VII. MECHANISM OF OXIDATION IN THE PLANT. PART III.

PEROXYDASE. OBSERVATIONS ON THE THERMO- STABILITY OF THE PEROXYDASE OF THE MANGOLD.

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IN a previous communication [Gallagher, 1924] an examination was made of various substances which, like the enzyme peroxydase, have the property of activating the oxygen of peroxides. The results of this study indicate that the activity of such substances is associated with the presence of an oxide group of the type $R = O$. A group of this type appears to be present in the monoperoxides of aliphatic aldehydes and, as a rule, in the oxides of the heavy metals. In the present paper the investigation is extended to the natural enzyme with a view to determining its possible relationship to the compounds already examined.

The peroxydase chosen for special study was that of the mangold root. The manner of preparing the enzyme by the method of alcoholic precipitation has already been described [Gallagher, 1923].

Peroxydase possesses properties which appear to justify its classification with typical enzymes. Its activity is destroyed on heating, though as a rule a higher temperature is usually required for its complete destruction than is the case with most other enzymes. Its power is also markedly influenced by variation of hydrogen potential, and it is readily precipitated by the common enzyme precipitants. Although, as Kastle and Loevenhart [1901] have pointed out, oxidising enzymes are practically uninfluenced by such enzyme poisons as mercuric chloride and silver nitrate, traces of such substances as hydroxylamine, hydrocyanic acid or aniline, are known to have a marked inhibiting influence. Hydroxylamine in the dilution of 1 : 70,000 was found to have a quite definite influence on a solution of mangold peroxydase.

ACTION OF HEAT ON THE PEROXYDASE OF THE MANGOLD.

If a few cc. of fresh mangold juice be brought to boiling for two minutes and if, on cooling, it be immediately tested with guaiacum and peroxide, the peroxydase is found to have been destroyed. A somewhat remarkable feature of the juice of this root is that if it be allowed to remain in the cold after undergoing the above treatment it is found to regain its activity. Specimens of peroxydase obtained by alcoholic precipitation behave in a like manner. The present work deals chiefly with a study of this reactivation.

Comparative colorimetric method for estimating peroxydase.

In the first place it was sought to determine quantitatively in what manner and to what extent reactivation took place after a certain degree of heating. For this purpose a relatively rapid method of estimation was essential. A colorimetric method was adopted which was based on the use of guaiaconic acid. This substance appears to be the most generally sensitive of oxydase reagents. It possesses the disadvantage, however, of yielding a relatively unstable colouring matter. In consequence of this it is found impossible to prepare standard solutions of the oxidation product for comparison. Euler and Bolin [1909] recommend indigo blue as a standard. This was found unsatisfactory. The standard used was a 20 % solution of copper nitrate to which 2 cc. of saturated cobalt nitrate was added per 100 cc. of solution. The colour of this solution matches that of the oxidation product of guaiacum extremely well. Yet again there was the disadvantage that while guaiaconic acid is insoluble in water, peroxydase is insoluble in alcohol. This difficulty was overcome by allowing the reaction to take place in 50 % alcohol.

The following reagents are required in dealing with peroxydase solutions approximating in strength to that of natural juices:

- (i) A 2.5 % solution of *guaiaconic acid* in 50 % alcohol.
- (ii) A stock solution of pure neutral 2.5 % *hydrogen peroxide*.
- (iii) A standard solution of *peroxydase* containing a concentration of enzyme not more than about twice that of natural plant juices such as those of the potato or the mangold.
- (iv) A *standard tint* consisting of a 20 % solution of copper nitrate to which a saturated cobalt nitrate solution has been added in the ratio of 2 cc. of cobalt nitrate for every 100 cc. of copper solution.

It is first necessary to prepare standardisation curves with these reagents by comparing the colour of certain definite thicknesses of the standard tint with colorations given under specified conditions by different dilutions of the peroxydase solution. Convenient dilutions of the peroxydase solution for this purpose are 1, $\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{4}$, $\frac{1}{10}$, $\frac{1}{20}$. The procedure adopted in carrying out the oxidation of guaiaconic acid consists in first adding 10 cc. of 50 % alcohol to a 50 cc. measuring flask. 1 cc. of guaiaconic acid solution is then added, followed by 1 cc. of hydrogen peroxide solution, and finally by 1 cc. of

peroxydase solution. The time at which the peroxydase is added is noted and the contents of the flask are gently shaken for one minute, after which addition of rectified spirit to the 50 cc. mark is quickly made. After thoroughly mixing the contents of the flask the solution is allowed to stand for a further five minutes in a dark place. The colour of the solution is then quickly compared with that of the copper-cobalt solution in a colorimeter. This procedure is repeated with the different dilutions of the peroxydase solution and the colorations obtained as compared with, say, 1 cc. of the standard tint, are plotted on a curve against the various strengths of peroxydase used. It is advisable to take readings with two or three different thicknesses of the standard tint with each solution so that the values obtained from the different curves may be checked against one another.

