LXIII. MECHANISM OF OXIDATION IN THE PLANT.

PART I. THE OXYGENASE OF BACH AND CHODAT: FUNCTION OF LECITHINS IN RESPIRATION.

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In the ordinary guaiacum test for plant oxydases two types of reaction are met with according to the variety of plant examined. Several species, notably *Russula* and *Lactarius*, are capable of reacting directly with a fresh alcoholic solution of guaiacum resin with production of its characteristic blue oxidation product. On the other hand, the majority of plant species only give the guaiacum reaction in presence of hydrogen peroxide, or of such substances as peroxidised turpentine, etc. In an attempt to correlate these two types of reaction Bach and Chodat [1903, 1] suggested the existence of two enzymes in the plant oxidative system: (i) a substance which they termed "oxygenase," which has the property of fixing atmospheric oxygen in such a manner as to produce a peroxide, and (ii) a "peroxydase" which catalyses the decomposition of the peroxide, with liberation of active oxygen. The view of these authors has been rather generally, though not universally, accepted. It is admittedly questionable as to whether all the so-called direct oxydases are resolvable into the two types of constituents suggested. At the same time it will be realised that if plants which give only the indirect, or peroxydase, reaction are, in the ordinary course of metabolism, to effect oxidations at all comparable to that of guaiacum or of the other reagents usually employed, some substitute within the plant for the hydrogen peroxide which must be added in the ordinary laboratory test, will be admittedly essential.

Existence of Peroxides in Plant Juices.

The existence of peroxides in plant juices has given rise to a certain amount of controversy. De Clermont [1875] reported the existence of hydrogen peroxide in vegetable juices. His observation was supported by Griesmayer [1876], Béchamp [1882], and by Bert and Regnard [1882]. Bellucci [1878] could not confirm the observation of these authors by means of the ordinary chromic acid reaction. It is to be observed, however, that the chromic acid reaction, though sensitive for hydrogen peroxide, is rarely given by other peroxides. Generally speaking, it is a matter of no little difficulty to distinguish between hydrogen peroxide and peroxide derivatives when the substance in question is present in relatively small quantity. The reactions, therefore,

which the above authors attributed to hydrogen peroxide may possibly have been given, instead, by organic peroxides present in the cell-sap. Bach and Chodat [1902] showed that the juice of *Lathraea squamaria* contained a substance which turned potassium iodide-starch paper blue. It could be precipitated with 1 % baryta solution. On washing the precipitate and decomposing with weak acid the product was found to be non-reactive towards titanium sulphate, but continued to react with potassium iodide-starch paper. The solution gave no reaction for nitrous acid. They therefore concluded that the reactive substance present was a peroxide. Onslow, M. W. [1919, 1920] also brought forward evidence in favour of the formation of peroxides in various plant extracts.

Evidence obtained in the course of the present work indicates that a substance, or substances, of a peroxide nature is formed in plant extracts in contact with air. It is a matter of common experience that the outer portion of vegetable roots is found to give the direct oxydase reaction with guaiacum, while the inner portion of the root only gives the indirect or peroxydase reaction. If, for instance, a thin section be cut from the surface of a potato and the portion of the tuber so exposed be treated with fresh guaiacum tincture, an immediate intense blue coloration is obtained. If a tuber be cut in two through the centre, a solution of guaiacum poured over the fresh surface produces an immediate blue coloration only in the portion near the skin. Subsequent addition of hydrogen peroxide results in the production of a blue colour of nearly uniform intensity over the whole surface. It is frequently found that if a section of tuber, cut through the centre as in the last instance, be allowed to remain exposed to air for some time before treatment with guaiacum, a more or less uniform coloration over the whole surface is obtained. It is not, however, always a simple matter to demonstrate peroxide production in a plant tissue by this latter method. Moore and Whitley [1909] suggest that the occasional failure of this test is due to the destructive action of excess of oxygen on the peroxide first formed. Since, however, the test is nearly always positive in winter and frequently negative when tissues are examined in the summer, a more likely explanation would seem to be that at a time of relatively great vegetative activity the peroxide is used up as quickly as it is formed.

Various authors have suggested that the difference between the direct oxydase reaction and the indirect, or peroxydase, reaction is one of degree only, depending on the relative concentrations of a single oxidising enzyme. The fact that peroxydase enzyme preparations of very different concentrations are not known to show any difference in their manner of reacting with guaiacum would appear to be strong evidence against this view. When a vegetable extract has been made capable, by exposure to air, of directly causing the oxidation of guaiacum (without special addition of peroxide) the change accomplished is found to have taken place in a constituent other than the peroxydase. The following observations make this point clear.

