Interrelationship of Iron and Manganese Supply in Growth, Chlorophyll, and Iron Porphyrin Enzymes in Barley Plants [1]

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Introduction

Somers and Shive (37) suggested that the physiological availability of iron in plants is determined by the relative manganese supply and that the optimum growth and yield of plants depend on a certain definite proportion rather than the absolute level of iron and manganese supply. These workers also held that iron deficiency is identical with manganese excess and iron excess with manganese deficiency, the 2 elements having a mutually antagonistic effect. Twyman (40) and Weinstein and Robbins (42) observed a close similarity between the effect of iron deficiency and manganese excess but the experimental evidence obtained by Berger and Gerloff (8), Hewitt (15, 16), and Morris and Pierre (24) indicated that in many plants the effects of the 2 were quite distinct.

To find out if a definite ratio of iron and manganese supply is necessary for optimal growth of barley, and to learn if the deficiency of iron is identical with manganese excess, we investigated the interaction of iron and manganese as it relates to the growth, visual pathological effects, chlorophyll, nutrient uptake, sugars, protein, and nonprotein nitrogen, and iron porphyrin enzymes in barley. This paper describes the interaction of iron and manganese as it relates to the growth, dry matter yield, chlorophyll content, and the iron porphyrin enzymes. In mature barley plants efforts to measure cytochrome oxidase were not successful and the study of iron porphyrin enzymes was confined to catalase and peroxidase.

Materials and Methods

Barley (*Hordeum vulgare* L. var. K 12) plants were raised in refined sand culture at 3 levels of manganese, 0.0055 ppm (low), 0.55 ppm (normal), and 5.5 ppm (excess). At each level of manganese supply there were 6 levels of iron supply, 0.056 ppm, 0.28 ppm, 0.56 ppm, 1.4 ppm, 5.6 ppm, and 28 ppm. The 18 iron manganese combinations represented varying iron/manganese ratios (table I). For each treatment there were 16 pots arranged in 2 blocks. The treatments were randomized in each block. Plants were raised in 8-inch clay flower pots with a drainage hole and painted 3 times with bitumen. The silica sand used for growing plants was purified by 3 hot acid and 2 hot alkali treatments. The details of sand purification procedure have been described elsewhere (3). Prior to use, the acid washed sand was leached with appropriately purified 4 mM calcium nitrate, till on standing overnight the pH of the sand was 6.5 to 7. Deionized water which contained < 0.0005 ppm each of iron and manganese was used for culture. Macronutrient stock solutions were prepared from A. R. grade salts and were purified against iron and manganese by the phosphate adsorption technique described by Hewitt (18). Stock solutions for manganese, copper, boron, zinc, and molybdenum were prepared from recrystallized A. R. grade salts. Manganese was supplied as manganese sulphate, purified against iron by boiling with sodium bicarbonate according to the method described by Partington (29). Ferric citrate was prepared from ferric chloride, purified against manganese by the procedure described by Hewitt (18) and twice recrystallized citric acid. The purified ferric citrate still contained 0.001 μg manganese per ppm iron. This has not been considered except in working out the iron-manganese ratios. Nutrient solution containing differential iron-manganese supply, 4 mM calcium nitrate, 4 mM potassium nitrate, 2 mM magnesium sulphate,, 1.33 mM sodium dihydrogen phosphate, and micronutrient supplement containing 0.064 ppm copper, 0.065 ppm zinc, 0.37 ppm boron, 0.05 ppm molybdenum, and 0.005 ppm of each cobalt and nickel, was supplied daily except on week ends when pots were flushed with deionized water. The pH of the nutrient solution which drained out of the culture vessels ranged from 6.7 to 7.

