MANGANESE AS A FUNCTIONAL COMPONENT OF CHLOROPLASTS

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Summary

A series of experiments has been carried out to determine whether manganese is directly or indirectly involved in the photochemical reactions (as measured by the Hill reaction) of chloroplasts of higher plants.

It has been established that manganese is a constituent of tomato chloroplasts, and that isolated chloroplasts contain a manganese fraction which is not readily removed by repeated washing, by lysis, or by purification using density-gradient centrifugation. Further, the Hill reaction activity of isolated chloroplasts was found to be proportional both to the level of manganese supplied in the external nutrient and, more importantly, to the manganese content of the isolated chloroplasts.

It has been shown that the manganese of chloroplasts is not removed by treatment with a range of organic solvents but that it is removed by treatment with metal-complexing agents, including cyanide. A high concentration (5×10^{-2}M) of KCN is required to remove manganese from isolated chloroplasts. A high cyanide concentration is also needed to inhibit the Hill reaction of isolated chloroplasts.

These results are consistent with the suggestion that manganese is directly involved in the Hill reaction system of chloroplasts.

I. Introduction

Kessler (1955) found that manganese-deficient, intact algae (*Ankistrodesmus*) show reduced photosynthesis (O_2 evolution) but not reduced photoreduction (H_2 uptake) per unit of chlorophyll. This finding led to the suggestion that manganese does not take part in the primary photochemical process of photosynthesis but rather that it is involved as a cofactor in the oxygen-evolving sequence (Kessler 1957).

Further evidence indicating that manganese may have a specific role in photosynthesis comes from the work of Arnon (1954) who has shown that there is a rapid (20 min) restoration of photosynthesis upon the addition of manganous salts to manganese-deficient cultures of *Scenedesmus*. He has suggested that this occurs in far less time than that required for any new cell division or even for the formation of protein.

In higher plants it has been shown by Eyster *et al.* (1958) with *Lemna minor*, and by Spencer and Possingham (1960) working with tomato, that chloroplasts isolated from manganese-deficient plants have a greatly reduced Hill reaction activity. In our experiments we also obtained evidence which indicated that manganese deficiency impairs the hydroxyl sequence which terminates in the evolution of molecular oxygen. Further, we established that the addition of manganous salts to chloroplasts isolated from manganese-deficient plants brought about no increase

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in the rate of their Hill reaction. From these experiments and others in which the effects of all the mineral deficiencies, except chlorine, on the Hill reaction were studied, it was concluded that there was no unequivocal evidence of the direct participation of any element, including manganese, in the photochemical reactions of higher plants. The observed effects of nutrient deficiencies could have arisen as an indirect consequence of the nutrient's direct action elsewhere in the cell's metabolism.

The present experiments were designed to determine whether manganese is directly involved in the photochemical reactions of plants. Data are presented which show that manganese is a normal constituent of higher plant chloroplasts and that there is a close correlation between the manganese content of chloroplasts and their Hill reaction activity. The manganese of chloroplasts is not thought to act as a free inorganic ion but rather as a part of a complex.

II. Methods

(*a*) *Water Culture Methods*

Tomato (*Lycopersicon esculentum* L.) plants were grown in nutrient solutions in a glasshouse in which the air temperature was controlled to approximately 25°C by day and 20°C by night. Seedlings were germinated in vermiculite and transferred, after approximately 14 days, to nutrient solutions of the composition described by Tsiu (1948). Each culture vessel held 12 plants. Solutions deficient in manganese were prepared by the method of Stout and Arnon (1939).

In the experiments involving radioactive manganese (^{54}Mn) the normal 3-l. culture vessels were replaced by 1-l. dishes at the time the radioactive solutions were supplied.

(*b*) *Preparation of Chloroplasts, Assay of Hill Reaction Rates, and Estimation of Chlorophyll*

Chloroplasts were isolated by the procedures outlined earlier (Spencer and Possingham 1960). Leaves were homogenized in a solution containing 0·3M sucrose, 0·05M potassium phosphate (pH 8·5), 0·01M KCl, and 0·01M ethylenediaminetetraacetate (EDTA) and separated by differential centrifugation. Routinely the chloroplasts were twice washed by resuspension in buffer consisting of 0·3M sucrose, 0·05M potassium phosphate (pH 7·3), and 0·01M KCl.

