LXXII. OXIDATIONS BY HYDROGEN PEROXIDE IN PRESENCE OF SULPHYDRYL COMPOUNDS.

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It has been shown recently by Thurlow [1925] and by Harrison and Thurlow [1926] that during certain biological oxidations such as that of hypoxanthine and succinic acid in presence of their respective oxidising enzymes, or sulphhydryl compounds in presence of traces of iron, the secondary or induced oxidation of substances such as nitrites, lactic acid and β -hydroxybutyric acid can be brought about. These secondary oxidations were shown to occur only in presence of a suitable peroxidase, *e.g.* milk peroxidase or ferrous iron. Thurlow [1925] gave evidence that these secondary oxidations were brought about by hydrogen peroxide formed during the oxidation of the primary system by the combination of active hydrogen with molecular oxygen. In the case of the primary oxidising system hypoxanthine with xanthine oxidase, hydrogen peroxide was actually detected in the solution. The hydrogen peroxide in presence of a suitable peroxidase or activator can then bring about the secondary oxidation of other substances. During the oxidation of the sulphhydryl compound, however, positive tests for the presence of hydrogen peroxide in the solution could not be obtained. This was explained by the fact that hydrogen peroxide can readily oxidise the sulphhydryl group and that, consequently, hydrogen peroxide formed during the oxidation of the sulphhydryl compound by oxygen would be capable of bringing about the oxidation of a further quantity of the sulphhydryl compound, and hence would rapidly be used up. Similarly, hydrogen peroxide formed in other primary oxidising systems such as hypoxanthine with xanthine oxidase can bring about the oxidation of a further quantity of the oxidisable substance hypoxanthine, though in this latter case the rate of disappearance of the hydrogen peroxide is slower, requiring the presence of the xanthine oxidase. It was shown, however, by Thurlow [1925] and Harrison and Thurlow [1926] that, in spite of the fact that part of the hydrogen peroxide formed during the primary oxidation is used up in oxidising a further quantity of the oxidisable substance, nevertheless, in presence of a peroxidase another part of this hydrogen peroxide can bring about a secondary oxidation of various substances such as nitrites and lactic acid. Evidence of the same kind was given in the case of a number of oxidising systems, including those of the sulphhydryl compounds cysteine and glutathione.

It was shown by Abderhalden and Wertheimer [1923] that cysteine is rapidly oxidised to cystine by hydrogen peroxide. These authors used a large excess of hydrogen peroxide in their experiments. In a recent paper, Szent-Györgyi [1926] has shown that a dilute solution of reduced glutathione is oxidised fairly rapidly by excess of hydrogen peroxide. In consequence, he considers that the presence of the sulphydryl group in the tissues will interfere with secondary oxidations brought about in presence of peroxidase by hydrogen peroxide; in other words that peroxidase cannot compete with the oxidising sulphydryl group for a share of the hydrogen peroxide to a sufficient extent to make the secondary oxidations important. This criticism does not appear to be a valid one. In the first place, the conditions in Szent-Györgyi's experiments are not physiological, since excess of hydrogen peroxide is employed and the hydrogen peroxide is all added at once. In the living cell hydrogen peroxide is formed by the primary oxidising systems themselves and is hence produced only in small quantities at a time. If it were present to a greater extent, the cell enzymes, including peroxidase itself, would be destroyed, and the widespread distribution of catalase in the tissues appears to be an additional safeguard against the accumulation of hydrogen peroxide in toxic quantities.

In the second place it is actually possible, as the following experiment will show, for a secondary oxidation by hydrogen peroxide and peroxidase to occur in presence of the sulphydryl group, and even when the hydrogen peroxide is slowly added to the system instead of being formed by the system itself. It was considered worth while to perform this experiment in order to meet Szent-Györgyi's criticism, because the mechanism involved in secondary oxidations brought about by peroxidase and hydrogen peroxide seems too important to be laid aside without due consideration. The following experiment was devised to show that even when hydrogen peroxide is added continuously to a sulphydryl system containing peroxidase together with a suitable oxidisable substance such as a nitrite, the hydrogen peroxide serves not only to oxidise the sulphydryl compound but also, activated by the peroxidase, to bring about the oxidation of the nitrite.

Broadly speaking, the principle of the experiment was slowly to add dilute solutions of cysteine, peroxidase and hydrogen peroxide to a solution of peroxidase containing a small quantity of sodium nitrite, and then to test for the disappearance of the nitrite by the Griess-Ilosvay reagent. The solutions were added in such a way that a small excess of the sulphydryl compound was present in the solution throughout the experiment. Oxidation of the cysteine by the air was entirely prevented by bubbling purified nitrogen through the solution, this also sufficing to keep the solution well stirred.

The cysteine solution containing 13.3 mg. of free cysteine per cc. (neutralised with sodium hydroxide) was quickly poured into a burette, the entry of air into the latter being prevented throughout the experiment by a stream of nitrogen. The peroxidase was prepared from milk by the method of

Thurlow [1925], and a saturated solution in phosphate buffer (p_H 7.6) was used (the solution being filtered and the necessary amount of sodium hydroxide being added to the solution to maintain the p_H at 7.6). The hydrogen peroxide solution was made up to be equivalent to the cysteine solution. The strength of the hydrogen peroxide solution was checked at the beginning of the experiment by a direct titration of the cysteine solution, a known volume of the latter being made alkaline with ammonium hydroxide, warmed, and titrated with the hydrogen peroxide, using the nitroprusside test as an external indicator.