Chlorophyll, catalase, peroxidase, tissue iron, and

Table I. *The Fe/Mn Ratios at the Different Combinations of Iron Manganese Supply to Barley Plants Grown in Sand Culture*

Manganese supply*	ppm iron supply					
	0.056	0.28	0.56	1.4	5.6	28.0
Mn_1	10	48	92	203	505	835
Mn_2	0.1	0.5	1.0	2.5	10	48
Mn_3	0.01	0.05	0.10	0.25	1.0	5

* Mn_1, Mn_2, and Mn_3 denote the 3 levels of manganese supply, 0.0055 (Mn_1), 0.55 (Mn_2), and 5.5 (Mn_3) ppm plus the manganese contribution from the iron supply, 0.001/μg Mn per ppm of iron supply.

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protein nitrogen were estimated in leaves at 3 stages of growth, viz. 24, 52, and 80 days. At 24 days growth the aforementioned estimations (except protein nitrogen) were made separately in the leaf formed first, first leaf, and those formed subsequently, other leaves. At 52 and 80 days growth only the 2 youngest fully unrolled leaves were taken for the different estimations. Dry matter yield was determined at 24, 52, 80, and 112 days growth.

Chlorophyll was estimated colorimetrically in acetone extracts by the method of Petering et al. (31). The calibration curve for chlorophyll was prepared by the method of Comar and Zscheile (11).

Catalase and peroxidase were estimated in crude tissue extracts. Finely chopped plant material was chilled and ground with purified silica sand in a chilled pestle and mortar with 0.005 M potassium phosphate buffer pH 7. The temperature during the grinding procedure was maintained below 5°. The crude extracts were strained through twofold muslin.

Catalase was estimated manometrically by an adaptation of the method described by Chance and Maehly (9). The main compartment of the Warburg flask contained 1 ml of suitably diluted enzyme preparation and 2 ml phosphate buffer pH 7. The side arm of the flask contained 0.2 ml of 0.1 M hydrogen peroxide. Evolution of O_2 was followed for 5 minutes and catalase activity is expressed as μl O_2 evolved/g fresh tissue in 5 minutes. Within the range of zero to 120 μl O_2 evolved/5 minutes, catalase activity was found to be linear with the enzyme concentration and catalase activity measurements were made within this range.

Peroxidase was estimated by the method of Willstäter and Stoll as modified by Sumner and Gjessing (38). Peroxidase activity is expressed as milligrams purpurogallin formed/100 mg fresh tissue in 5 minutes. Within the range of zero to 20 mg purpurogallin formed/5 minutes, peroxidase activity was found to be linear with enzyme concentration: peroxidase activity measurements were made within this range.

Protein nitrogen was estimated by the semimicro-kjeldahl method. Fresh plant material was fixed in boiling 80 % enthanol. After being ground the alcohol insoluble material was separated and digested by the method of Chibnall et al. (10). Ammonia produced was distilled by steam distillation in a markham apparatus into a boric acid buffer and estimated by titration with N/140 H_2SO_4 containing Conway and O'Malley indicator (12).

Total iron was determined colorimetrically as ferrous-orthophenanthroline complex (19) after wet digestion of the oven-dried samples with nitric-perchloric acid (32).

Dry matter yield was determined by drying the plants in a forced draft oven at 70° for 24 hours. Since the dry matter was required for the estimation of the different nutrient elements, fresh matter kept for drying was thoroughly cleaned against surface contamination by first being washed in running deionized water for a short duration and then rinsed with glass distilled water.

All estimations were made in duplicate and analytical results have been tested statistically for significance at 5 % level of probability.

Results

Growth and Visual Effects. Visual differences in the growth of plants due to differential iron-manganese supply were not observed up to 18 days growth. Thereafter, growth differences appeared and gradually became pronounced. In the entire range of iron supply at 0.0055 ppm manganese supply and within the range of 0.056 to 0.56 ppm iron supply at 0.55 and 5.5 ppm manganese supply growth was depressed. At 0.056 ppm iron supply at the low manganese level, and 0.056 to 0.56 ppm iron at the normal and the excess manganese levels, the number of the expanded leaves, leaf area, particularly the width of the lamina, the length of the internodes, and the tillering of plants was severely restricted. At 0.056 ppm iron supply, inflorescence was not formed at the low and the excess manganese supply, and was delayed and poorly formed at the normal manganese level. At 0.28 to 1.4 ppm iron at the low manganese level emergence of ears and ripening of grains was considerably delayed.

