fifteen minutes to remove traces of uncombined ozone. The contents of the tube were then poured into 20 cc. of well-cooled rectified spirit. A few drops of bromocresol purple were added as indicator and addition of *N* alkali was made, drop by drop, till the solution changed to a green colour. A little of the neutralised solution was now poured into two test-tubes, one of which contained a fresh alcoholic solution of guaiaconic acid and the other a mixture of guaiaconic acid solution and 1.5 % ethyl hydroperoxide. In the second tube a blue colour soon appeared which became quite intense in a few minutes. The tube which contained guaiaconic acid alone only began to develop a faint blue colour after 20–30 minutes. Since guaiaconic acid is

readily oxidised by free ozone the latter test is essential to ensure that no free ozone be present in the aldehyde solution.

In making the above experiment it is necessary to keep the ozonised aldehyde well cooled when neutralising; otherwise the ozonide is rapidly decomposed by the excess of free acid or alkali as the case may be.

The experiment was repeated with *n*-valeric aldehyde with similar results. Control experiments in which ordinary oxygen was passed through a quantity of the aldehyde under similar conditions gave practically no coloration with guaiaconic acid and ethyl hydroperoxide.

Discussion.

This experiment seems to offer strong proof that the peroxydase reaction given by the aldehydes examined is due, not to the aldehyde itself, nor to a compound of the aldehyde with hydrogen peroxide as Woker suggests, but to a relatively uncommon class of peroxides—a monoperoxide or ozonide. Harries and Langheld [1906] represent the structure of the substance formed by the action of ozone on oenanthyl aldehyde by the formula

The formation of substances of this type among organic compounds seems to be characteristic of the carbonyl group.

The association of aldehyde with peroxydase enzymes.

In view of the association of the peroxydase reaction with specimens of the common aldehydes it is interesting at this stage to note certain observations made on the possible aldehyde nature of natural peroxydase.

(i) Preparations of peroxydase made by alcoholic precipitation from some common vegetables (potato, horse radish and mangold) give a strong coloration with Schiff's reagent. If a section of mangold root or potato tuber be treated with Schiff's reagent, the location of the coloration which takes place is the same as when a section is treated with tincture of guaiacum and peroxide. The reaction in both cases takes place at the tracheides and at the epidermis.

(ii) Peroxydase preparations obtained by alcoholic precipitation likewise give an intense reaction for aldehyde when treated with diazobenzenesulphonic acid and sodium amalgam in presence of alkali [Fischer and Penzoldt, 1883].

(iii) The peroxydase solutions examined likewise give Lewin's reaction for aldehydes [Cohn, 1916]; when a peroxydase solution is added to a mixture of piperidine and sodium nitroprusside solution a blue colour develops. This changes to bluish-green on adding glacial acetic or dilute hydrochloric or sulphuric acid. The original blue colour changes to rust-colour on adding strong mineral acids, and the blue colour is restored on dilution. A rust-colour is likewise obtained on adding caustic soda to the original solution.

The possible aldehyde nature of plant peroxydases will be dealt with further in a subsequent paper on the enzyme itself.

PEROXYDASE ACTIVITY OF METALLIC OXIDES.

Bertrand [1897] in his investigation of laccase suggested that the activity of this substance is due to its manganese content. He found that all the salts of manganese which he examined were capable of causing the oxidation of guaiacum in presence of oxygen. He observed that the salts of weak acids had considerably greater oxidising power than those of strong acids, and concluded that the active substance was manganous oxide formed by hydrolysis. It is known that the presence of free acid markedly inhibits the action of oxydases and peroxydases. For this reason, and in view of the fact that the peroxydase activity of aldehydes has been shown to be due to a substance of oxide nature, the present study of peroxydase activity among inorganic compounds has been confined chiefly to metallic oxides. The results are recorded in the table given on p. 34.

The tests were made by adding a small quantity of the oxide (sufficient to cover the tip of a pen-knife blade) to a few cc. of a 1 % solution of guaiaconic acid in 50 % alcohol. Each oxide was tested with the reagent alone, and in presence of a drop of 2.5 % hydrogen peroxide. The test in each case was repeated with indophenol mixture (1 % *p*-phenylenediamine and 1 % α -naphthol in 50 % alcohol). Only such colorations are recorded (p. 34) as were really distinct, appearing in at least one minute.

