haem of cytochrome *a* of ox-heart muscle and further studies on its characteristics were made on material from this more accessible source.

4. The general properties of this haem and its reactions with pyridine, cysteine, carbon monoxide and hydroxylamine were investigated.

5. The free porphyrin, like the haem from cytochrome *a*, reacted with cysteine and hydroxylamine.

6. The results of the tests described indicate that this haem possesses at least one aldehyde group.

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The Oxidation of Manganese by Plant Extracts in the Presence of Hydrogen Peroxide

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Manganese is known to be an essential micronutrient of plants, although its function is unknown. It apparently plays a part in plant respiration; Lundegårdh (1939) found that the oxygen uptake of manganese-deficient wheat roots was raised by 155–470 % by the addition of 5×10^{-5} M manganese chloride. Such an effect might be brought about by the activation of certain enzyme systems, e.g. arginase (Waldschmidt-Leitz & Purr, 1931), phosphoglucomutase (Cori, Colowick & Cori, 1938), leucylpeptidase (Berger & Johnson, 1939) or by a system in which Mn^{++} undergoes alternate oxidation and reduction. It has been shown that soil micro-organisms can oxidize Mn^{++} (e.g. Beijerinck, 1913; Gerretsen, 1937; Leeper & Swaby, 1940; Mann & Quastel, 1946). No satisfactory evidence has been put forward to show that Mn^{++} is oxidized in higher plants and the present work was undertaken to investigate whether such oxidation does take place.

MATERIALS AND METHODS

Preparation of plant extracts. The roots were scrubbed free from soil and minced twice in a meat mincer. The mince was weighed and the juice squeezed out by hand through madapollam. Water to one-quarter of the original weight of the mince was added to the residue, which was then ground in a mortar with sand and squeezed through madapollam. This treatment was repeated twice. The extracts were combined and filtered through Whatman no. 1 filter paper, and stored in a refrigerator. Most of the work was done with horse-radish (*Cochlearia armoracia*); it was found that extracts of this root showed little loss of activity over a period of several weeks. In view of the possibility of contamination, however, fresh extracts were frequently made. Extracts of other roots were made and used on the same day. This method was originally adopted with horse-radish as benzidine-H_2O_2 tests showed satisfactory extraction of peroxidase.

Catalase preparation. The caps of the Basidiomycete, *Marasmius oreades*, were ground with sand, squeezed through madapollam and centrifuged at 3500 rev./min.

(1500 *g.*) for 0·5 hr. The catalase activity (Katalasefähigkeit) of the supernatant liquid, estimated by the method of Sumner & Somers (1943), was about 80. The catalase activity diminished rapidly and consequently the preparation was made and used on the same day.

Peroxidase preparation. The peroxidase preparation was made by the method of Keilin & Mann (1937) from horse-radish. After precipitation with $(NH_4)_2SO_4$, followed by fractional precipitation with ethanol and further purification with tricalcium phosphate gel, a preparation of Purpurogallinzahl (P.Z.) 290 was obtained. The P.Z., i.e. mg. purpurogallin formed by 1 mg. enzyme preparation in 5 min. from pyrogallol and H_2O_2 under fixed conditions, was determined by the method of Keilin & Mann (1937).

Preparation of hydrated manganese dioxide. The suspension of hydrated MnO_2 was prepared by the method of Heintze & Mann (1949), which is based upon the dismutation of manganipyrophosphate in alkaline solution. The valency, as determined from the Mn content of the preparation and its oxidizing capacity towards oxalic acid, was 4·08.

Manometric measurements were carried out in the Warburg apparatus at 25°. The volume of the reaction mixture was 3 ml. and KOH was present in the centre cup unless otherwise stated.

EXPERIMENTAL AND RESULTS

Colorimetric evidence of manganese oxidation

In preliminary experiments with certain types of root extracts colour reactions were obtained which indicated the formation of manganic complexes. The addition of $MnSO_4$ and H_2O_2 to the extracts in the presence of citrate at pH 7 caused the development of a greenish yellow colour, while in pyrophosphate at this pH a pink colour was obtained. On acidification of the pyrophosphate solution the colour changed to a brighter pink; both the pink and greenish colours were rapidly discharged by the addition of acid H_2O_2 or hydrazine sulphate. These reactions are typical of manganipyrophosphate and citrate complexes. The colour reactions were not given by extracts which had been heated for 5 min. in a boiling water bath, nor were they given in absence of H_2O_2 or Mn^{++}.

Reaction mixtures consisting of 0·4 ml. root extract, 0·2 ml. 0·1 M-$MnSO_4$, 2 ml. 0·1 M-citrate (pH 7) or 2 ml. 0·5 M-pyrophosphate (pH 7) and 0·2 ml. water were made up in test tubes and 0·2 ml. 0·05 M-H_2O_2 added. The rate of development and the intensity of the pink or greenish yellow colour was followed for a 15 min. period. The strongest reactions were given by extracts from horse-radish (*Cochlearia armoracia*) and turnip (*Brassica campestris* L.), with which the colour developed within a minute of adding H_2O_2. Weaker reactions were given by extracts of carrot peel (*Daucus carota*) and spinach beet (*Beta vulgaris*); dandelion (*Taraxacum officinale*) extracts were inactive. Difficulty was experienced with some extracts, e.g. potato which, owing to the presence of strong direct oxidase systems, rapidly became so dark as to obscure any colour due to manganic complex formation.

