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Iron Deficiency and the Structure and Physiology of Maize Chloroplasts[1]

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ABSTRACT

The ultrastructure of mesophyll chloroplasts of maize (*Zea mays* L.) was more severely affected by iron deficiency that induced mild chlorosis than was the ultrastructure of bundle sheath plastids. Ferredoxin and ribulose diphosphate carboxylase levels were severely decreased by iron deficiency. Malic enzyme was less affected, and phosphoenolpyruvate carboxylase activity remained high even under severe iron deficiency. Iron deficient leaves fixed carbon into malic and aspartic acids but the rate of entrance of carbon into the sugar phosphates and sucrose was greatly reduced compared to the control. Chlorophyll *a*/*b* ratios ranged from low values of less than 2 in severely iron deficient leaves to high values exceeding 4 in leaves showing little iron deficiency.

In C_4 plants such as maize, CO_2 is first fixed through β-carboxylation of PEP[2] into the C_4-dicarboxylic acid, oxaloacetic acid, and then rapidly appears in aspartic and malic acids. It is generally believed that this process takes place in the mesophyll cells of the leaf, and that malate and aspartate then are transported to the bundle sheath cells where they are decarboxylated and the CO_2 that is released is fixed by the action of ribulose diP carboxylase in the normal C_3 cycle (11, 12).

Such a mechanism involves a spatial separation of activities between mesophyll and bundle sheath cells in the leaf, but at the same time necessitates a considerable and rapid movement of small molecules such as malate, aspartate, pyruvate, glycerate-3-P, and trioses out of one type of plastid across a series of membrane barriers and into a second plastid type in another cell. Although such a rapid shuttling of material between the two adjacent cell types (mesophyll and bundle sheath) seems feasible (18), the exact situation still requires considerable clarification. One method of studying the problem would be to inhibit preferentially the activity of one of these cell types *in vivo* and study the effects on the carbon fixation pathway.

Plants suffering from Fe deficiency are characterized by having leaves showing various degrees of interveinal chlorosis. Even though the two cell types are in physical contact, one might expect that, on the average over the whole leaf, the mesophyll cells would be suffering from a more severe deficiency of Fe than the bundle sheath cells, which are more closely associated with the veins. De Kock (7) has already observed, by radioautography of plants fed ^{59}Fe, that veinal areas contain more Fe, but that under the stress of Fe deficiency, Fe decreases disproportionately faster in veinal than in the interveinal areas. A number of reports have indicated that the bulk of the Fe in the leaf is in the chloroplasts, and ultrastructural studies such as those of Bogorad (4) have shown the drastic effects of the deficiency on the lamellar system of the plastids. These effects are chiefly expressed as a loss of grana and an extensive formation of parallel lamellae within the plastids.

Price (19) reviewed the physiological effects of Fe deficiency and concluded that Fe deficiency produces a massive derangement of chloroplasts both ultrastructurally and physiologically. Thus, in addition to the more obvious decrease in Chl, other chloroplast constituents such as ferredoxin have been shown to be strongly decreased under Fe deficiency.

I was particularly interested in determining whether mesophyll and bundle sheath cells respond differently to Fe deficiency and whether Fe deficiency altered the kinetics of carbon fixation in leaves of C_4 plants.

MATERIALS AND METHODS

Plant Material. Maize grains (*Zea mays* L., var. Pioneer 3369 A MF) were germinated in sand moistened with half strength Hoagland's solution. Four days after germination, the seedlings were transferred to full strength Hoagland's solution in glass containers in the greenhouse. Iron deficiency was obtained by supplying Fe, in the form of Fe chelate, at rates of 0, 1.4, 2.8, 5.6, and 7.6 mg Fe/l of nutrient solution. Deficiency symptoms began to appear in about one week. The plants were used after they were three or four weeks old. At this time, the series showed deficiency symptoms ranging from almost yellow leaves, through decreasing degrees of interveinal chlorosis, to healthy green leaves on the high Fe plants.

