EFFECTS OF IRON AND CHELATING AGENTS ON DARK CARBOXYLATION REACTIONS IN PLANT HOMOGENATES [1]

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A previous study in this laboratory (12) indicated that leaves and excised roots of bush beans grown under conditions of Fe deficiency fixed larger quantities of $C^{14}O_2$ from an enriched atmosphere than did those receiving adequate amounts of Fe. For this reason, studies have been made to determine the effect of Fe and chelating agents on PEP (phosphoenolpyruvate) carboxylase (1, 9, 13), PEP carboxykinase (9), and the carboxylation enzyme (17) systems that fix CO_2.

MATERIALS AND METHODS

The reagents were prepared for use as follows: The K salt was prepared by dissolving the Ag-Ba salt of PEP in 0.1 M HNO_3 and precipitating the Ag and Ba with equivalent amounts of KCl and K_2SO_4, respectively, and a stock solution of 0.01 M was prepared in 0.2 M TRIS (tris(hydroxymethyl) aminomethane) buffer, pH 8.0. The K salts of G6P (glucose-6-phosphate) and R5P (ribose-5-phosphate) were prepared from the Ba salt of each by precipitating the Ba with an equivalent amount of K_2SO_4. These reagents were centrifuged to remove the precipitates, and stock solutions of 0.02 M were prepared in TRIS buffer as above. Stock solutions of 0.01 M Na salts of ethylenediaminetetraacetic acid (EDTA) and ethylenediamine di(*o*-hydroxyphenolacetic acid) (EDDHA) (6) were prepared and made to pH 8.0.

In the systems studied the addition of 1 micromole (μM) of EDDHA into the reaction mixtures appeared to provide close to a maximum increase in the rate of the reactions, consequently this level was used throughout the experiments.

Soybean and barley seeds were germinated in sand and allowed to grow about 2 inches in length or about 10 days. The barley seedlings were used directly in the preparation of homogenates. The soybean seedlings were transferred into aerated solution cultures for about 3 weeks with nutrient solutions containing as m.e. per liter, $Ca(NO_3)_2$, 10; KNO_3, 1; $MgSO_4$, 8; K_2SO_4, 4; and $NH_4H_2PO_4$, 2; and as μM per liter, $MnSO_4$, 18; $ZnSO_4$, 3.8; H_3BO_3, 9.2; and $(NH_4)_6\ MO_7O_{24}$, 0.10. The Fe level was varied to give green plants, moderately deficient plants, and severely deficient plants by the use of 10^{-3}, 10^{-4}, and 2×10^{-5} M $FeSO_4$, respectively, in the solutions. Trifoliate leaves of equal age were removed from these treatments for assay. Recently matured trifoliate orange leaves which were used in some studies were obtained from trees in the UCLA orchard.

The assays were made as follows: Leaf homogenates were produced by grinding 1 weight of fresh leaves with 1 volume of TRIS buffer, 0.2 M, at pH 8.0, with a mortar and pestle at 0° C. The homogenate was strained through 2 layers of cheesecloth and kept at 0° C until use which never exceeded one half hour. All the reaction mixtures received 140 μM TRIS buffer pH 8, 20 μM $KHC^{14}O_3$ containing 90,-800 cpm as the $BaCO_3$ precipitate with a conventional thin window Geiger-Müller counter, and 0.1 ml enzyme crude homogenate preparation which was added last. Total volume of all reaction mixtures was 1.0 ml. The mixtures were incubated 10 minutes at 37°C, the enzyme killed with 0.1 ml 1 *N* HCl, which also expelled unreacted HCO_3^-, and the mixtures centrifuged. Aliquots of 0.2 ml of the supernatant were then dried in forced air at room temperature in Pyrex or plastic planchets (aluminum and stainless steel planchets were unsatisfactory) and counted with a Geiger-Müller counter. This method was essentially described by Jackson (4) as being a modification of that employed by Bandurski and Greiner (1) and Saltman et al (13).

The coefficient of variability of this method was found to be less than 4 % and this value is taken into account in the discussion of the results. All studies were repeated several times and data are included that represented consistent trends.