Isolation of manganese dioxide

In neutral or acid pyrophosphate, MnO_2 dissolves on the addition of $MnSO_4$ to give a pink manganipyrophosphate complex (Heintze & Mann, 1946, 1949). At pH values 'much above 8' the complex dismutes into hydrated MnO_2 and manganopyrophosphate (Lingane & Karplus, 1946):

$$Mn_2O_3 \rightleftharpoons MnO + MnO_2.$$

By making use of the dismutation in alkaline solution an attempt was made to prove that the colour formation with root extracts and H_2O_2 was due to the formation of manganic complexes.

To a reaction mixture containing 455 ml. 0·5 M-pyrophosphate buffer (pH 7), 25 ml. horse-radish extract and 20 ml. 0·5 M-$MnSO_4$, H_2O_2 (5 ml., 0·05 M) were added at 10 min. intervals, with stirring, until the solution became a deep red and further addition of H_2O_2 did not appear to intensify the colour. In all, 25 or 30 ml. of 0·05 M-H_2O_2 were added; 20 min. after the last addition, 0·15 ml. catalase preparation was added. (No H_2O_2 could be detected in the solution at this stage. It was necessary, however, to add the catalase preparation to control (*B*) and consequently the reaction mixture was similarly treated.) After a further 20 min. the mixture was adjusted to pH 10 (glass electrode) by the addition of 2 N-NaOH. During the addition of NaOH a brisk current of N_2 was blown through the solution to assist mixing and exclude atmospheric O_2, thus preventing autoxidation of the remaining $MnSO_4$ due to local excess of alkali.

The mixture, which was now a dark brown, was allowed to stand for 3 hr. to ensure complete dismutation and then centrifuged. The brown sediment was washed on the centrifuge 4 times with 50 ml. 0·2 M-pyrophosphate (pH 9·8), 3 times with 50 ml. 0·2 M-pyrophosphate (pH 7·0) and finally twice with 50 ml. water, to remove Mn^{++} which is absorbed by hydrated MnO_2. The sediment was ground thoroughly

Table 1. *Valency of manganese in oxidation product*

(Mn content was estimated colorimetrically; oxidizing capacity by manometric measurement of O_2 output: 0·5 ml. suspension, 2·3 ml. N-H_2SO_4 in main vessel and 0·2 ml. 0·5 M-H_2O_2 in 0·1 N-H_2SO_4 tipped from side arm.)

Preparation no.	Total 0·05 M-H_2O_2 added in preparation (ml.)	Manganese (mg./ml. of suspension)	Oxygen (μl. O_2/ml. of suspension)	Valency	Total MnO_2* isolated (mg.)	Yield of MnO_2 based on total H_2O_2 added (%)
1	25	0·775	304	3·92	29·5	27
2	30	1·254	518	4·03	51·8	40

* Calculated from oxidizing capacity.

in a mortar and made up in water to 25 ml. Portions were taken for colorimetric determination of manganese and the oxidizing capacity was estimated manometrically with acid H_2O_2. Two preparations were made: the valency of the manganese was found to be 3·92 in one case and 4·03 in the other (Table 1).

Two control experiments were made, one in which no H_2O_2 was added (*A*), and the other in which horse-radish extract previously heated for 10 min. at 100° (*B*); these mixtures were worked up similarly to the reaction mixture, except that no catalase was added to control (*A*). Immediately after the addition of catalase to (*B*), a sample was transferred to a Warburg vessel and the evolution of O_2 was followed. This was complete in 25 min. after which 0·2 ml. MnO_2 suspension (2·6 mg. MnO_2/ml.) was tipped from the side arm. No further evolution of O_2 took place showing that the H_2O_2 had been completely decomposed by the catalase. After adjustment to pH 10 and standing for 3 hr. the control (*B*) was yellow brown and control (*A*) yellow, indicating the presence of a small amount of colloidal MnO_2. The colour was, however, less brown than the supernatant obtained by centrifuging the reaction mixture at this stage and no sediment was obtained on centrifuging.

It was clear from the experiments that the oxidation depended on the presence of H_2O_2 and a thermolabile factor in the plant extract.

Evidence for the oxidation of manganese based on the accumulation of manganipyrophosphate

It is well known that MnO_2 decomposes H_2O_2 with evolution of O_2; in water the reaction is catalytic, but in acid solution reduction of MnO_2 may take place and under suitable conditions of acidity (e.g. in 0·1 N-H_2SO_4) the reaction is stoicheiometric,

$$MnO_2 + H_2O_2 = MnO + O_2 + H_2O.$$

Experiments were carried out to find out whether the decomposition of H_2O_2 by manganipyrophosphate was catalytic or stoicheiometric under the conditions used. The behaviour of hydrated MnO_2 and $MnSO_4$ under the same conditions was also investigated.

Decomposition of H_2O_2 by MnO_2 and manganipyrophosphate. Known amounts of MnO_2, or manganipyrophosphate and H_2O_2 were allowed to react and the output of O_2 measured manometrically (Table 2). In water the H_2O_2 was decomposed catalytically by MnO_2, but in the other media the reaction was generally stoicheiometric. In orthophosphate at pH 7 the decomposition was somewhat larger than stoicheiometric, but there was also more decomposition in the control. With manganipyrophosphate the reaction was stoicheiometric. The spontaneous decomposition of H_2O_2 was slight, and not significantly affected by the presence of 110 μg. Mn^{++}.

