3. Tryptochrome and its precursor, iodoprotryptochrome, have been isolated, and provisional formulae are assigned to them.

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The Oxidation of Manganese by Peroxidase Systems

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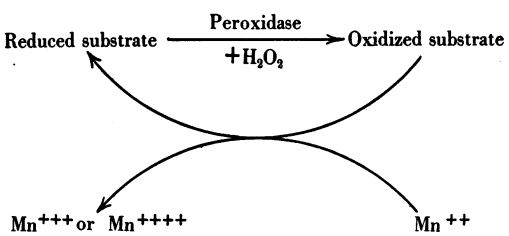
Kenten & Mann (1949) demonstrated the oxidation of manganese by certain root extracts in the presence of added hydrogen peroxide; the oxidizing system consisted of a thermolabile and a thermostable factor, together with hydrogen peroxide. Partially purified peroxidase preparations, in the presence of certain phenolic peroxidase substrates and hydrogen peroxide, were found to oxidize added manganous



ions (Mn^{++}) and it was suggested that the thermolabile and thermostable factors in the root extracts are peroxidase and peroxidase substrate respectively. The hypothesis was advanced that intermediate products of the oxidation of certain phenolic substances by peroxidase and hydrogen peroxide can bring about the oxidation of manganese, according to the above scheme which involves the substrate in a cycle of oxidation and reduction. The present work was undertaken to establish the oxidation of manganese by plant peroxidase preparations, in the presence of phenolic substrates and hydrogen peroxide, and to investigate the properties of the system.

MATERIALS AND METHODS

Peroxidase preparations of different purity were obtained from horseradish and turnip, while following the method of Keilin & Mann (1937). The 'Purpurogallinzahl' (P.Z.) of these preparations was determined by the method of Keilin & Mann (1937).

Catalase was prepared from horse liver by the dioxane method of Sumner & Dounce (1937). The 'Katalasefähigkeit' (Kat.f.) of the preparation, as determined by the method of Sumner & Somers (1943), was 20,000.

Hydrogen peroxide (0·05 M). H_2O_2 (2·7 ml. of A.R. 20 vol.) was diluted to 100 ml. A fresh solution was prepared for each experiment, and estimated manometrically with acid MnO_2. (See Tables 3 and 4.)

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. The gas space was air unless otherwise stated.

EXPERIMENTAL AND RESULTS

Colorimetric evidence of manganese oxidation

Kenten & Mann (1949) found that on addition of $MnSO_4$ and H_2O_2 to certain plant extracts in pyrophosphate at pH 7, oxidation of manganese took place with the formation of pink manganipyrophosphate. Similar colour reactions were obtained with partially purified horseradish peroxidase preparations and *p*-cresol on addition of $MnSO_4$ and H_2O_2. With horseradish extracts MnO_2 was isolated from the reaction mixtures by allowing the manganipyrophosphate to dismute at pH 10.

A variety of phenolic peroxidase substrates have now been tested colorimetrically and the results obtained with horseradish peroxidase of P.Z. 210 are given in Table 1. Similar results were obtained with a turnip peroxidase preparation of P.Z. 90. Colorimetric evidence of manganese oxidation was obtained with all the monohydric phenols tried except tyrosine, which was apparently not oxidized under these conditions. None of the dihydric and trihydric phenols tested, with the exception of resorcinol, gave evidence of manganese oxidation.

Where positive tests were obtained the normal oxidation product of the phenol did not accumulate. This is in agreement with the hypothesis that the manganese is oxidized by oxidation products of the phenols. Experiments were made with reaction mixtures as in Table 1, using phenol and *p*-cresol as phenolic substrate, in which the $MnSO_4$ was added after the oxidation of the phenol had been allowed to proceed for 10 min. No colorimetric evidence of manganese oxidation was obtained under these conditions. This suggests that it is intermediate and not final oxidation products of the phenols which oxidize the manganese.

