

Investigations of the Role of Iron in Chlorophyll Metabolism

I. Effect of Iron Deficiency on Chlorophyll and Heme Content and on the Activities of Certain Enzymes in Leaves^{1, 2}

H. V. Marsh, Jr.³, H. J. Evans⁴, and G. Matrone

North Carolina State College, Raleigh

Introduction

For over 100 years it has been known that iron is essential for the growth of higher plants and that it is involved in some manner with chlorophyll metabolism. Iljin (17, 18, 19) demonstrated that iron deficiency in plants resulted in decreased dry weight, salt, and protein nitrogen contents and increased contents of amino and organic acids. Extensive studies (8, 9, 25, 33) have confirmed Iljin's observations, but despite the keen interest in iron chlorosis, no clear evidence of the precise role of iron in chlorophyll metabolism has been obtained.

Evans (11) and DeKock et al. (7) found a positive correlation between the iron content of the nutrient medium and the chlorophyll and heme contents of leaves. The activities of enzymes containing heme prosthetic groups, including catalase, peroxidase, and cytochrome oxidase, have been reported (7, 11, 33) to be less in iron-deficient than in normal tissues. There is no evidence for a direct role of the heme enzymes in chlorophyll metabolism; however, there is strong evidence that the porphyrin moieties of heme and chlorophyll are formed by the same biosynthetic system (2). The fact that the activities of heme-containing enzymes and the contents of both heme and chlorophyll in leaves are influenced in a similar manner by the iron content of nutrient solutions that some step in chlorophyll and heme synthesis is dependent on an adequate iron supply.

Studies (22, 32) on porphyrin biosynthesis by microorganisms and animals have provided evidence suggesting a role of iron in this process. Small amounts of iron (32) have been reported to stimulate porphyrin and heme formation in the blood of iron-deficient ducks. According to Lascelles (22) the formation of porphyrins in *Rhodopseudomonas*

spheroides also is stimulated by iron additions. Studies show that insufficient iron results in a decreased rate of conversion of coproporphyrinogen III to protoporphyrin (23, 28). Thus iron appears to be important in porphyrin metabolism in several types of organisms.

Our experiments were initiated in an effort to identify in iron-deficient plants metabolic abnormalities that may be related directly or indirectly to chlorophyll metabolism. Experiments were conducted to determine the influence of the iron supply on the activities of certain enzymes and on the content of heme and protoporphyrin in leaves. The postulation was tested that the activities of PPNR⁵ and of cytochromes are decreased by iron deficiency, thus decreasing the energy supply which may be involved in chlorophyll biosynthesis (24).

Materials and Methods

Plant Materials. Cowpea plants (*Vigna sinensis* L.) were grown in the greenhouse in an aerated Hoagland nutrient solution (16). Iron in the form of hydrogen ferric ethylenediamine di-(*O*-hydroxyphenylacetate) (Geigy Chemical Co.) was supplied at concentrations of 0.05, 0.25, and 2.50 mg per liter. When the plants were approximately 3 weeks old, the immature leaves of those grown on the 2 lower iron levels showed symptoms of iron deficiency. While mature leaves grown on all 3 iron levels remained relatively normal in appearance, prolonged deficiency resulted in necrosis of the first immature trifoliolate leaves (counted down from the terminal bud). Unless otherwise noted, therefore, the second trifoliolate leaves (about half the area of mature leaves) were used for the various experiments. Leaves from the 6 plants in each vessel were harvested and the composite treated as one sample.

Isolation and Determination of Heme. The method used for the isolation of heme was adapted from a procedure described by Kiese and Kurz (21). Second trifoliolate leaves (15 g) were collected and homogenized in a Waring blender with 75 ml

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³ Present address: Central Research Laboratories, United Fruit Company, Norwood, Massachusetts.

⁴ Present address: Department of Botany and Plant Pathology, Oregon State University, Corvallis.

