THE MODE OF ACTION OF PLANT PEROXIDASES

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(WITH TWO FIGURES)

In a recent paper[1] it was shown that while colloidal platinum markedly accelerates the rate of oxidation of various substances by hydrogen peroxide, it does not ordinarily bring about oxidation in the absence of hydrogen peroxide. Experiments were described, however, which show that when the colloidal metal is charged with oxygen (by making it an anode) it rapidly brings about a certain amount of oxidation. By repeating the charging process at sufficiently frequent intervals colloidal platinum might be made to bring about the oxidation of various substances at a rate approximating that affected by hydrogen peroxide and colloidal platinum. From this it was concluded that the action of the colloidal metal in accelerating oxidation by hydrogen peroxide (that is, its peroxidase action) is due to the taking of oxygen from the peroxide by the metal to form a compound which is a more efficient oxidizing agent than the original peroxide.

This information, gained from a study of a simple peroxidase reaction where the constitution of the catalyzer was known, has made possible an analogous investigation of the more significant and complicated problem of the nature of the peroxidases produced in living tissue. Since, as has frequently been pointed out, the peroxidase action of colloidal platinum closely resembles that of the plant peroxidases, it seemed probable that the mechanism of the reactions must be similar. Accordingly, the experiments which proved fruitful in a study of the platinum reaction have been repeated, as nearly as the material permitted, with certain plant peroxidases.

The very active ferment of horseradish root was first investigated. About 150 gm. of the finely chopped tissue was mixed with twice its volume of distilled water and allowed to stand for 24

[1] Reed, G. B., Mechanism of oxidase action. Bot. Gaz. 62:53–64. 1916.

hours. On filtering, a clear, pale yellow extract was obtained, which exhibited very active peroxidase properties.

The peroxidase activity of this extract was determined quantitatively by the method of BACH and CHODAT.[2] To 100 cc. of a solution containing 0.2M potassium iodide and 0.05M acetic acid 20 cc. of the horseradish peroxidase extract was added; the mixture was kept well stirred and at a constant temperature of 15° C., and at frequent intervals portions of 5 cc. were removed and titrated with 0.01M sodium thiosulphate solution (with starch indicator) for the iodine liberated in the oxidation. The values obtained in this way are plotted in the curve *OA*, fig. 1. The rate of oxidation of hydrogen peroxide in the absence of a ferment is shown by the curve *OB* of the same figure. By subtracting the ordinates of the curve *OB* from the ordinates of the curve *OA* we obtain the curve *OC*, which expresses the effect of the catalyzer. For subsequent comparisons the curve *OC* is also plotted as *OC*, fig. 2.

