The Oxidation of Manganese by Illuminated Chloroplast Preparations

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It has long been known that manganese is an essential micronutrient of plants. Although the precise functions of manganese are unknown there is evidence that it may be concerned in chlorophyll synthesis, photosynthesis, respiration and nitrate assimilation. Decreased photosynthesis under conditions of manganese deficiency was originally attributed to the diminished chlorophyll content, but more recent investigations suggest the possibility of a direct effect of manganese on photosynthesis. The evidence, which is conflicting, is reviewed by Ruck & Bolas (1954). Arnon (1954) and Gerretsen (1937, 1949, 1950) obtained evidence in favour of a direct effect of manganese on photosynthesis. Gerretsen (1950) concluded from potentiometric measurements that H_2O_2 was formed when chloroplast preparations were illuminated in the presence of Mn^{2+} . It was suggested that Mn^{2+} functions as an activator of oxygen and that a photosensitive oxidation-reduction system is present in chloroplasts in which there is equilibrium between Mn^{2+} and Mn^{3+} or Mn^{4+} , this equilibrium moving in the direction of oxidation during illumination. Mehler (1951*a, b*) demonstrated the formation of H_2O_2 by illuminated chloroplast suspensions by showing that addition of catalase to such suspensions resulted in the coupled oxidation of added ethanol. Kenten & Mann (1950) showed that peroxidase systems catalyse the oxidation of Mn^{2+} . If chloroplast preparations contain peroxidase the hydrogen peroxide formed on illumination might be used for the oxidation of Mn^{2+} . The present work was undertaken to find out whether illuminated chloroplast preparations oxidize Mn^{2+} and whether such oxidation is due to peroxidase systems.

MATERIALS AND METHODS

Plant material. Most of the plants used were glasshouse-grown in a John Innes potting compost.

Leaf extracts. These were made from leaves of tobacco (*Nicotiana tabacum* L.), spinach (*Beta vulgaris* L.), pea (*Pisum sativum* L.) and broad bean (*Vicia faba* L.). The leaves (20 g.) were ground with sand and 100 ml. 0.04M-phosphate pH 7 at 0° in a chilled mortar. The macerate was squeezed by hand through strong cotton cloth into a chilled tube, centrifuged for 2 min. at 2000 r.p.m. (900 g) and the precipitate discarded. The values given for

g refer to the centrifugal force at the bottom of the centrifuge tubes.

Chloroplast preparations. The leaf extract (100 ml.) was centrifuged at 10000 r.p.m. (11000 g) for 15 min. in an MSE (Measuring and Scientific Equipment Ltd.) angle-head centrifuge at 0–4°. The precipitate was suspended in 100 ml. 0.04M phosphate, pH 7, at 0° and centrifuged again at 10 000 r.p.m. for 15 min. at 0–4°. The precipitate of chloroplast material was suspended in 0.04M phosphate, pH 7, to a convenient volume and stored at 0°.

Peroxidase. This was prepared from horseradish roots by the method of Kenten & Mann (1954). The *Purpurogallinzahl* (P.Z.) (Willstätter & Stoll, 1918) of the preparation, i.e. mg. purpurogallin formed by 1 mg. enzyme preparation in 5 min. from pyrogallol and H_2O_2 under fixed conditions was estimated by the method of Keilin & Hartree (1951) using an EEL (Evans Electroselenium Ltd.) colorimeter with Ilford Bright Spectrum Blue filter 622 (maximum transmission at λ 465 m μ). The preparation of P.Z. 1040 used was about 90% peroxidase.

Catalase. This was prepared from ox liver by the method of Sumner & Dounce (1937) and the activity of the preparation was estimated as described previously (Kenten & Mann, 1952*b*). The *Katalasefähigkeit* (Kat.f.) was 11000, suggesting a purity of about 20%. From the equation of Chance & Herbert (1950) that $Kat.f. = 520 k'_1/M$, where M is the molecular weight of catalase (230 000), the velocity constant of the preparation, $k_1 = 4.9 \times 10^6 M^{-1} \text{ sec}^{-1}$.