Conditions leading to the accumulation of manganipyrophosphate. Since manganipyrophosphate reacts stoicheiometrically with H_2O_2 its accumulation as an oxidation product must depend on its rate of formation being greater than its rate of reduction by H_2O_2, but during the oxidation of Mn^{++} by horse-radish root extract and H_2O_2 in presence of pyrophosphate an evolution of oxygen would be expected owing to reduction of part or all of the manganipyrophosphate by the H_2O_2. If there is no accumulation of manganipyrophosphate and no side reaction the total O_2 evolved should be equivalent to the H_2O_2 initially present. If manganipyrophosphate accumulates the total oxygen output will be lower since part of the H_2O_2 is used up in this accumulation. These reactions can be followed manometrically as shown in Fig. 1.

Table 2. *The decomposition of H_2O_2 by MnO_2 and manganipyrophosphate*

(With MnO_2 the reaction mixtures consisted of 106 μg. MnO_2 (hydrated) and 0·0033 M-H_2O_2, in 0·033 M-buffer at pH 7 or in water, with or without the addition of horse-radish extract (0·4 ml., heated 10 min. at 100°). The manganipyrophosphate was preformed by the addition of 110 μg. Mn^{++} to 106 μg. MnO_2 in pyrophosphate at pH 7. The H_2O_2 was tipped from the side arm except when horse-radish extract was used when the MnO_2 was tipped. Control experiments were made with 110 μg. Mn^{++}. Theoretical O_2 output for complete catalytic decomposition of H_2O_2: 112 μl., in absence of catalysis, 24·4 μl.)

	Oxygen output (μl. O_2)				
Time (min.)	Water (MnO_2)	Ortho-phosphate (MnO_2)	Pyro-phosphate (MnO_2)	Horse-radish extract heated at 100° (MnO_2)	Mangani-pyro-phosphate
10	104·5	23·0	23·8	25·3	23·5
20	108·5	23·0	24·4	26·0	24·9
60	109·5	29·7	26·1	29·0	27·6
120	111·0	35·3	28·4	29·5	30·3
	Control with 110 μg. Mn^{++}				
120	9·0	12·0	3·0	3·0	3·0

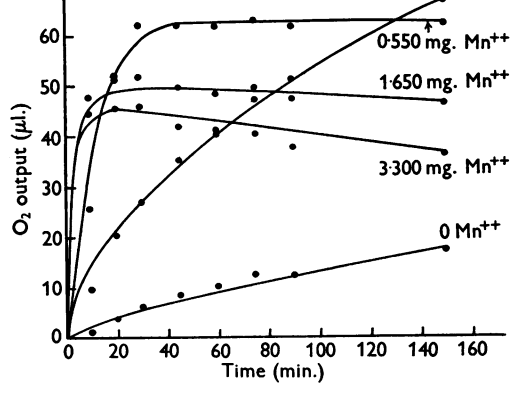


Fig. 1. The accumulation of manganipyrophosphate when horse-radish extract is treated with H_2O_2 in the presence of added Mn^{++}. The reduction in O_2 output at the higher levels of Mn^{++} indicates manganipyrophosphate accumulation; theoretical O_2 output: 112 μl.

In this experiment the reaction mixtures consisted of 0·4 ml. horse-radish extract in presence of 0·45 M-pyrophosphate at pH 7 and 0·2 ml. of 0·05 M-H_2O_2 tipped from the side arms, the Mn^{++} added being varied from 0 to 3·3 mg. The slow evolution of O_2 which occurred with the root extract alone was markedly increased on addition of Mn^{++}. The initial output was more rapid with the higher amounts of Mn^{++}, but the total output was greatest with the lowest amount of Mn^{++}. In absence of horse-radish extract the output was negligible. These results confirm the view that two reactions are proceeding, the oxidation of the added Mn^{++} by the root extract and H_2O_2, and the reduction of the oxidation product by H_2O_2 with evolution of O_2. That the lower total outputs with the higher concentrations of Mn^{++} were due to the accumulation of manganipyrophosphate was supported by the fact that the most intense pink colour was in the flask with the highest concentration of Mn^{++}.

The system may catalyse the oxidation of manganese to the manganic form or to MnO_2. In orthophosphate at pH 7 a stable manganic complex cannot be formed and breakdown will occur into MnO_2 and MnO, the final product being MnO_2. If a stable manganic complex can be formed, as in pyrophosphate, it will accumulate provided that the rate of formation is greater than that of reduction by the H_2O_2. Increasing the concentration of Mn^{++} would therefore favour accumulation by accelerating the rate of oxidation. The fact that Mn^{++} is much less soluble in orthophosphate (pH 7) than in pyrophosphate (pH 7) may partly explain why no accumulation of MnO_2 has been observed in orthophosphate.

Assuming that the primary oxidation product of Mn^{++} is MnO_2, the following reactions may take place in the pyrophosphate reaction mixture:

(1) $MnO \rightarrow MnO_2$;
(2) $MnO_2 + H_2O_2 \rightarrow MnO + O_2 + H_2O$;
(3) $MnO + MnO_2 \rightarrow Mn_2O_3$;
(4) $Mn_2O_3 + H_2O_2 \rightarrow 2MnO + O_2 + H_2O$.