Some freshly-peeled potatoes or mangold roots are finely minced and the pressed-out juice is treated with not less than five times its volume of rectified spirit. This ensures fairly complete precipitation of the peroxydase. After standing for a day the precipitate is filtered off. The peroxydase contained therein is purified somewhat by dissolving in water and reprecipitating. A solution of peroxydase so obtained gives no coloration when added to fresh guaiacum tincture. If a few cc. of the fresh alcoholic filtrate be diluted with an equal volume of distilled water and then added to the mixture no oxidation of the guaiacum will be found to take place. It will be found, however, on storing the two fractions obtained from the plant extract, in contact with air, that the alcohol-insoluble fraction shows no alteration in the manner of its reacting with guaiacum, while the alcohol-soluble fraction gradually acquires the power of reacting with guaiacum in presence of peroxydase. This alteration which takes place in alcoholic extracts of plants on storing in contact with air would seem to be the most reliable method of demonstrating the formation of peroxide from plant material. The percentage of alcohol (nearly 80 %) in the extracts is such as to eliminate bacterial action.

These results lend very considerable support to the Bach-Chodat view as to the dual nature of the oxidative system of the plants examined. The question arises as to what extent the classification by these authors of the production of peroxide as an enzyme reaction, as ordinarily understood, is justified. An investigation as to the nature of the substance concerned was therefore undertaken.

Nature of the Peroxide-forming Constituent of the Plant-cell.

Moore and Whitley [1909] in a study of oxidising enzymes demonstrated the presence of peroxides in plant juices and their formation by action of atmospheric oxygen. They disputed, however, the intervention of a special enzyme in the production of these peroxides, as had been suggested by Bach and Chodat. Onslow, M. W. [1919, 1920] asserted that the peroxide was produced by a derivative of catechol present in the plant, and that the fixation of oxygen to this body was catalysed by an "oxygenase" also said to be present. Prior to the publication of the Bach and Chodat theory Kastle and Loevenhart [1901] stated that oxydase is not a true ferment, but an organic peroxide. They explained its activity on Baeyer and Villiger's theory in that it acts as a carrier of oxygen in the same way as does benzaldehyde, but is not a true catalytic agent. The Baeyer and Villiger theory referred to is in reality that of Bach [1897]. In dealing with the well-known phenomenon of autoxidation Bach suggested that the substance undergoing autoxidation united with whole molecules of oxygen to form a peroxide. The peroxide thus formed, or the hydrogen peroxide in the so-called nascent state resulting from its decomposition, was held to be the active oxidising agent in such a case, for instance, as the oxidation of indigo simultaneously with the autoxidation of benzaldehyde or of oil of turpentine. A peroxide of benzaldehyde of the type suggested

by Bach was subsequently prepared by Baeyer and Villiger [1900]. It will be noted that Kastle and Loevenhart regarded oxydase as a single active substance. Their statement, that organic peroxides give an immediate blue coloration with guaiacum, could not be confirmed in the present work, other than in the case of benzoyl peroxide. Organic peroxides in general, like hydrogen peroxide, only cause a rapid oxidation of guaiacum in presence of a catalyst, such as various salts of manganese or iron, or in presence of natural peroxydase. The necessity for some such catalyst is at present fairly generally accepted[1].

In connection with the mechanism of the production of peroxide in the plant, it will be seen that the view of Kastle and Loevenhart, in which it is suggested that one of the plant constituents is of an autoxidisable nature is relatively more simple and in keeping with the facts than the assumption of a special ferment. The known autoxidisable organic compounds are invariably substances of an unsaturated nature. The production of a peroxide as an essential stage in autoxidation phenomena, though difficult to demonstrate in some cases, has, none the less, been confirmed in many instances. Thus Engler and Weissberg [1904] have succeeded in preparing, in a pure condition, peroxides of fulvene formed by the action of molecular oxygen. Harries and Muller [1902] describe similar preparations. In many other cases, though the actual peroxide has not been isolated, its presence can be shown by means of suitable reagents. Unsaturated substances present in oil of turpentine give rise to peroxide derivatives in presence of air. As is well known, aerated turpentine may be substituted for hydrogen peroxide in the ordinary guaiacum test for peroxydases. The same is true of aerated benzaldehyde or linseed oil, though in these cases but slight amounts of peroxide seem to be present.