The peroxides of calcium, barium, strontium, sodium and potassium had no action on guaiaconic acid with, or without, hydrogen peroxide, but in presence of a substance which behaves as a peroxydase it was found that these peroxides, when added in small quantities, were capable of replacing hydrogen peroxide in the oxidation of guaiaconic acid. The general structural formula given for these peroxides is $M \begin{smallmatrix} \diagup O \\ \diagdown O \end{smallmatrix}$.

In each case where an oxide was found to behave as an oxydase or peroxydase a distinct evolution of oxygen from hydrogen peroxide was also found to take place.

Discussion.

Relatively little of a really decisive nature appears to be known of the structural formulae of inorganic substances. Most of the formulae given in the tables, however, are those generally accepted for the oxides in question. Metallic peroxides fall into two main classes corresponding to the ozonides and antozonides of Schönbein or the polyoxides and superoxides of Mendeléef. Schönbein's ozonides include the peroxides of the heavy metals and his antozonides those of the alkali metals and of the alkaline earths. The former, it was assumed, were derived from ozone and the latter from hydrogen peroxide. It is worthy of note in the present connection that the metallic oxides which are capable of oxidising guaiaconic acid, with or without addition of hydrogen peroxide, are those of the metals which form peroxides of the so-called ozonide or polyoxide type. On the other hand, Mendeléef's

Copper.

Oxide	Guaiaconic acid	Guaiaconic acid and H_2O_2	Indophenol mixture	Indophenol mixture and H_2O_2
Cupric oxide $\text{Cu}=\text{O}$	No action	Coloration	No action	Coloration

Lead.

Lead monoxide $\text{Pb}=\text{O}$	No action	Coloration	No action	Coloration
Lead dioxide $\text{Pb} \begin{smallmatrix} \diagup \text{O} \\ \diagdown \text{O} \end{smallmatrix}$ or $\text{Pb}=\text{O}=\text{O}$	Coloration	—	Coloration	—

Manganese.

Manganous oxide $\text{Mn}=\text{O}$. Prepared by heating carbonate in a current of nitrogen	} Coloration on brief shaking with air	No coloration	No coloration	Coloration
Manganese dioxide $\text{Mn} \begin{smallmatrix} \diagup \text{O} \\ \diagdown \text{O} \end{smallmatrix}$ or $\text{Mn}=\text{O}=\text{O}$		—	Immediate coloration	—
Mangano-manganic oxide $\text{Mn} \begin{smallmatrix} \diagup \text{O} \\ \diagdown \text{O} \end{smallmatrix} \text{Mn} \begin{smallmatrix} \diagup \text{O} \\ \diagdown \text{O} \end{smallmatrix} \text{Mn}$	} No action	No action	No action	No action
Prepared by heating MnO_2 at a white heat for six hours				

Iron.

Ferrous oxide $\text{Fe}=\text{O}$. Prepared by heating iron in a current of carbon dioxide	} No action	Coloration	No action	Coloration
Ferric oxide $\text{O} \begin{smallmatrix} \diagup \text{Fe}=\text{O} \\ \diagdown \text{Fe}=\text{O} \end{smallmatrix}$ or $\text{Fe} \begin{smallmatrix} \diagup \text{O} \\ \diagdown \text{O} \end{smallmatrix} \text{Fe}$		No action	No action	No action
Magnetic iron oxide $\text{Fe} \begin{smallmatrix} \diagup \text{O}-\text{Fe}=\text{O} \\ \diagdown \text{O}-\text{Fe}=\text{O} \end{smallmatrix}$	} No action	Coloration	No action	Coloration

Nickel.

Nickelous oxide $\text{Ni}=\text{O}$. Prepared by heating nickel carbonate in a current of nitrogen	} No action	No action	No action	Coloration
Nickelic oxide $\text{O}=\text{Ni}-\text{Ni} \begin{smallmatrix} \diagup \text{O} \\ \diagdown \text{O} \end{smallmatrix} ?$		—	Immediate coloration	—
Prepared by gently heating nickel carbonate in air				

Cobalt.