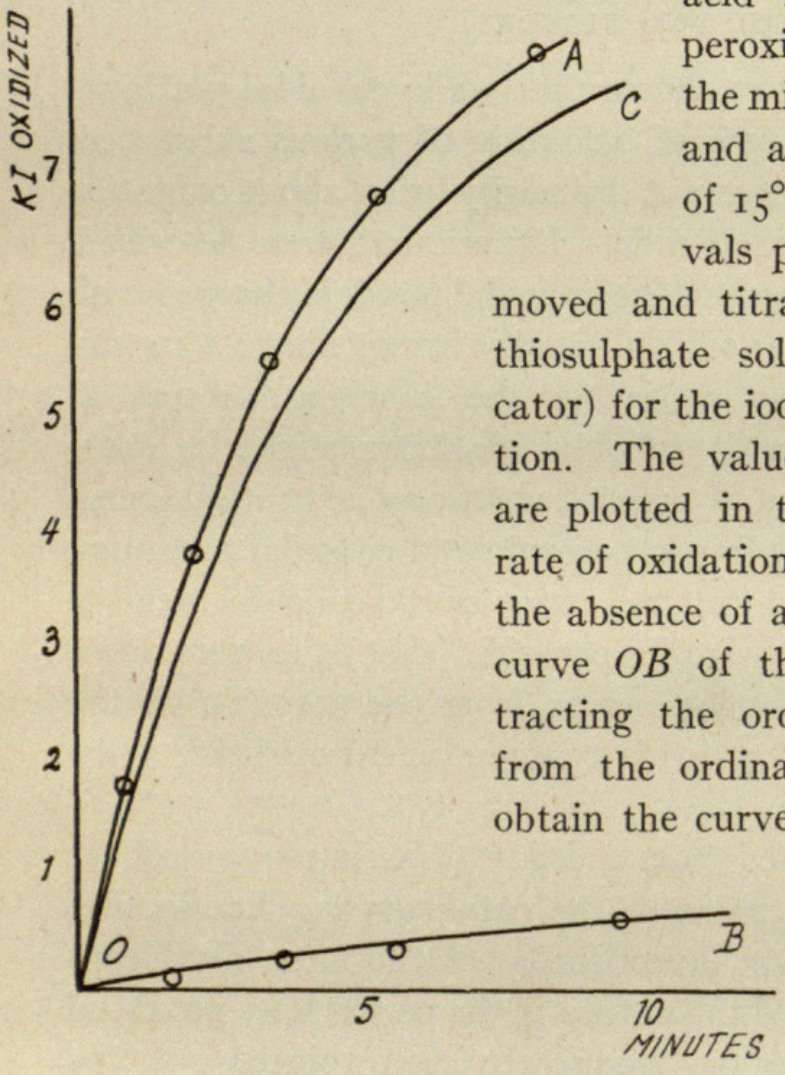

FIG. 1.—Curves of oxidation of potassium iodide by hydrogen peroxide: *OA*, in presence of horseradish peroxidase; *OB*, in absence of a catalyzer; *OC*, the curve resulting when the ordinates of *OB* are subtracted from those of *OA;* ordinates represent number of cc. of 0.01M $Na_2S_2O_3$ required to combine with the iodine in 5 cc. of reaction mixture; abscissae represent time in minutes.

The fresh peroxidase extract in the absence of a peroxide showed no oxidative activity; on adding portions to solutions of potassium iodide and starch or to gum guaiac free from peroxide no oxidation took place. In this respect it was analogous to an ordinary colloidal platinum solution. The platinum may be made

[2] BACH, A., and CHODAT, R., Ber. Deutsch. Chem. Gesells. **35**: 2466–2470, 3943–3946. 1902.

active by charging it with oxygen, either electrically (by making it an anode) or by a chemical method (exposing it to potassium permanganate). Obviously it was not possible to charge the plant peroxidase with oxygen electrically; hence the chemical method was adopted. To about 100 cc. of the same peroxidase solution as that used in the previous experiment concentrated potassium permanganate[3] solution was added drop by drop until the permanganate was no longer reduced, as shown by the color. A small excess of fresh extract was then added to reduce any free potassium permanganate present. The mixture was then filtered free from a small amount of brown precipitate consisting of manganic hydroxide and probably also some organic manganese compounds. In this way a clear solution, somewhat deeper yellow in color than the original extract, was obtained; but this solution possessed a very marked oxidative activity. Solutions of potassium iodide, gum guaiac, or pyrogallol mixed with portions of it, were rapidly oxidized. This new compound was very unstable; when heated rapidly to 60° C. its ability to oxidize gum

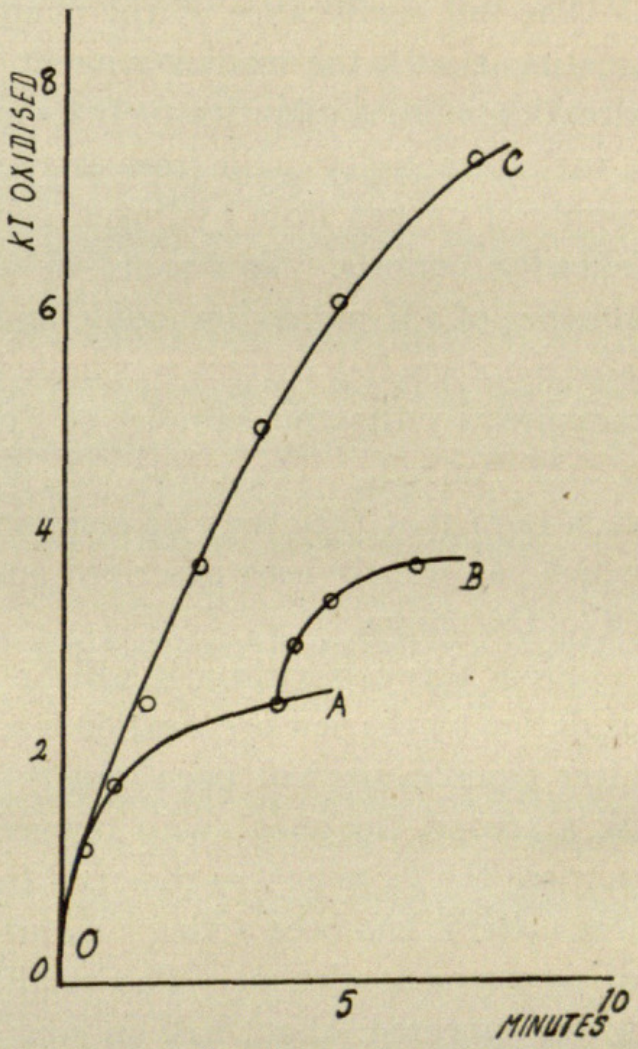

FIG. 2.—Curves showing oxidation of potassium iodide; lower curves *OA* and *AB* represent successive oxidations in the absence of hydrogen peroxide by horseradish peroxidase which had been treated with potassium permanganate at the beginning of each oxidation; curve *OC* represents oxidation by hydrogen peroxide in presence of untreated horseradish peroxidase; ordinates represent number of cc. of 0.01M $Na_2S_2O_3$ required to combine with the iodine in 5 cc. of reaction mixture; abscissae represent time in minutes.