Manganipyrophosphate solution. This was prepared by the procedure of Heintze & Mann (1949), which depends on the reaction

Excess of hydrated MnO_2 was added to solutions of $MnSO_4$ in 0.1M pyrophosphate at pH 7. The mixtures were allowed to stand with occasional shaking at room temperature for 18 hr. The residual MnO_2 was then removed by centrifuging. The manganipyrophosphate in the supernatant solutions was estimated by titration against standard $FeSO_4(NH_4)_2SO_4$.

Solutions of manganipyrophosphate in 0.1M pyrophosphate at pH 7 absorb ultraviolet light strongly, with an absorption maximum at 258 m μ . (Fig. 2). At this wavelength such solutions of manganipyrophosphate obey Beer's law at least over the range of concentration of 2–7 $\mu g.$ $Mn^{3+}/ml.$ (Fig. 1). Manganipyrophosphate in experimental reaction mixtures was estimated by measuring the absorption at 258 m μ , or manometrically with hydrazine sulphate according to the method of Kenten & Mann (1949). The results of Table 1 show a close agreement between the two methods of estimation.

Spectrophotometry. Absorption data were determined by the use of a Unicam model SP. 500 quartz spectrophotometer.

Chlorophyll. This was estimated spectrophotometrically by the method of Arnon (1949).

Illumination. An Osram Daylight fluorescent tube was the source of light.

Fig. 1. Effect of manganipyrophosphate concentration on the absorption of ultraviolet light at 258 mμ. 0.1M-pyrophosphate, pH 7.

Fig. 2. Spectrophotometric evidence for the oxidation of Mn²⁺ by illuminated chloroplast preparations. Samples of a tobacco chloroplast preparation were incubated with and without MnSO₄ in light (100 ft.c.) and in darkness for 3 hr. After clarification by centrifuging the ultraviolet absorption curves of the solutions were determined by using the mixture incubated in darkness without added MnSO₄ in the blank cell. ●, in light with MnSO₄; +, in darkness with MnSO₄; Δ, in light without MnSO₄; —, manganipyrophosphate (3.5 μg. Mn²⁺/ml.) in 0.1M pyrophosphate, pH 7.

Table 1. *Estimation of manganipyrophosphate by the spectrophotometric and manometric methods*

The concentration of the manganipyrophosphate solution (300 μg. Mn³⁺/ml.) was established by titration against standard FeSO₄·(NH₄)₂SO₄·6H₂O. For the manometric estimation each vessel contained 2.8 ml. of the undiluted manganipyrophosphate solution. After equilibration 0.2 ml. of saturated hydrazine sulphate solution was added from the side arm. Gas phase, N₂; temp. 28°. The Mn²⁺ present was calculated from the N₂ outputs after correction for 105% recovery as described by Kenten & Mann (1949). For the spectrophotometric estimation, the absorption at 258 mμ. of suitable dilutions of the manganipyrophosphate solution in 0.1M pyrophosphate, pH 7, were measured.

Mn ³⁺ present (μg./ml.)	Mn ³⁺ found (μg./ml.)	
	Spectro- photometric method	Manometric method
1.5	1.38	—
3	2.95	—
6	6.20	—
300	—	300
300	—	286
300	—	286

EXPERIMENTAL AND RESULTS

Oxidation of Mn²⁺ by illuminated chloroplast preparations

Colorimetric evidence. Kenten & Mann (1952a) showed that when certain plant extracts were incubated with added Mn²⁺ and pyrophosphate at pH 7, the oxidation of Mn²⁺ and the accumulation of the oxidation product in the form of manganipyrophosphate could be demonstrated by means of the intense blue coloration which this compound gives with benzidine. In preliminary experiments in the present work positive tests of this nature were obtained by the use of illuminated leaf extracts and chloroplast preparations in the presence of Mn²⁺ and pyrophosphate pH 7. The tests were made in 50 ml. beakers with reaction mixtures consisting of 2 ml. leaf extract from tobacco or spinach leaves, 3 ml. 0.1M pyrophosphate (pH 7) with and without 1–2 ml. 0.02M-MnSO₄ and water to 6 ml. The reaction mixtures were illuminated (100 ft.c.) for 2–3 hr. at room temperature. Control reaction mixtures were incubated in the dark. Benzidine acetate (a few drops of 0.2% solution in 10% v/v acetic acid) was then added. Strong blue colorations were obtained with those reaction mixtures containing added Mn²⁺ which had been illuminated, whereas in the absence of added Mn²⁺, or of illumination, negative or only weakly positive tests were obtained. The results suggest the possibility that some of the positive