If (2) is more rapid than (1) MnO_2 cannot accumulate, but under conditions where (3) is favoured at the expense of (2), i.e. at high Mn concentration, Mn_2O_3 will be formed, and will accumulate if (4) is slow in comparison with (3).

When hydrated MnO_2 was allowed to react with dilute H_2O_2 in pyrophosphate at pH 7, it was noticed that after the disappearance of the brown MnO_2 the reaction mixture turned pink, and in spite of the presence of excess H_2O_2 this colour faded only slowly. The intensity of this pink colour, which was presumably due to manganipyrophosphate, could be increased if $MnSO_4$ was added together with the H_2O_2 to the MnO_2. The formation of manganipyrophosphate in the experiment with horse-radish extracts may therefore be due to reaction (3), particularly when large additions of Mn^{++} are made initially.

Since, as is shown below (Fig. 2), reaction (2) is more rapid than (4), the conversion of MnO_2 to manganipyrophosphate favours the accumulation of oxidized Mn.

The reaction between hydrated MnO_2 and H_2O_2 in pyrophosphate at pH 7 is very rapid, particularly at high H_2O_2 concentration, and although that with manganipyrophosphate is slower it is difficult to demonstrate this manometrically.

The conditions finally chosen as satisfactory were as follows: 0·264 mg. MnO_2 (hydrated) in 0·16 M-pyrophosphate at pH 7 in one vessel, and 0·264 mg. MnO_2 (hydrated) plus 0·1 ml. 0·05 M-$MnSO_4$ (to give manganipyrophosphate) in the other vessel. KOH was omitted from the centre cup and the volume of the reaction mixtures was 5 ml. After equilibration, H_2O_2 to give a concentration of 0·0006 M was tipped into both vessels simultaneously by two operators, and the O_2 output was measured at 2 min. intervals.

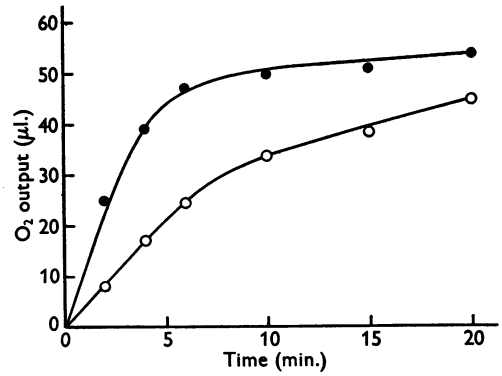


Fig. 2. A comparison of the velocity of the reactions between $MnO_2 + H_2O_2$ and manganipyrophosphate + H_2O_2; ●—●, $MnO_2 + H_2O_2$; ○—○, manganipyrophosphate + H_2O_2.

With MnO_2 most of the H_2O_2 was decomposed in the first 5 min. with a velocity 2 to 3 times that with manganipyrophosphate (Fig. 2). After 1 hr. the output was the same in both cases within experimental error. The comparison is not strictly between MnO_2 and manganipyrophosphate, since manganipyrophosphate would be formed as the reaction between MnO_2 and H_2O_2 proceeded. This probably explains the slow output in the later stages where MnO_2 was used. Furthermore, the hydrated MnO_2 was in the form of a suspension and the rate of the reaction would depend on the particle size, whereas the manganipyrophosphate was in solution.

Estimation of manganipyrophosphate by hydrazine. It is known that MnO_2 reacts quantitatively with hydrazine in the following way:

$$2MnO_2 + N_2H_4 \rightarrow N_2 + 2MnO + 2H_2O.$$

Manganipyrophosphate also reacts with hydrazine giving a gas output slightly higher than that required by theory (Table 3), and can be estimated in this way. Experiments in which the horse-radish extract was allowed to react with H_2O_2 in 0·67 M-pyrophosphate and in the presence of varying concentrations of $MnSO_4$ were carried out manometrically in an atmosphere of N_2. When the O_2 output ceased, hydrazine was tipped from the side arm, and a rapid evolution of gas, presumably N_2,

occurred. The output was greatest where the highest accumulation of manganipyrophosphate was expected and was accompanied by discharge of the pink colour. If the reactions in the first part of the experiment led only to the evolution of O_2 and the accumulation of manganipyrophosphate, then the combined O_2 and N_2 gas outputs should be equivalent to the H_2O_2 added. In experiments using 0·4 ml. horse-radish extract, the combined gas outputs were considerably lower than this, but were only 5–10% lower when 0·1 ml. extract was used (Table 4). The reduction in the amount of extract used led to a decrease in the accumulation of manganipyrophosphate. The fact that the output of N_2 was less than the theoretical suggests either that part of the H_2O_2 was lost in a side reaction, or that part of the MnO_2 or manganipyrophosphate formed was reduced by the extract. Reduction of MnO_2, and to a less extent of manganipyrophosphate, takes place when these compounds are incubated with the horse-radish extract (Table 3).

The properties of the manganese oxidizing system

It has been demonstrated in the previous experiments that horse-radish extracts in presence of H_2O_2 can bring about the oxidation of Mn^{++}. To identify and estimate the oxidation product it was necessary to carry out the experiments under conditions in which a stable manganic complex could accumulate, i.e. in pyrophosphate at pH 7 and with a high concentration of Mn^{++}. If the system is of physiological significance, however, oxidation should take place at low concentration of Mn^{++}. The oxidation product does not accumulate under these conditions because it reacts with H_2O_2 with evolution of O_2 as rapidly as it is formed. The oxidation can therefore be demonstrated manometrically.