[3] A concentrated solution was used so that the amount added was not sufficient to dilute appreciably the peroxidase extract.

guaiac or potassium iodide directly was destroyed, but its peroxidase activity was still maintained, as shown by a vigorous action on the addition of hydrogen peroxide.

The full significance of the change effected in the extract by treatment with the oxidizing agent was only apparent, however, after determining quantitatively its oxidizing ability and comparing it with the activity of the fresh extract in the presence of a constant supply of oxygen from hydrogen peroxide. This was done in the following manner. To 100 cc. of a solution consisting of equal volumes of 2M potassium iodide and 2M acetic acid 20 cc. of the *treated* horseradish extract was added and the amount of oxidation measured by titrating samples of 5 cc., at frequent intervals, with 0.01M $Na_2S_2O_3$. The curve *OA*, fig. 2, expresses the results in such form that they may be compared with the peroxide reaction which has already been described and which is plotted in the curve *OC* of this figure.

From this comparison it will be apparent that the amount of oxidation by the new preparation is small; but it was found possible after equilibrium had been nearly reached to renew its activity by a second treatment with potassium permanganate. For this purpose the ferment was removed from the potassium iodide solution (after it had been acting 5 minutes) by adding about 2 volumes of 95 per cent alcohol. The small amount of white precipitate which appeared (about half an hour after adding the alcohol) contained the greater part of the peroxidase. This was centrifuged out, washed in 80 per cent alcohol, dissolved in a few cc. of water, and again treated with potassium permanganate in precisely the same manner as before. On adding this preparation to a potassium iodide solution as previously used, oxidation followed at a rate indicated by the curve *AB* (fig. 2). The smaller amount and somewhat slower rate of oxidation are probably due to loss of ferment in the precipitation and subsequent treatment. An attempt to repeat the process a third time was not successful, only a faint cloudiness appearing on the addition of alcohol to the reaction mixture.

By an inspection of the curves (fig. 2) it is apparent that if it were mechanically possible to repeat the process indefinitely the oxidation could probably be indefinitely prolonged. Moreover, by

subjecting the peroxidase to the oxidizing action of the permanganate at sufficiently frequent intervals, it is apparent that the reaction could be made to proceed as rapidly as the curve *OC* (fig. 2), which represents the hydrogen peroxide reaction catalyzed by untreated peroxidase.

Hence we may conclude that just as platinum is recharged with oxygen by hydrogen peroxide, as soon as some of the oxygen has been removed from the platinum by a reducing agent, so the horseradish peroxidase is recharged by hydrogen peroxide under similar conditions.

An investigation of potato peroxidase gave exactly similar results. It may be of interest to note that by treating the peroxidase from the inner tissues of the potato with permanganate it could be made to behave like the oxidase (that is, peroxidase in combination with the oxygenases) from the outer part of the tuber.

It may be concluded, therefore, that in oxidation processes catalyzed by peroxidases two reactions are involved. The peroxidase combines with oxygen from the oxygenases (or from hydrogen peroxide, or possibly from some other source, since it is capable of taking it from potassium permanganate) to form an intermediate compound which is a more energetic oxidizing agent than the original source of the oxygen. The final stage in the oxidation is then affected by this intermediate compound.

A new light is thus thrown on the mechanism of oxidation in living tissues which has so long proved baffling to investigators. The difficulties which beset this field of research are clearly illustrated by the fact that a great amount of careful investigation was necessary before the conceptions of oxygenase and peroxidase could be established. The next step was to elucidate the connection between these entities. This now seems to be accomplished, but it would probably not have been done so soon or so satisfactorily without first making a careful study of the simpler conditions to be found in oxidations which are catalyzed by platinum.

In this connection it is of interest to recall that VAN SLYKE and CULLEN[4] have shown recently that in the fermentation of urea by soy bean urease there is a combination between urea and ferment

[4] VAN SLYKE, D. D., and CULLEN, G. E., Jour. Biol. Chem. **39**:141–180. 1914.

which eventually splits to form carbon dioxide and ammonia. From the course of the reaction these authors have devised a formula which applies to several enzyme reactions which had previously been measured, particularly the hydrolytic action of invertase and lactase. It seems possible that it may prove to be generally true that the catalyzer combines in a definite manner with the reacting substances.

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