Even before plants showed any differences in growth, visual pathological symptoms appeared—manganese excess effects on the first leaf and the iron and manganese deficiency effects on the second leaf. The excess manganese effects, paling followed by the appearance of minute brown spots on the distal half of the first leaf, were conspicuous only in the range of 0.056 to 1.4 ppm iron supply. Iron deficiency effects were seen at all the 3 levels of manganese supply up to an iron supply of 0.56 ppm. At 0.056 ppm iron supply the entire leaf was chlorotic whereas at 0.28 and 0.56 ppm iron supply the chlorosis was of the intervenal type. Mild chlorosis was also observed at the low manganese level at 1.4 and 5.6 ppm iron supply. This seemed to be due to the deficiency of manganese.

Later, symptoms of manganese excess, iron deficiency and manganese deficiency intensified. At 0.28 to 0.56 ppm iron supply effects of manganese excess appeared on the other leaves. In the intervenal chlorotic areas, leaves developed minute brown spots which spread and coalesced into more or less continuous stripes of dark necrotic tissue. Brown necrotic lesions due to manganese excess also developed on leaf sheaths, nodes, and internodes. The chlorosis due to iron deficiency spread to the entire foliage within the range 0.056 to 0.56 ppm iron supply. At the lowest level of iron supply the leaf apices became totally bleached, showed a collapse of the mesophyll, withered, and hung down. Within the range 0.28 to 1.4 ppm iron supply, faint chlorotic mottling and necrosis of leaves appeared due to low manganese supply.

Yield. The yield of plants increased with an in-

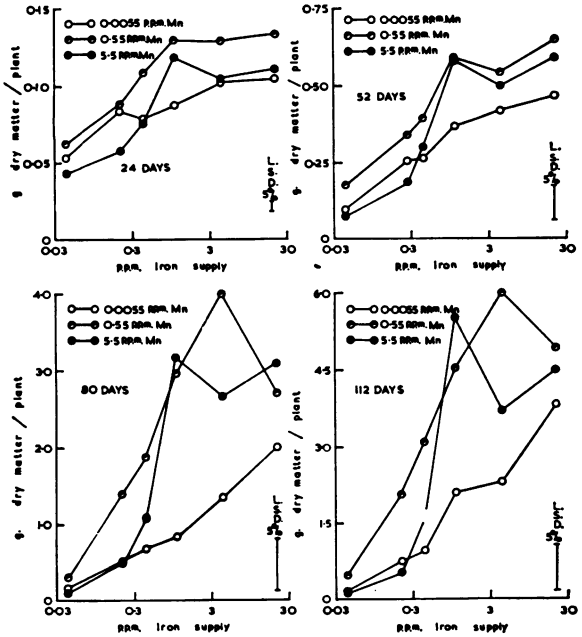

FIG. 1–4. Effect of iron and manganese supply on yield of barley.

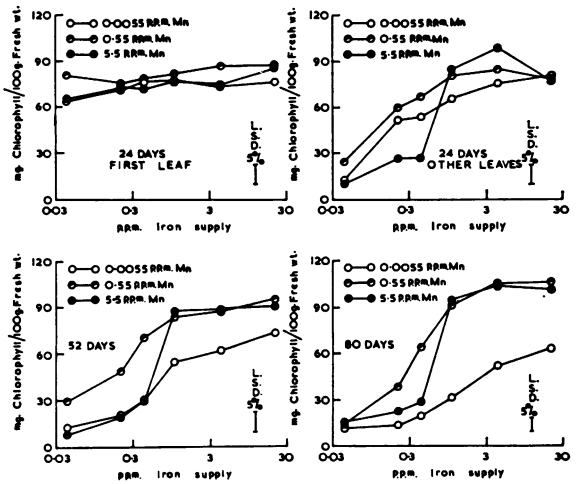

FIG. 5–8. Effect of iron and manganese supply on chlorophyll content of barley.