tests for manganese oxidation obtained by Kenten & Mann (1952*a*) with leaf extracts may have been dependent on illumination. Washed chloroplast suspensions prepared from the leaf extracts also gave strongly positive tests for manganese oxidation in light but not in darkness, while the supernatants from the centrifuged leaf extracts (15 min. at 10 000 r.p.m.) gave negative or, only occasionally, slightly positive tests. The colorimetric tests were negative with extracts or chloroplast preparations which had been heated for 5 min. in a boiling-water bath. The fact that the positive colorimetric tests were due to the accumulation of manganipyrophosphate was subsequently confirmed by the manometric estimation of this compound in the reaction mixtures by using the method of Kenten & Mann (1949). But for much of the work with the chloroplast preparations, particularly where low concentrations of Mn^{2+} were used, the spectrophotometric method of estimation of manganipyrophosphate was found more suitable.

Spectrophotometric evidence. The fact that the Mn^{2+} is oxidized by chloroplast preparations in the presence of light was confirmed by experiments in which the accumulated manganipyrophosphate was identified and estimated by the spectrophotometric method. Fig. 2 illustrates the results of one such experiment.

Reaction mixtures consisting of 0.5 ml. washed tobacco chloroplasts (1.67 mg. chlorophyll/ml.), 3 ml. 0.1 M pyrophosphate, pH 7, with and without 0.6 ml. 0.01 M- $MnSO_4$ in a total volume of 6 ml. were incubated either in light (100 ft.c.) or in darkness for 3 hr. Each reaction

mixture was then made up to a total volume of 8 ml. and centrifuged at 10 000 r.p.m. for 20 min. The supernatants were diluted with 4 vol. 0.1 M pyrophosphate pH 7, and the ultraviolet absorption curves were determined, using a suitable mixture in the blank cell. The supernatant solution from the illuminated reaction mixture containing added Mn^{2+} gave an absorption curve closely resembling that of manganipyrophosphate. The absorption, like that of manganipyrophosphate, was destroyed within a few minutes by added hydrazine sulphate (0.1 ml. 0.1 M- $N_2H_4 \cdot H_2SO_4$ /3.5 ml.). From the absorption at 258 m μ , it was calculated that a total of 116 μ g. Mn^{3+} had been formed in this reaction mixture. The amount of added Mn^{2+} (0.6 ml. of 0.01 M soln.) was 330 μ g. The supernatants from the other reaction mixture showed comparatively little absorption of ultraviolet light. Little evidence of Mn^{2+} oxidation was obtained when the chloroplast suspensions were illuminated before addition of Mn^{2+} and then incubated in the dark.

Comparison of spectrophotometric and manometric methods of estimation of the manganese oxidation product (manganipyrophosphate)

The amount of manganipyrophosphate formed when chloroplast preparations are illuminated in the presence of added Mn^{2+} was often sufficient to impart a pink colour to the supernatant solution. Under these conditions, provided that sufficient of the supernatant solution was used, it was possible to estimate the manganipyrophosphate by the manometric method. The results obtained by this method generally agreed well with those obtained by the more sensitive spectrophotometric method (Table 2).

Table 2. *Spectrophotometric and manometric estimation of manganipyrophosphate formed by illuminated chloroplast preparations*

Reaction mixtures consisting of washed tobacco or spinach chloroplast preparations (0.2–2 ml.), 1.8 ml. 0.02 M- $MnSO_4$, 3 ml. 0.1 M pyrophosphate, pH 7, in a total volume of 6 ml. were illuminated for 18 hr. at 100 ft.c. Control reaction mixtures were incubated in darkness for the same period. The reaction mixtures were then diluted to 8 ml., centrifuged, and the manganipyrophosphate present in the supernatants was estimated by the spectrophotometric and manometric methods.