M. W. Onslow [1919, 1920] claims to have shown that the blackening of plant tissues on injury is due to the oxidation of a substance which contains a catechol group, and it is stated that this same phenolic substance functions as the medium of peroxide production in plant tissues. The *ortho*-diphenol concerned has not been characterised further than by the green coloration given by certain plant extracts with ferric chloride. For the purpose of investigating the generalisations mentioned an examination was made in the present work of the root of the common mangold, *Beta vulgaris*. The mangold root, on mincing, first becomes brown and then intensely black on exposure to air. An extract of the root, prepared as described further on (Experimental Part, Section 3) was found to give a blue-black precipitate with a trace of ferric chloride. On adding excess of the salt a green coloration was obtained. On further examination, the extract was found to give many reactions charac-

[1] The guaiacum solution used in the present work was made by dissolving 0·5 g. of translucent resin in 5 cc. of boiling alcohol. An equal volume of water was then added. No coloration should be obtained on addition of a pure peroxydase; nor should any oxidation take place on addition of peroxide in absence of peroxydase. It will usually be found that only fresh solutions of the reagent fulfil both of these conditions.

teristic of the tannins. It is thus evident that the coloration obtained with ferric chloride is due to a substance of this nature. The substance present in the mangold appears to be a gallo-tannin, and the question arises as to whether the catechol derivative referred to above be not, in fact, a catechol tannin.

Peroxide-forming property of Tannin-fraction from the Mangold.

After allowing the tannin-containing extract to remain in the bottom of a stoppered Erlenmeyer flask for a couple of weeks it was found that on adding a quantity to a mixture of the peroxydase enzyme and fresh guaiacum tincture, a slight oxidation of the guaiacum took place. The solution was subsequently stored in a dark place for some months. A black amorphous substance was deposited. After two months the test with a mixture of peroxydase and guaiacum was found to give quite an intense blue coloration. At this stage, however, it was noted that the solution no longer gave any coloration whatsoever with ferric chloride. Quantitative study here will be necessary to determine definitely whether production of peroxide continues after the disappearance of the tannin reactions. It would appear from the qualitative study made that the production of peroxide tends noticeably to increase as the phenolic reaction disappears.

"Anti-oxygen" substances.

It will be seen from the foregoing that the view that catechol derivatives are the peroxide-forming substances of the plant is based on insufficient evidence. The inhibitory influence of phenols on the fixation of oxygen to autoxidisable substances, as recently studied by Moureu and Dufraisse [1922] renders this view of the function of phenols in the plant rather untenable. The results of these authors show that the addition of phenols or tannins to autoxidisable compounds prevents the formation of peroxide, and they ascribe to such substances the rôle of anti-oxydases or oxydation buffers (anti-oxygènes).

The Blackening of Vegetable Juices on Exposure to Air.

When the fresh tannin-containing fraction from the mangold root is mixed with a solution of the peroxydase fraction from the same root the blackening characteristic of the fresh juice is obtained on exposure to air. It was found, however, that the aqueous residue which remained after extraction of the tannin portion with ether-alcohol mixture likewise gave this blackening even to a much stronger degree. It was found on exhaustively extracting this aqueous portion with ether that the substrate of the blackening process still remained in the aqueous layer after the substance which gave the ferric chloride coloration was completely removed. This clearly indicates that the compound which gives rise to the black oxidation products must be other than that which gives the ferric chloride coloration. Closer examination of the blackening process led to the conclusion that the characteristic blackening of the juice of the mangold is, in fact, due to the action of tyrosinase on tyrosine. A study of the darkening of the juice of the potato led to a similar conclusion (see

Experimental Part, Section 2). Bertrand [1896] attributed the blackening of the sap of beetroot, potato and dahlia to the oxidation of tyrosine by tyrosinase.

The Peroxide-forming Constituent of the Potato.

The bulk of the evidence would seem to indicate that the oxygenase of Bach and Chodat is a substance of an autoxidisable nature. Experiments were consequently carried out with a view to the isolation of such substance or substances from the plant. The material chosen for special study was the potato tuber. In this particular isolation there existed the disadvantage that the substance sought for gave no direct characteristic reaction which would enable its location after a series of operations to be rapidly and easily ascertained. It was necessary to adopt the procedure of starting with material which gave no reaction for peroxide when treated with a mixture of fresh guaiacum tincture and peroxydase solution and subsequently carrying out operations as far as possible in absence of oxygen. It was thus possible to ascertain whether any particular fraction obtained during the course of the treatment contained a constituent of peroxide-forming properties by exposing a portion of it in solution to the action of air or oxygen and subsequently testing this with guaiacum-peroxydase mixture.