Since Fe is known to decarboxylate β-ketodicarboxylic acids (14), the following procedure was used to insure a measurement of the true effect of Fe on the PEP carboxylase system: Ethanol and DPN were added to the reaction mixtures to furnish sufficient DPNH to convert a maximum amount of the OAA (oxalacetate) formed to malate. After the 10-minute incubation period, 0.1 ml of 2,4-dinitrophenylhydrazine in 1 *N*-HCl was added to each reaction mixture to convert any OAA present to the hydrazone form. Such a system was found to almost completely prevent decarboxylation of labeled products during the period the planchets were dried prior to counting. Protein determinations were made with Folin's reagent (7).

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TABLE I

EFFECT OF Fe PRETREATMENTS ON DARK FIXATION OF CO_2 IN HOMOGENATES FROM SOYBEAN LEAVES WITH DIFFERENT SUBSTRATES

REACTION SYSTEM*	PRETREATMENT		
	VERY DEFICIENT Fe	MODERATELY DEFICIENT Fe	ADEQUATE Fe
		cpm	
Blank + Mg	...	94	44
ATP + Mg	...	75	40
PEP + Mg	303	600	381
PEP+Mg+EDDHA	567	692	677
PEP+ATP+Mn	510	496	392
PEP+ATP+ Mn+EDDHA	507	489	393
G6P+ATP+Mg	...	330	118
G6P+ATP+ Mg+EDDHA	...	543	223
R5P+ATP+Mg+TPN	778	490	232
R5P+ATP+Mg+ TPN+EDDHA	1240	860	1070

* The general procedure was outlined under Materials and Methods. The amounts of the other cofactors and of substrates where indicated were as follows: PEP, 1 μM; G6P, 4 μM; R5P, 2 μM; ATP, 4 μM; TPN, 0.2 μM; EDDHA, 1 μM; Mg, 20 μM; Mn, 6 μM. It was found previously that the PEP carboxykinase reaction proceeded as well in the crude homogenates with ATP as with ADP. Endogenous ATP+Mn resulted in some, but slight activity only.

RESULTS AND DISCUSSION

Table I shows the results of experiments in which soybeans were grown at 3 Fe levels. In general all reactions studied resulted in greater activity at a moderately deficient Fe level than at an adequate Fe level. Sometimes with a chelating agent, however, this was not so. At a very deficient Fe level the PEP carboxylase reaction was decreased, while the PEP carboxykinase reaction was essentially unchanged. Under the same condition the reactions with R5P as the substrate were increased; however, a consistent decrease occurred as the Fe level in the treatments increased. Assays of the homogenates for total protein indicated that the results would not be materially different on that basis of calculation. These effects of Fe could be analogous to some of the enzyme activities obtained by Nicholas and Goodman (10) for 4 different Fe levels where, in some systems, they showed increases for moderate deficiency and decreases for extreme deficiency.

Fixation of CO_2 with R5P as a substrate is quite probably through the reaction catalyzed by the carboxylation enzyme with ribulose diphosphate as a substrate (17). If this were the case then the phosphoriboisomerase (2) and the phosphoribulokinase (2) enzymes were active in the crude homogenates. The inhibitory effect of Fe actually could have been on any of the 3 enzymes involved.

The fixation of CO_2 with G6P as a substrate is suspected to be through the same reaction as R5P. In all aspects studied here and in many unpublished experiments this appeared to be so. Actually, in the 1st experiments made by us with G6P, G6P with TPN and Mn was used as a means of driving the fixation of CO_2 catalyzed by isocitric dehydrogenase with α-ketoglutaric acid as a substrate (11). It was soon found, however, that more CO_2 was fixed with G6P when α-ketoglutaric acid was absent than with, and that Mg rather than Mn resulted in greater activity.

TABLE II

EFFECT OF EDDHA, GSH AND TPN ON DARK FIXATION OF CO_2 THROUGH R5P IN HOMOGENATES FROM BARLEY LEAVES

REACTION SYSTEM*	CPM
ATP+Mg	27
R5P+ATP+Mg	424
R5P+ATP+Mg+TPN	444
R5P+ATP+Mg+EDDHA	660
R5P+ATP+Mg+EDDHA+TPN	805
R5P+ATP+Mg+GSH	647
R5P+ATP+Mg+GSH+TPN	807

* The procedures were outlined under Materials and Methods and in table I. Amount of GSH was 1.0 μM.