The effect of small amounts of Mn^{++} *on the decomposition of* H_2O_2 *by horse-radish extract.* With most of the horse-radish root extracts, but not with other root extracts, an increase in the rate of decomposition of H_2O_2 occurred on addition of very small amounts of $MnSO_4$. The experiments were carried

Table 3. *Reaction of* MnO_2 *and manganipyrophosphate with hydrazine in presence and absence of horse-radish extract*

(The MnO_2 content of a suspension of hydrated MnO_2 was determined by oxalate titration. 0·4 ml. portions (0·984 mg. MnO_2) were suspended in 0·67 M-pyrophosphate buffer in Warburg vessels. The gas space was then filled with N_2. After equilibration 0·2 ml. saturated aqueous $N_2H_4 . H_2SO_4$ was tipped from the side arm; the gas output was measured. Similar experiments were carried out with manganipyrophosphate formed by adding 0·2 ml. of 0·1 M-$MnSO_4$ to the reaction mixtures. Experiments were also made to determine the effect of additions of 0·4 ml. horse-radish extract on the estimation.)

Method of estimation	Reaction mixture	Gas output (μl.)	MnO_2 (mg.)	Recovery (%)
Oxalate titration	MnO_2	—	0·984	—
Acid H_2O_2 (manometric)	MnO_2	248·5	0·965	98·0
N_2H_4 (manometric)	MnO_2	128·0	0·994	101·0
	MnO_2 tipped into extract + N_2H_4	125	0·972	98·5
	MnO_2 + extract incubated 30 min.	116·0	0·901	91·5
	MnO_2 + extract incubated 60 min.	110·8	0·861	87·5
	Manganipyrophosphate	133·5	1·037	105·0
	Manganipyrophosphate + extract incubated 30 min.	131·0	1·02	103·5
	Manganipyrophosphate + extract incubated 60 min.	127·0	0·987	100·5

Table 4. *Accumulation of manganipyrophosphate*

(Reaction mixtures consisted of horse-radish extract and $MnSO_4$ as below in 0·67 M-pyrophosphate at pH 7; 0·2 ml. 0·05 M-H_2O_2 was tipped from the side arm and the O_2 output was measured. When the O_2 output stopped 0·2 ml. saturated aqueous $N_2H_4 . H_2SO_4$ was tipped from the second side arm and the N_2 output measured. Gas space N_2.)

	Extract *A* (0·4 ml.)			Extract *A* (0·1 ml.)		Extract *B* (0·2 ml.)				
$MnSO_4$ added (mg. Mn)	0·55	1·65	2·75	1·65	2·75	0·55	1·375	2·75	5·50	8·25
(1) O_2 output (μl.)	66·2	49·0	49·5	85·8	79·3	76·2	75·6	71·0	62·5	55·3
(2) N_2 output (μl.)*	25·1	43·6	48·5	25·7	27·9	22·9	30·2	36·2	41·8	46·8
(3) $O_2 + N_2$ output (μl.)	91·3	92·6	98·0	111·5	107·2	99·1	105·8	107·2	104·3	100·3
(4) Theory for $O_2 + N_2$ output (μl.)†	116·0	116·0	116·0	116·0	116·0	112·8	112·8	112·8	112·8	112·8

* Uncorrected for 105% recovery (see Table 3).

† Half O_2 output obtained by tipping the H_2O_2 into excess hydrated MnO_2 in N-H_2SO_4.

out manometrically with 0·4 ml. root extract in presence and absence of 0·033 M-ortho- or pyro-phosphate buffer, 0·2 ml. of 0·05 M-H_2O_2 being tipped from the side arm after equilibration. The results of a set of experiments in the absence of buffer are given in Fig. 3. In absence of added Mn^{++} a slow decomposition of H_2O_2 occurred. This was in general increased by the addition of 2·2 μg. Mn^{++}, and increased progressively with increasing amounts of Mn^{++}. In general, the experiments were carried out in air, but similar results were obtained when the gas space was filled with N_2, showing that direct oxidases were not concerned in the reaction.

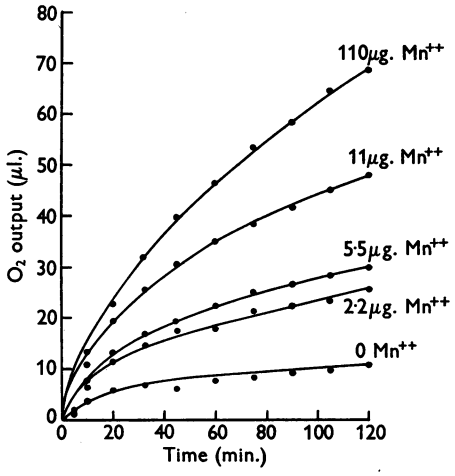


Fig. 3. The effect of added Mn^{++} on the rate of decomposition of H_2O_2 by horse-radish extract.