The experimental details of the method, as finally adopted, are given further on. Briefly, the method consists in finely mincing the tubers and in extracting for 2 hours with alcohol on the water-bath at 50–60°. The product is filtered and evaporated down nearly to dryness under reduced pressure. The aqueous residue in the flask is then extracted with ether. The ether extract is filtered and evaporated down to small bulk. It is then poured into a large excess of acetone. The precipitate having been allowed to settle, the acetone is filtered off. The precipitated material is purified by frequent repetition of the process of precipitation from ethereal solution by means of acetone. The substance thus obtained proved to be of phosphatide nature. The acetone washings from each precipitate were found, after standing some hours in presence of air, to develop the peroxide reaction towards guaiacum-peroxydase mixture. This phenomenon was observed even after repeated precipitation. After removal of all traces of ether and acetone in a vacuum, an alcoholic solution of the substance exposed to air also showed peroxide formation. The final product from the acetone precipitations was precipitated from alcoholic solution as a cadmium chloride double compound. On decomposing this the regenerated lipin continued to give evidence of peroxide formation in presence of air.

Peroxide-forming property of Potato juice associated with the Lipins.

From these results it is evident that the production of peroxide in the potato is intimately associated with the lecithin of the tuber. The substance from which the peroxide is derived may either be the lecithin itself or a compound intimately associated with it. While bearing in mind the latter possi-

bility, it may be pointed out that, generally speaking, lecithins are unsaturated substances owing to their containing unsaturated fatty acids in the molecule, and autoxidation is very generally associated with unsaturation. Erlandsen [1907] gives some extremely interesting details as regards the autoxidisable nature of a lecithin which he examined. The freshly prepared substance proved to have the formula $C_{71}H_{125}NP_2O_{21}$. After storage for some time in dry air it was found to have altered to $C_{71}H_{125}NP_2O_{30}$. Storage of the material for one month in an unevacuated desiccator resulted in an increase in weight of nearly 9 %. The iodine value of the fresh material he found to be 101; after combination with oxygen it was found to have fallen to 22. This change of iodine value appears to indicate alteration of the fatty acid radicles under the influence of atmospheric oxygen. Thunberg [1911] makes the observation that lecithin is oxidised in air by ferrous ammonium sulphate. It would thus appear to form a peroxide after the manner of benzaldehyde (Bayliss).

Function of Lecithins in Respiration.

Palladin and Stanewitsch [1910] pointed out an apparent relationship between the lecithins and plant respiration. They treated wheat embryos with organic solvents and then soaked them for a short time in water, estimating the amount of carbon dioxide evolved during definite intervals. They found that the respiration energy was lowest after treatment with those solvents which extracted most lipin. Vernon [1912, 1914] claims to have demonstrated the dependence of the action of oxydases in the animal organism on the cell lipins. He concluded that the effect of the oxydase is dependent on the lipin membrane which, he suggested, holds together the tissue oxygenase and peroxydase and makes possible their mutual enzymic activity. If, however, as the present research on the potato indicates, the lipin be itself an oxygenase the results of Vernon, as also those of Palladin and Stanewitsch, are readily explicable.

Lecithins are known to be of very general occurrence in animal and vegetable cells, and their function, or functions, has so far remained rather obscure. The property of lecithins of being able to combine readily with molecular oxygen to produce a peroxide would therefore seem to be of rather general importance from the point of view of biological oxidation. Dakin [1922] has pointed out the marked resemblance between oxidation within the animal body and oxidations effected in the laboratory by means of hydrogen peroxide. Kostytschew [1910] claims to have succeeded in oxidising certain degradation products of glucose by the simultaneous action on these substances of a vegetable peroxydase and hydrogen peroxide. Free hydrogen peroxide appears to be practically absent from both vegetable and animal tissues. It is known to be relatively toxic to both, and the ferment catalase, of very general occurrence, prevents its accumulation. According to Bach and Chodat [1903, 2] catalase is entirely without action on "oxygenase" or on ethyl hydroperoxide. It is thus evident that a substituted peroxide of the type of ethyl hydroperoxide

must be the active oxidising agent in the cell rather than free hydrogen peroxide. Such peroxides, as a rule, are found to be very weak oxidising agents and require the addition of a catalyst before oxidations such as that of guaiacum, benzidine, etc., can take place at all readily. In the plant the catalyst is usually a peroxydase. It yet remains to be definitely determined whether the lecithin of animal tissues is capable, by the production of peroxide, of causing the oxidation of other substances present[1]. The well-known peroxydase reaction of haemoglobin with respect to guaiacum, benzidine, etc., is of interest in this respect, and suggests that haemoglobin or some closely related substance in animal tissues may be capable of fulfilling a catalytic rôle comparable to that of peroxydase in the plant.

Plant Constituents other than the Lipins which may function as "Oxygenases."