Since then we have used G6P as a substrate for CO_2 fixation with the crude homogenates. Likewise, it was found that CO_2 fixation resulting from G6P hindered efforts to demonstrate CO_2 fixation through "malic" enzyme with pyruvate as a substrate (11). In addition to the Mg requirement the responses of the G6P and R5P reactions were parallel for ATP, TPN, and GSH (glutathione) as cofactors and were parallel for NaF inhibition. NaF did not inhibit the PEP reactions, but it did inhibit the G6P and R5P reactions. Since the activity obtained when ATP and Mg were added without a substrate was also inhibited by NaF, it is possible that this endogenous reaction, at least in part, was similar to G6P and R5P.

Effects of chelating agents on CO_2 fixation are given in tables I, II, III, and IV. In all cases except possibly for the reaction catalyzed by PEP carboxy-

TABLE III

EFFECT OF CHELATING AGENTS ON DARK FIXATION OF CO_2 WITH HOMOGENATES FROM TRIFOLIATE ORANGE LEAVES

REACTION SYSTEM*	CPM
Blank+Mg	23
PEP+Mg	2180
PEP+Mg+EDDHA	3630
G6P+ATP+Mg	536
G6P+ATP+Mg+EDDHA	647
G6P+ATP+Mg+EDTA	628
R5P+ATP+Mg	12900
R5P+ATP+Mg+EDDHA	14700

* The procedures were outlined under Materials and Methods and in table I. Amount of EDTA was 0.1 μM.

TABLE IV

EFFECT OF Fe AND CHELATE SEPARATELY AND TOGETHER ON DARK FIXATION OF CO_2 IN TRIFOLIATE ORANGE LEAF HOMOGENATES

ADDITIONS IN μM	REACTION SYSTEM* PEP + Mg	PEP + ATP + Mn	R5P + ATP + Mg
		cpm	
Nothing	1350	1580	4270
1 Fe	1090	1320	3620
1 EDDHA	2010	2060	4950
1 Fe + 1 EDDHA	1320	1490	4240

* The procedures were outlined under Materials and Methods and in table I.

kinase a chelating agent was necessary for maximum activity. This is analogous to the findings of Weissbach et al for the carboxylation enzyme (17). In all cases there was a greater percentage of response to chelate at an adequate Fe level than at a slightly deficient Fe level. This provides some evidence that the effect of the chelating agent is in chelation of Fe. Additional evidence for this is in table IV, where the addition of EDDHA to the reaction mixture overcame the inhibiting effect of the Fe added, while added Fe overcame the enhancing effect of the chelate. Chelating agents increased activity also for homogenates from barley and soybeans. These data also suggest a possible reason why synthetic chelating agents have a growth-promoting effect on plants other than by supplying micronutrients as has been reported (8).

It was of interest to note that stimulation with a chelating agent on the PEP carboxylase reaction as observed in several experiments was less consistent with roots than with leaves.

Part of the chelating agent effect with R5P in table I may be the result of TPN which was included in the reaction mixture with and without the chelating agent. In a large number of tests it was shown that when TPN was added to a reaction mixture containing R5P+Mg+ATP it had no effect, but did have a consistent enhancing effect when added to R5P+Mg+ATP+EDDHA (table II).

To further test the inhibitory effect of Fe, it was added to reaction mixtures with PEP, G6P, and R5P as substrates. Each was found to be significantly inhibited (table V).

To determine the nature of the inhibitory effect of Fe, it was added to reaction mixtures containing PEP+Mg, and double reciprocal curves were plotted. The enzyme was a crude preparation from trifoliate orange leaves. The curves in figure 1 indicated that Fe may be a competitive inhibitor of PEP carboxylase. This observation is at slight variance with that of Walker (16), who found that 10^{-3} M Fe completely inhibited the PEP reaction in certain Crassulacean plants by 100% precipitation of the enzyme. In the present work Fe at 4×10^{-2} M did completely inactivate the system from trifoliate orange leaf.