In ortho- and pyro-phosphate at pH 7 the rate of decomposition of H_2O_2 by the extract was higher than in the unbuffered extract (pH 4–4·5), but a similar effect of Mn^{++} was found. In orthophosphate at pH 7 with 11 μg. Mn^{++} added the output stopped in 80 min., the total output was 70 μl. O_2, about 63% of that required for complete decomposition of the H_2O_2 added (112 μl. O_2). No residual H_2O_2 could be detected in the reaction mixture and some H_2O_2 must therefore be lost in side reactions. In orthophosphate at pH 5 the rate of decomposition of H_2O_2, both in presence and absence of added Mn^{++}, was less than at pH 7. In general 0·4 ml. of extract produced the effects described above; with smaller amounts the response to added Mn^{++} was less, and usually with 0·05 ml. of extract little or no increase in the rate of decomposition of H_2O_2 was observed with additions of 110 μg. Mn^{++}. Some extracts gave little response to additions of 11 μg. Mn^{++} even when 0·4 ml. was used.

Control experiments (Table 3) showed that in all the media used the decomposition of H_2O_2 in absence of extract was not appreciably affected by addition of Mn^{++} and was not sufficiently great to affect the results of the above experiments.

The rate of decomposition of H_2O_2 by the horse-radish extract in different media with and without added Mn^{++} was reduced by previous heating at 100°. The effect of heat was somewhat variable and even after heating for 10 min. a slight activity remained in some cases.

It has already been shown that when large amounts of Mn^{++} are added to horse-radish extract and H_2O_2 the manganese is oxidized and the oxidation product reacts with H_2O_2 with evolution of O_2. It is considered therefore that the increase in rate of decomposition of H_2O_2 with small amounts of Mn^{++} is likewise due to oxidation of manganese. Since under the conditions of the experiments the reaction of MnO_2 and manganipyrophosphate with H_2O_2 is stoicheiometric (Table 2) it is obvious that if small amounts of Mn^{++} cause a large increase in O_2 evolution the Mn^{++} must undergo a cycle of oxidation and reduction.

The effect of various metals on the decomposition of H_2O_2 by horse-radish extract. The effect of Mn^{++}, Co^{++}, Cu^{++}, Zn^{++}, Ni^{++}, Fe^{++} and Fe^{+++} on the decomposition of H_2O_2 by the horse-radish extract was tested. At the concentration used (22 μg./3 ml.) only Mn^{++} produced an increase in the rate of decomposition.

Effect of variation in H_2O_2 concentration. The effect of varying the H_2O_2 concentration on the decomposition of H_2O_2 by horse-radish extract with 22 μg. added Mn^{++} in 0·033 M-orthophosphate at pH 7 was tested. The system was active over the range 0·02–0·00083 M-H_2O_2. With the higher concentrations an inhibitory effect was apparent in time. There was no reduction in the initial velocity, even with the lowest concentration.

Activation by an ether-soluble factor. The response of relatively inactive extracts, previously mentioned

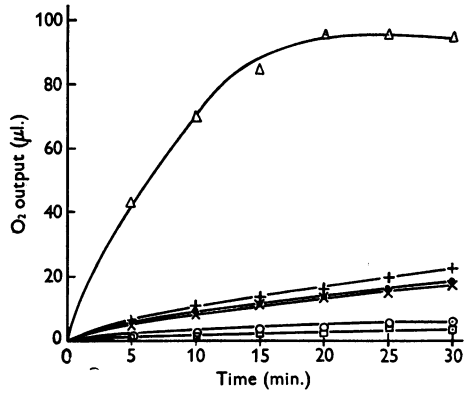


Fig. 4. The activation of horse-radish extract + Mn^{++} by an ether-soluble factor in the extract; ●—●, extract; +—+, extract + 55 μg. Mn^{++}; △—△, extract + ether-soluble factor + 55 μg.-Mn^{++}; ×—×, extract + ether-soluble factor; ⊡—⊡, ether-soluble factor + 55 μg. Mn^{++}; ⊙—⊙, 55 μg. Mn^{++}.

(p. 260), was increased considerably by the addition of an ether extract of acidified active horse-radish extract. The extract used was made from a residue from a peroxidase preparation and had previously been saturated with $(NH_4)_2SO_4$ and filtered; 200 ml. was acidified to pH 3 with N-H_2SO_4 and shaken

3 times with 30 ml. ether; the combined ether extracts were taken to dryness at room temperature and the residue suspended in 5 ml. water. The effect of 0·5 ml. of this suspension on the rate of decomposition of H_2O_2 by horse-radish extract which gave little response to 55 μg. Mn^{++} was tested in 0·033 M-orthophosphate at pH 7 in the presence and absence of Mn^{++} (Fig. 4). While the ether-soluble factor or Mn^{++} alone caused little or no increase in the activity of the extract, together they gave a marked increase. The ether-soluble factor was unaffected by heating for 15 min. in a boiling water bath, but was destroyed by ashing. It was clear therefore that a thermostable factor was a component of the manganese oxidation system.

The oxidation of manganese by a peroxidase system

The fact that H_2O_2 was necessary for the oxidation suggested that the thermolabile factor was a peroxidase. It was found that, on precipitation with $(NH_4)_2SO_4$ and dialysis, peroxidase preparations, obtained by the method of Keilin & Mann (1937), gave no evidence of manganese oxidation by colorimetric tests. Positive tests, however, were obtained with these and more highly purified peroxidase preparations, when the ether-soluble factor, previously described, or certain peroxidase substrates, such as *p*-cresol, were added. It was noted that whereas in the absence of manganese, *p*-cresol was oxidized by the peroxidase and H_2O_2 to give an insoluble white oxidation product, in the presence of Mn^{++} the solution remained clear and the coloured manganic complex appeared. These results suggested that the ether-soluble factor of the horse-radish extracts is a substrate for peroxidase and that manganese reduces the oxidized substrate and is thereby itself oxidized.