Unsaturated substances, as has already been pointed out, are very frequently found to be autoxidisable. It is thus reasonable to expect that unsaturated compounds, other than the lipins, present in a plant may, according to circumstances, function as "oxygenases." The formation of peroxide by oil of turpentine, itself a plant product, is well known. It appeared interesting, therefore, to submit to examination a number of other terpenes with a view to ascertaining whether they behave in a similar manner. Specimens supplied by British Drug Houses, Ltd., were examined as delivered. The following gave a strong blue coloration with guaiacum-peroxydase mixture: limonene, cumene, cedrene, phellandrene, terpineol, terebene, terpinol, linalol and carvone. Pinene and carvene gave no coloration on preliminary examination. On placing a small quantity of each in test-tubes and passing a current of oxygen through for about four hours they were found, however, to have acquired the property of causing the oxidation of guaiacum in presence of peroxydase. On the other hand, samples of caryophyllene, citral, citronellal, camphene and terpene hydrate, even after oxygen had been passed through them for eight to nine hours, gave scarcely any coloration with guaiacum-peroxydase mixture. Like pinene and carvene, a specimen of oleic acid gave no reaction for peroxide until oxygen had been bubbled through it for three to four hours. The production of peroxide by oleic acid is of especial interest in view of its frequent occurrence in the molecule of lecithin-like substances.

These observations on the occurrence of peroxide among the terpenes examined are provisional. It is possible that in individual cases where a positive reaction for peroxide was obtained, the reaction may be due to an autoxidisable impurity present; or on the other hand, in those instances where no peroxide appears to be formed, the negative result may be due to an inhibiting substance. The results as given are none the less interesting as indicating the ease with which the terpenes, as a class, give rise to peroxides. It appears quite likely, in consequence, that these substances are thus capable of taking part in the respiration of the plants in which they are found.

[1] A specimen of egg-lecithin in alcoholic solution was found, after several days' exposure to air, to acquire the power of oxidising guaiacum in presence of peroxydase.

Experimental Part.

1. *Preparation of Solutions of Peroxydase from the Mangold root and the Potato.*

The solutions of peroxydase employed in the course of the present work were invariably prepared from the root of the mangold. The most suitable variety of root for the preparation is that known as "yellow globe." The product obtained from the mangold is not a pure peroxydase, but contains a considerable proportion of tyrosinase. The same is true of that from the potato tuber. It would appear that most tyrosinase preparations so far studied give the peroxydase reaction with guaiacum, but whether this is due to admixture with ordinary peroxydase or a property of tyrosinase itself it is impossible to say.

For the purpose of preparing the enzyme solution the plant material is first finely minced. The juice is then pressed out from the minced material through a double layer of fine muslin, and is treated with about five times its volume of rectified spirit. The mixture is allowed to stand overnight. The precipitate which collects is then filtered off. It is purified somewhat by redissolving in water and reprecipitating the enzyme by the addition of more alcohol in approximately the same proportion as above. The precipitate obtained in this case is likewise allowed to settle. After again filtering off, it is dissolved in a quantity of distilled water approximately equivalent to one-fifth the volume of the original juice.

The solution of peroxydase thus obtained from the mangold root, or from the potato, is a clear liquid of slight brown colour. In presence of hydrogen peroxide it oxidises guaiacum, guaiacol, benzidine, *p*-phenylenediamine, α-naphthol, etc. In the absence of hydrogen peroxide it is without action on these substances. A mixture of peroxydase and one of these substances is consequently an excellent reagent for detecting hydrogen peroxide, either in the free condition, or combined as a peroxide derivative. In the course of the present work use has been made of a mixture of peroxydase solution and fresh guaiacum tincture for this purpose. For this test to be reliable it is essential that care be taken to use a guaiacum solution free from peroxide, otherwise the mixture of guaiacum solution and peroxydase alone will yield the characteristic blue colour. It is likewise essential to note that the substance under examination be incapable of oxidising guaiacum in the absence of peroxydase[1].

[1] Guaiacum is readily oxidised by ozone, nitrogen peroxide, free nitrous and nitric acids, permanganates and chromates. In each of these cases, however, the action takes place in the absence of peroxydase. In the case of solutions, therefore, where guaiacum is oxidised only when peroxydase is also added there is ample evidence for believing that the solution contains either hydrogen peroxide or other peroxides of similar constitution. The guaiacum reaction, as described, would thus appear to be a specific test for peroxides. This cannot be said of the potassium-iodide-starch test which has been commonly employed in dealing with these substances. Guaiacum-peroxydase mixture appears to be considerably more sensitive to organic peroxides than mixtures of peroxydase and other common oxydase reagents (such as benzidine etc.) which are sometimes employed.

2. *Blackening of the Juice of the Mangold and of the Potato due to the action of Tyrosinase on Tyrosine.*

The influence of tyrosinase on the blackening of the juice of the potato may be demonstrated as follows.