crease in iron supply. The extent of the increase and the range of iron supply in which yield increased was determined by the manganese supply. At the low manganese supply the increase occurred up to the highest, 28 ppm iron supply, at excess manganese up to 1.4 ppm iron, and at the normal manganese supply up to 28 ppm at 24 and 52 days and 5.6 ppm iron supply at 80 and 112 days growth. Within these limits the increase in yield due to increase in iron supply was almost linear (fig 1, 2, 3, 4); the yield curves at the low manganese supply having the least slope and that at excess manganese the highest. Further, owing to a continued restriction of growth at 0.056 to

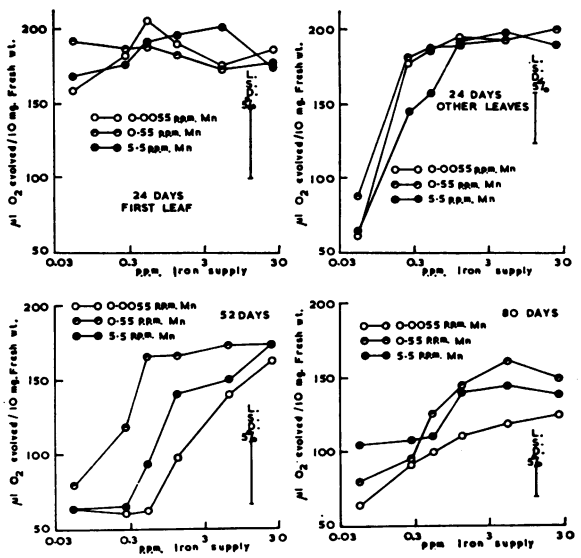

FIG. 9–12. Effect of iron and manganese supply on catalase in barley.

0.56 ppm iron supply and increase at 1.4 to 28 ppm iron supply, the yield curves became steeper with increase in growth period, intensifying the differences between the minimum and the maximum yield.

In the entire range of iron supply low manganese depressed the yield of plants. The depression was marked and statistically significant within the range 0.28 to 28 ppm iron supply. Except at 1.4 ppm iron supply at 80 and 112 days growth and 28 ppm iron supply at 80 days growth, excess manganese also depressed the yield of plants. The depression in yield due to excess manganese supply was in most cases marked and statistically significant.

Chlorophyll. In the first leaf chlorophyll was only slightly affected by variation in iron and manganese supply (fig 5). In the leaves other than the first, chlorophyll was related to iron and manganese supply. At all the 3 levels of manganese, increase in iron supply in the range 0.056 to 1.4 ppm brought about marked increase in chlorophyll, significant at $P < 0.05$ (fig 6, 7, 8). At the low and normal manganese supply increase in chlorophyll was gradual but at the excess manganese supply there was an abrupt, over twofold, increase between 0.056 to 1.4 ppm iron supply. Above 1.4 ppm iron supply increase in chlorophyll was appreciable only at the low manganese level.

In the entire range of iron supply, low manganese depressed chlorophyll, the depression being marked and generally statistically significant at 52 and 80 days growth. The effect of the excess manganese supply was largely determined by the iron supply. Excess manganese depressed chlorophyll within the range of iron supply 0.056 to 0.56 ppm but not above 1.4 ppm.

