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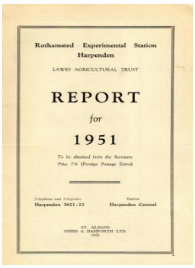

Review of Work on Manganese Oxidation in Higher Plants

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REVIEW OF WORK ON MANGANESE OXIDATION IN HIGHER PLANTS

By P. J. G. Mann

The work to be reviewed was undertaken in the Biochemistry Department at Rothamsted in 1948 with the object of investigating whether manganese undergoes a cycle of oxidation and reduction in higher plants. The work developed from the observation that with water extracts of certain plant roots in presence of pyrophosphate at pH 7 a pink colour was obtained within a few minutes of adding manganous sulphate and hydrogen peroxide; this suggested that manganipyrophosphate had been formed. Except in strongly acid solutions manganic ions are unstable and dismute to manganous ion and hydrated manganese dioxide :—

$$2\,Mn^{+++} + 2H_2O \longrightarrow Mn^{++} + MnO_2 + 4H^+ \quad \ldots \quad (1)$$

Some complexions of manganic manganese are more stable. Thus manganipyrophosphate is stable up to pH 9 but dismutes at more alkaline reactions. By making use of this dismutation Kenten and Mann (1949) isolated manganese dioxide from large-scale reaction mixtures of plant extract, manganous sulphate and hydrogen peroxide and thus proved that the colour reactions obtained were indeed due to the formation of manganipyrophosphate. The manganese dioxide isolated in such experiments was equivalent to only 25-40 per cent of the hydrogen peroxide added. A manometric study of the reaction showed that these low yields were due to the fact that manganipyrophosphate reacts stoicheiometrically with hydrogen peroxide under the experimental conditions. The oxidation therefore causes oxygen evolution and the reactions taking place in the pyrophosphate media may be represented by the equations :—

$$2MnO + H_2O_2 \longrightarrow Mn_2O_3 + H_2O \quad \ldots \quad \ldots \quad (2)$$

$$Mn_2O_3 + H_2O_2 \longrightarrow 2MnO + H_2O + O_2 \quad \ldots \quad (3)$$

The accumulation of manganipyrophosphate therefore depends on reaction (2) being more rapid than reaction (3). No accumulation of manganese oxidation product could be demonstrated in orthophosphate media. Here a stable manganic complex cannot be formed and oxidation would result in the formation of manganese dioxide either directly or through the dismutation of an unstable manganic complex. Manganese dioxide does not accumulate in the orthophosphate media because the rate of its reduction by hydrogen peroxide (4) is as rapid as its formation :—

$$MnO_2 + H_2O_2 \longrightarrow MnO + H_2O + O_2 \quad \ldots \quad (4)$$

Under these conditions the rate of decomposition of the hydrogen peroxide is a measure of the rate of manganese oxidation. In manometric experiments it was shown that the addition of as little as 1 μg Mn^{++}/ml extract increased the rate of decomposition of hydrogen peroxide by plant extracts. Indirect evidence was thus obtained that manganese oxidation by the system proceeded readily at physiological concentrations of Mn^{++}.

Analysis of the oxidizing system showed that it consisted of a thermolabile and a thermostable factor in addition to hydrogen peroxide. Evidence was obtained that the thermolabile factor is

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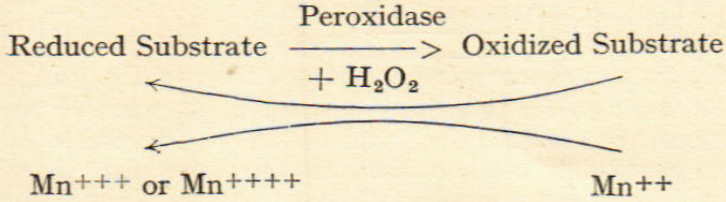
the enzyme peroxidase and the thermostable factor a peroxidase substrate. It was suggested, as a working hypothesis, that intermediate products of the oxidation of certain peroxidase substrates can bring about manganese oxidation according to the scheme below which involves the substrate in a cycle of oxidation and reduction.



The concentrations of hydrogen peroxide used in the preceding experiments were necessarily high compared with those likely to be present *in vivo*. It was suggested that *in vivo* the manganese oxidation product would react preferentially with plant metabolites other than hydrogen peroxide thus involving the manganese in an oxidation-reduction cycle which could be responsible for its known effect on plant respiration.