A quantity of potato tubers is finely minced and the pressed-out juice is quickly filtered or centrifuged. About 10 cc. of the clear juice is then transferred to a boiling-tube by means of a pipette, care being taken that the juice falls directly to the bottom and does not spill along the sides of the tube. The boiling-tube is then immersed in a water-bath at 75° and the juice is stirred with a thermometer. When the juice has attained the temperature of the water-bath the time is noted and heating is continued for 10 minutes at this temperature. The contents of the tube are then cooled.

It will now be found that although the contents of the tube still give a strong peroxydase reaction with guaiacum, no darkening in colour takes place on exposure to air. If a quantity of tyrosine or of *p*-cresol be added to a portion of the juice so heated, the characteristic colorations given by these substances in presence of tyrosinase are no longer obtained, although fresh potato juice gives this reaction. It is thus evident that the tyrosinase has been destroyed on heating. If now a solution of tyrosinase, prepared in the ordinary manner from any plant containing it, be added to another portion of potato juice which has been heated as described, the darkening in colour characteristic of fresh potato juice is again obtained. Addition of a pure peroxydase preparation, such as that from the horse-radish, produces no change. That the actual substrate in this blackening process in the case of potato juice is free tyrosine is rendered very probable by the isolation from the potato of considerable quantities of this amino acid in the free condition. The substance was isolated incidentally in another portion of this work (see Section 6, Experimental Part) and considering that it was obtained in an alcohol-ether extract of a concentrate of the juice, the yield of 0·3 g. from 2½ kilos. of fresh tuber is very probably far from quantitative.

A similar destruction of the tyrosinase in the mangold root results in the prevention of the blackening of the juice. The tyrosinase of the mangold is usually found to be somewhat more resistant to heat than that of the potato, and a rather longer period of heating than 10 minutes at 75° is found necessary to complete its destruction in some samples. The peroxydase is also relatively more resistant to heat in this case than in that of the potato.

3. *Study of the Tannin of the Mangold root.*

The alcoholic filtrates obtained in the preparation of the peroxydase of the mangold were now studied in connection with the suggestion made by Onslow, M. W. [1919, 1920] that the peroxide-forming constituent in the plant as well as the substrate in the blackening process of the juice was a derivative of catechol. The alcoholic solution (six litres) obtained after removing

the peroxydase-tyrosinase fraction of the juice was concentrated *in vacuo* at 40–50°. When all of the alcohol had been removed and the aqueous residue suitably reduced in bulk the distillation was stopped. The residue was then extracted with ether containing about 20 % of alcohol. The ether was distilled off from the extract. A residue consisting of a solution in a mixture of water and alcohol was thus obtained. With ferric chloride it gave a blue-black coloration or precipitate, which disappeared on adding excess of the salt, yielding a green solution. On submitting the extract to further examination the following reactions were observed:

(i) Addition of alkali causes the solution to darken in colour.

(ii) With a solution of ammonium picrate it gives a red colour, which changes to green.

(iii) On adding a little sodium sulphate, and then treating with a dilute solution of iodine a purple-red colour results.

(iv) With a solution of potassium cyanide scarcely any change takes place.

(v) A purple colour is obtained on adding, first, ammonia, and then nitric acid, to the solution.

(vi) On treating 2 cc. of the solution with three drops of 20 % thymol solution and then adding 3 cc. of strong sulphuric acid a deep red coloration is obtained.

(vii) Lime-water gives a greyish precipitate which rapidly turns blue.

These reactions are characteristic of the tannins, and it is thus evident that the coloration given by ferric chloride is due to a substance or substances of this type. It is interesting to note, moreover, that tannins, as a rule, are said to have an inhibiting influence on oxydase action.

4. *Peroxide-forming character of Alcoholic Extract of Plants, and Action of Phenols thereon.*

The production of peroxide by a substance in plant tissue may best be demonstrated by exposing an alcoholic extract of the tissues to air. An alcoholic extract for the purpose may conveniently be prepared as follows.

Fresh mangold roots, or a quantity of potato tubers, are peeled and then finely minced. The minced material is quickly strained through several layers of muslin, 500 cc. of the juice thus obtained are then added to 2 litres of rectified spirit. The mixture is well shaken and after being allowed to stand overnight, is filtered. The alcoholic extract thus obtained produces no change when added to a mixture of peroxydase and fresh guaiacum tincture. In making the test 5 cc. of the alcoholic plant extract is diluted with an equal volume of distilled water before adding it to a mixture of peroxydase, prepared as in Section 1, and a few drops of a 5 % solution of guaiacum in 50 % alcohol. The dilution of the extract with water is necessary to prevent precipitation of peroxydase. On storing the alcoholic extract in presence of air, no change in its behaviour relative to a guaiacum-peroxydase mixture is observed till after a certain length of time. In the case of an extract of potato, 50 cc. of solution in an uncorked