A solution of 0.1 mg. of sodium nitrite in 20 cc. of peroxidase was contained in a thick glass boiling-tube, a bulb being blown in the upper part of the latter to increase its capacity and to reduce frothing of the protein-containing solution. The tube was fitted with a rubber stopper through which passed an entry and narrow exit tube for nitrogen and three short pieces of glass tubing each drawn out to a fine jet. Three burettes containing the cysteine, hydrogen peroxide and peroxidase respectively, were attached by short pieces of rubber tubing to the three glass tubes the jets of which had previously been adjusted to dip well under the surface of the liquid in the boiling-tube. Before putting in the rubber stopper, the burettes were turned on until all the air in the glass tubes was displaced by liquid. After drying the outside of the tubes with filter paper, the stopper was put into the boiling-tube and a stream of nitrogen (purified by passing through four vessels of pyrogallol) was bubbled through the solution. After all the air had been driven out 1.0 cc. of cysteine was added from the burette, and the tube was immersed in a water-bath at 37°. An excess of cysteine was thus present at the beginning and the excess was maintained throughout the experiment. The cysteine, peroxidase and hydrogen peroxide were now run in from the burettes in successive small quantities in the following order, namely, 0.2 cc. cysteine, 0.5 cc. peroxidase, 0.2 cc. hydrogen peroxide. (The peroxidase was added to replace that which was destroyed during the experiment.) The duration of the experiment was about one and a half hours, and in all 7.5 cc. cysteine, 20.0 cc. peroxidase and 7.5 cc. hydrogen peroxide were added. The tube was now removed from the water-bath and 4.0 cc. of water were added (by removing the nitrogen exit tube), making the total volume of the solution up to 60 cc. In the tests described below, this solution will be called solution B. A fairly rapid stream of nitrogen was maintained to prevent the entry of oxygen, and quantities of the solution were removed for testing by replacing the nitrogen exit tube by a pipette. A test portion of the solution gave a strong nitroprusside test at the end of the experiment, showing that excess of cysteine was present.

In order to test samples of the solution for the disappearance of nitrite, it was necessary first to remove the excess of cysteine, as the latter was found to inhibit the development of the colour in the Griess-Ilosvay test. This was effected by making the solution alkaline and adding hydrogen peroxide, after

destruction of the peroxidase in the solution by heat or by treatment with concentrated hydrochloric acid. (Haas and Lee [1924] have shown that nitrites are not oxidised by hydrogen peroxide in the absence of peroxidase.)

Three tests were carried out for the disappearance of nitrite as follows.

1. A control solution containing 40 cc. peroxidase and 0.1 mg. sodium nitrite was made up to 60 cc. with water. This would represent the concentration of nitrite in the experimental solution if none had been oxidised. 2.5 cc. of this control solution A and 2.5 cc. of the experimental solution B from the boiling-tube were pipetted respectively into two test tubes, each containing 1 cc. of concentrated hydrochloric acid which destroyed the peroxidase. After shaking, the solutions were made alkaline with 1 cc. of concentrated ammonium hydroxide and 0.1 cc. of the hydrogen peroxide solution was added to each to oxidise the cysteine remaining in solution B. (This hydrogen peroxide cannot oxidise any nitrite present since the peroxidase has been destroyed.) After shaking, and confirming the absence of cysteine in B, 2.0 cc. of Griess-Ilosvay reagent were added to each, and the tubes were allowed to stand a few minutes for development of colour. The solution B gave a completely negative test for nitrite, whereas the control solution A gave a definitely positive reaction for nitrite.

2. 2.5 cc. of the control solution A and of the test solution B were taken and the peroxidase in the two solutions was destroyed by boiling. After cooling, 0.1 cc. of ammonium hydroxide and 0.1 cc. of hydrogen peroxide were added. The solutions were well shaken, the coagulated protein being broken up with a glass rod, and 1 cc. of Griess-Ilosvay reagent was added to each. After standing for a few minutes, the control solution A gave a strongly positive test for nitrite, while the experimental solution B gave a completely negative reaction for nitrite.

3. Two quantities of 2.5 cc. of the solution B were pipetted from the boiling-tube and to one was added 0.05 cc. of sodium nitrite solution containing 0.00084 mg. The two solutions were then treated in the manner described in Test 2. The solution, to which 0.05 cc. of nitrite had been added before testing, gave a definitely positive test for nitrite, while the other solution, which had originally contained five times this amount of nitrite, gave a completely negative test. This last test showed that one-fifth of the quantity of nitrite which was originally present in the solution could readily be detected by this method of testing. Further, this last test gives confirmation that the disappearance of nitrite is not due to its oxidation by the hydrogen peroxide added during the test, the peroxidase having previously been destroyed.

The whole experiment was repeated under the same experimental conditions and identical results were obtained.

It is evident, therefore, that hydrogen peroxide in presence of peroxidase brings about the oxidation of nitrites in a solution containing a sulphydryl compound. No attempt was made to find the maximum quantity of nitrite

which could be oxidised by a given quantity of hydrogen peroxide and peroxidase. It was desired merely to show that such an oxidation actually could take place to a measurable extent *in vitro* in the presence of a sulphydryl compound. In our present state of knowledge it is impossible fully to imitate *in vitro* the effects on chemical reactions produced *in vivo* by the specialised structure of the living cell, and such factors as selective permeability and selective adsorption may well play an important part in determining in what proportion the hydrogen peroxide is shared between oxidising sulphydryl compounds and between bringing about, in presence of peroxidase, secondary oxidations in the living cell.

SUMMARY.

Experimental evidence is given to show that the presence of sulphydryl compounds does not prevent the oxidation of sodium nitrite by hydrogen peroxide in presence of milk peroxidase.

The bearing of this on secondary oxidations by hydrogen peroxide in the living cell is discussed.

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