Table 1. *Colorimetric evidence of manganese oxidation by peroxidase systems*

(Reaction mixtures consisting of horseradish peroxidase of P.Z. 210 (10 μg. in 0·5 ml. water), 0·1 ml. of 0·5 M-$MnSO_4$, 2 ml. M-pyrophosphate at pH 7, and 0·3 ml. of 0·1 M-phenolic substrate, were made up in test tubes and 0·2 ml. of 0·05 M-H_2O_2 were added. Control experiments were carried out in the absence of $MnSO_4$. The colour which had developed within 1 min. of the addition of the H_2O_2 was noted.)

Phenolic substrate	Colour in absence of $MnSO_4$	Colour in presence of $MnSO_4$
None	Nil	Nil
Phenol	Pale yellow-brown	Pink
p-Cresol	Milky	Pink
o-Cresol	Yellow-green	Pink
Tyrosine*	Nil	Nil
Catechol	Green-brown	Green-brown
Quinol	Yellow-brown	Yellow-brown
Resorcinol	Light brown	Pink
Pyrogallol	Brown	Brown
3:4-Dihydroxy-cinnamic acid* (caffeic acid)	Brown	Brown

* 1·5 mg. solid added to each tube 10 min. before adding H_2O_2.

Isolation of manganese dioxide

Reaction mixtures containing 150 ml. of M-pyrophosphate buffer (pH 7), 30 ml. 0·1 M-phenolic substrate, 20 ml. 0·5 M-$MnSO_4$, 4 mg. horseradish peroxidase of P.Z. 105, or 5 mg. turnip peroxidase of P.Z. 90, and 100 ml. water, were made up. A brisk current of N_2 was blown through the mixture and 20 ml. of 0·05 M-H_2O_2 was added. The solution rapidly became dark red with phenol and *p*-cresol, and dark brown with pyrogallol and catechol. Ten minutes after the H_2O_2 addition the manganipyrophosphate was converted to MnO_2, by the procedure of Kenten & Mann (1949). In these experiments, however, the MnO_2 flocculated readily and was spun off 10 min. after the addition of alkali. On making the catechol and pyrogallol reaction mixtures alkaline (pH 10) and centrifuging, a black sediment was obtained and the supernatant remained a dark colour.

Control experiments were made with reaction mixtures in which either the phenolic substrate or peroxidase or H_2O_2, was omitted. These control mixtures were worked up

Table 2. *Analysis of oxidation products isolated*

(The washed sediments were ground in a mortar and made up in water to 25 ml. The Mn content was estimated colorimetrically and the oxidizing capacity by manometric measurement of O_2 output by 0·5 ml. suspension in 2·3 ml. N-H_2SO_4 (in main vessel) from 0·2 ml. of 0·5 M-H_2O_2 in 0·1 N-H_2SO_4 (added from sidearm).)

Phenolic substrate	Mn (mg./ml. of suspension)	O_2 (μl./ml. of suspension)	Apparent valency of Mn in oxidation product	Total MnO_2* isolated (mg.)	Yield of MnO_2 (% based on total H_2O_2 added in preparation)
p-Cresol	0·826	324·0	3·92	31·5	36
p-Cresol†	0·800	305·5	3·87	29·7	34
Phenol	0·640	255·4	3·96	21·9	25
Catechol	0·001 (approx.)	2·0	—	—	—
Pyrogallol	0·001 (approx.)	2·5	—	—	—

* Calculated from oxidizing capacity.
† In this experiment a turnip peroxidase preparation of P.Z. 90 was used.

similarly to the complete reaction mixtures, except that 1 mg. catalase (Kat.f. 20,000) was added to remove the H_2O_2 where present. This was necessary to parallel the complete reaction mixtures in which all the H_2O_2 had been used up. No hydrated MnO_2 could be isolated from the control mixtures.

The analysis and amounts of hydrated MnO_2 isolated from the various reaction mixtures are given in Table 2.

Manometric studies of manganese oxidation by peroxidase systems

The oxidation of manganese by peroxidase systems has been studied manometrically in two ways (cf. Kenten & Mann, 1949): (1) In pyrophosphate at pH 7. A stable manganic complex can be formed and under conditions where the rate of formation of manganipyrophosphate is greater than its rate of decomposition by the H_2O_2, the manganipyrophosphate accumulates and can be estimated. (2) In orthophosphate at pH 7. A stable manganic complex cannot be formed and the manganese oxidation product reacts with the H_2O_2 as rapidly as it is formed, and there is no accumulation of oxidized manganese.