250 cc. flask, placed so as to be exposed to daylight, only began to oxidise guaiacum-peroxydase mixture after two days' exposure. An extract of the mangold exposed in a similar manner required over a week's exposure before the presence of peroxide could be detected. There is thus evidence of a certain latent period in the production of peroxide, and this is probably due, in part at any rate, to the presence of phenolic substances such as tannins. The inhibiting influence of such substances on the course of autoxidation has been demonstrated by Moureu and Dufraisse [1922]. In the course of normal plant metabolism the proportion of these inhibiting substances present is probably controlled by the peroxydase, the known oxidising power of the latter enzyme relative to phenolic substances being responsible for its being commonly classed as phenolase.

The rate at which peroxide is produced in the plant alcoholic extracts appears to be very much accelerated by the action of light. Light, however, is not essential to the production of peroxide, since extracts stored in the dark were also found to have acquired it. As might be expected, the free admission of oxygen has also a marked accelerating influence.

5. *Action of the "Anti-oxygènes" of Moureu and Dufraisse on the production of Peroxide in Plant extracts.*

The following table shows the influence of traces of some common phenols on the rate of peroxide formation in alcoholic extracts of the mangold, prepared as described in Section 4.

100 cc. alcoholic extract treated with phenol in proportion of 1 : 100,000	Test with guaiacum-peroxydase mixture after one week's exposure to air	Test with guaiacum-peroxydase mixture after two weeks' exposure to air
1. Quinol	No coloration	Faint blue coloration
2. Gallotannic acid	„	„ „
3. Pyrocatechol	Blue coloration	Very strong blue coloration which quickly faded
4. Control	„	Strong blue coloration

The inhibiting influence of traces of quinol and of gallotannic acid on peroxide formation is seen to be distinctive. That the action of these substances is to prevent the production of peroxide is shown by the fact that subsequent addition of peroxide to the solutions containing them, after treatment with guaiacum and peroxydase, gives rise to the usual intense blue colour. Pyrocatechol appears to be without inhibiting action in the dilution employed. In the case of potato extract, *p*-cresol was likewise found to be without inhibiting action when added in traces similar to the above. On the other hand, a trace of pyrogallol was found to be quite effective in preventing peroxide formation.

6. *Preparation of the Peroxide-forming Constituent of the Potato.*

The method by which this substance was first prepared consisted in finely mincing 2½ kilos. of freshly-peeled potato tubers. The minced material was then added to 7½ litres of rectified spirit and the mixture was allowed to digest in the cold for 24 hours. The solid matter was then filtered off on the Buchner

funnel. The filtrate was evaporated down *in vacuo* at 40–50° until 400–500 cc. of aqueous residue remained. A quantity of waxy material separated. This was removed by thorough extraction with ether. This ether extract was subsequently found to contain the bulk of the peroxide-forming constituent of the tuber. The aqueous portion remaining after the ether extraction was then shaken with a mixture of equal parts of alcohol and ether. On concentrating this extract somewhat, a yield of 0·3 g. of free tyrosine was found to separate. That this amount of tyrosine is by no means indicative of the total amount of this amino acid present in the original tuber is evident from a consideration of the solubility of tyrosine in the solvents employed, as also from the fact that tyrosine continued to separate slowly, in a rather impure condition, from the aqueous solution remaining after the extraction. The ether extract obtained above from the original concentrate was then worked up. On distilling off the ether a wax-like material was obtained. This was redissolved in 15–20 cc. of ether. On addition of about 250 cc. of alcohol a light yellow solid was precipitated. The precipitate was redissolved in a small quantity of ether and then poured into about a litre of acetone, with stirring. A precipitate, more nearly colourless than that given by alcohol was thus obtained. On allowing the acetone washings from this material to remain exposed to air for some hours and then testing them with peroxydase-guaiacum mixture a blue colour resulted, indicative of the formation of peroxide in the liquid. The precipitate obtained with acetone was again redissolved in a small quantity of ether and again poured into a large excess of acetone (500–600 cc.). This method of purification was repeated six times. In each case the acetone washings continued to give a peroxide reaction after exposure to air for some time. The final product, on drying off the acetone, became quite brown in colour, and contracted to a wax-like mass. On drying it in a vacuum desiccator it became quite brittle and could be easily reduced to powder. It proved to be relatively slightly soluble in alcohol. In alcoholic solution production of peroxide was also noted. About 0·2 g. of the substance was dissolved in alcohol by warming to 50–60°. 5 cc. of a saturated alcoholic solution of cadmium chloride were added. After standing for some time the precipitate which separated was filtered off. It was placed in a beaker and about 20 cc. of ether added. After acidifying with a drop of 10 % hydrochloric acid, the cadmium was precipitated by hydrogen sulphide and the suphide was filtered off. Excess of hydrogen sulphide was then removed with a current of air and the ethereal solution remaining was washed by shaking in a separating funnel, first with a saturated aqueous solution of sodium bicarbonate and finally with distilled water. The remaining ethereal solution was then poured into excess of acetone. The product thus obtained continued to show peroxide-forming properties. After prolonged action of air it was found capable of liberating iodine from potassium iodide, even in the absence of acid.