For some experiments the chloroplasts were further purified using the density-gradient centrifugation technique of James and Das (1957). Gradients were set up in 16-ml plastic test tubes and consisted of a lower layer of 4·0 ml of 60/40 (v/v) glycerol–buffer* on top of which was layered 8·0 ml of 25/75 (v/v) glycerol–buffer followed by 3–4 ml of chloroplast suspension. The tubes were spun in the cold at 1000 *g* for 12 min, and the chloroplasts which had congregated in a thick band at the position of interpenetration of the two glycerol/buffer layers were collected. This collection was made by puncturing the plastic centrifuge tubes

* Composition 0·3M sucrose, 0·05M phosphate, 0·01M KCl.

with a fine needle just below the dark band and allowing the chloroplast to drip out slowly. These chloroplasts were given a further wash in buffer to remove traces of glycerol before use.

Hill activity measurements, using 2,3',6-trichloroindophenol, and chlorophyll estimations were made by the methods described earlier (Spencer and Possingham 1960).

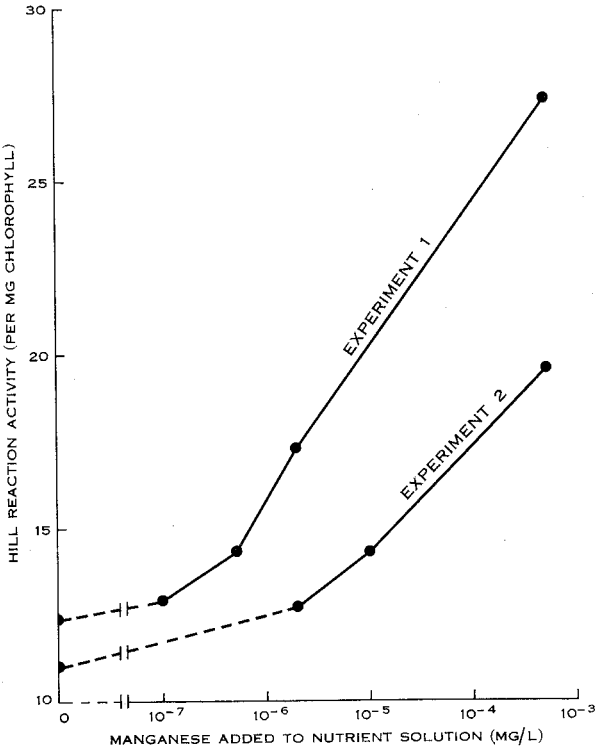

Fig. 1.—Hill reaction activity of chloroplasts isolated from manganese-deficient plants which were supplied with various amounts of manganese 48 hr prior to harvest.

(*c*) *Estimation of Radioactive Manganese*

Chloroplast suspensions were either counted directly in an M6 liquid Geiger tube or, after drying out on planchets, under an end-window Geiger tube. No corrections for self-absorption were necessary as ^{54}Mn has only hard gamma irradiation. Very low counting efficiencies of the order of 0·5% were obtained with the counting assemblies available but this efficiency was adequate provided long counting times were employed.

(*d*) *Estimation of Total Chloroplast Manganese*

Chloroplasts were ashed at 600°C in a muffle furnace. The ash was dissolved in a small volume of conc. hydrochloric acid, and water was added to give a final

volume of 5·0 ml. The manganese content of this solution was determined by atomic absorption spectrophotometry using an apparatus of the type described by David (1960). The absorption of the manganese line at 2794 Å was measured in these assays.

III. Experimental and Results

(*a*) *Effect of Nutrient Levels of Manganese on the Hill Reaction*

Tomato plants were grown in nutrient solutions deficient in manganese and after 25 days graded amounts of manganese were added to the deficient cultures. After a further growing period of 2 days the plants were harvested and the Hill reaction activities of the isolated chloroplasts were measured. The results of two such experiments (Fig. 1) show that under conditions of suboptimal manganese nutrition the Hill activity of the isolated chloroplasts is dependent on the level of the manganese supplied to the growing plant. This result serves to confirm with tomato the relationship established by Eyster *et al.* (1958) with *Lemna* and a number of algae.