⁵ Abbreviations: PPNR, photosynthetic pyridine nucleotide reductase; δ -AL, δ -aminolevulinic acid.

of 92 % acetone. Acetone was removed by vacuum filtration and the powder washed with 92 % acetone until the tissue was devoid of chlorophyll. A portion of the acetone solution was reserved for chlorophyll determination. The acetone powder was extracted twice with absolute acetone to remove persisting carotenoids and finally was extracted 3 times with anhydrous ether. The amount of dry powder obtained by this procedure ranged from 60 to 90 mg per g of fresh leaves.

Heme was extracted from the dry powder by acidified acetone. Approximately 50 ml of 80 % acetone were added to the entire sample of powder and the slurry was adjusted to pH 3 with concentrated HCl. The mixture was allowed to stand with occasional stirring for 90 minutes at 0 to 4°. After this, the acetone was removed by vacuum filtration, the residue extracted again with acidified acetone for 30 minutes, and the extracts combined. A third extraction failed to yield appreciable heme.

The acidified acetone solution was carefully adjusted to pH 6.5 with concentrated NH_4OH and the precipitate collected by centrifugation. A negligible amount of heme remained in the supernatant fraction and therefore it was discarded. Ten to fifteen ml of acetone were added to the precipitate and the slurry again allowed to stand for 30 minutes at 0 to 4° and the supernatant fraction collected by centrifugation. The acetone solution containing the heme was evaporated to dryness in vacuo and the heme was dissolved in 5 ml of an alkaline pyridine solution (0.5 ml pyridine, 0.25 ml 1.0 N NaOH, and 1.75 ml water) for spectrophotometric analysis. In the chromatography experiments heme was dissolved in pyridine.

The heme concentration was calculated from the difference between the absorbancy of the reduced (by excess $\text{Na}_2\text{S}_2\text{O}_4$) and oxidized pyridine hemochromogen, determined on Beckman DU spectrophotometer at 555 $m\mu$ using the extinction coefficient of 22 $\text{cm}^{-1} \text{mM}^{-1}$ (5). An authentic sample of hemin was obtained from Nutritional Biochemicals Corporation.

Isolation and Measurement of Porphyrins. Porphyrin synthesis was studied in normal and chlorotic tissue by floating 10 g each of normal and chlorotic immature leaf sections for 8 to 10 hours at room temperature in the dark in 100 ml of 1.2 mM δ -AL, pH 7 (Nutritional Biochemicals Corporation). After incubation, porphyrins were extracted by a method adapted from the procedure of Dresel and Falk (10). The tissue was homogenized for 2 minutes with a Waring blender containing 40 ml of 1.3 N acetic acid. Ethyl acetate (50 ml) was added, and the blending was continued for another 2 minutes. Ethyl acetate (200 ml) was added to the homogenate, the pH was adjusted to 3.1 with glacial acetic acid, and the slurry allowed to stand overnight. The emulsion which formed was broken by centrifugation and the organic fraction was removed with a pipette. The aqueous fraction was extracted a second time with 100 ml of ethyl acetate and the ethyl acetate ex-

tracts were combined and concentrated to 15 ml. The ethyl acetate solution was extracted by shaking with equal volumes of 15 % HCl previously saturated with ethyl acetate. The acid extracts were combined and adjusted to pH 3.1 with a saturated solution of sodium acetate. The porphyrins were quantitatively extracted from the aqueous solution into peroxide-free ether and the ether extracts were combined and concentrated to 15 ml. The ether solution was extracted exhaustively first with ether-saturated 0.2 % HCl and then with ether-saturated 10 % HCl. Coproporphyrin was extracted from the ether by 0.2 % HCl and protoporphyrin by 10 % HCl (10). In this experiment most of the porphyrin was recovered in the 10 % HCl fraction. A trace with the absorption spectra of coproporphyrin was found in the 0.2 % HCl fraction. The absorption spectra of the porphyrin in the 10 % HCl fraction and of its methyl ester derivative agreed with the spectral data of protoporphyrin and its methyl ester (12). On the basis of this evidence the porphyrin is referred to as protoporphyrin. The concentrations of porphyrin in the 10 % HCl solutions were determined from the absorbancy at 408-, 383-, and 433 $m\mu$, according to the method of Rimington and Sveinsson (29), using the extinction coefficient, $E_1^{1\%}$ cm, 4900 (10).