Catalase. In the first leaf catalase was not affected by variation in iron-manganese supply (fig 9). In leaves other than the first, irrespective of the man-

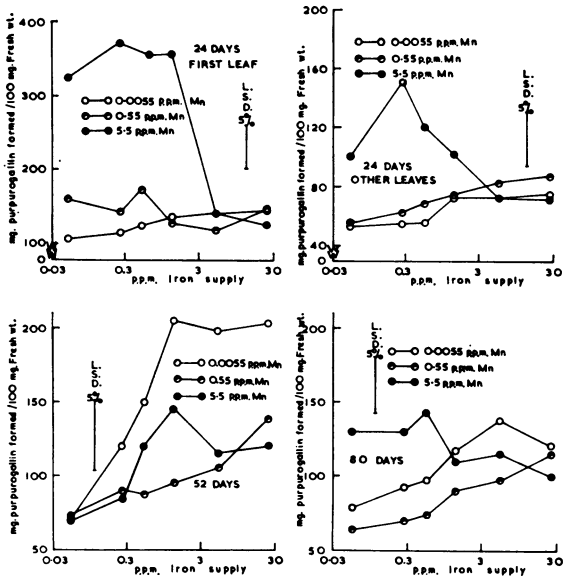

FIG. 13–16. Effect of iron and manganese supply on peroxidase in barley.

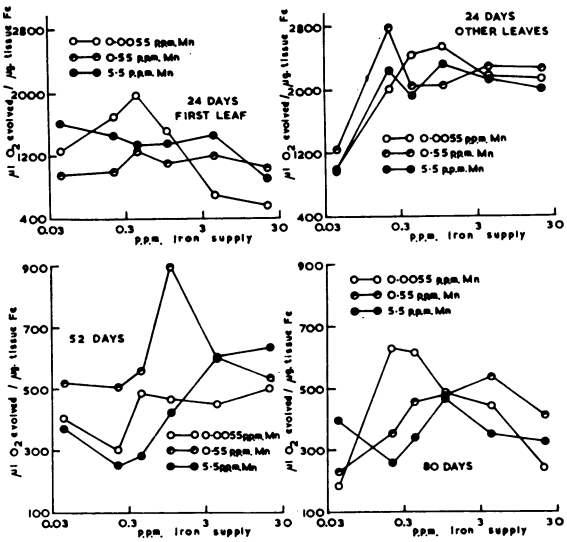

FIG. 17–20. Effect of iron and manganese supply on catalase. Catalase expressed on tissue iron basis.

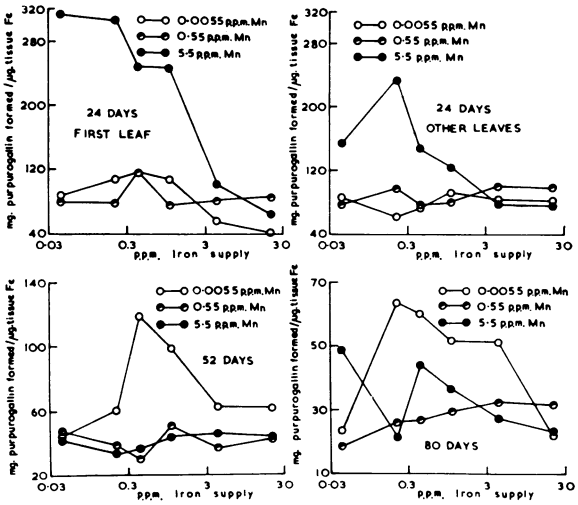

FIG. 21–24. Effect of iron and manganese supply on peroxidase. Peroxidase expressed on tissue iron basis.

ganese supply, catalase was appreciably depressed at 0.056 to 0.56 ppm iron supply (fig 10, 11, 12). The depression at 0.056 ppm iron and often at 0.28 ppm iron supply was pronounced and statistically significant. The optimum concentration of catalase was found in the range 1.4 to 28 ppm iron supply.

Almost in the entire range of iron supply catalase was generally higher at 0.55 ppm manganese than at 0.0055 ppm and 5.5 ppm manganese supply (fig 10, 11, 12), the effects being in many cases statistically significant.