Table 1

Relationship between Hill activity and manganese content of isolated tomato chloroplasts

Chloroplast Source	Hill Reaction Activity (ΔO.D. at 620 mμ/mg chlorophyll/45 sec)	Manganese Content (μg Mn/mg chlorophyll)
Manganese-deficient plants	8·55 (58%)*	1·242 (66%)*
Control plants	14·75 (100%)	1·891 (100%)

* As percentage of control.

(*b*) *Relationship between Hill Reaction Activity and the Manganese Content of Isolated Tomato Chloroplasts*

Two experiments were carried out in which the total manganese content and the Hill reaction activity of isolated chloroplasts were measured. In the first chloroplasts were isolated from the leaves of 34-day-old control and manganese-deficient plants. Aliquots were taken for Hill activity and chlorophyll measurements and the bulk of the chloroplasts ashed for the determination of manganese content. The results of this experiment (Table 1) showed that manganese deficiency reduced Hill activity and manganese content of chloroplasts to approximately the same extent.

In the second experiment 26-day-old, manganese-deficient plants were treated with various concentrations of manganese and allowed to recover for 40 hr. At this time samples were taken from the treated plants, from the untreated manganese-deficient plants, and from the healthy control plants. Chloroplast suspensions were prepared and aliquots taken for Hill activity, chlorophyll, and manganese estimations.

The results of this experiment are presented in Figure 2 where it can be seen that there is a close relationship between the manganese content of isolated chloroplasts and their Hill activity. This relationship is particularly direct for chloroplasts isolated from plants receiving suboptimal amounts of manganese.

(c) *Incorporation of* ^{54}Mn *into Tomato Chloroplasts* in vivo

A series of experiments was carried out to establish whether there was any significant incorporation of ^{54}Mn into the chloroplasts of tomato when this isotope

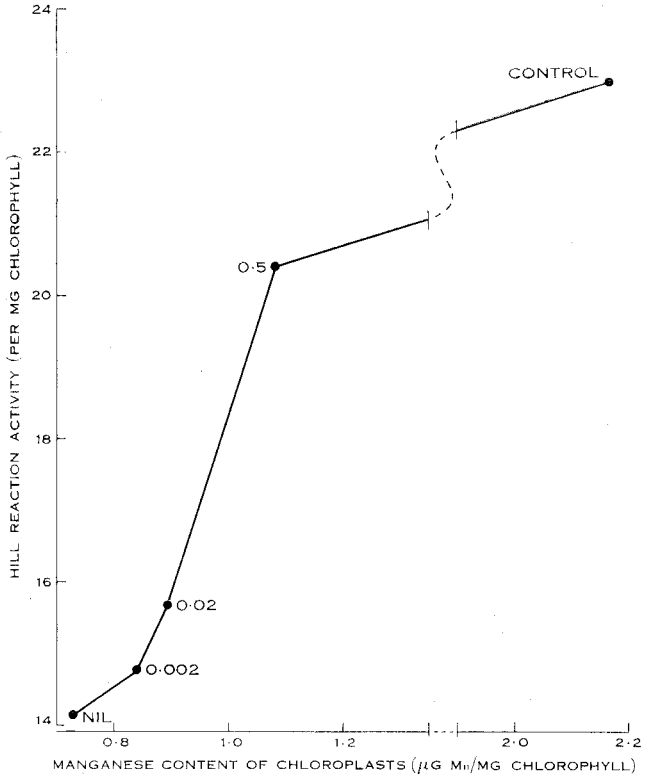

Fig. 2.—Relationship between Hill reaction activity and manganese content of isolated tomato chloroplasts. Chloroplasts isolated from 26-day-old, manganese-deficient plants which had received manganese supplements of nil, 0·002, 0·02, and 0·5 p.p.m. Mn for 40 hr prior to harvest, and from 26-day-old control plants.

was supplied to manganese-deficient plants. Deficient plants were supplied with varying amounts (4–25 μc) of $^{54}MnCl_2$ in the nutrient culture solutions. After varying recovery periods the plants were harvested and chloroplasts isolated in the normal way. Table 2 shows that there was a significant incorporation of ^{54}Mn into the chloroplast fraction of leaves. These results also indicate that the amount of

manganese incorporated into chloroplasts varies, as would be expected, with the quantity of ^{54}Mn supplied and with the length of the recovery period. ^{54}Mn-labelled chloroplasts prepared in this way were used in the subsequent experiments described below.