Enzyme Assays. The activity of PPNR was estimated by the method of San Pietro and Lang (30), in which the increase in absorbancy at 340 $m\mu$ is measured. An acetone powder was prepared from 7.5 to 10 g of fresh cowpea leaves, and a crude preparation of the enzyme was made by grinding 0.25 g of powder with 5 ml of 0.005 M Tris pH 8.0. The homogenate was centrifuged at $12,000 \times g$ for 15 minutes and the supernatant fluid was used as the enzyme. Chloroplast fragments were prepared from spinach by the method of Giovanelli and San Pietro (14). The reaction mixture contained 2.5 mmoles of potassium phosphate buffer, pH 7.0; chloroplast fragments equivalent to 105 μg of chlorophyll; 10 μmoles of glutathione; 1.35 μmoles of TPN; enzyme; and water to a final volume of 3 ml. The light source was a 100-w tungsten lamp approximately 7.5 cm from the cuvette. A complete reaction mixture in a cuvette wrapped in tinfoil served as the dark control. The samples were incubated for 10 minutes at 12°.

Catalase activity was estimated by the perborate method of Feinstein (13). Fresh cowpea leaves (1 g) were ground with 5 ml of 0.067 M potassium phosphate buffer, pH 6.8. The homogenates of the normal and chlorotic tissues were diluted 1:25 and 1:10, respectively, and were used as the enzyme preparations. The reaction mixture, containing 8 ml of 1.5 % $\text{NaBO}_3 \cdot 4\text{H}_2\text{O}$, pH 6.8; 1.5 ml of 0.067 M potassium phosphate buffer, pH 6.8; and 0.5 ml of enzyme, was incubated at 37° for 5 minutes and the reaction was stopped by the addition of 10 ml of 2 N H_2SO_4 . The mixture was titrated with standardized KMnO_4 and the equivalents of perborate destroyed were computed.

Other Determinations. Chlorophyll was determined by the method of Mackinney (26) and the iron content of leaves was determined by the method of Parks et al. (27). Dry weight was estimated from acetone powder of fresh material.

For the determination of protein and ATP in leaves, the first (immature) and the second (about half-mature) trifoliolate leaves were harvested and treated as separate samples. One-gram samples were withdrawn from these composites and the ATP content was determined on one sample by the luciferin-luciferase assay (31). For the determination of protein, a 1-g sample was ground in 1 ml of 10% trichloroacetic acid. The precipitate was collected by centrifugation and washed twice with 5% trichloroacetic acid, then digested by the Kjeldahl procedure. Nitrogen was determined by nesslerization on duplicate portions of the digest.

All spectra were determined with a Cary model 11 recording spectrophotometer.

Results

General Effects of Iron Deficiency. The data in table I indicate the effect of iron level in culture

Table I

Effect of Different Levels of Iron in the Nutrient Solutions on Dry Weight and on Chlorophyll and Iron Contents of Second Trifoliolate Cowpea Leaves

Plants were grown on a standard nutrient solution with the indicated iron concentrations. Second trifoliolate leaves (counted down from terminal bud) of 6 plants in each culture vessel were harvested and the composite was treated as 1 sample. The data are means of 4 replicate cultures.

Iron in nutrient solution mg/l	Iron mg/g fr wt	Dry wt* mg/g fr wt	Chlorophyll mg/g fr wt
0.05	19.9	88.1	0.52
0.25	19.5	104.3	1.12
2.50	26.5	132.6	2.34
LSD-0.05	6.5	17.7	0.29
LSD-0.01	9.9	26.7	0.44

* Computed from weight of an acetone powder prepared from fresh material.

solutions on the dry weight and on the contents of chlorophyll and iron in the second trifoliolate leaves of cowpea plants. The iron content of the leaves is poorly correlated with the iron level of the nutrient solution and, therefore, the amount of chlorophyll in leaves utilized in various experiments is presented as an index of the iron status of plants.