Experiments in pyrophosphate: the accumulation and estimation of manganipyrophosphate

The manganipyrophosphate which accumulates when the reactions are carried out at high $MnSO_4$ concentration in pyrophosphate at pH 7 can be estimated manometrically with hydrazine (Kenten & Mann, 1949). This method has been used to study the effect of variation in the peroxidase, the phenolic substrate, and the H_2O_2 concentration on the accumulation of manganipyrophosphate.

The effect of variation in the peroxidase concentration. Provided that the concentration of phenolic substrate is sufficient, an active manganese oxidizing system can be demonstrated with a few μg. of the peroxidase preparation, P.Z. 105. The effect of variation in the peroxidase concentration on the accumulation of manganipyrophosphate is shown in Table 3. On adding the H_2O_2 a rapid evolution of O_2 took place, the rate increasing with increasing enzyme concentration, and the evolution of gas being complete in all cases within 10 min. The total O_2 output decreased with increasing peroxidase concentration and varied from 71 to 22% of the theoretical output over the range of 10–160 μg. of peroxidase of P.Z. 105. This decrease in O_2 output was due to increase in manganipyrophosphate accumulation. The most intense pink colour was in the vessel containing the largest amount of peroxidase, and in the subsequent estimation of manganipyrophosphate with hydrazine the N_2 evolution was highest in this vessel. The combined O_2 and N_2 output was in every case close to that required by theory. Under these conditions, with purified enzyme preparations, no H_2O_2 is lost in side reactions, whereas with horseradish extract the sum of the O_2 and N_2 outputs was less than theoretical (Kenten & Mann, 1949). Calculation from the N_2 output showed that the yield of manganipyrophosphate was 30–82% of theory. Under the conditions used 10 μg. horseradish peroxidase of P.Z. 105 gave about 460 μg. Mn_2O_3. Control experiments gave no evidence of any significant manganese oxidation when either peroxidase or *p*-cresol was omitted from the reaction mixtures. Since the purity of the peroxidase preparation was only of the order of 10% (Keilin & Mann, 1937; Theorell, 1942), it is apparent that an active manganese oxidizing system can be obtained at very low peroxidase concentrations.

The effect of variation in p*-cresol concentration.* An active system can also be produced with a few μg. of *p*-cresol provided the concentration of peroxidase is sufficient. This is shown by the results of Table 4. Here 0·85 mg. of horseradish peroxidase of P.Z. 210 was used and the *p*-cresol was varied from 0 to 20 μg. The rate of evolution of O_2 increased with increasing *p*-cresol concentration. The output was complete in 3 min. with 20 μg., and in 15 min. with 1 μg. *p*-cresol. The total O_2 output decreased with increasing *p*-cresol concentration and varied from 61 to 17% of the theoretical output over the range 1–20 μg. *p*-cresol. As noted in the previous experiment the decrease in O_2 output was associated with manganipyrophosphate accumulation, shown by the relative intensities of the pink colorations and by the subsequent gas outputs on adding hydrazine. The sum of the O_2 and N_2 outputs was again close to that required by theory. In control experiments a comparatively small N_2 output was obtained when either *p*-cresol or Mn^{++} was omitted. These control values were greater than in the previous experiment (Table 3), and seem to be associated

Table 3. *Effect of variation in the peroxidase concentration on the accumulation of manganipyrophosphate*

(Reaction mixtures consisted of 0·2 ml. of 0·5 M-$MnSO_4$ (i.e. 5500 μg. of Mn^{++}), 0·3 ml. of 0·1 M-*p*-cresol and varying amounts of horseradish peroxidase of P.Z. 105 in 0·5 M-pyrophosphate at pH 7. Gas space, N_2. 0·2 ml. of 0·05 M-H_2O_2 was added from the sidearm and the O_2 output measured. When the O_2 output stopped the manometers were regassed with N_2 and 0·2 ml. saturated aqueous N_2H_4, H_2SO_4 was added from the second sidearm and the N_2 output measured after 10 min.)