TABLE 2

IN VIVO INCORPORATION OF ^{54}Mn INTO TOMATO CHLOROPLASTS

Age of Manganese-deficient Plants (days)	Amount of ^{54}Mn Supplied (μc)	Recovery Period (hr)	^{54}Mn Content (counts/min/mg chlorophyll)*
29	5	40	116
29	25	40	368
29	5	72	249
29	4	96	356

* Using an M6 liquid Geiger tube.

To test the possibility that a non-specific adsorption of ^{54}Mn ions onto chloroplasts occurs during isolation, leaves from manganese-deficient plants were ground in the normal solution to which was added high specific activity $^{54}MnCl_2$. Chloroplasts were then isolated by the routine procedures. In three separate experiments no significant radioactivity could be detected in chloroplasts isolated under these conditions.

TABLE 3

^{54}Mn CONTENT OF CHLOROPLASTS PURIFIED BY DENSITY-GRADIENT CENTRIFUGATION

Treatment of Chloroplasts	^{54}Mn Content (counts/min/mg chlorophyll)					
	Expt. 1*	Expt. 2*	Expt. 3*	Expt. 4†	Expt. 5†	Expt. 6†
Original	756	1426	2213	249	116	356
Once-purified	649	1246	2073	200	107	371
Twice-purified					98	357

* Expts. 1–3, ^{54}Mn assayed using an end-window Geiger tube.

† Expts. 4–6, ^{54}Mn assayed using a liquid Geiger tube.

(*d*) *^{54}Mn Content of Chloroplasts Purified by Density-gradient Centrifugation*

As a check that in chloroplasts as routinely prepared the manganese was associated with chloroplasts and not with other contaminating cell particles which might be present, standard chloroplast preparations were subjected to further purification by density-gradient centrifugation. Routinely prepared, twice-washed,

^{54}Mn-labelled chloroplasts were purified by passage through one, and in some cases two, density-gradient centrifugations. ^{54}Mn content per milligram of chlorophyll was measured before and after purification.

These experiments (Table 3) showed that even repeated purification of standard chloroplast preparations resulted in only a slight lowering of the ^{54}Mn content per unit of chlorophyll.

(e) *Preliminary Characterization of the Manganese of Choroplasts*

(i) *Non-extractability of ^{54}Mn from Labelled Chloroplasts.*—^{54}Mn-labelled chloroplasts were subjected to repeated cycles of centrifugation and resuspension in sucrose–phosphate–KCl solution. The ^{54}Mn content remained approximately constant throughout four additional cycles (Table 4) indicating that the ^{54}Mn

TABLE 4

EFFECT OF WASHING WITH SUCROSE ON HILL REACTION ACTIVITY AND ON ^{54}Mn CONTENT OF ISOLATED CHLOROPLASTS

No. of Washings of Chloroplasts	Hill Reaction Activity*	^{54}Mn Content (counts/min/mg chlorophyll)
2 (routine)	15·43	290
3	15·60	282
4	16·60	264
5	14·61	258
6	14·45	276

* Expressed as ΔO.D. at 620 mμ/mg chlorophyll/45 sec.

present in chloroplasts is not readily diffusible. It is possible, of course, that chloroplasts contain a further manganese fraction which is removed during isolation. The Hill reaction activity of chloroplasts also remained approximately constant throughout the above manipulations.

(ii) *Extraction of ^{54}Mn-labelled Chloroplasts with Water, Organic Solvents, and Metal-complexing Agents.*—^{54}Mn-labelled chloroplasts were extracted with a range of solvents for 30 min at room temperature or at 0°C. The insoluble fraction was then removed by centrifugation or filtration and the clear supernatant solutions were counted in a liquid Geiger tube. These counts were related to the total counts recorded in similar chloroplast aliquots diluted in buffer and counted in the same assembly. Values for the percentage of total activity removed by different solvents and treatments are given in Table 5.