Expt. no.	Plant	Volume of chloroplast preparations (ml.)	Chlorophyll present (μ g.)	Total Mn^{3+} formed (μ g.)	
				Spectro- photometric method	Manometric method
1	Spinach	2	440	560	560
2	Spinach	0.2	290	216	230
	Spinach	0.5	740	430	460
	Spinach	1	1470	610	610
3	Spinach	1	1830	790	760
	Spinach	1	1830	780	860
	Spinach	1	1830	770	780
4	Tobacco	2	540	410	430
	Tobacco	2	540	400	380
5	Tobacco	2	580	420	—
	Tobacco	2*	580	18	—

* Chloroplast suspensions previously heated for 10 min. at 100°.

*Reduction of manganipyrophosphate by
chloroplast preparations*

The chloroplast preparations were found to reduce manganipyrophosphate. This was clearly shown in reaction mixtures incubated in darkness (Table 3). With chloroplasts in the light the fall in manganipyrophosphate concentration was comparatively small and was usually observed clearly only in the first 2 hr. of the incubation. The apparent oxidation measured in this work is, therefore, the resultant of both oxidation and reduction. If the amount of this reduction in light is no greater than that in darkness it is insufficient to affect the results greatly.

chloroplast material was used within 48 hr. of preparation.

Conditions of illumination. The reaction mixtures (6 ml.) in 50 ml. beakers had a depth of about 5 mm. and were only occasionally shaken. Under these conditions the intensity of illumination of the bottom of the reaction mixture would be much less than that at the surface, particularly when large amounts of chloroplast preparation were present. Accordingly, the effect of continuous stirring and of varying the depth of the reaction mixtures was investigated. When the reaction mixtures were stirred by bubbling with wet air, with the same intensity of incident light (100 ft.c.), the rate of Mn^{2+} oxidation increased by about 25 %. When the

Table 3. *Reduction of manganipyrophosphate by chloroplast preparations*

Complete reaction mixtures consisting of 2 ml. washed tobacco chloroplast preparation (560 $\mu g.$ chlorophyll/ml.) and 130 $\mu g.$ Mn^{2+} in 0.05 M pyrophosphate, pH 7, were incubated for periods of 2 and 18 hr. in light (100 ft.c.) and darkness. Control reaction mixtures consisting of chloroplasts without added Mn^{2+} and of manganipyrophosphate alone were incubated under the same conditions. The manganipyrophosphate remaining at the end of the incubation periods was estimated spectrophotometrically.

Components present	Conditions of incubation	Total Mn^{2+} finally present ($\mu g.$)
Manganipyrophosphate	2 hr. dark	132
Manganipyrophosphate	2 hr. light	130
Manganipyrophosphate	18 hr. dark	132
Manganipyrophosphate	18 hr. light	126
Chloroplasts + manganipyrophosphate	2 hr. dark	98
Chloroplasts + manganipyrophosphate	2 hr. light	112
Chloroplasts + manganipyrophosphate	18 hr. dark	84
Chloroplasts + manganipyrophosphate	18 hr. light	124

*Effect of various factors on the oxidation of
 Mn^{2+} by illuminated chloroplasts*

Type and age of chloroplast preparation. It is known that the inclusion of sucrose in the extraction medium helps to preserve the morphological integrity of chloroplasts; phosphate-sucrose is usually preferred to sucrose alone in the isolation of intracellular structures. The manganese-oxidizing activities of chloroplast preparations made with 0.5 M sucrose solution containing 0.04 M phosphate at pH 7 were therefore compared with those of chloroplast preparations made with phosphate alone. The rate of manganese oxidation was less with the chloroplast preparations made with phosphate-sucrose. Sucrose (0.5 M) added to the reaction mixtures had little effect on the rate of manganese oxidation.

The effect of the age of the chloroplast preparation, stored at 0°, on the manganese-oxidizing activity was very variable. With some preparations the activity persisted almost unchanged for up to 3 days; with others decreases of up to 50 % were observed in this period. For most of the work the

average depth of the reaction mixtures was reduced to 0.4 mm. by using Petri dishes as reaction vessels the rate of Mn^{2+} oxidation was about 40 % higher than in unstirred reaction mixtures in beakers. When open Petri dishes were used the rate of Mn^{2+} oxidation was also higher than with the unstirred reaction mixtures in beakers, but the results were erratic, possibly owing to the rapid evaporation.