The dry weight of the tissue (table I) is closely correlated with the degree of chlorosis ($r = 0.889$), a phenomenon reported by others (7, 11, 19). The

Table II

Effect of Different Levels of Iron in the Nutrient Solution on Adenosine Triphosphate and Protein Contents of Immature Cowpea Leaves

Plants were grown and leaves were harvested as indicated in table I. The data are means of 4 replicate cultures.

Iron in nutrient solution mg/l	ATP μ moles/g fr wt	Protein mg/g fr wt	Chlorophyll mg/g fr wt
First trifoliolate leaves			
0.05	0.324	62.2	0.33
0.25	0.344	57.1	1.64
2.50	0.408	66.5	2.09
LSD-0.05	0.102	7.1	0.36
LSD-0.01	0.154	10.8	0.57
Second trifoliolate leaves			
0.05	0.223	23.6	0.98
0.25	0.295	36.4	1.78
2.50	0.313	46.9	2.17
LSD-0.05	0.229	9.8	0.38
LSD-0.01	0.347	14.8	0.57

protein content (table II) of the half-mature, second trifoliolate leaves was closely correlated with the degree of chlorosis, whereas the protein content of the first trifoliolate leaves from plants receiving the 3 iron treatments was nearly the same, despite the striking differences in the chlorophyll contents. The ATP content (table II) of the immature leaves was not correlated with the degree of chlorosis. The data suggest a trend in the ATP content in the first trifoliolate leaves, although it is not statistically significant at the 5% level. In the more mature second trifoliolate leaves the ATP content exhibited the same trend as the protein content, an observation which is consistent with those reported by Sisler and Klein (31).

Table III

Effect of Different Levels of Iron in the Nutrient Solution on Activities of Photosynthetic Pyridine Nucleotide Reductase and Catalase of Second Trifoliolate Cowpea Leaves

Details of the reaction mixture and assay conditions are given in the section on Material and Methods. The data are means of 4 replicate cultures.

Iron in nutrient solution mg/l	PPNR activity*	Catalase activity**	Chlorophyll mg/g fr wt
0.05	0.01	0.24	0.50
0.25	0.16	0.98	1.65
2.50	0.32	2.52	2.32
LSD-0.05	0.04	1.94	0.31
LSD-0.01	0.06	2.93	0.47

* Measured by optical density change (340 $m\mu$) in 10 min/mg of protein.

** Measured by meq of perborate destroyed in 5 min/mg of protein.

Enzymatic Studies. The activities of both PPNR and catalase were reduced by iron deficiency (table III). PPNR activity was detected in only 25 % of the cultures grown on the lowest iron level, whereas catalase activity was demonstrated in all tissue. The striking increase in PPNR activity of leaves which paralleled the increased iron supply is consistent with the report (personal communication with Dr. A. San Pietro) that iron is a constituent of the enzyme. As indicated in table III the level of iron supplied to cultures markedly influenced the catalase activity of tissues. This observation is in agreement with several reports in the literature (7, 11, 33) of this relationship.

Heme Content. By use of the methods already described a heme component of cowpea leaves was identified by spectrophotometric and chromatographic procedures. In figure 1 the spectrum of the pyridine hemochromogen of an authentic recrystallized sample of heme (16 μM) is compared with the spectrum of the pyridine hemochromogen of the heme isolated from cowpea leaves. The reduced pyridine hemochromogen of both compounds showed absorption