The bulk of the manganese of chloroplasts remained with the particulate fraction when chloroplasts were lysed with water. Furthermore, lysis in the presence of 10^{-2}M $MnCl_2$ did not cause a significantly greater loss of ^{54}Mn than did water alone. Treatment of chloroplasts with a range of organic solvents also failed to remove the incorporated ^{54}Mn.

However, treatment with dilute acids and metal-complexing agents, including *o*-phenanthroline, 8-hydroxyquinoline, and potassium cyanide largely removed the ^{54}Mn from labelled chloroplasts.

(iii) *Effect of Extraction with Cyanide on ^{54}Mn Content and Hill Reaction Activity of Isolated Chloroplasts.*—In this experiment ^{54}Mn-labelled chloroplasts prepared in the usual way were suspended either in further sucrose–phosphate–KCl or in this solution containing various concentrations of cyanide. After standing for 1 hr at 0°C the chloroplasts were spun down and aliquots of the supernatant liquid were taken for ^{54}Mn counts. The chloroplasts from all treatments were resuspended and washed in further buffer to remove residual cyanide. Their Hill reaction activity was then measured.

TABLE 5

EXTRACTION OF ^{54}Mn FROM LABELLED CHLOROPLASTS

Treatment	% of Total ^{54}Mn Removed	Mean % Removal
Lysis in:		
Water (0°C)	6, 11, 11	9
10^{-2}M $MnCl_2$ (0°C)	15, 9	11
Extraction with:		
80% acetone (room temp.)	4, 10, 6, 4, 11	7
80% acetone (0°C)	10, 8, 6, 6	7
80% ethanol	2, 0, 2	1
Butanol	0, 2	1
Methanol	0	0
Chloroform	0, 0, 0	0
Petroleum ether	0, 2	1
Chloroform containing 1% 8-hydroxyquinoline	93, 70, 87	83
80% ethanol containing 0·1M *o*-phenanthroline	60, 75	68
Buffer containing 5×10^{-2}M KCN	83, 105, 76	88
1N HCl	87, 90	89
1% trichloroacetic acid	98, 105	100
1N NaOH	62	62

The results of this experiment are given in Table 6, where the amounts of ^{54}Mn removed by washing have been expressed as a percentage of the total activity present in the original chloroplast sample. Cyanide does not displace a significant quantity of manganese from isolated chloroplasts until the high concentration of 5×10^{-2}M KCN is added. High concentrations of cyanide were also required to bring about a significant reduction in Hill reaction activity. Essentially similar results were obtained when ^{54}Mn-labelled chloroplasts were dialysed in the cold (0–4°C) for 24 hr against sucrose–phosphate–KCl solution containing various concentrations of cyanide.

IV. Discussion

The results reported in this paper are all consistent with the suggestion that manganese is directly involved in the photochemical reactions of chloroplasts, as measured by the Hill reaction. Earlier experiments (Arnon 1954; Kessler 1955; Eyster, Brown, and Tanner 1958; Spencer and Possingham 1960, 1961) which demonstrated an inhibitory effect of manganese deficiency on various chloroplast activities did not permit a decision on whether manganese as such was directly involved. The observed effects could merely have been the indirect consequence of a lack of manganese elsewhere in the cell's metabolism.

Table 6

EFFECT OF WASHING LABELLED CHLOROPLASTS IN POTASSIUM CYANIDE ON HILL REACTION ACTIVITY AND ON MANGANESE CONTENT

Chloroplasts Washed in:	Hill Reaction Activity*	Inhibition of Hill Activity† (%)	Removal of ^{54}Mn (%)
Sucrose alone	17·1	0	9·3
Sucrose containing 1×10^{-3}M KCN	15·9	7	9·3
Sucrose containing 1×10^{-2}M KCN	13·8	19	12·1
Sucrose containing 5×10^{-2}M KCN	1·9	89	41·1

* Expressed as ΔO.D. at 620 mμ/mg chlorophyll/45 sec.

† Percentage inhibition of Hill activity relative to that of chloroplasts washed in buffer alone.