TABLE V

EFFECT OF Fe ON DARK FIXATION OF CO_2 IN TRIFOLIATE ORANGE LEAF HOMOGENATES

REACTION SYSTEM*	CPM
Blank + Mg	34
PEP + Mg	2360
PEP + Mg + Fe	1050
ATP + Mg	135
ATP + Mg + Fe	66
G6P + ATP + Mg	536
G6P + ATP + Mg + Fe	164
R5P + ATP + Mg	12300
R5P + ATP + Mg + Fe	6820

* The procedures were outlined under Materials and Methods and in table I. Six micromoles of Fe as $FeSO_4$ were added where indicated.

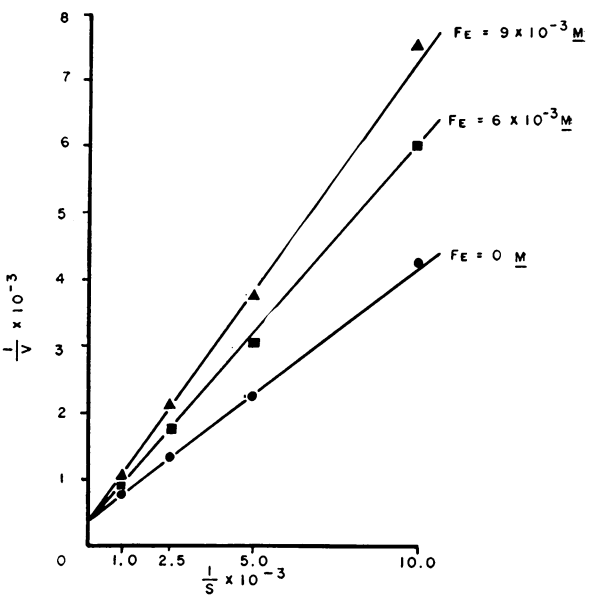

FIG. 1. Double-reciprocal plot of the effect of Fe on $C^{14}O_2$ fixation by crude homogenates of trifoliate orange leaves through the reaction catalyzed by PEP carboxylase. S is molar concentration of PEP in the reaction mixture, and V is cpm C^{14} in 0.2 ml reaction mixture. Fe as added as $FeSO_4$.

The indication that PEP carboxylase activity is greater in plants in which Fe is insufficient may offer an explanation of the observations that certain organic acids (3, 12), K, and sometimes Ca (15) are increased in Fe-deficient plants. As previously stated, dark fixation of CO_2 by this system leads directly to organic acid formation and accumulation in some plant species (1, 9, 13, 16). This in turn may induce increased cation uptake (4, 5). The observation that extreme Fe deficiency resulted in less CO_2 fixa-

tion than did a moderate Fe deficiency which in turn resulted in more CO_2 fixation than did an adequate Fe level may explain the observations that in lime-induced chlorosis, organic acid contents increased and then decreased as the disorder advanced (3, 12).

Summary

Fixation of CO_2 with PEP (carboxylase and carboxykinase systems each included) and R5P as substrates was greater in homogenates of plants grown at a slightly deficient Fe level than in those grown at an adequate Fe level. For severe Fe deficiency the fixation catalyzed by PEP carboxylase was decreased. A chelating agent sometimes increased the amount of CO_2 fixation with the PEP carboxykinase reaction. A chelating agent was necessary in the reaction mixture for maximum activity of the reaction catalyzed by PEP carboxylase as well as for the carboxylation enzyme. Addition of Fe to assay mixtures inhibited CO_2 fixation through PEP. Kinetic studies indicated this to be a competitive inhibition. Fe also inhibited CO_2 fixation when R5P and G6P were used as substrates and activity was increased with both substrates by use of chelating agents.

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LOSS OF PHOSPHORUS-32 BY PLANT ROOTS AFTER FOLIAR APPLICATION [1,2]

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The fact that plant roots are capable of losing ions to the ambient medium was recognized as early as the nineteenth century, and has been the subject of intermittent attention since that time. Excellent reviews of the early work in this field were compiled by Merrill (10) and True (14). Definite conclusions in these early experiments were often lacking since the work was limited solely to the use of electrolytic or chemical techniques. Such techniques prevented accurate determination of very low concentrations, as well as detection of ion movement in a direction opposite to that of net flow. Recent development of radioisotope methods has made such measurements possible, and has provided a new approach to the study of ion loss by roots.

Evidence exists to indicate that root loss of ions may play an important role in the overall nutrient

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