Period of illumination. The relation between the period of illumination and manganipyrophosphate formation was found to be linear during the early stages of the reaction. In most experiments the rate was approximately linear for at least 1 hr., but then decreased with time until a stage was reached (after 6-24 hr.) when further prolonged illumination produced little or no increase in the amount of manganipyrophosphate present. The rate of manganipyrophosphate formation, the duration of the linear rate, and the total amount of manganipyrophosphate formed, varied widely with different chloroplast preparations even when the amounts of these used were such as contained equal amounts of chlorophyll. These experiments were carried out by

using reaction mixtures similar to those of Table 2. The manganipyrophosphate formed was estimated spectrophotometrically by measuring the difference in absorption at 258 $m\mu$. between the reaction mixtures in light and dark.

Light intensity. The light intensity was varied over the range 50–200 ft.c. by placing the beakers or Petri dishes containing the reaction mixtures at suitable distances from the source of light. The

increase of chloroplast preparation. The effect of this factor on the manganipyrophosphate formation was also studied in Petri dishes by using a short reaction period (30 min.). The results (Fig. 4) show that under these conditions the manganipyrophosphate formed was approximately proportional to the chlorophyll concentration up to about 100 μg . chlorophyll/ml. Further increases in chlorophyll concentration (up to 250 μg ./ml.) produced progressively smaller increases in the rate of manganipyrophosphate formation.

Fig. 3. Effect of light intensity on the oxidation of Mn^{2+} by two different tobacco chloroplast preparations illuminated for 1 hr. in beakers Δ , $\circ$; a broad-bean chloroplast preparation illuminated for 30 min. in Petri dishes, $\square$. Reaction mixtures were similar to those of Table 2 except that 0.9 ml. 0.01 M- $MnSO_4$ was used.

differences in temperature of the reaction mixtures were negligible under the conditions used. The experiments were of short duration (0.5–1 hr.), so that the measurements were made during the period when the rate of formation of manganipyrophosphate was rapid. Results obtained with three different chloroplast preparations are shown in Fig. 3. Although the amount of manganipyrophosphate formed varies with the chloroplast preparation used, all three preparations showed the same general trend of increase in activity with increase in light intensity. In other experiments a decrease was sometimes observed at an intensity of 200 ft.c. with the reaction mixtures in Petri dishes.

Concentration of chloroplast preparation. It is clear from the results of Table 2 (Expt. 2) that the manganipyrophosphate formation increases with

Fig. 4. Effect of varying the concentration of broad-bean chloroplast preparation on the rate of oxidation of Mn^{2+} . The reaction mixtures, in Petri dishes, were similar to those of Table 2 and were illuminated for 30 min. at 100 ft.c.

Concentration of Mn^{2+} . The effect of this factor on manganipyrophosphate formation was tested over a range of concentrations of Mn^{2+} of 1–550 μg ./ml. In these experiments, in order to keep the higher amounts of Mn^{2+} in solution, the amount of buffer used was increased to 1.2 ml. M pyrophosphate. The results of one experiment are shown in Table 4. With the reaction mixtures containing the lowest concentrations of added Mn^{2+} it was necessary to measure the absorption directly on the supernatants without dilution. Under these conditions the results obtained were erratic. The method finally adopted throughout these experiments was to measure the decrease in absorption at 258 $m\mu$. produced by the addition of hydrazine sulphate. This decrease in absorption was taken as a measure of the manganipyrophosphate present. At the lower concentrations of Mn^{2+} the results do not represent the effect of Mn^{2+} concentration on the reaction velocity

since all the added Mn^{2+} was oxidized. This result is of particular interest since it shows that the reaction can take place at physiological concentrations of Mn^{2+} . Oxidation of added Mn^{2+} was complete up to a concentration of Mn^{2+} of 11 $\mu g./ml.$ Further increases of Mn^{2+} increased the oxidation though not in proportion to the increase in concentration of Mn^{2+} .