The current experiments have demonstrated that manganese is a normal constituent of tomato chloroplasts. The Hill reaction activity of isolated chloroplasts was shown to be proportional to both the level of manganese supplied in the external nutrient solution, and, more importantly, to the manganese content of the isolated chloroplasts. This latter constitutes the strongest evidence to date in favour of the direct participation of manganese in the Hill reaction.

It was possible to characterize to some extent the manganese fraction of isolated chloroplasts using chloroplasts isolated from plants which had been supplied with ^{54}Mn. Manganese was found to be tightly bound to chloroplasts and was not removed by repeated washing of chloroplasts in sucrose (Table 4) or by lysis in water or in non-radioactive $MnCl_2$ (Table 5). This latter result indicates that the bound fraction of chloroplast manganese is primarily associated with the grana. The failure of acetone to extract chloroplast manganese (Table 5) suggests that this element is not part of the lipid fraction.

Extremely high (5×10^{-2}M) concentrations of KCN were required to extract chloroplast manganese, presumably as the manganocyanide complex. A high concentration of KCN was required for the inhibition of most of the Hill activity (Table 6), thus providing another correlation between the presence of manganese and Hill activity. Manganese was removed by extraction of chloroplasts with the

metal-chelating agents 8-hydroxyquinoline and *o*-phenanthroline in chloroform and ethanol respectively. This evidence suggests that manganese is firmly bound in the organized structure of chloroplasts in such a way that it is not readily accessible to aqueous solutions. The high concentration of KCN required may reflect this inaccessibility. It is possible of course that at such high concentrations, cyanide is acting primarily in some other way unrelated to its property of complexing heavy metals.

It should be emphasized that in the present experiments we may be concerned with only a fraction of the total manganese of normal chloroplasts. In the course of isolation, chloroplasts undergo extensive dilution, and it is possible that a less firmly bound manganese fraction is removed in this process. This is especially likely in the present experiments since the tomato leaves were initially ground in a solution containing 10^{-2}M EDTA. However, the fact that the Hill reaction of chloroplasts from manganese-deficient plants is not reactivated by the addition of manganous salts (from 3×10^{-6} to 3×10^{-4}M) indicates that the observed inhibition of this reaction by manganese deficiency is not due to lack of free ionic manganese (Spencer and Possingham 1960). This suggests that the manganese important in the Hill reaction is part of a more complex molecule which cannot be formed *in vitro*. It must be pointed out that the work of Habermann (1960) on the Mehler reaction (Mehler 1951), and the electron-spin resonance studies of Tanner *et al.* (1960) and Treharne *et al.* (1960) suggest that a physiological role for manganese ions may exist in chloroplast reactions beyond those encompassed by the Hill reaction as measured by the reduction of trichloroindophenol.

In one respect our experience is at variance with a brief report by Brown, Eyster, and Tanner (1958) that the Hill reaction of chloroplasts from pokeweed, honey locust, and spinach could be reduced by repeated washing in distilled water, or by treatment with EDTA. Addition of manganese salts to washed chloroplasts caused up to 40% recovery of Hill reaction activity. We have attempted to repeat these experiments with both tomato and spinach chloroplasts but in no case was the activity enhanced by the addition of manganous salts over the range 10^{-6} to 10^{-2}M. In fact, increasing inhibition of Hill activity was observed over this range of concentrations. Also, as noted earlier, the Hill activity of chloroplasts from manganese-deficient plants is not enhanced by the addition of manganous ions (Spencer and Possingham 1960). In addition, no increase in Hill activity could be found when manganese ions were added to chloroplasts from which much of the manganese had been removed by cyanide treatment. No explanation for this discrepancy in results can be offered. It may be that treatments which displace manganese are so damaging to chloroplast organization that reactivation is not possible.

There is now a considerable body of evidence (Kessler 1955; Bishop 1958; Spencer and Possingham 1960, 1961) that the part of the Hill reaction which is impaired by manganese deficiency is the oxygen-evolving sequence. The present report provides correlative evidence which is consistent with the suggestion that manganese is directly involved in the Hill reaction. These two lines of evidence suggest that manganese is an essential component of the oxygen-evolving sequence of chloroplasts.

V. Acknowledgments

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