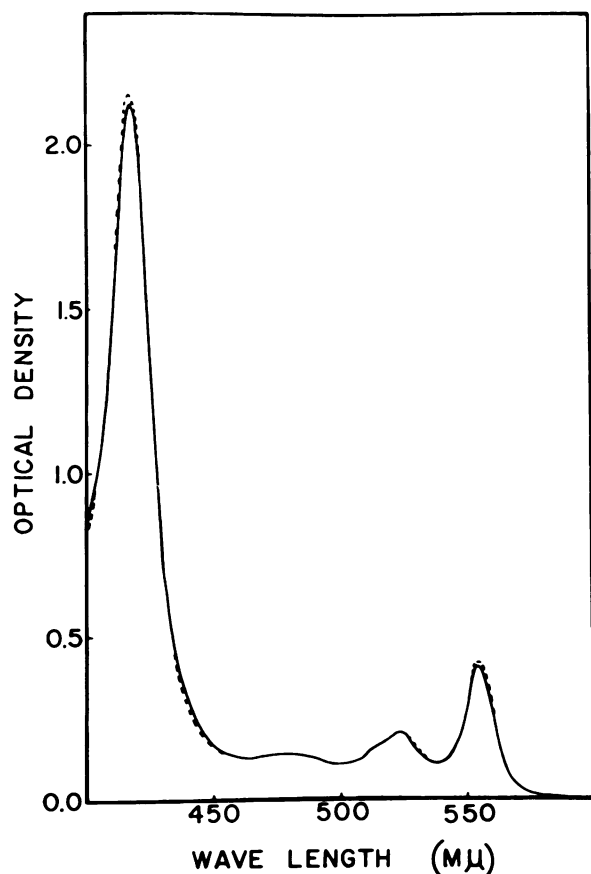


FIG. 1. Absorption spectra of reduced heme isolated from cowpeas (—) and reduced, recrystallized, authentic heme (---). The spectra were determined in an alkaline pyridine solution.

Table IV

Effect of Different Levels of Iron in the Nutrient Solution on Heme Content of Second Trifoliolate Cowpea Leaves

Conditions were the same as noted in table I. The data are the means of 2 experiments with 4 replicate cultures per iron level in each experiment.

Iron in nutrient solution mg/l	Heme $\mu\text{g/g}$ fr wt	Chlorophyll mg/g fr wt
0.05	0.83	0.72
0.25	2.12	1.69
2.50	2.81	2.54
LSD-0.05	0.55	0.33
LSD-0.01	0.83	0.50

peaks at 555-, 512-, and 418 $\text{m}\mu$. The spectral identification of the isolated heme was verified by paper chromatography (6).

The effect of iron level on the heme content of leaves of cowpea plants was studied in 2 different experiments. Statistical analysis of the results of the 2 comparable experiments showed no significant differences in response of either chlorophyll or heme to the different iron levels in the 2 experiments and therefore the data from the 2 experiments were combined. The data (table IV) indicate that the heme content was reduced significantly by the inadequate iron supply. The overall linear trend of increasing heme concentrations with increasing iron levels was highly significant ($P \leq 0.05$). Furthermore, there was a highly significant positive correlation between the heme and chlorophyll contents.

Porphyrin Synthesis. The tissues used in these experiments were from cowpea plants grown on 2.50 and 0.05 mg of iron per liter. Care was taken to select half-mature leaves comparable to the ones used for high and low iron

Table V

Porphyrin Formation by Normal and Iron-deficient Cowpea Leaves

In experiments II-47 and III-2, 10-g samples of segments of cowpea leaves were incubated in the dark in 100 ml of water, pH 7, at room temperature. δ -AL was added as indicated. In experiment II-75, normal and deficient cowpea plants were kept in the dark at room temperature for 10 hours. From these plants 120 g of leaves comparable to those used in the other experiments were harvested and porphyrins were extracted.

Experiment No.	Incubation Time hr	δ -AL added μmoles	Protoporphyrin formed $\mu\text{moles/g}$ fr wt	
			Normal	Deficient
II-47	10	none	0.00*	0.00*
	10	120	0.61	1.52
III-2	8	120	1.67	5.38
II-75	10	none	0.006	0.006

* None detected.

levels in the preceding experiments. Segments of the leaves were incubated in the dark with 1.2 mM δ -AL, and from these a porphyrin tentatively identified as protoporphyrin was extracted (table V, experiments II-47 and III-2). Up to threefold more of this porphyrin was isolated from the iron-deficient than from the normal tissue. The higher yields in experiment III-2 resulted from a more thorough extraction with acetic acid-ethyl acetate. Other porphyrins (15) farther along the biosynthetic pathway may have accumulated in this tissue, but if so, they were masked by the chlorophyll and could not be detected. From these data it is concluded that there is no metabolic block in the synthesis of protoporphyrin from δ -AL.