In subsequent work Kenten and Mann (1950) established the fact that peroxidase in presence of certain of its substrates, catalyses the oxidation of Mn^{++}. The reaction mixtures used in this work were similar to those previously described with the exception that the plant extracts were replaced by partially purified peroxidase preparations together with peroxidase substrates such as phenol or p-cresol. Manganese dioxide was isolated from large-scale reaction mixtures. In manometric experiments systems active in manganese oxidation were produced by the use of a few μg of peroxidase preparation and phenolic substrate. The catalytic activity of the phenolic substrates in the oxidation was illustrated by the fact that in one experiment the addition of 1 μg p-cresol to the system led to an accumulation of manganipyrophosphate equivalent to 605 μg Mn_2O_3. Probably the most important results of the manometric work were those obtained in a study of the effect of variation in the hydrogen peroxide concentration. Under the conditions used, the accumulated manganipyrophosphate, calculated as a percentage of that theoretically possible, increased with decreasing concentration of hydrogen peroxide from 30 per cent at 0·0066 M. hydrogen peroxide to 80 per cent at 0·0017 M. which was the lowest concentration practicable with the technique used. This result supported the suggestion previously made that with sufficiently low hydrogen peroxide concentrations all the manganese oxidation product would be available for the oxidation of plant metabolites other than hydrogen peroxide.

The possibility that the oxidizing capabilities of peroxidase systems towards inorganic compounds are not confined to compounds of manganese was investigated by Kenten and Mann (1951). The results of this work showed that peroxidase in presence of suitable phenolic substrates catalyses the oxidation of ferrocyanide by hydrogen peroxide and possibly that of molybdates, vanadates and tungstates, though in the latter cases direct proof of the oxidation was not obtained. It was found that under certain conditions the rate of oxidation of Mn^{++} by peroxidase systems is markedly

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increased by catalytic concentrations of these compounds. Thus a clear effect of ammonium molybdate was obtained at a concentration of 10^{-6} M. (2·4 μg Mo/3ml). This was attributed to catalysis by peroxidase systems, of the oxidation of molybdates, tungstates and vanadates by hydrogen peroxide to the corresponding peroxy compounds according to the general equation :—

$$X{\cdot}OH + H_2O_2 \rightleftharpoons X{\cdot}O{\cdot}OH + H_2O \quad \ldots \quad (5)$$

It was shown that these peroxy compounds rapidly oxidise Mn^{++}.

In the work so far described with plant extracts or peroxidase preparations, the oxidations studied were dependent on added hydrogen peroxide. Such oxidations can take place in plants *in vivo* only if hydrogen peroxide is formed by the plant tissues. The work of Kenten and Mann (1952a) showed that Mn^{++} is oxidized by plant extracts in absence of added hydrogen peroxide and that such oxidation forms a useful test for the presence in the extracts of enzyme systems producing hydrogen peroxide. The blue coloration obtained when benzidine and hydrogen peroxide are added to most plant and animal tissues is known to be due to the catalysis by peroxidase, or by the peroxidatic action of haem or haematin derivatives, of the oxidation of benzidine by hydrogen peroxide. Therefore a blue coloration obtained with tissue and benzidine in the absence of added hydrogen peroxide suggests the formation of hydrogen peroxide by the tissue. Using this test for the production of hydrogen peroxide by plant extracts Kenten and Mann (1952a) obtained negative or only weakly positive results. But extracts of many plants gave positive benzidine tests after incubation with Mn^{++} and pyrophosphate at pH 7. Evidence was obtained that this was due to oxidation of the Mn^{++} and accumulation of manganipyrophosphate which readily oxidizes benzidine. The manganese oxidation was shown to be dependent on the formation of hydrogen peroxide by the plant extracts. Hydrogen peroxide cannot accumulate to any extent in the extracts owing to their strong catalatic activity.

In attempts to identify the enzyme systems producing hydrogen peroxide it was shown that pea seedling extracts (*Pisum sativum* L.) oxidize both mono- and di-amines according to the general equation :

$$R{\cdot}CH_2{\cdot}NH_2 + O_2 + H_2O \longrightarrow R{\cdot}CHO + H_2O_2 + NH_3 \quad \ldots \quad (6)$$

Some evidence was also obtained of the presence of an aldehyde oxidase catalysing the oxidation of phenylacetaldehyde with hydrogen peroxide formation. It seems of particular interest that tryptamine is among the amines attacked by the amine oxidase in view of the evidence that tryptamine can function as a precursor of indoleacetic acid in the plant. The action of the amine oxidase on tryptamine presumably results in the formation of indoleacetaldehyde. It was suggested that indoleacetaldehyde is a substrate of the aldehyde oxidase present in the extracts and that indoleacetic acid could be formed from tryptamine by the action of the amine oxidase followed by that of the aldehyde oxidase.