Electron Microscopy. Observations of the effects of Fe deficiency on the ultrastructure of mesophyll and bundle sheath cells were made on 1-cm square samples taken from the midregion of leaves and including minor veins and areas between major veins. The samples were cut into smaller sections and fixed at 4 C in 6% (v/v) glutaraldehyde (0.1 M potassium phosphate buffer, pH 7.2). The tissue was washed in buffer for 1 hr (three 20-min changes) and postfixed overnight in 2% osmium tetroxide (0.1 M potassium phosphate buffer, pH 7.2). Specimens were dehydrated through an ethanol series and embedded in Araldite-DDSA plastic. Sections were cut on a Porter-Blum MT-1 ultramicrotome with a Dupont diamond knife, stained with 2% aqueous uranyl acetate and lead citrate, and viewed on a Hitachi HU-11 electron microscope at 75 kv.

Kinetics of Carbon Fixation. Strips about 20 cm long with-

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[2] Abbreviation: PEP: phosphoenolpyruvate.

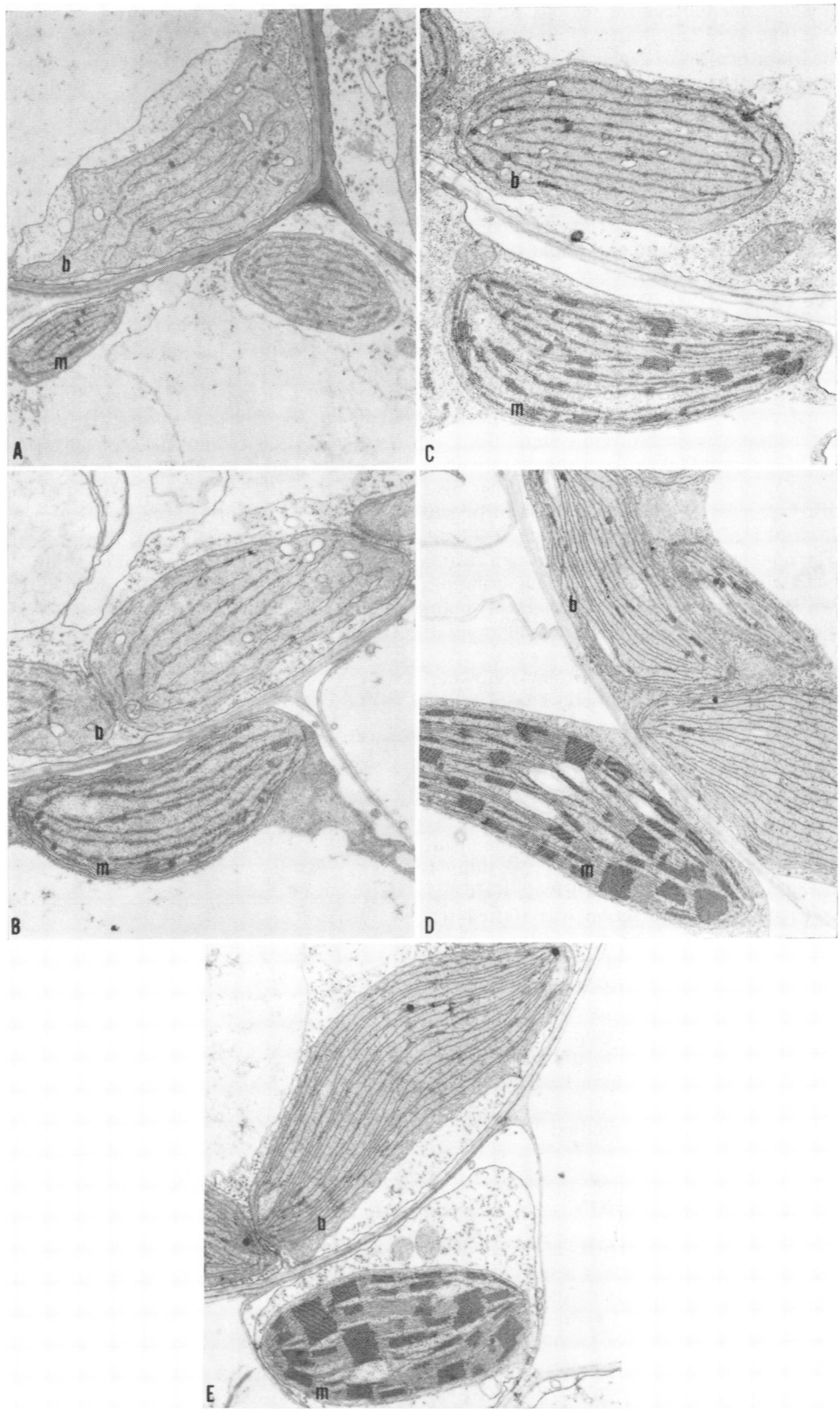

FIG. 1. Chloroplasts from Fe deficiency series. Concentration of Fe in mg Fe/l of culture solution. A: 0.0; B: 1.4; C: 2.8; D: 5.6; E: 7.6. × 12,000. Note bundle sheath chloroplasts (b) and mesophyll chloroplasts (m).

out midveins were taken from the central regions of mature leaves. Four strips at a time were hung vertically in a Plexiglas feeding chamber. The lower one cm of each strip dipped in water in the bottom of the chamber. The top centimeter extended through close fitting soft rubber lips at the top of the chamber. The chamber was attached to a $^{14}CO_2$ generating system from which the gas mixture could be circulated either through the chamber or by the chamber. After various times of exposure to $^{14}CO_2$, leaf strips were pulled through the rubber