Effect of catalase. With reaction mixtures similar to those of Table 2 added catalase (2 mg.) caused a 20–40 % inhibition of manganese oxidation. The chloroplast suspensions contained both intact chloroplasts and chloroplast fragments of varying sizes. The fact that the inhibition by catalase was only partial may have been due to insufficient penetration of the catalase to the site of the oxidation, particularly with the intact chloroplasts and larger fragments. This was tested with two different types of chloroplast preparation. After removal of the precipitate obtained by centrifuging a tobacco leaf extract at 2000 r.p.m. (900 g) two fractions

were obtained from the supernatant, one by spinning for 10 min. at 3200 r.p.m. (2200 g) and the other by spinning the 3200 r.p.m. supernatant for 10 min. at 10 000 r.p.m. (11 000 g). The pellets were suspended in 0.04M orthophosphate (pH 7) and the chlorophyll contents estimated. Samples containing the same amount of chlorophyll were used in the subsequent experiments. It was found (Table 5) that the inhibiting effect of catalase was slightly more marked on the chloroplast preparations obtained by high-speed centrifuging, though in the absence of added catalase the activity of these preparations was similar to those obtained by centrifuging at lower speeds. In the reaction mixtures used the catalatic activity due to the chloroplasts present was of the order of one-hundredth of that of the added catalase.

Effect of peroxidase. The results given in Table 6 show that added peroxidase increases both the rate and total amount of Mn^{2+} oxidation. In agreement with the known properties of peroxidase, rather prolonged heat treatment was necessary to eliminate the peroxidase effects. The amounts of added peroxidase necessary to produce the maximum increase in rate were large in comparison with the amounts of peroxidase in the chloroplast preparations. Those preparations tested showed weak peroxidatic activity in colorimetric tests with benzidine and H_2O_2 , but the activity was usually insufficient to allow satisfactory estimations of the P.Z. by the method of Keilin & Hartree (1951). In the attempts to estimate the peroxidase activity by this method P.Z. values of 0–0.2 were obtained.

Effect of phosphate. When phosphate was substituted for pyrophosphate in the reaction mixtures strongly positive benzidine tests were obtained after illumination, but, on centrifuging, the supernatants had little absorption at 258 $m\mu$. These results were due to the formation of an oxidation product insoluble in phosphate. When the precipi-

Table 4. *Effect of concentration of Mn^{2+} on the formation of manganipyrophosphate by illuminated chloroplast preparations*

Reaction mixtures consisted of 0.4 ml. washed tobacco chloroplast preparation (420 $\mu g.$ chlorophyll), varying amounts of $MnSO_4$ and 1.2 ml. M pyrophosphate, pH 7, in a total volume of 6 ml. The reaction mixtures were illuminated for 3 hr. at 100 f.t.c.

Mn^{2+} added ($\mu g.$)	Mn^{2+} formed ($\mu g.$)
5.5	6
11	9.5
22	24.5
44	45
66	62
82.5	64
495	78
1490	106
3300	167

Table 5. *Effect of catalase on the oxidation of Mn^{2+} by illuminated chloroplast preparations*

The chloroplast preparations used were prepared as described in the text by low speed (LS) followed by high speed (HS) centrifuging of tobacco leaf extracts. The reaction mixtures were similar to those of Table 2 except where the catalase preparation (2 mg.) was added.

Expt. no.	Time of illumination (hr.)	Chloroplast preparation	Chlorophyll present ($\mu g.$)	Mn^{2+} formed ($\mu g.$)		
				No catalase added	Catalase added	Heat-treated (10 min. at 100°) catalase added
1	1.5	HS	240	70	—	—
	1.5	LS	240	76	—	—
2	18	HS	970	198	—	—
	18	LS	970	190	—	—
3	3.5	HS	730	126	60	—
	3.5	LS	730	118	72	—
4	3	HS	590	78	40	64
	3	LS	590	72	56	72

tates obtained by centrifuging were extracted with pyrophosphate containing $MnSO_4$ the extracts obtained gave the characteristic absorption curve of manganipyrophosphate and positive tests with benzidine. Pyrophosphate containing $MnSO_4$ was used as solvent, since although MnO_2 is insoluble in neutral pyrophosphate it dissolves when Mn^{2+} is also present to give manganipyrophosphate according to equation 1. Although Mn^{2+} was added to the pyrophosphate for the routine extractions it was found that, in some cases at least, this addition was unnecessary since that part of the Mn^{2+} added to the reaction mixtures which remained unoxidized during illumination of the chloroplasts was largely retained by the precipitate on centrifuging. The