With reagents of the strengths given above measurable differences of coloration are obtained by variations of one or two per cent. in the peroxydase strength of the juice of common vegetables. If it be necessary to deal with more concentrated preparations these must first be suitably diluted before accurate readings can be obtained with reagents of the above concentration.

It will be seen that the special value of this method in dealing with the reactivation of peroxydase after heating is that the strength of the reactivated solution relative to that of the original solution may readily be determined. To arrive at reasonable accuracy it is necessary that the estimations be always carried out under the same conditions of treatment and temperature, etc.

Extent to which the reactivation of mangold peroxydase takes place under varying conditions.

Using the quantitative method just outlined, the extent to which a solution of mangold peroxydase underwent reactivation after different degrees of heating was determined. The specimen used required a little less than two minutes at 100° for its total temporary inactivation. About 10 cc. of the solution was added to a boiling-tube by means of a pipette. The tube was then immersed in boiling water. The solution was well stirred by means of a thermometer, and when a temperature of 100° was attained the time was noted and heating at this temperature was continued for one minute. While continuing to stir the solution the tube was then immersed in cold water for one minute. The activity of the peroxydase present was then estimated. Similar readings were taken at suitable intervals during six hours. The experiment was repeated with specimens of the same peroxydase solution which had been heated in a similar manner for three and five minutes, respectively. The progress of the reactivation is shown in all three cases in Fig. 1. The strengths of the reactivated solutions are expressed in percentages of that of the original solution.

It will be seen that after heating to 100° for one minute the activity of the peroxydase is reduced to 9 % that of the original solution. After standing

at room temperature for six hours this same solution had increased in activity to 40 % that of the original. After heating for three minutes no measurable coloration was obtained with guaiacum at the beginning. After standing for six hours the strength of this solution was 14 % that of the original, while after heating for five minutes the final strength attained was 10 %.

Fig. 1.

When a specimen of peroxydase solution, subsequent to heating, was stored in a sealed tube, reactivation appeared to take place to the same extent as when air had free access.

Discussion.

The reaction curves show that the rate of reactivation is greatest at the beginning and that it falls off considerably after the first hour. Equilibrium is attained more readily the longer the period of heating has been. The evidence suggests that the reaction is bimolecular and that one of the substances taking part undergoes decomposition on prolonged heating. An explanation which suggests itself at this stage is that we are dealing with the interaction of a zymogen and a second substance which is capable of transforming it into peroxydase.

Before proceeding to an account of some experiments which appear to throw some light on the nature of the substances to which the regeneration of per-

oxydase is due it is necessary to recall results arrived at in an investigation of substances which are capable of behaving as peroxydases [Gallagher, 1924].

It was shown that the aldehyde monoperoxides and various oxides of the heavy metals were the main types of synthetic compounds with which the property of oxidising guaiacum in presence of peroxide is found to be associated. It was observed that the preparations of peroxydase examined gave (a) Schiff's reaction, (b) Fischer and Penzoldt's and (c) Lewin's reactions for aldehyde. The test for aldehyde is found to remain positive after the peroxydase is temporarily destroyed by heating. The elements of the heavy metal class sought for in the peroxydase solutions under examination were manganese and iron. Manganese was found to be practically absent from preparations from the mangold root, and it was found that the iron content of solutions from this root, prepared as already described, was usually in the region of 1 mg. of iron per 100 cc. of the solution. An observation made to the effect that traces of iron added to aldehydes, such as formic or valeric, results in a distinct increase in peroxydase activity, would seem to be of especial interest in connection with the present problem.

INFLUENCE OF IRON ON THE PEROXYDASE ACTIVITY OF ALIPHATIC ALDEHYDES.

In the quantitative treatment of this subject a colorimetric method of analysis was employed which was similar to that used in dealing with the plant enzyme. Certain modifications, however, were found to be essential. The active derivatives of the common aldehydes are incapable of carrying out the oxidation of guaiacum in presence of much hydrogen peroxide. An organic peroxide must therefore be used. Ethyl hydroperoxide was chosen, chiefly on account of its solubility.

Note on the use of ethyl hydroperoxide as a reagent for peroxydases.

Although hydrogen peroxide has been made use of in previous sections of this work, subsequent experience proves that an aqueous solution of ethyl hydroperoxide is very much to be preferred, not only in quantitative, but also in qualitative work with oxydases. Its action very probably approximates more nearly to reactions in the plant cell than does that of hydrogen peroxide. Unlike most organic peroxides this reagent is fairly soluble in water and it does not appear to be affected by catalase. Unlike hydrogen peroxide it does not seem to have much injurious effect on peroxydase, even when present in excess. It is found to be sensitive, not alone in dealing with weak solutions of peroxydase but also in the investigation of peroxydase substances of relatively weak character such as the solutions of iron and aldehyde at present under investigation. The use of hydrogen peroxide in the estimation of relatively strong peroxydases is only justified when the method used is strictly comparative.