lips and plunged into liquid N_2. In this way, one strip at a time could be removed rapidly from the feeding chamber without disturbing the remaining strips. About one sec was required to remove a strip and plunge it into liquid N_2. Frozen leaves were dried without thawing under vacuum.

The dried plant material was extracted five times each with 80% (v/v) ethanol at 80 C and with H_2O at 80 C. Chromatographic separation of the ^{14}C-labeled compounds was carried out by two-dimensional paper chromatography with phenol-water and butanol-water-propionic acid solvent systems (2).

Enzyme Assays. A 0.5-g sample taken from the mature section of the leaves was ground rapidly to complete cellular disintegration in a TenBroeck glass homogenizer in 10 ml of ice cold extraction medium (0.1 M tris-chloride buffer, pH 7.8; 1 mM dithiothreitol). The homogenate was filtered through nylon mesh and used immediately for enzyme assays. This grinding procedure gave essentially complete disintegration of both mesophyll and bundle sheath cells. No evidence of differential grinding was observed.

Ribulose diP carboxylase was assayed at 28 C by following the conversion of $KH^{14}CO_3$ into stable products. The reaction mixture (0.5 ml) contained the following in μmoles; tris-chloride buffer, pH 7.8, 50; dithiothreitol, 1; $MgCl_2$, 3; ribulose diP, 0.5; $KHCO_3$ containing 2.5 μCi of ^{14}C. The reaction was started by adding 0.1 ml of the plant extract (5).

Phosphoenolpyruvate carboxylase was assayed at 28 C by the same procedure. The reaction mixture (0.5 ml) contained the following in μmoles: tris-chloride buffer, pH 7.8, 50; dithiothreitol, 1; $MgCl_2$, 3; sodium glutamate, 2.5; PEP, 1; $KHCO_2$, 4.5 containing 0.25 μCi of ^{14}C. The reaction was started by adding 0.1 ml of plant extract.