Small but equal amounts of protoporphyrin were isolated from normal and chlorotic tissues incubated in the dark without δ -AL (table V, experiment II-75). No protoporphyrin was found, however, in samples, incubated in the light. When tissue which had accumulated protoporphyrin in the dark from added δ -AL was transferred to the light, no protoporphyrin could be detected after 1 hour, the shortest time period checked. Unfortunately, no efforts were made to determine whether the chlorophyll concentration increased significantly under these conditions.

Discussion

The pronounced effect of iron deficiency on the heme content and catalase activity reported here and elsewhere (7, 11) indicates that the level of activities of heme enzymes of leaf tissue as well as the chlorophyll content, is markedly influenced by the iron supply. Reduced activities of the heme enzymes undoubtedly would affect metabolism in general, and perhaps may result in indirect effects on chlorophyll metabolism. The correlation between chlorophyll and heme contents suggests, however, that iron chlorosis is an expression of a regulatory influence exerted by the supply of iron on porphyrin synthesis.

The fact that sections of leaves from iron-deficient cowpea plants have the capacity to synthesize protoporphyrin from δ -AL indicates that this portion of the porphyrin biosynthetic pathway does not require iron. These results may be contrasted with those obtained from experiments with bacteria (23, 28) which indicated that an insufficient iron supply resulted in a decreased rate of conversion of coproporphyrinogen III to protoporphyrin. This apparent difference between plants and bacteria may mean that bacteriochlorophyll is formed by a reaction sequence different from that of chlorophyll, as was suggested by Bogorad (2).

The effect of iron deficiency on the δ -AL-dependent accumulation of protoporphyrin is of special interest. Protoporphyrin accumulated only when the tissues were incubated in the dark with δ -AL. When tissues were transferred to the light, the porphyrin rapidly disappeared. These results may be related

to those of Granick (15) who found that dipyrindyl stimulated porphyrin accumulation in etiolated barley incubated with δ -AL. These observations suggest that iron functions in the conversion of protoporphyrin to chlorophyll. On the other hand, since protoporphyrin is also the precursor of heme, it is entirely possible that the greater accumulation of porphyrin resulted from the concentration of iron being rate-limiting in the conversion of protoporphyrin to heme.

The accumulation of an equal amount of protoporphyrin in normal and iron-deficient leaves not incubated with δ -AL is noteworthy. If the low heme content of iron-deficient tissue was caused entirely by insufficient iron to convert the protoporphyrin to heme, then porphyrins should have accumulated in the dark in the iron-deficient tissue. Since porphyrins did not accumulate, utilization of the porphyrins formed in chlorotic tissue from endogenous substrates apparently is not seriously impaired by iron deficiency. The possibility of a block between protoporphyrin and chlorophyll resulting from iron deficiency cannot be eliminated on the basis of this evidence alone. The data reported here and elsewhere (3, 4, 22, 32), however, are consistent with the premise that iron functions in the synthesis of δ -AL. A limited rate of formation of this key precursor of porphyrins (2) would account for the observed correlation between the heme compounds and chlorophyll.

The striking reduction of PPNR activity associated with decreasing iron levels in the nutrient solution and increasing severity of iron chlorosis suggests that iron is an important cofactor of this enzyme. San Pietro (personal communication) has pointed out that the purified enzyme contains non-heme iron. Thus the PPNR data presented here suggest that iron deficiency may drastically reduce the activities of nonheme-iron enzymes as well as of the heme enzymes. Bacon et al. (1) have reported that the activity of aconitase shows a striking response to the level of iron in the culture medium.