The curves shown in Fig. 2 were obtained by adding to 10 cc. of 50 % alcohol (a) 1 cc. of 1.5 % aqueous ethyl hydroperoxide, (b) 1 cc. of 2.5 % guaiaconic acid solution and (c) 2 cc. of the mixture of aldehyde and an iron salt of a weak acid (ferric oxalate). The mixture, after shaking, was allowed to stand for five minutes and a further 10 cc. of 50 % alcohol was added before taking the colorimetric readings. The readings were taken after suitable intervals against the same copper-cobalt solution as previously used during

six hours in all. The standardisation curves were prepared immediately at the end of the experiment by quickly taking readings from various dilutions of the solution corresponding to curve 1. The strength of the solution after six hours was given the value 100, and the other readings are expressed in percentages accordingly.

Fig. 2.

I 20 % formaldehyde containing 3.5 mg. Fe per 100 cc.
 II 20 % " " " 1.125 " "

It will be seen that the rate of increase in peroxydase activity is roughly proportional to the amount of iron present. The curve of the reaction in each case is practically a straight line. A 1 % solution of formaldehyde containing 3.5 mg. of iron per 100 cc. was found to increase in activity somewhat more rapidly than the solution containing the same amount of iron with 20 % formaldehyde. Since the aldehyde used contained a small amount of free acid this unexpected difference is no doubt partly due to the lesser quantity of acid in the weaker aldehyde solution. Fig. 3 shows the behaviour of weak solutions of formic and valeric aldehydes in presence of iron. In making the colorimetric determinations in this case 1 cc. only of the peroxydase solution was employed as compared with 2 cc. in the case of the solutions previously dealt with. The method of estimation was otherwise similar to that used in preparing Fig. 2.

The p_H of the formaldehyde solution, as determined colorimetrically at the commencement of the experiment was found to be 4.6 and that of the valeric aldehyde solution 4.4. These values appeared to vary but little throughout

the experiment. A special feature of the experiment made with valeric aldehyde is the manner in which the reaction falls off after three hours. This aldehyde is but slightly soluble in water, so that the half-saturated aqueous solution contains a relatively small quantity of the substance in solution. This would appear to explain the attainment of an early equilibrium.

Fig. 3. --- represents 1 % formaldehyde containing 1.75 mg. of iron per 100 cc. solution.
— represents a half-saturated aqueous solution of *n*-valeric aldehyde containing 1.75 mg. of iron per 100 cc. solution.

Discussion.

In view of the observations already recorded that mangold peroxydase solutions, even after destruction of the peroxydase, contain a substance of aldehyde nature as well as an iron content of the same order as that used in dealing with formic and valeric aldehydes, the above experiments appear to constitute a remarkable parallel to the reactivation of the natural enzyme after its destruction by heat. The fact that the interaction between formaldehyde and iron is linear in the instances studied may be explicable on the grounds that such a small quantity of active product is formed that the relative proportions of the interacting substances do not vary appreciably. A more likely explanation for the linear nature of the curves lies in the fact that excess of this aldehyde was used in all cases, while the iron probably acts catalytically. In the case of valeric aldehyde a lower ratio of the aldehyde was employed and the rate of activation decreased after three hours. Here, it would seem, we have attained a condition of affairs more nearly approximating that of the enzyme solution. It has already been shown [Gallagher, 1924] that the derivative of aldehydes which is responsible for the peroxydase

reaction is very probably a substance of ozonide or mono-peroxide nature, $R \cdot CH = O = O$. In the formation of this substance iron probably acts as an oxygen carrier. Whether the action is due to ferric compounds only has not yet been definitely determined. Specimens of ferrous oxalate were found capable of activating formaldehyde, but here, it will be realised, the problem of entirely excluding ferric compounds is extremely difficult.

It is proposed to investigate further the influence of mineral substances on the peroxydase activity of aldehydes with a view to determining whether the interaction described constitutes, in all respects, a true parallel to the plant enzyme.

SUMMARY.

The destruction of the peroxydase of the mangold on heating is shown to be of temporary character. Reactivation of the enzyme is found to take place when the solution is subsequently allowed to remain some time in the cold. The progress of the reactivation has been studied quantitatively by means of a comparative colorimetric method.

The peroxydase solutions examined were found to contain iron. They also gave several reactions for aldehyde. The aldehyde character of the solution was maintained after the peroxydase had been temporarily inactivated by heat.

It is shown that traces of iron added to aqueous solutions of aliphatic aldehydes give rise to an increase of peroxydase activity on standing in the cold.

The most probable explanation for the reactivation of solutions of the enzyme after heating is that the zymogen or precursor of the enzyme is an aldehyde and that the enzyme is produced by the catalytic action of iron.

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