obtained by centrifugation from such reaction mixtures was suspended in phosphate instead of pyrophosphate for the manometric estimations, the results obtained by the method were higher than those by the spectrophotometric method. Kenten & Mann (1952b) demonstrated the oxidation of Mn^{2+} by coupling enzyme systems producing H_2O_2 with peroxidase systems. Here also the oxidation product in phosphate media reacted much more slowly than manganipyrophosphate or hydrated MnO_2 with the hydrazine but finally gave a total N_2 output greater than that required by theory. The reason for these findings is not yet clear, since MnO_2 reacts quantitatively with hydrazine in phosphate media.

Table 6. *Effect of peroxidase on the oxidation of Mn^{2+} by illuminated chloroplast preparations*

The reaction mixtures were similar to those of Table 2, except that 0.9 ml. 0.02M $MnSO_4$ was used in Expt. 1.

	Time of illumination (hr.)	Chlorophyll present ($\mu g.$)	Peroxidase added ($\mu g.$)	Mn^{3+} formed ($\mu g.$)
Expt. 1				
Tobacco chloroplasts	1	520	0	75
	1	520	1	85
	1	520	5	100
	1	520	50	125
Expt. 2				
Spinach chloroplasts	18	940	0	280
	18	940	10	404
	18	940	10*	320
	18	940	10†	304

* Previously heated for 15 min. at 100°.

† Previously heated for 30 min. at 100°.

Table 7. *Comparison of the oxidation of Mn^{2+} by illuminated chloroplast preparations in phosphate and pyrophosphate media*

The reaction mixtures were similar to those in Table 2 except that 0.9 ml. 0.02M $MnSO_4$ was used in Expt. 2, and, where indicated, phosphate buffer was used in place of pyrophosphate. The reaction mixtures were illuminated for 18 hr. at 100 ft.c.

		Chlorophyll present (μ g.)	Manganese oxidized (calculated as μ g. Mn^{3+})		
Buffer			Spectro- photometric method	Manometric method	Manometric method in phosphate
Expt. 1					
Broad-bean chloroplasts	Phosphate	1350	220	240	—
	Pyrophosphate	1350	250	240	—
Expt. 2					
Tobacco chloroplasts	Phosphate	1120	270	—	390
	Pyrophosphate	1120	220	240	—

results of Table 7. suggest that there is little difference in the amount of manganese oxidation, whether phosphate or pyrophosphate is used to buffer the reaction mixtures. This is supported by the results both of the spectrophotometric and manometric estimations. When the manometric method was used with phosphate reaction mixtures without prior centrifuging, or when the precipitate

DISCUSSION

The results of the present work show that Mn^{2+} is oxidized by illuminated chloroplasts. This oxidation occurs at physiological concentrations of Mn^{2+} , though, to facilitate the estimation of the oxidation product, the experiments were generally made at relatively high concentrations of Mn^{2+} . Mn^{2+} is

known to be present in chloroplasts, though it is apparently not concentrated therein. Thus Whatley, Ordin & Arnon (1951) found that the concentration of Mn^{2+} in the chloroplasts of sugar-beet leaves was approximately equal to that expected on the basis of random distribution in the leaf. In their chloroplast preparations for each g. atom Mn, 12.8 moles chlorophyll were found, which corresponds to 4.4 $\mu g.$ $Mn^{2+}/mg.$ chlorophyll.