	Complete system						Controls	
							Mn omitted	*p*-Cresol omitted
Peroxidase of P.Z. 105 added (μg.)	0	10	20	40	80	160	160	160
O_2 output (μl.)	−0·6	76	65·7	51·8	38·4	23·6	−1·5	1·6
N_2 output* (μl.)	3·5	32·4	49·7	59·5	73·4	87·6	1·0	8·8
O_2+N_2 output (μl.)	—	108·4	115·4	111·3	111·8	111·2	—	—
Theory for O_2+N_2 output† (μl.)	—	107·0	107·0	107·0	107·0	107·0	—	—

* Corrected for 105% recovery (see Kenten & Mann, 1949).

† Half O_2 output obtained by adding the H_2O_2 used to excess hydrated MnO_2 in N-H_2SO_4.

with the larger amounts of peroxidase used. It must be remembered, however, that in the complete systems all the H_2O_2 was used up in the first half of the experiment, whereas most of the controls contained residual H_2O_2 and part of the output observed on adding hydrazine may have been due to partial decomposition of this H_2O_2. Calculations from the N_2 outputs showed that the yield of manganipyrophosphate was 37–82% of theory. Under the conditions used 1 μg. *p*-cresol gave 605 μg. Mn_2O_3. These results support the hypothesis that the phenolic substrate undergoes a cycle of oxidation and reduction during the oxidation of manganese in this system.

were calculated as Mn_2O_3 from the N_2 outputs and are plotted against the molar concentration of the H_2O_2 used (Fig. 1). The amounts of manganipyrophosphate which accumulated increased with increasing H_2O_2 concentration up to 0·003 M; further increase in H_2O_2 concentration caused little increase in manganipyrophosphate accumulation. The accumulated manganipyrophosphate calculated as a percentage of that theoretically possible, decreased with increasing H_2O_2 concentration from 80% at 0·0017 M-H_2O_2 to 30% at 0·0066 M-H_2O_2. This suggests that at sufficiently low H_2O_2 concentrations all the H_2O_2 would be used in the accumulation of manganipyrophosphate.

Table 4. *Effect of variation in the* p-*cresol concentration on the accumulation of manganipyrophosphate*

(Reaction mixtures consisted of 0·85 mg. horseradish peroxidase of P.Z. 210, 0·2 ml. 0·5 M-$MnSO_4$ (i.e. 5500 μg. of Mn^{++}) and varying amounts of *p*-cresol as below in 0·5 M-pyrophosphate at pH 7. Gas space N_2. 0·2 ml. of 0·05 M-H_2O_2 was added from the sidearm and the O_2 output measured. When the O_2 output stopped the manometers were regassed with N_2 and 0·2 ml. saturated aqueous N_2H_4, H_2SO_4 was added from the second sidearm and the N_2 output measured after 10 min.)

	Complete system						Controls: Mn omitted	Controls: Peroxidase omitted
p-Cresol added (μg.)	0	1	2	5	10	20	20	20
O_2 output (μl.)	9·0	71·5	60·8	43·0	26·3	19·7	9·3	0·6
N_2 output* (μl.)	11·5	42·8	56·3	75·7	88·6	95·7	11·0	4·1
O_2+N_2 output (μl.)	—	114·3	117·1	118·7	114·9	115·4	—	—
Theory for O_2+N_2 output† (μl.)	—	116·5	116·5	116·5	116·5	116·5	—	—

* Corrected for 105% recovery (see Kenten & Mann, 1949).
† Half O_2 output obtained by adding the H_2O_2 used to excess hydrated MnO_2 in N-H_2SO_4.