The physiological significance of the inactivity of PPNR can be deduced from the reaction known to be catalyzed by the enzyme. Thus, some of the symptoms of iron chlorosis can be ascribed to restricted energy reserve. Reduced PPNR activity presumably would reduce formation of TPNH and ATP during photosynthesis as would the low chlorophyll content of iron-deficient plants. Oxidative phosphorylation also should be limited by reduced heme enzyme activity. The accumulation of free amino acids and decreased protein content and dry weight can be explained by a limited energy supply without postulating a specific function of iron in protein metabolism.

Summary

The effect of iron deficiency on the formation of porphyrin compounds in cowpea leaves was in-

vestigated. There was a positive correlation between the concentration of either heme or chlorophyll and the level of iron in the nutrient solution. Protoporphyrin, the precursor of heme, did not accumulate from endogenous substrates in the iron-deficient or normal tissues. The synthesis of protoporphyrin from δ -aminolevulinic acid proceeded just as rapidly in iron-deficient as in normal tissues. These observations suggest that iron deficiency results in the limited formation of δ -aminolevulinic acid and that this block is one rate-limiting step in chlorophyll synthesis.

Significantly more protoporphyrin accumulated in iron-chlorotic than in normal tissue incubated with δ -aminolevulinic acid in the dark. The hypothesis is proposed that this accumulation results from insufficient iron which is needed to form heme from the protoporphyrin.

The activity of photosynthetic pyridine nucleotide reductase in the leaves of cowpea plants is strikingly decreased by a limited iron supply in the nutrient medium.

The data suggest that such symptoms of iron deficiency as reduced dry weight, protein, and ATP contents are indirect results of the deficiency.

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Investigations of the Role of Iron in Chlorophyll Metabolism II. Effect of Iron Deficiency on Chlorophyll Synthesis^{1,2}

H. V. Marsh, Jr.³, H. J. Evans⁴, and G. Matrone

North Carolina State College, Raleigh

Introduction

Since iron deficiency in plants results in severe chlorosis of leaves, it has been assumed by many that iron is an essential cofactor in the biosynthesis of chlorophyll. No definitive information is available, however, showing a precise role of iron in chlorophyll metabolism.

Previous investigations (7, 10, 15) established that the concentrations of heme and chlorophyll in leaves are correlated with each other and with the level of iron in the nutrient medium. It was reported (15) that the activities of the enzymic systems involved in the synthesis of protoporphyrin from δ -aminolevulinic acid (δ -AL) were not reduced by iron deficiency, yet protoporphyrin failed to accumulate in iron-deficient leaves unless the tissue was incubated in the dark with δ -AL. Since protoporphyrin is a precursor of both chlorophyll and heme (12), these observations suggest that a depressed

rate of δ -AL synthesis may be one cause of the low chlorophyll content of iron-deficient plants. An iron requirement for δ -AL formation in *Rhodospseudomonas spheroides* (14) and in avian blood (5, 6, 17) has been suggested. The reaction mediated by aconitase may limit the rate of δ -AL synthesis in iron-deficient tissues of animals (9, 16) and plants (3).

Since significantly more protoporphyrin was identified in iron-chlorotic than in normal cowpea leaves previously incubated in the dark with δ -AL (15), it is suggested that iron may be a cofactor in the enzymic system converting protoporphyrin to chlorophyll.

The present investigation was initiated in an attempt to locate possible sites where a deficiency of iron might limit chlorophyll synthesis. Studies were made of the rates of incorporation of certain C¹⁴-labeled organic acids into chlorophyll in normal and in iron-deficient leaves. It was assumed that a reduced rate of incorporation of the isotope into chlorophyll would indicate a block in chlorophyll biosynthesis.

Materials and Methods

The following compounds were obtained from the California Corporation for Biochemical Research: δ -AL-2-C¹⁴, citric acid-1,5-C¹⁴, and α -ketoglutarate-5-C¹⁴ (Na salt). Succinic acid-2,3-C¹⁴ was obtained from Volk Radiochemical Company, and all other materials were standard laboratory reagents.

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³ Present address: Central Research Laboratories, United Fruit Company, Norwood, Massachusetts.

⁴ Present address: Department of Botany and Plant Pathology, Oregon State University, Corvallis.