The oxidation of manganese by systems containing peroxidase can be followed by manometric measurement of the O_2 evolved. In the experiments shown in Fig. 5 the oxidizing system was: in the main vessel, 7 μg. of a peroxidase preparation with P.Z. 290, 0·00033 M-*p*-cresol in 0·033 M-orthophosphate at pH 7 to which varying additions (5·5–33 μg.) of Mn^{++} were made; 0·2 ml. 0·05 M-H_2O_2 was tipped from the side arm after equilibration. In absence of added Mn^{++} no evolution of oxygen took place, but with increasing amounts of Mn^{++} increases in the velocity and in the total output were observed. The theoretical output (112 μl. O_2) was obtained with 33 μg. added Mn^{++}.

In the reaction mixture without Mn^{++}, a white precipitate was present, indicating that the *p*-cresol had been oxidized. This precipitate was smaller in the vessels with 5·5–11 μg. Mn^{++} and was not visible with 22–33 μg. Mn^{++}. No significant decomposition of H_2O_2 was observed in control experiments. These results are in agreement with the conclusion previously reached that manganese reduces an oxidation product of *p*-cresol and is itself oxidized. In the absence of sufficient Mn^{++} the oxidation product accumulates. In orthophosphate at pH 7 the oxidized manganese reacts stoicheiometrically with H_2O_2. In presence of sufficient Mn^{++}, therefore, accumulation of the oxidation product does not occur and the H_2O_2 is completely decomposed.

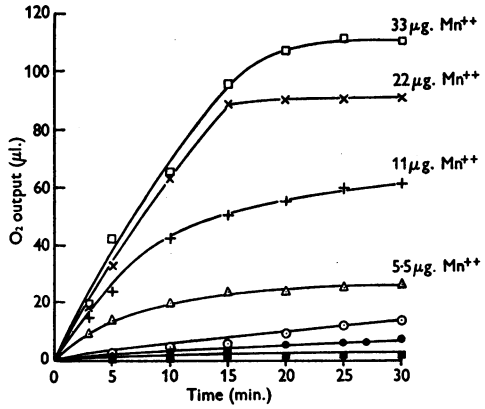


Fig. 5. The decomposition of H_2O_2 by the system peroxidase, *p*-cresol and Mn^{++}. △—△, +—+, ×—×, ⊡—⊡, peroxidase + *p*-cresol with varying Mn^{++} additions; ⊙—⊙, peroxidase heated 10 min. at 100° + *p*-cresol + 22 μg. Mn^{++}; ●—●, peroxidase + 22 μg. Mn^{++} or *p*-cresol + 22 μg. Mn^{++}; ■—■, peroxidase + *p*-cresol; buffer, orthophosphate.

DISCUSSION

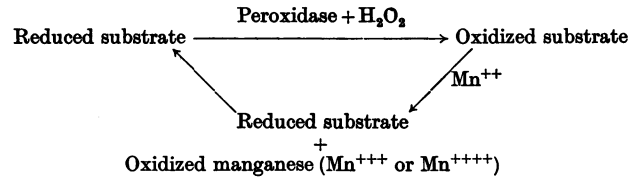
The results obtained provide conclusive evidence that a system is present in horse-radish extract which in the presence of H_2O_2 oxidizes manganese. Colour tests with other root extracts suggest that this reaction is a general phenomenon. The valency of the manganese oxidation product has not been established, but under conditions where manganic manganese is stable the product accumulates in this form. This may arise by a reaction between MnO and MnO_2.

In addition to H_2O_2 a thermolabile and a thermostable factor, which are probably peroxidase and a peroxidase substrate respectively, are necessary for the oxidation. The thermolabile properties of the natural system agree with those attributed to peroxidase by Gallagher (1924) and Herrlinger & Kiermeier (1944), in that prolonged heating at 100° is necessary for complete inactivation. Also, of the root extracts tested those such as horse-radish and turnip, which are good sources of peroxidase, appear to be the most active and the maximum activity as shown by the decomposition of H_2O_2 is reached at low H_2O_2 concentrations.

On the basis of the results obtained with the partially purified peroxidase preparations the working hypothesis is advanced that manganese oxidation is brought about by the oxidized substrate

of the peroxidase, which is involved in a cycle of oxidation and reduction.

The results of the present work suggest a connexion between manganese and plant peroxidase.



It is necessary to postulate such a cycle since only catalytic amounts of substrate are required experimentally. Preliminary experiments suggest that not all peroxidase substrates are active in the cycle. Thus manganese oxidation has been obtained with the system using *o*- and *p*-cresol but not with pyrogallol. The experiments with *p*-cresol indicate that it is an intermediate and not the final oxidation product which brings about the oxidation of manganese.

Comparison of the activities of the horse-radish extracts, used with that of the peroxidase preparation in presence of *p*-cresol, suggests that the activity of the extracts is limited by the amount of the peroxidase substrate present. In agreement with this, activation of comparatively inactive horse-radish extracts could be brought about by the addition of an ether-soluble factor from active extracts.