Peroxidase. The effects of iron and manganese supply on the peroxidase were interrelated. In the first leaf, peroxidase was generally not affected by variation in manganese supply at 5.6 and 28 ppm iron supply. But at 0.056 to 0.56 ppm iron supply low manganese depressed peroxidase and at 0.056 to 1.4 ppm iron supply excess manganese increased peroxidase, the increase being very marked and statistically significant (fig 13). In leaves other than the first, at the normal manganese supply, peroxidase was depressed within the range 0.056 to 0.56 ppm iron supply and, but for one exception, not affected within the range 1.4 to 28 ppm iron supply (fig 14, 15, 16). At the excess manganese supply very often peroxidase was markedly higher at an iron supply of 0.056 to 0.56 ppm than at 5.6 and 28 ppm.

In general the effect of manganese on peroxidase was more pronounced than that of iron and varied in both nature and extent at the different stages of growth. Thus, almost in the entire range of iron supply peroxidase at the low manganese supply was slightly depressed at 24 days growth and markedly and significantly enhanced at 52 days and 80 days growth. Within a certain range of iron supply, 0.056 to 0.56 ppm at 24 and 80 days, and 0.56 and 1.4 ppm iron at 52 days, excess manganese very markedly and significantly increased peroxidase.

Discussion

Barley plants supplied 5.5 ppm manganese developed symptoms of manganese toxicity, brown necrotic lesions on leaves, nodes, internodes, and leaf sheaths at 0.28 and 0.56 ppm iron supply. As in potato (8) Lespedeza (24) and certain other plants (41) but not in soybeans (37) oats and tomato (40) and sunflower (42) these effects were distinct from chlorosis of the younger growths resulting from iron deficiency. Contrary to the findings of Somers and Shive (37) and Twyman (40) but in agreement with

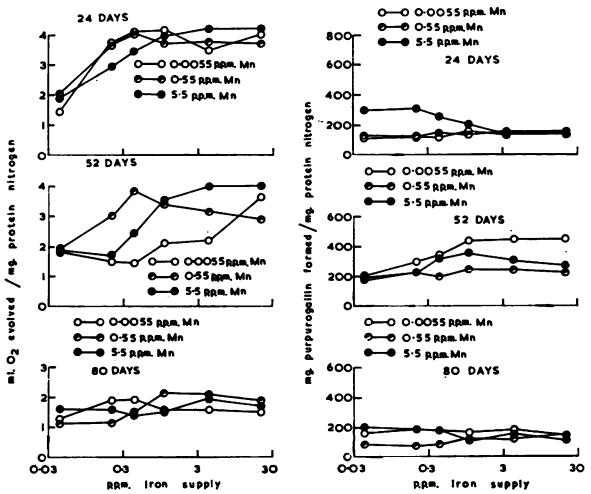

FIG. 25–27 (*left*). Effect of iron and manganese supply on catalase. Catalase expressed on protein nitrogen basis.

FIG. 28–30 (*right*). Effect of iron and manganese supply on peroxidase. Peroxidase expressed on protein nitrogen basis.

Weinstein and Robbins (42) no visual effects due to iron excess were obtained in barley plants.

The yield of plants showed a significant interaction of iron and manganese supply. In contrast to the findings of Pugliese (34) for wheat, Scharrer and Schropp (35) for maize, and Somers and Shive (37) for soybeans, yield of barley plants was found to be determined more by the absolute levels of iron or manganese supply than by their relative proportions. The Fe/Mn ratio for maximum yield at the 3 levels of manganese varied widely—835 at low manganese, 10 at normal manganese, and 0.25 at excess manganese. Moreover, the same ratio of iron to manganese (Fe/Mn = 10) produced the lowest yield when the actual supplies of iron and manganese were 0.056 and 0.0055 ppm respectively and the highest when the supplies of the 2 nutrients were raised hundredfold (5.6 ppm iron, 0.55 ppm manganese). That absolute levels of iron and manganese supply rather than the Fe/Mn ratio were more important in determining yield of oats has been earlier shown by Twyman (40).