The effect of variation in the hydrogen peroxide concentration. This was studied in N_2, reaction mixtures containing 40 μg. horseradish peroxidase of P.Z. 105, 0·01 M-*p*-cresol, 5500 μg. $MnSO_4$ in 0·5 M-pyrophosphate at pH 7. H_2O_2 was

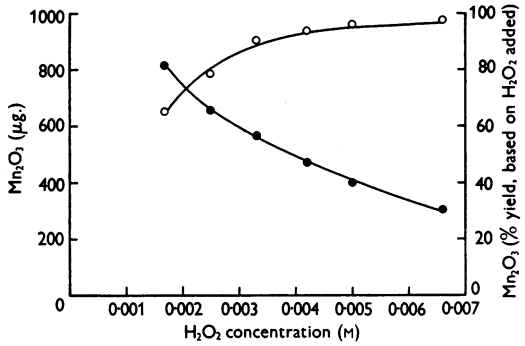


Fig. 1. The effect of variation in the H_2O_2 concentration on the accumulation of manganipyrophosphate; O—O as μg. Mn_2O_3; ●—● as percentage yield of Mn_2O_3 based on H_2O_2 added. Pyrophosphate buffer at pH 7 and 5500 μg. Mn^{++}.

added from the sidearm to give initial concentrations of 0·0017–0·0066 M, and the O_2 output measured. When the O_2 output stopped, the manometers were regassed with N_2 and 0·2 ml. saturated aqueous N_2H_4, H_2SO_4 was added from the second sidearm and the N_2 outputs measured after 10 min. The sum of the O_2 and N_2 outputs was close to that required by theory. The amounts of manganipyrophosphate formed

Experiments in orthophosphate: the decomposition of hydrogen peroxide by peroxidase systems in the presence of Mn^{++}

It has previously been shown that under conditions where a stable manganic complex cannot be formed the manganese oxidation product reacts stoicheiometrically with H_2O_2, and the oxidation of manganese can be followed manometrically by observation of the decomposition of H_2O_2. The oxidation of manganese by horseradish extracts and by a peroxidase preparation in presence of *p*-cresol has already been followed in this way (Kenten & Mann, 1949).

In the above accumulation experiments it was necessary to use a comparatively high concentration of Mn^{++}. In the following experiments, where the decomposition of H_2O_2 served as a measure of the oxidation of manganese, the properties of the system could be studied at manganese concentrations approaching those likely to be present *in vivo*.

The activity of different phenolic substrates. The activity of some of the phenolic substrates previously tested colorimetrically (Table 1) has been studied manometrically. The results obtained with phenol, *o*-cresol, catechol, resorcinol, and caffeic acid are shown in Fig. 2.

Reaction mixtures consisted of 20 μg. horseradish peroxidase of P.Z. 105, 55 μg. Mn^{++}, and 0·001 M-phenolic substrate in 0·033 M-orthophosphate at pH 6·5. After equilibration 0·2 ml. 0·05 M-H_2O_2 was added from the sidearm.

A rapid evolution of gas was observed with phenol, resorcinol and *o*-cresol. This output was complete in about 10 min. with phenol and resorcinol and in 30 min. with *o*-cresol. The theoretical output for complete decomposition of the H_2O_2 used was 109 μl. O_2. The outputs observed in presence of phenol, resorcinol and *o*-cresol were 108, 87 and

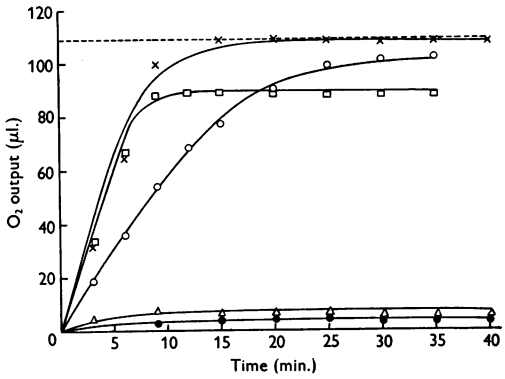


Fig. 2. The activity of different phenolic substrates in the oxidation of manganese by peroxidase and H_2O_2, demonstrated by the catalatic activity produced. Orthophosphate buffer at pH 6·5 and 55 μg. Mn^{++}. ×—×, phenol; □—□, resorcinol; ○—○, *o*-cresol; △—△, catechol; ●—●, caffeic acid. The dotted line shows the theoretical output.

102 μl. O_2 respectively. With catechol and caffeic acid no significant output was observed. Pyrogallol, quinol and tyrosine were also tested and showed slight uptakes of 12, 4 and 4 μl. O_2 respectively in 40 min. Owing to the low solubility of tyrosine 3 mg. of solid was weighed into the vessel. The substrates whose presence led to a rapid decomposition of the H_2O_2 were those previously found to give positive colorimetric tests for manganese oxidation (Table 1).