At the concentrations of H_2O_2 used experimentally part or all of the manganese oxidation product is reduced by the H_2O_2. This involves the Mn^{++} as well as the peroxidase substrate in a cycle of oxidation and reduction. In manometric experiments with small amounts of added Mn^{++} this results in decomposition of the H_2O_2 with evolution of O_2. These experiments were carried out at concentrations of H_2O_2 relatively high compared with those that would be present *in vivo*. At very low concentrations of H_2O_2 it appears probable that the oxidized Mn would react preferentially with metabolites other than H_2O_2. This would lead to a transfer of oxygen from H_2O_2 to plant metabolites without evolution of O_2. Such a transfer would involve the manganese in a cycle of oxidation and reduction and the hypothesis is put forward that this cycle may be responsible, at least in part, for the effect of manganese on plant respiration shown by Lundegårdh (1939). At very low concentrations of H_2O_2, if oxidation is rapid compared with reduction, the oxidation product would accumulate. It is possible that such conditions exist in soils where microbiological oxidation of Mn^{++} leads to an accumulation of manganese higher oxides. It has been suggested that the underlying factor of Mn-Fe antagonism in the growth of plants is the oxidation of ferrous iron to ferric by manganese (Hopkins 1930; Shive 1941). Such a mechanism depends on the presence in the plant of a system oxidizing manganese.

If manganese can also be oxidized through the agency of the peroxidase-like activity of haem or haematin derivatives, or by milk peroxidase, it is possible that manganese oxidation may occur in animal tissues.

It is known that H_2O_2 is formed in several oxidation reactions catalysed by enzymes, e.g. xanthine oxidase, amino-acid oxidase and glucose oxidase. Such H_2O_2 can be used in promoting secondary or coupled oxidations; thus Thurlow (1925) and Harrison & Thurlow (1926) used an enzyme system to provide H_2O_2 for the peroxidase system and Keilin & Hartree (1936, 1945) similarly demonstrated oxidation of alcohols to aldehydes by means of catalase together with H_2O_2 formed by the previously mentioned enzyme systems.

In preliminary experiments colorimetric evidence has been obtained that H_2O_2 formed by the xanthine oxidase—hypoxanthine system may be used to bring about the oxidation of manganese by the horse-radish extract. It seems possible that an enzyme system producing H_2O_2 provides a suitable means of maintaining a low concentration of H_2O_2 and would facilitate the study of the role of the oxidized Mn in plant respiration. Further work along these lines is in progress.

Lastly, it should be pointed out that the system may be able to oxidize metallic ions other than Mn^{++}. It has been possible to follow the oxidation of manganese manometrically, as the oxidation product decomposes H_2O_2, with evolution of O_2. The fact that the other metallic ions so far tested have not shown such activity is no proof that they are not oxidized by the system.

SUMMARY

1. A system which oxidizes manganese in the presence of hydrogen peroxide has been demonstrated in horse-radish root extracts. This system also exists in other root extracts.

2. Under suitable conditions (i.e. in pyrophosphate or citrate at pH 7) the oxidation product accumulates as a coloured manganic complex. Using horse-radish root extract, manganese dioxide was isolated by the dismutation of manganipyrophosphate at weakly alkaline reaction. The oxidation product decomposes hydrazine and can be estimated manometrically by means of this reaction.

3. An increase in the rate of decomposition of hydrogen peroxide was observed on adding small amounts of manganous sulphate to horse-radish extracts. This effect was attributed to manganese oxidation.

4. The manganese oxidizing system in horse-radish extract consists of a thermolabile and a thermostable factor together with hydrogen peroxide. Partially purified peroxidase preparations, in the presence of certain peroxidase substrates and hydrogen peroxide, oxidize Mn^{++}. It is suggested that the thermolabile and thermostable factors in the extract are peroxidase and peroxidase substrate respectively.

5. The hypothesis is advanced that the manganese reduces the oxidized peroxidase substrate and thereby is itself oxidized. This involves the substrate in a cycle of oxidation and reduction. At low hydrogen peroxide concentrations, the manganese oxidation product may react with plant metabolites other than hydrogen peroxide; this would involve the manganese in an oxidation-reduction cycle, which may explain its effect on plant respiration.

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The Biochemistry of Locusts

1. THE CAROTENOIDS OF THE INTEGUMENT OF TWO LOCUST SPECIES (*LOCUSTA MIGRATORIA MIGRATORIOIDES* R. & F. AND *SCHISTOCERCA GREGARIA* FORSK.)

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A research programme on the biochemistry of locust pigmentation, with special reference to the problem of swarming, has recently been initiated. An essential preliminary to the study of carotenoid metabolism in these insects (Goodwin, 1949) was the identification of the pigments concerned. Lederer (1935), quoting unpublished work carried out by Volkonsky and himself, reported the presence of a mixture of xanthophylls and carotene in adult *Schistocerca gregaria* Forsk.; Chauvin (1941) noted an unidentified pink pigment in hoppers and a mixture of α- and β-carotenes in adult insects of the same species. No data on *Locusta* species have been recorded.

We have found that hoppers and adult insects of both *Locusta migratoria migratorioides* R. & F. and *Schistocerca gregaria* yielded, on extraction with acetone after removal of the alimentary tract, a mixture of two carotenoids. These pigments have been identified as β-carotene and astaxanthin. Small amounts of lutein (xanthophyll) and chlorophyll were occasionally encountered, but they probably originated in small amounts of unremoved alimentary tract or in small pieces of grass which can become tightly lodged in the buccal cavity and the claws of the forelegs.

The pigments were identified by their chromatographic and spectrographic properties; insufficient