Cobaltous oxide $\text{Co}=\text{O}$. Prepared by heating cobalt carbonate in current of nitrogen	} No action	Coloration	No action	Coloration
Cobaltic oxide $\text{O}=\text{Co}-\text{Co} \begin{smallmatrix} \diagup \text{O} \\ \diagdown \text{O} \end{smallmatrix} ?$		—	Immediate coloration	—
Prepared by gently heating cobalt carbonate in air				
Cobalto-cobaltic oxide $\text{Co} \begin{smallmatrix} \diagup \text{O}-\text{Co}=\text{O} \\ \diagdown \text{O}-\text{Co}=\text{O} \end{smallmatrix}$	} No action	Coloration	No action	Coloration
Prepared by heating cobalt carbonate at white heat for six hours				

superoxides or Schönbein's antozonides are found to be capable of replacing hydrogen peroxide in the guaiacum reaction. When we consider the formulae of the oxides (such as cupric oxide, lead monoxide, ferrous oxide, ferroso-ferric oxide, cobaltous oxide and cobalto-cobaltic oxide) which behave as peroxydases, it will be seen that the common feature in each is an oxygen atom linked thus, $=O$. It has already been pointed out that this is the type of oxide group which characterises the active derivatives of aldehydes.

The behaviour of manganous oxide in the foregoing experiments deserves special notice. When added to a solution of guaiaconic acid a coloration rapidly appears on shaking with air in the absence of hydrogen peroxide. When hydrogen peroxide is present the guaiaconic acid is not oxidised, while a rapid decomposition of the peroxide takes place with evolution of oxygen. It is known that hydrogen peroxide in neutral or alkaline solutions readily transforms manganous hydroxide into the dioxide which catalytically decomposes the hydrogen peroxide. Although manganese dioxide alone is capable of immediately oxidising guaiaconic acid it is found that in presence of excess of hydrogen peroxide only the catalytic interaction of the dioxide and hydrogen peroxide takes place. If guaiaconic acid be present it is not attacked by the dioxide under these conditions. Thus, if a small quantity of manganese dioxide be placed in a test-tube and a couple of cc. of 2.5 % hydrogen peroxide be added, guaiaconic acid quickly added afterwards is not oxidised. In support of the above explanation of the behaviour of manganous oxide it is found that if an aqueous solution of ethyl hydroperoxide be used in place of hydrogen peroxide, manganous oxide immediately causes the oxidation of guaiacum. With organic peroxides, therefore, manganous oxide seems capable of behaving as a peroxydase.

The relative ease with which manganous oxide causes the oxidation of guaiaconic acid in presence of air is no doubt due to the facility with which the manganous oxide oxidises in air to manganese dioxide. The dioxide, as will be seen above, is itself capable of oxidising guaiaconic acid.

The marked difference between the manner in which manganous oxide reacts towards guaiaconic acid and that in which it behaves towards indophenol mixture remains to be considered. No coloration is obtained with indophenol mixture in the absence of hydrogen peroxide. The inhibiting influence of phenolic substances on autoxidation has been referred to in a previous paper [Gallagher, 1923]. There appears to be little doubt that the presence of α -naphthol in the reagent inhibits the autoxidation of the manganous oxide. This explanation is supported by the observation that if manganous oxide be added to guaiaconic acid solution containing a little α -naphthol no oxidation of guaiaconic acid takes place on shaking with air. Addition of a little quinol or catechol was likewise found to prevent the oxidation of guaiaconic acid by manganous oxide. It was noted that the phenol itself gradually underwent oxidation when present in the mixture. On the other hand indophenol mixture, unlike guaiaconic acid, is oxidised by manganous

oxide in presence of hydrogen peroxide. This appears to be due to the fact that indophenol mixture is much more easily oxidised by hydrogen peroxide than is guaiacum, and a relatively weaker stimulus is required to accelerate the oxidation. The case of nickelous oxide is a further illustration of the difference between guaiacum and indophenol mixture in this respect.

The apparent inhibitory influence of phenolic substances on the autoxidation of manganous oxide recalls observations made in the previous paper of this series in regard to the action of these substances on peroxide formation in plant tissues. The question arises as to whether the action of oxygen on manganous oxide results in the introduction of a peroxide group similar to that formed in the case of lecithins, terpenes and other common autoxidisable substances. The peroxides formed in such cases appear to be structurally related to hydrogen peroxide. They are capable of replacing hydrogen peroxide in testing for peroxidases. Does manganese dioxide, for instance, contain

the group $\begin{array}{c} -O \\ | \\ -O \end{array}$ which appears to be characteristic of peroxides of the hydrogen peroxide type? As already pointed out, it is formed in the action of hydrogen peroxide on manganous oxide. Lead dioxide, which resembles manganese dioxide in oxidising properties, is likewise formed from the monoxide by the action of a neutral or alkaline solution of hydrogen peroxide. Cupric oxide reacts with hydrogen peroxide to form an unstable dioxide of the formula CuO_2 , H_2O or CuO , H_2O_2 . This compound is probably structurally represented by

The evidence suggests that the dioxides of manganese and lead in their hydrated forms are of similar structure.