The effect of variation in phenol concentration. The effect of variation in phenol concentration was studied over the range 0·00033–0·02 M in 0·033 M-orthophosphate at pH 7 using 55 μg. Mn^{++} and 20 μg. horseradish peroxidase P.Z. 105. On adding 0·2 ml. 0·05 M-H_2O_2 a rapid evolution of O_2 took place in all vessels; it was complete with the higher phenol concentrations in about 5 min. Attempts to slow down the rate of reaction at the higher phenol concentrations by using less peroxidase gave erratic results. With the lower phenol concentrations, where the reaction was sufficiently slow for several 3 min. readings to be taken, a plot of O_2 output against time showed a linear relationship between these quantities until about 85% of the H_2O_2 had been decomposed. The straight lines so obtained when extrapolated did not pass through the origin but cut the time axis at about 1 min. Consequently the O_2 outputs after 3 min. have been plotted against phenol concentration in Fig. 3. The curve shows that a maximum velocity was reached at about 0·007 M-phenol. At this concentration an output of 83 μl. O_2/3 min. was obtained with 20 μg. peroxidase of P.Z. 105, giving a Q_{O_2} of 83,000 μl./mg. dry wt./hr. Pure peroxidase under these conditions would therefore give a Q_{O_2} of about 1,000,000. The total O_2 outputs were close to theory in all cases (Table 5).

With 3 ml. of 0·007 M-phenol the introduction of one oxygen atom per molecule of phenol requires 235·2 μl. O_2. It is clear, therefore, that no significant amount of the phenol can finally have been present in the dihydric or higher oxidation forms. In agreement with this the contents of the flasks remained colourless at the end of the experiment.

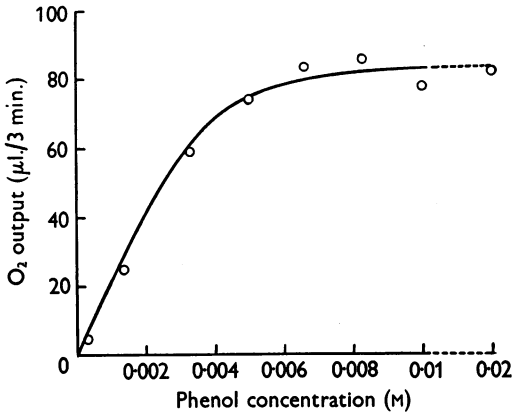


Fig. 3. The effect of variation in phenol concentration on the oxidation of manganese by peroxidase and H_2O_2. Orthophosphate buffer at pH 7 and 55 μg. Mn^{++}.

Table 5. *Decomposition of* H_2O_2 *by horseradish peroxidase and* $MnSO_4$ *in presence of varying concentrations of phenol*

(Reaction mixtures consisting of 20 μg. horseradish peroxidase of P.Z. 105, 55 μg. Mn^{++}, in 0·033 M-orthophosphate at pH 7, and varying amounts of phenol as below. 0·2 ml. of 0·05 M-H_2O_2 was added from the sidearm and the O_2 output measured. Theoretical output for complete decomposition of H_2O_2 111·5 μl. O_2.)

Concentration of phenol (M)	Total O_2 output: Time (min.)	Total O_2 output: Vol. of O_2 (μl.)
0	40	6
0·00033	30	105
0·00133	10–15	110
0·002	10	110·5
0·0033	10	109
0·01	5	107
0·02	5	109·5