Because the enzyme analyses were made on leaves from a number of separate Fe deficiency series, considerable scattering of the points occurs. The data, expressed as enzyme activity per mg of protein plotted against the Chl content per gram of fresh weight of leaf sample were subjected to computer analysis. The regression line representing the curve best fitting the data is plotted for each set of data.

Chlorophyll was determined by Arnon's method (1) and protein by the Biuret method (14).

RESULTS AND DISCUSSION

Chloroplast Ultrastructure and Chlorophyll. Mild Fe deficiency during the development of maize leaves results in interveinal chlorosis. Consequently a wide variety exists, at different locations in the leaf, in the ultrastructural expression of this deficiency. Care was taken to note the exact location and general appearance of the leaf sections. In general, mesophyll chloroplasts from areas showing Fe deficiency symptoms fail to develop normal grana but rather the lamellar system may extend the length of the chloroplast (Fig. 1, A, B, and C). These lamellae may occur in groups. This is in agreement with the observations of earlier investigators (4). On the other hand, the ultrastructure of bundle sheath plastids seems to be less drastically affected. Under mild deficiency, these plastids are characterized by having developed a somewhat less extensive lamellar system, some swelling of the interlamellar spaces is evident, and some small vesicles appear (Fig. 1C). With progressively more severe deficiency, the differences in response between the two kinds of plastids become even more pronounced (Fig. 1B). In advanced stages of Fe deficiency, there is a drastic reduction of the lamellar systems in both types of plastids. The peripheral reticulum is greatly reduced or absent, and many of the interlamellar spaces are swollen (Fig. 1A).

It has been suggested that bundle sheath plastids lacking extensive grana are characterized by a high Chl *a*/*b* ratio and little photosystem II activity. Nitrogen and sulfur deficiencies in maize result in plastids with increased grana stacking (10) and increased photosystem II activity (3). In contrast to the expected result of a high Chl *a*/*b* ratio in Fe-deficient leaves with chloroplasts in which the grana are poorly developed (Fig. 1, A and B), these severely deficient leaves had very low Chl *a*/*b* ratios. Only when the deficiency symptoms were moderate to slight did the Chl *a*/*b* ratio reach a maximum exceeding the level of 3.9 found in normal corn leaves (Table I).

Protein and Enzymes. Under increasingly severe Fe deficiency, the total leaf protein/chl ratio increased from approximately 25 for normal maize leaves to over 100 in leaves showing severe deficiency (Fig. 2). This is a reflection of the massive derangement of chloroplasts induced by Fe deficiency which, as pointed out by Price (19), is consistent with the notion that Fe

Table I. *Influence of Fe Deficiency on Chl a/b Ratio in Maize Leaves*

Level of Fe in Culture Solution	Chl *a*/*b*	
	Series 1	Series 2
mg Fe/l	*ratio*	
0.0	1.6	2.8
1.4	2.8	3.5
2.8	4.9	3.9
5.6	4.5	4.0
7.6	3.9	3.9