The oxidation of added Mn^{++} by plant extracts in absence of added hydrogen peroxide was attributed by Kenten and Mann (1952a) to the presence in the extracts of enzyme systems producing hydrogen peroxide. In subsequent work Kenten and Mann (1952b) working with partially purified enzyme preparations demonstrated

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the oxidation of Mn^{++} by enzyme systems producing hydrogen peroxide coupled with peroxidase. The enzyme systems used to provide hydrogen peroxide were the D-amino-acid oxidase and xanthine oxidase from animal sources and the plant amine oxidase systems already described. The reactions catalysed by these enzymes may be represented by the general equation :—

$$RH_2 + O_2 \longrightarrow R + H_2O_2 \quad \ldots \quad \ldots \quad (7)$$

If the hydrogen peroxide formed is decomposed by catalase according to the equation :—

$$H_2O_2 \longrightarrow \tfrac{1}{2}O_2 + H_2O \quad \ldots \quad \ldots \quad (8)$$

the apparent oxygen uptake is halved and the net reaction is :—

$$RH_2 + \tfrac{1}{2}O_2 \longrightarrow R + H_2O \quad \ldots \quad \ldots \quad (9)$$

It is well known that the hydrogen peroxide produced by these enzyme systems may be used for secondary or coupled oxidations catalysed by peroxidase. Where catalase is present such coupled oxidations cause increases in the apparent oxygen uptake which may reach 100 per cent if all the hydrogen peroxide is used in the coupled oxidation rather than decomposed by catalase. In manometric experiments with these enzyme systems in both pyrophosphate and orthophosphate buffers it was shown that, in presence of catalase, the addition of peroxidase and manganous sulphate together with catalytic amounts of p-cresol caused a doubling of the oxygen uptake of the primary reaction suggesting that all the hydrogen peroxide was used for manganese oxidation. This was confirmed by the finding that the manganese oxidation products which accumulated were equivalent to the hydrogen peroxide produced in the primary reactions. Manganese oxidation was also observed in systems composed of the plant enzyme α-hydroxyacid oxidase and its substrates, lactic or glycollic acids, with peroxidase. Proof was thus obtained that hydrogen peroxide is formed in oxidations catalysed by α-hydroxyacid oxidase. This has since been confirmed by more conventional methods (Kenten and Mann, 1952c).

In the preceding experiments, designed to demonstrate quantitative accumulation of manganese oxidation products, Mn^{++} was added in amounts more than equivalent to the hydrogen peroxide formed by the primary enzyme system. It was shown that Mn^{++} when present in low concentrations was also oxidized by adding oxalate to the system which reduced the manganese oxidation product as fast as it was formed and thus allowed the Mn^{++} to undergo a cycle of oxidation and reduction. The oxidation of these small amounts of Mn^{++} with accumulation of the oxidation product produced little increase in the oxygen uptake. Where oxalate was present in addition to Mn^{++}, however, large increases in oxygen uptake were observed due to the manganese oxidation-reduction cycle.

In this attempt to reconstruct with purified enzyme preparations the type of systems which causes manganese oxidation in plant extracts the significance of the results lies not merely in the fact that manganese oxidation was observed but also that the oxidation product accumulated quantitatively. In previous results with added hydrogen peroxide the accumulation of manganipyrophosphate was not quantitative and no accumulation could be demonstrated in orthophosphate owing to reduction of the manganese

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dioxide by hydrogen peroxide (equations 3 and 4). Provided the hydrogen peroxide concentration is sufficiently low this reduction is negligible and all the oxidation product would be available in the plant for the oxidation of metabolites other than hydrogen peroxide as suggested by Kenten and Mann (1949). Under such conditions the Mn^{++} would undergo, in the plant, a cycle of oxidation and reduction as demonstrated *in vitro* in the experiments with oxalate as reductant.

The results obtained may throw light not only on the physiological action of manganese but also on that of peroxidase. Peroxidase is widely distributed in higher plants but its functions are unknown. It has not been shown to catalyse the oxidation of any compound recognized as an intermediate in the main pathways of metabolism. Since peroxidase catalyses the oxidation of Mn^{++} the range of compounds which peroxidase systems can oxidize will be extended by addition of Mn^{++} to include those compounds capable of oxidation by the manganese oxidation product. Thus, in work as yet unpublished, it has been shown that in presence but not in absence of Mn^{++} peroxidase systems oxidize not only oxalate but also oxaloacetate, ketomalonate and dihydroxytartrate.

Manganese oxidation has not yet been demonstrated in the plant *in vivo* under normal conditions of manganese supply. In preliminary experiments with plants grown in water culture with high manganese concentrations evidence has been obtained of the accumulation of manganese oxidation products in the tissues. This can generally be shown in the pea plant (*Pisum sativum* L.) with concentrations of Mn^{++} of the order of 50 p.p.m. in the culture solution. It has already been shown that the oxidation takes place *in vitro* with physiological concentrations of Mn^{++}. At such concentrations of Mn^{++} *in vivo* it is possible that the oxidation product is reduced by plant metabolites as fast as it is formed and that only under conditions of manganese toxicity is the rate of oxidation faster than the rate of reduction. This suggests the possibility that the symptoms of manganese toxicity may be due, at least in part, to the deposition of higher oxides of manganese in the plant tissues.

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