The effect of variation in the peroxidase concentration. Reaction mixtures consisted of 0·001 M-phenol and 22 μg. Mn^{++} in 0·033 M-orthophosphate at pH 7 and varying amounts (5–1500 μg.) of horseradish peroxidase of P.Z. 105. The evolution of O_2 on adding the H_2O_2 is plotted in Fig. 4. As the peroxidase added was increased from 0 to 80 μg. the rate of O_2 output increased and the total output, where it was reached in the experimental time, was theoretical. Further increases in peroxidase concentration up to 1500 μg. caused a progressive decrease in the total O_2 output. Thus with 150, 500 and 1500 μg. peroxidase the total outputs were 93, 83 and 67% of theory, respectively. The reaction mixtures which contained the lower concentrations of

enzyme remained colourless at the end of the experiment, showing that the rate of reduction of the effective phenol oxidation product by the manganese is of the same order as its rate of formation. The reaction mixtures which contained the higher enzyme concentrations, producing a lower O_2 output, were brown in colour and contained a black precipitate. Under these conditions the rate of oxidation of the phenol is faster than the rate of reduction of the oxidation product by the manganese, and phenol oxidation products accumulate.

An active system can be demonstrated with a few μg. of peroxidase preparation or phenolic substrate. Decrease in the H_2O_2 concentration favours accumulation of the manganese oxidation product, and the results suggest that by coupling with a system such as hypoxanthine-xanthine oxidase, which would provide a constant supply of H_2O_2 at very low concentration, all this H_2O_2 could be used in the formation and accumulation of manganese oxidation product. When the oxidation of manganese is followed manometrically by the decomposition of H_2O_2, under suitable conditions the rate of reaction remains constant for 10 min. or longer, possibly owing to the fact that phenolic oxidation products do not accumulate. It may be possible, therefore, to develop a manometric method of peroxidase estimation based on this reaction, alternative to that of Ettori (1949).

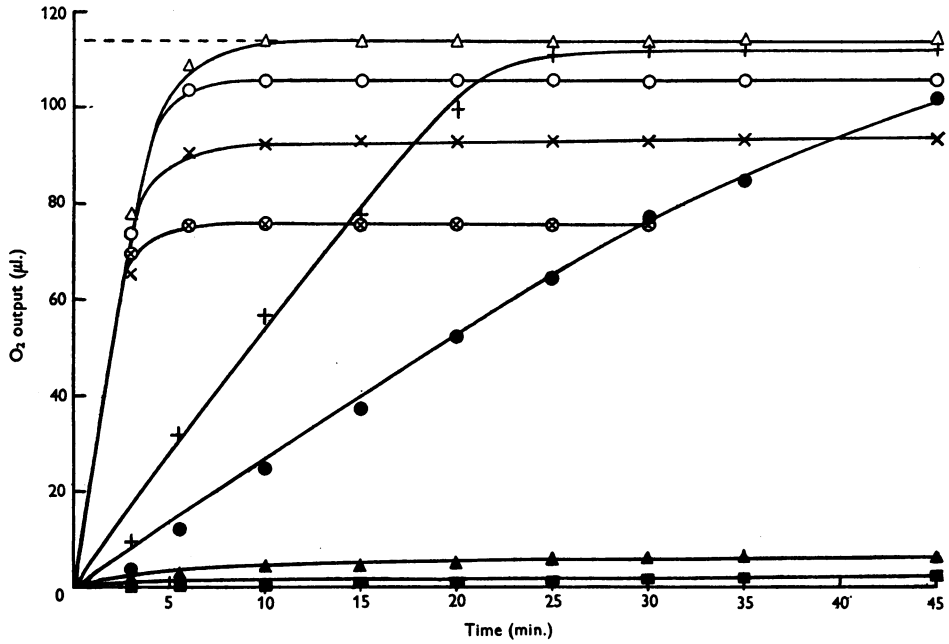


Fig. 4. The effect of variation in the peroxidase concentration on the oxidation of manganese. Orthophosphate buffer at pH 7 and 22 μg. Mn^{++}. Peroxidase added (P.Z. 105), ⊗—⊗, 1500 μg.; ×—×, 500 μg.; ○—○, 150 μg.; △—△, 80 μg.; +—+, 10 μg.; ●—●, 5 μg. Controls: ▲—▲, 80 μg. peroxidase, Mn omitted; ■—■, 80 μg. peroxidase, phenol omitted. The dotted line shows the theoretical output.