Although it is not possible to completely discount the possibility, it appears extremely unlikely that the differences in the results presented here and that of Somers and Shive (37) were due to the form in which iron was supplied. Somers and Shive (37) supplied iron as ferrous sulphate whereas iron was supplied as ferric citrate in the experiments described here. Somers and Shive (37) postulated that owing to the high redox potential for the oxidation of Mn^{++} to a higher valency form, manganese was able to cause the oxidation of most of the ferrous form of iron to the ferric state which was considered to be physiologically inactive, the ferrous form of iron being the physiologically active iron. Kenten and Mann (20, 21, 22, 23) have established the presence in plants of a system which would bring about the oxidation of Mn^{++} to Mn^{+++}. From this it does not necessarily follow that Mn^{+++} oxidizes Fe^{++} to Fe^{+++}. In addition to ferrous iron a number of other substances are present in plants which could reduce trivalent manganese to divalent manganese. Pirson (33) has concluded that there is no indication of an oxidation-reduction system between these 2 elements. Hewitt (17) has concluded that the activity of heavy metals to induce iron deficiency effects cannot be explained on the relative oxidation-reduction potentials of the heavy metals and that there is no justification in assigning a unique position to manganese in this respect. In view of the fact that most iron porphyrin systems involve the reversible reaction ferric to ferrous, Arnon (5) considered it doubtful if plants can utilize only ferrous iron. Further, if the assumption that ferrous iron is the physiologically active iron is correct, it would follow that there is an active system in plants which normally converts Fe^{+++} to Fe^{++}, otherwise it would not be possible to grow plants with ferric iron—a form in which iron is usually supplied (Hewitt, 18). Somers and Shive (37) also held that there is an active system in plants for converting Fe^{+++} to Fe^{++} iron.

In the light of the hypothesis proposed by Somers and Shive (37) one would expect a similarity in the effects of iron deficiency and manganese excess and a mitigation of the effects of the former by manganese deficiency. The effect of iron deficiency and manganese excess was identical on chlorophyll and catalase. But peroxidase was depressed by iron deficiency and often markedly enhanced by manganese excess. Also the effect of iron deficiency on chlorophyll, catalase, and peroxidase was often enhanced rather than mitigated by manganese deficiency.

In respect to the depression in both catalase and peroxidase at the deficient levels of iron supply, 0.056 to 0.55 ppm at the normal manganese supply, our results are in accord with DeKock et al. (13) and Nicholas and Goodman (28); in respect of catalase only with those of Weinstein and Robbins (42), Banerjee (7), and Agarwala and Sharma (2). The depression in chlorophyll at low iron levels has also been reported by Sideris and Young (36), Weinstein and Robbins (42), DeKock et al. (13), and Agarwala and Sharma (2). The depression in chlorophyll catalase and peroxidase at low levels of iron supply could be a result of the depression in the synthesis of a precursor, common to chlorophyll and the heme part of catalase and peroxidase which was earlier suggested to be dependent on the level of iron supply (2) rather than the tissue iron. The effect of the variation in iron and manganese supply in barley plants on catalase and peroxidase content of leaves cannot be attributed to the tissue concentration of iron and protein nitrogen. In many instances at levels of iron supply at which clear definite visual iron deficiency effects were seen catalase was relatively less and peroxidase relatively more than would

be expected on the basis of tissue iron (fig 17–24).