It is evident from these results that the formation of peroxide is due to the autoxidation of a lecithin-like substance. It has been pointed out by

various workers on plant lecithins that these substances are not very readily extracted from the tissues by cold solvents. Schulze and his co-workers, who investigated many lecithins of this type, carried out their extractions with alcohol at 50–60°. The extraction of a further quantity of lecithin from the potato tuber was accordingly carried out as follows.

Three kilos. of fresh, finely minced tubers were treated with five litres of 90 % alcohol and the mixture was digested on the water-bath for two hours. To prevent autoxidation at this temperature a current of nitrogen was passed through the liquid during the digestion. The extract was then pressed out from the solid material through a couple of layers of fine muslin. The liquid thus obtained was cloudy, owing to solid matter in suspension. On allowing it to stand for about a quarter of an hour the solid material readily settles down. The liquid may then be decanted and the remainder filtered on a Buchner funnel. The liquid was evaporated down as in the previous preparations and the residue was then thoroughly extracted with ether. After dehydrating the extract with anhydrous sodium sulphate the liquid was filtered and the greater part of the ether was distilled off. The ethereal solution remaining (about 50 cc.) was then added to a litre of acetone. The liquid was allowed to stand in the dark for about an hour and the lecithin which separated out was then filtered off and dried in a vacuum desiccator. The yield of lecithin thus obtained from three kilos. of fresh tuber was 1·6 g. The substance thus obtained may be further purified as in the previous preparation.

A specimen of the substance obtained in the present work was submitted to hydrolysis for the purpose of characterisation. 0·5 g. of the substance was boiled for five minutes with 12·5 cc. of 6 % sodium hydroxide solution. 2 cc. of glacial acetic acid were then added, and boiling was continued for a further minute. The acid liquid was allowed to cool. It was filtered through a moistened filter paper. The presence of phosphate in the filtrate was proved by precipitates given by ammonium molybdate and magnesium sulphate. By treatment with a solution of iodine in potassium iodide, no evidence of the production of "Florence crystals" said to be characteristic of choline [Struve, 1900] could be obtained, but microscopic crystals unlike those obtained from choline were precipitated. These may possibly be due to betaine, which substance is said to replace choline in some plant phosphatides. It is hoped in further work to continue the study of this phosphatide.

Summary.

The present work deals with the nature of the "oxygenase" in the Bach and Chodat theory.

It is shown that plant juices, on exposure to air, form peroxides. Alcoholic extracts of plant tissues, containing a sufficiently high content of alcohol to prevent bacterial action, are found to serve best for demonstrating this production of peroxide.

The formation of peroxide by the action of atmospheric oxygen on plant

extracts appears to be markedly influenced by the addition of certain phenolic substances such as quinol or gallotannic acid. Minute traces of such substances appear usually, though not always, to prevent the fixation of oxygen.

The suggested relation of catechol to the plant oxydase system is discussed. It is shown that the blackening of aqueous extracts of the potato and of the mangold is due, not to the oxidation of a catechol derivative under the influence of peroxydase, but to the action of tyrosinase on tyrosine. A detailed examination of the extract of the mangold root which gave a ferric chloride coloration revealed the fact that the reactive substance present was a tannin. The tannins, in general, are said to have an inhibiting influence on oxydase action.

There appears to be no definite evidence that the production of peroxide in the plant is due to enzyme action; it seems more likely that the formation of peroxide is due to the presence of an autoxidisable substance in the tissues. A substance of this nature isolated from fresh potato tubers was found to bear a close relation to the lipins. In contact with air or oxygen a solution of this substance acquires the property of causing the immediate oxidation of guaiacum in presence of peroxydase. It would thus appear that the so-called oxygenase of the potato is in reality an autoxidisable lecithin-like substance.

A study of the terpenes as possible "oxygenases" in the plants in which they occur would be of interest. It is shown that these compounds as a class are capable of combining with oxygen in such a manner as to cause the oxidation of guaiacum in presence of peroxydase.

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