The effect of several hydrogen peroxide additions. In previous experiments the oxidation of manganese by the system has been limited by the amount of H_2O_2 added. When this H_2O_2 has been used up an active system can again be produced under suitable conditions by further addition of H_2O_2. In one experiment in orthophosphate, three additions of 0·05 M-H_2O_2 were made to an active system; the second and third additions were made after the output from the previous additions had stopped. All the H_2O_2 was rapidly decomposed, although some falling off in activity was observed with the third addition. The total O_2 output was only slightly less than that required for complete decomposition of the H_2O_2 added.

DISCUSSION

It is now clear that peroxidase preparations from horseradish and turnip in the presence of certain phenolic substrates and H_2O_2 can oxidize manganese.

The results of the present work support the hypothesis previously advanced that the manganese reduces an intermediate oxidation product of the phenolic substrate and thereby is itself oxidized. This involves the substrate in a cycle of oxidation and reduction. Provided sufficient manganese is present the accumulation of phenolic oxidation products is prevented. The catalytic activity of the phenolic substrate is demonstrated by the fact that

under suitable conditions 1 μg. *p*-cresol produced 605 μg. Mn_2O_3.

It is well known that quinones formed by the action of polyphenol oxidase can bring about secondary oxidations, e.g. coenzyme I (Dixon & Zerfas, 1940), coenzyme II (Kubowitz, 1937), ascorbic acid (Robinson & Nelson, 1944), amino-acids (Happold & Raper, 1925; James, Roberts, Beevers & De Kock, 1948). Evidence has been presented that tyrosinase may act as a terminal oxidase in plant respiration, the quinone acting as a hydrogen acceptor (e.g. Boswell & Whiting, 1938; Baker & Nelson, 1943). In this connexion Robinson & Nelson (1944) point out that if insufficient hydrogen donors are present the quinones give rise to inactive melanin products. Similarly, with the manganese oxidizing system, if insufficient manganese is present to reduce the intermediate oxidation product, more highly oxidized inactive products are formed.

The nature of the effective phenolic oxidation product is not yet clear. Of the phenolic substrates so far tested monohydric phenols and resorcinol alone have been shown to bring about manganese oxidation. It is possible that the oxidation of manganese is brought about by the system with dihydric and trihydric phenols as substrates, but that in these cases the oxidized manganese is rapidly reduced by the phenolic substrates.

Preliminary attempts to demonstrate manganese oxidation with *o*-benzoquinone, or with tyrosinase in presence of *p*-cresol have been unsuccessful. If the oxidation with the peroxidase system were brought about by the reduction of *o*-quinones to dihydric phenols, part of the oxygen of the H_2O_2 would be finally present in the dihydric phenol. But provided sufficient manganese is present to reduce the active phenolic oxidation product, all of the H_2O_2 is utilized in the oxidation of manganese. This suggests that the active oxidation product is reduced back to the monohydric phenol. Further work is necessary to elucidate the mechanism of the reaction.

If, as postulated, the oxidation is brought about by reaction between phenolic oxidation product and divalent manganese, such a reaction would be unspecific for the reductant. It is possible that other inorganic and organic substances may be oxidized by the system peroxidase + phenolic substrate + H_2O_2. Elliot (1932) has investigated the oxidations catalysed by peroxidase in the presence of H_2O_2, but little work appears to have been done on the secondary oxidations brought about by the complete system, although it has been shown to oxidize ascorbic acid (e.g. Szent-Györgyi, 1928; Tauber, 1936; Huszak, 1937). The oxidizing capabilities of the system are under investigation. Preliminary results suggest that it can oxidize ferrocyanide to ferricyanide.

SUMMARY

1. Plant peroxidase preparations from horseradish and turnip in the presence of certain phenolic substrates and hydrogen peroxide have been shown to oxidize manganous salts.

2. Oxidation of manganese could be demonstrated with phenol, *p*-cresol, *o*-cresol and resorcinol as the phenolic substrate, but not with quinol, catechol, pyrogallol and caffeic acid.

3. The effect of variation in the concentrations of peroxidase, phenolic substrate, and hydrogen peroxide have been studied. An active system could be demonstrated with 1 μg. of *p*-cresol.

4. The results are in agreement with the hypothesis previously advanced that the manganese reduces an oxidation product of the phenolic substrate and thereby is itself oxidized.

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