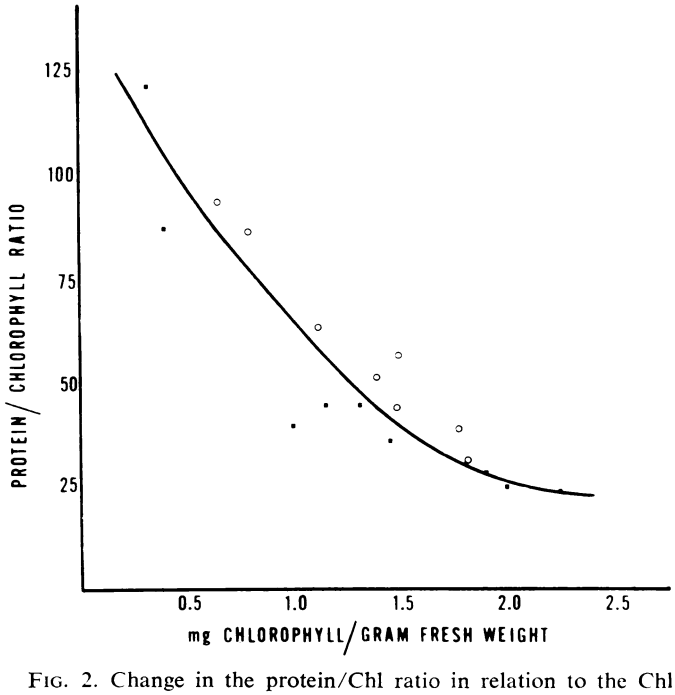

FIG. 2. Change in the protein/Chl ratio in relation to the Chl content of leaves in the Fe deficiency series.

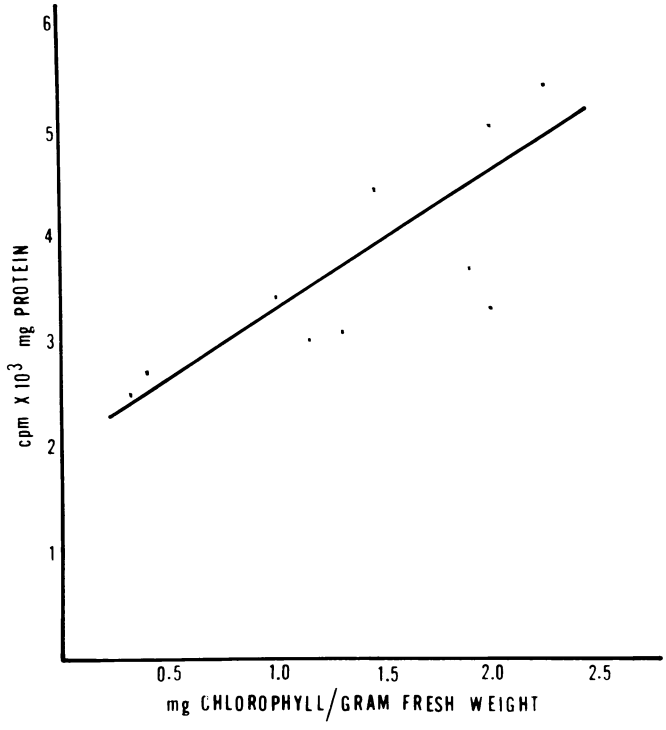

FIG. 3. RuDP carboxylase activity in relation to the Chl content of leaves in the Fe deficiency series.

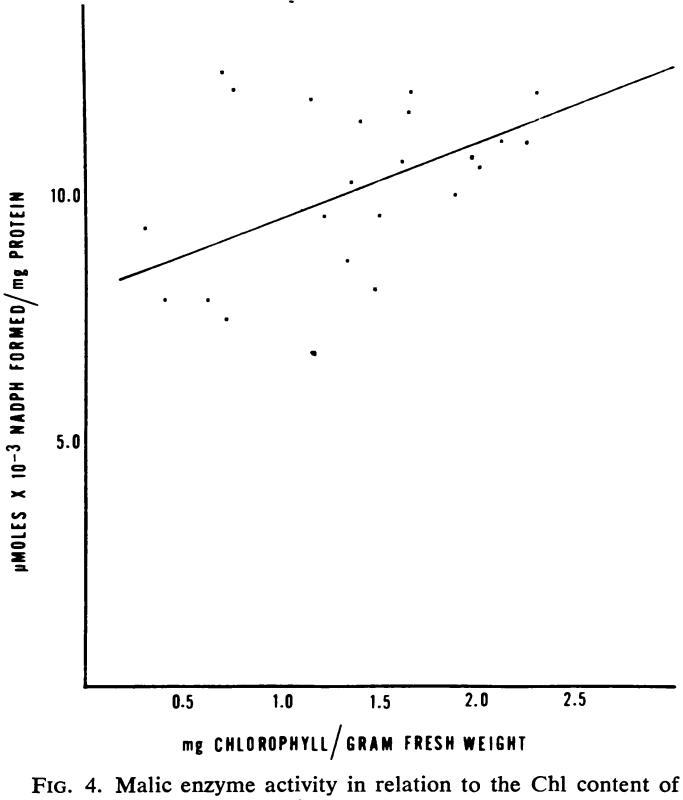

FIG. 4. Malic enzyme activity in relation to the Chl content of leaves in the Fe deficiency series.