The present survey of the subject, therefore, suggests that for the oxidation of substances such as those in use as oxydase reagents the presence of two different types of oxides is essential: (i) the ozonide or mono-peroxide type, $=\text{O}$, characteristic of peroxidases and (ii) the hydrogen peroxide type,

$\begin{array}{c} -O \\ | \\ -O \end{array}$, characteristic of the so-called "oxygenases." These may, or may not,

be found in the same plant constituent. The behaviour of manganous oxide is an illustration of how they may both be found in the same molecule. In a study of the catalytic decomposition of hydrogen peroxide Kastle and Loevenhart [1903] concluded that the first stage in the decomposition is the formation of an unstable additive higher oxide. It appears likely that some such additive compound is also the active oxidising agent in oxydase reactions, *e.g.*

The structural relationship of a substance of this type to ozone is significant

in view of the fact that ozone readily oxidises the reagents employed in testing for oxydases.

ORGANIC SUBSTANCES CAPABLE OF OXIDISING GUAIACONIC ACID.

Two organic substances which possess the property of oxidising guaiacum directly have been met with in the course of the present work, viz. quinone and benzoyl peroxide. It is interesting to note that a certain amount of controversy has existed as regards the constitution of these two substances. Quinone has been variously represented as

Benzoyl peroxide has been given the formulae:

[Willstätter and Hauenstein, 1909]

[Rius y Miró, 1920]

In view of the foregoing conclusions, the direct oxidation of guaiacum by these substances would suggest that each is a tautomeric mixture of both forms.

CONCLUSIONS.

The observations made on the peroxydase activity of inorganic oxides suggest that the presence of one or other of the heavy metals, such as manganese or iron, in a plant may undoubtedly have a direct bearing on oxydase activity. In this respect the relation between the behaviour of manganous oxide and the known activity of direct oxydases is significant. Iron, in the state of oxide, only appears to influence the oxidation of guaiaconic acid when in the ferrous condition. The inactivity of ferric oxide seems to favour the closed formula, $\text{Fe} \begin{smallmatrix} \text{O} \\ \diagup \quad \diagdown \\ \text{O} \end{smallmatrix} \text{Fe}$. X-ray spectra of ferric oxide likewise support this formula. On the other hand it has been observed that ferric salts frequently behave as peroxydases. It is known that ferric salts, even of strong acids, readily undergo hydrolysis with production of the corresponding oxyalts:

Here again, we have production of the oxide group typical of ferrous oxide.

Nonetheless there exists much evidence that the chief enzyme constituent in the plant is a substance which is separable from mineral matter. The aldehyde reactions given by partially purified peroxydase preparations, as already quoted, seem to be of considerable significance in this respect. In a study of the natural enzyme, experiments have been carried out which

appear to throw considerable light on the nature of peroxydase and its relation to the various types of substances studied in the present paper.

SUMMARY.

A study is made of a series of substances which, like the enzyme peroxydase, are capable of activating the oxygen of hydrogen peroxide and of similarly constituted peroxides.

It is shown that peroxydase activity is commonly found to be associated with aliphatic aldehydes. It is not a property of the pure aldehyde, but is due to a substance of oxide nature which is formed by the action of atmospheric oxygen. This oxide appears to be analogous with the monoperoxide formed by the action of ozone.

A series of reactions is given which show that the peroxydase enzyme preparations obtained by alcoholic precipitation contain a substance or substances of aldehyde nature.

The influence of various metallic oxides on the oxidation of guaiaconic acid and of indophenol mixture by peroxide was studied. The property of inducing the oxidation of these reagents is found to be fairly generally associated with derivatives of the so-called heavy metals.

The results in general indicate that substances behaving as peroxydases are characterised by the presence of an oxide group of the type $=O$. Such a group, in conjunction with the ordinary peroxide group in which oxygen is

linked to oxygen, $\begin{array}{c} -O \\ | \\ -O \end{array}$, gives rise to a higher oxidation potential than that possessed by either group alone. It is suggested that as a preliminary stage in the oxidation combination takes place between these two types of oxide yielding a substance of oxidation potential comparable to that of ozone:

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