A thermolabile factor is involved. Added peroxidase increases the rate of oxidation, though the amount necessary to produce significant increases is great in comparison with that present in the chloroplast preparations used. The oxidation is partially inhibited by added catalase. As with peroxidase comparatively large amounts are necessary to produce a significant effect. A possible explanation is that these enzymes penetrate only with difficulty to the site of the oxidation. Some evidence of this has been obtained in the experiments with added catalase. The results suggest that, in part at least, the oxidation is brought about by peroxidase systems. Mehler (1951*a, b*) and Mehler & Brown (1952) attribute the formation of hydrogen peroxide by illuminated chloroplast preparations to the action of oxygen as a Hill reagent. It is not yet clear how far this reaction takes place *in vivo*. Mehler (1951*a*) points out that oxygen may act as a Hill reagent only under abnormal conditions, but does consider the possibility that photo-oxidations of green plants may be catalysed by peroxidases after reduction of oxygen in the Hill reaction. The results of the present work suggest the possibility that manganese oxidation occurs in chloroplasts *in vivo* and that a manganese oxidation-reduction cycle may play a part in the reactions of photosynthesis. Work in progress with pea plants (*Pisum sativum* L.) in water culture has shown that manganese oxidation can take place in plants *in vivo*. At high manganese concentrations deposition of manganese higher oxides can be demonstrated mainly in the stems, but to a lesser extent in the leaves. In view of previous work on manganese oxidation by plant extracts and peroxidase systems (Kenten & Mann, 1949, 1950, 1952*b*) it is presumed that such oxidation is due to peroxidase systems. It may be that part of the oxidation observed in the leaf is due to hydrogen peroxide produced by the action of light on the chloroplasts.*

* Note added in proof. Since this work was submitted for publication Andreae (1955) described a photo-induced oxidation of Mn^{2+} by the use of riboflavin as light-sensitizer. The mechanism postulated could provide an explanation of the results of the present work. This possibility is under investigation.

SUMMARY

1. Chloroplast preparations oxidize Mn^{2+} in light but not, or to a much less extent, in darkness.
2. In pyrophosphate the oxidation product accumulated as a soluble manganipyrophosphate. In phosphate an insoluble oxidation product was formed which like MnO_2 on treatment with pyrophosphate containing Mn^{2+} gave a soluble manganipyrophosphate.
3. The oxidation products oxidize hydrazine and were estimated manometrically by this reaction or spectrophotometrically as manganipyrophosphate. A sensitive spectrophotometric method of estimating manganipyrophosphate has been described.
4. The effect of variation of light intensity, period of illumination, concentration of chloroplast material and of Mn^{2+} was studied.
5. Catalase partially inhibits the oxidation. Peroxidase increases the rate of oxidation.
6. It is suggested that the oxidation is due, at least in part, to peroxidase systems and that a manganese oxidation-reduction cycle may play a part in the reactions of photosynthesis.

REFERENCES

- Andreae, W. A. (1955). *Arch. Biochem. Biophys.* **55**, 584.
 Arnon, D. I. (1949). *Plant Physiol.* **24**, 1.
 Arnon, D. I. (1954). *Rep. and Comm. 8th Int. Bot. Congr., Paris*, Sect. 11-12, 73.
 Chance, B. & Herbert, D. (1950). *Biochem. J.* **46**, 402.
 Gerretsen, F. C. (1937). *Ann. Bot., Lond. (N.S.)*, **1**, 207.
 Gerretsen, F. C. (1949). *Plant & Soil*, **1**, 346.
 Gerresten, F. C. (1950). *Plant & Soil*, **2**, 159.
 Heintze, S. G. & Mann, P. J. G. (1949). *J. agric. Sci.* **39**, 80.
 Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
 Kenten, R. H. & Mann, P. J. G. (1949). *Biochem. J.* **45**, 255.
 Kenten, R. H. & Mann, P. J. G. (1950). *Biochem. J.* **46**, 67.
 Kenten, R. H. & Mann, P. J. G. (1952*a*). *Biochem. J.* **50**, 360.
 Kenten, R. H. & Mann, P. J. G. (1952*b*). *Biochem. J.* **52**, 125.
 Kenten, R. H. & Mann, P. J. G. (1954). *Biochem. J.* **57**, 347.
 Mehler, A. H. (1951*a*). *Arch. Biochem. Biophys.* **33**, 65.
 Mehler, A. H. (1951*b*). *Arch. Biochem. Biophys.* **34**, 339.
 Mehler, A. H. & Brown, A. H. (1952). *Arch. Biochem. Biophys.* **38**, 365.
 Ruck, H. C. & Bolas, B. D. (1954). *Ann. Bot., Lond. (N.S.)*, **18**, 267.
 Sumner, J. D. & Dounce, A. L. (1937). *J. biol. Chem.* **121**, 417.
 Whatley, F. R., Ordin, L. & Arnon, D. I. (1951). *Plant Physiol.* **26**, 414.
 Willstätter, R. & Stoll, A. (1918). *Liebigs Ann.* **416**, 21.