is required for some early step in chloroplast development, and that there is a more or less coordinated decrease in chloroplast components during iron deficiency. Known chloroplast components such as ribulose-1,5-diP carboxylase (Fig. 3) are strongly decreased under iron deficiency. Marsh *et al.* (16) observed a similar relationship for ferredoxin in *Vigna sinensis* leaves. Our estimations (unpublished data) of ferredoxin in Fe-deficient maize agree with these findings. In contrast, malic enzyme (17), which has been identified as being largely confined to bundle sheath cells (11, 12), is much less markedly affected by Fe deficiency (Fig. 4). However, although there is a wide scattering of these data, malic enzyme activity per unit of leaf protein also decreased under conditions of Fe deficiency.

It is interesting that PEP carboxylase, which has been identified as being located predominantly in mesophyll cell chloroplasts (11), although there is some disagreement on this point (15), shows an entirely different pattern of response to Fe deficiency (Fig. 5). In fact, the levels of PEP carboxylase activity in leaves suffering from Fe deficiency are more similar to the response expected of a protein not containing Fe and located outside of the chloroplasts than of an enzyme found in chloroplasts. Although these results do not necessarily show the intracellular distribution of any specific enzyme, the response of PEP carboxylase to Fe deficiency would strongly suggest that it may be a cytoplasmic rather than a chloroplast enzyme. Gibbs and co-workers (9) reported that PEP carboxylase was found in the supernatant fraction when chloroplasts were isolated from young *Zea mays* seedlings, Edwards *et al.* (8) concluded recently that PEP carboxylase is a cytoplasmic enzyme, and Lyttelton (15) previously reported that PEP carboxylase is located either in the cytoplasm or in the chloroplast envelope rather than within the mesophyll chloroplasts.

Carbon Fixation. It is logical to expect that the effects of Fe deficiency on the levels of some of the enzymes involved in photosynthesis would result in abnormalities in the carbon metabolism of leaves. The kinetics of labeling of the photosynthetic products in normal leaves and in leaves showing moderate interveinal chlorosis were studied over periods ranging from 15 sec to 16 min. During photosynthesis in $^{14}CO_2$, plants having

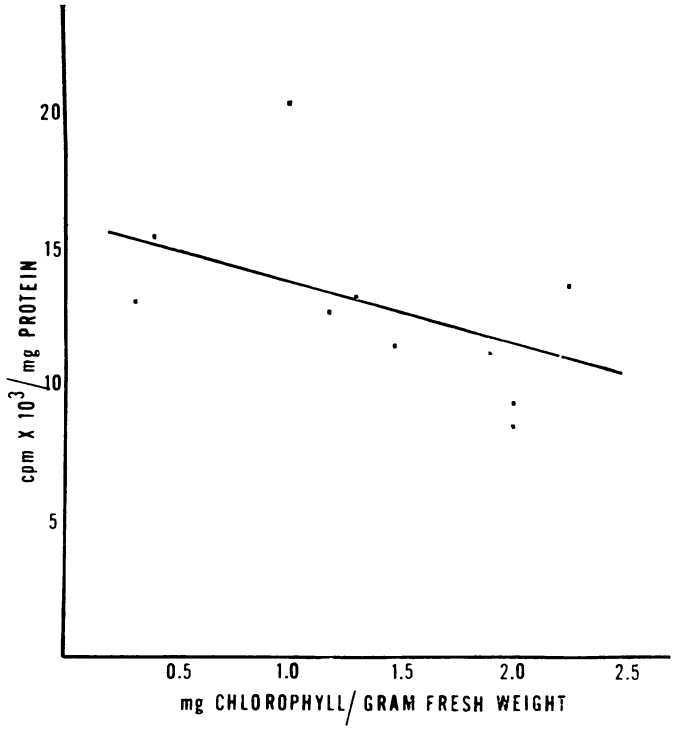

FIG. 5. PEP carboxylase activity in relation to the Chl content of leaves in the Fe deficiency series.