Some quantitative differences were noted between the magnitude of the effects of iron and manganese supply on catalase and peroxidase on the fresh weight basis and on the protein nitrogen basis (fig 10–12, 14–16, 25–30) and in general, the effects on protein nitrogen basis were less marked than on the fresh weight basis. But the main trends were almost similar on the 2 bases with the important exception that, at 80 days growth, catalase which was depressed by manganese deficiency on fresh weight basis showed an increase on the protein nitrogen basis at low levels of iron supply, 0.056 to 0.56 ppm iron. In any case, the changes in the concentration of protein nitrogen cannot explain the diverse trends in the 2 enzymes in relation to manganese supply as found here; if protein synthesis is involved in the change in enzyme concentration it would be the specific enzyme protein rather than the total protein.

The depression in chlorophyll and catalase due to manganese deficiency would suggest that manganese also affects the synthesis of the common precursor of chlorophyll and the heme of catalase. The depression in chlorophyll and catalase at the excess manganese supply may be due to the substitution of manganese in place of magnesium and iron in their respective porphyrin as suggested by Sideris and Young (36). But the effect of variation in manganese supply on peroxidase cannot be explained by suggesting that the synthesis of porphyrin is impeded at the low and excess manganese supply. Many instances were found where peroxidase was markedly increased at both low and excess manganese supply. This would mean that the synthesis of the 2 moieties of peroxidase, the specific protein and the heme, was stimulated. It would also show that iron in sufficient quantity was available for incorporation in protoporphyrin-9 to form the heme of peroxidase and the enzyme responsible for incorporation of iron in protoporphyrin (*27*) was not depressed. The suggestion of Sideris and Young (36) that manganese, when supplied in excess, competitively replaces iron in heme does not seem to explain the increase in peroxidase as manganese porphyrin, when combined with peroxidase protein in vitro, was found to show very feeble peroxidase activity (14, 39). Many instances are known where a deficiency or excess of an element increased an enzyme for which the element was not a specific cofactor (30, 25, 26, 4, 28). It is possible that the deficiency or the excess of an element, apart from other effects, upsets the balance in the synthesis of the different specific proteins. This is reflected in the increase of some enzymes and the decrease of others. This would also explain the diverse trends in catalase and peroxidase obtained in other cases (6, 1).

Summary

Barley (*Hordeum vulgare* L. var K 12) plants were raised at 3 levels of manganese, 0.0055 ppm (low), 0.55 ppm (normal), and 5.5 ppm (excess). At each level of manganese iron was supplied at 6 levels, 0.056, 0.28, 0.56, 1.4, 5.6, and 28 ppm to give 18 combinations of iron-manganese supply. Besides studying growth and visual pathological effects, plants raised at the differential iron-manganese supply were estimated for dry matter yield, chlorophyll, tissue iron, the iron porphyrin enzymes, catalase, peroxidase, and protein nitrogen.

Symptoms of manganese toxicity, brown necrotic lesions in intervenal chlorotic leaf areas and also on nodes, internodes, and leaf sheaths produced at excess manganese supply at the low levels of iron, 0.28 and 0.56 ppm, were quite distinct from chlorosis resulting from iron deficiency. Depression in plant yields was brought about by deficiency and excess of manganese and deficiency of iron but not by the excess iron supply. In fact, excess iron supply did not produce any characteristic effect.

The optimum Fe/Mn ratio for apparently normal growth and maximum yield of plants showed a wide variation depending upon the actual levels of iron and manganese supply.

Iron deficiency, manganese deficiency, and manganese excess depressed chlorophyll formation suggesting that synthesis of chlorophyll was determined by both iron and manganese.

Irrespective of manganese supply, catalase was depressed by iron deficiency. At deficiency levels of iron, peroxidase was depressed at normal and low manganese levels and generally increased at the excess manganese level. Almost in the entire range of iron supply both deficiency and excess of manganese depressed catalase. Deficiency of manganese slightly depressed peroxidase at 24 days growth and increased it at the later stages of growth. With a few exceptions excess manganese also increased peroxidase. The variation in catalase and peroxidase in the leaves of plants raised at the different levels of iron-manganese supply could not be attributed to the tissue concentration of iron or protein nitrogen.

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