the C_4 pathway rapidly incorporate ^{14}C into malic and aspartic acids. However, sugar phosphates and sucrose rapidly become labeled as ^{14}C enters the C_3 cycle (12, 13). Figure 6A shows the labeling pattern for the photosynthetic products in a normal maize leaf exposed to $^{14}CO_2$ for 2 min. At this time, 25.6% of the fixed ^{14}C was recovered in sucrose, 26.5% in sugar phosphates, and only 8.6% in malic and aspartic acids (Fig. 7). In contrast to the situation in the normal leaves, carbon incorporated into malic and aspartic acids during photosynthesis in Fe deficient maize leaves moved out of these acids very slowly (Fig. 6B). After 2 min, malate and aspartate still contained 84.6% of the total label, sugar phosphates, 4.8%, and sucrose only 1.8%.

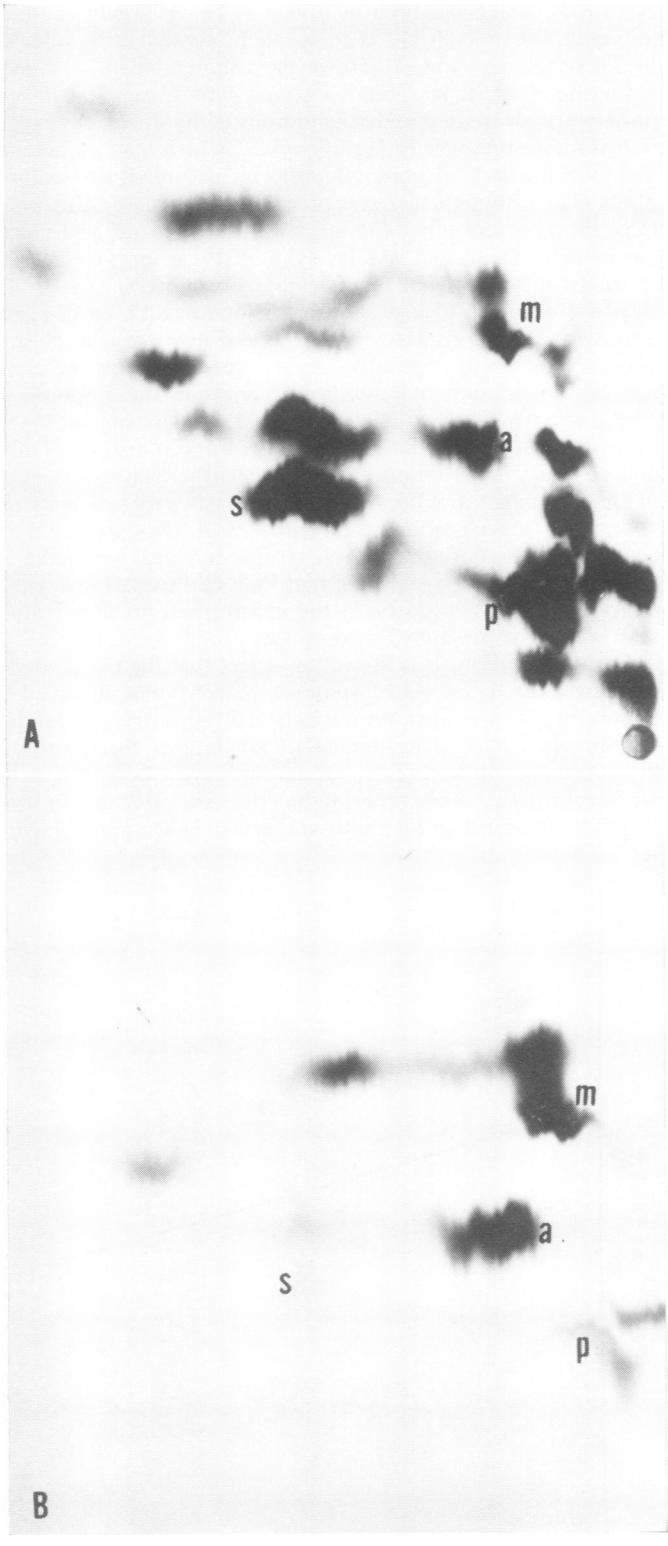

FIG. 6. Chromatograms of the radioactive compounds extracted from normal (A) and Fe deficient (B) leaves after treatment for 2 min with $^{14}CO_2$. Note sucrose (s), aspartic acid (a), malic acid (m), and sugar phosphates (p). Development of the chromatograms: butanol-propionic acid-water in the vertical direction; phenol-water in the horizontal direction.

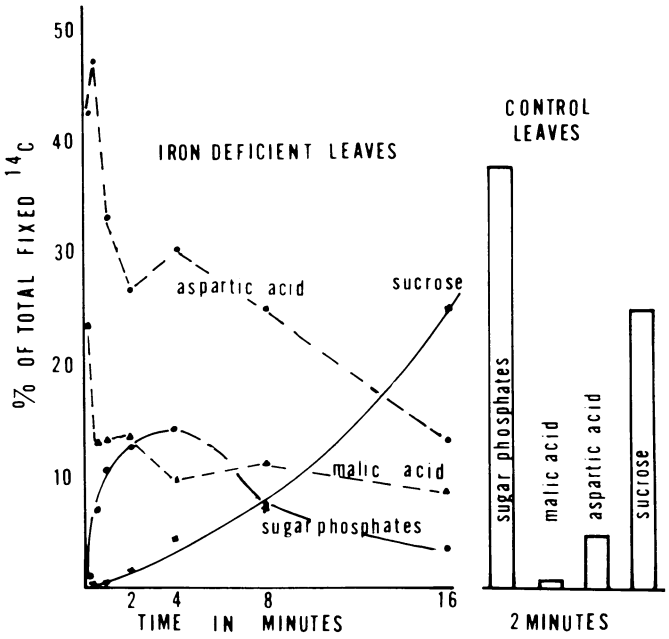

FIG. 7. Distribution of photosynthetic products in Fe deficient leaves (left) and in normal leaves (right). During the course of the experiment, the rates of CO_2 fixation for both control and iron deficient plants were essentially constant. Expressed as cpm per mg dry weight of tissue, the ^{14}C incorporation per min into the control leaves averaged 15,100 cpm and that for iron deficient leaves 2,400 cpm.

The rate of photosynthesis in the Fe-deficient leaves was approximately 16% of the rate for the control leaves when expressed on a unit of leaf dry weight basis. However, because the Chl levels of the Fe-deficient plants used in the photosynthesis experiments were approximately 38% of the levels in the control leaves, the rate of fixation of $^{14}CO_2$, on a Chl basis, for the Fe-deficient leaves averaged 42% of the control rate.

After 16 min of exposure to $^{14}CO_2$, when sucrose in the Fe-deficient leaves contained 25.5% of the total fixed carbon, malic and aspartic acids still contained 33.9% and sugar phosphates only 4.5% (Fig. 7). Thus, although the mechanisms for carbon fixation by PEP carboxylase and the synthesis of malic and aspartic acids remain active in Fe-deficient leaves, a drastic alteration in the part of the carbon cycle involving sugar phosphates and sucrose synthesis occurs.

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