

# USE OF SYNTHETIC CHELATING AGENTS IN PLANT NUTRITION AND SOME OF THEIR EFFECTS ON CARBOXYLATING ENZYMES IN PLANTS\*

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## *Introduction*

Synthetic chelating agents in recent years have become a reasonably satisfactory means of supplying micronutrients (iron, zinc, manganese) to plants. Sometimes chelating agents have effected growth responses beyond those that could be attributed to micronutrients. The postulated explanations include auxinlike effects and also improved micronutrient balance; another is an *in vivo* stimulation of some of the reactions relating to photosynthesis by chelating agents accumulated by plants. The carboxydismutase enzyme is extremely sensitive to heavy metals. Chelating agents protect against heavy-metal inactivation of a large number of enzymes in *in vitro* studies. In cell-free preparations many chelating agents not only increase the amount of CO<sub>2</sub> fixation, but also overcome the inhibition caused by the added heavy metals. Weissbach *et al.*<sup>1</sup> were first to report that chelating agents increased CO<sub>2</sub> fixation with the carboxydismutase enzyme.

A brief review of some of the behavior of synthetic chelating agents and their metal chelates in plants will clarify the studies in this laboratory on carboxylating enzymes.

## *Synthetic Chelating Agents in Plant Nutrition*

Synthetic chelating agents were used in the nutrition of microorganisms<sup>2</sup> before their use was developed for higher plants. EDTA and other chelating agents have given excellent results in maintaining adequate levels of micronutrient elements in nutrient substrates for plants over relatively long periods of time.<sup>3</sup> In addition, high or even moderate levels of micronutrient elements are less toxic to plants if a chelating agent is present in the nutrient solution.<sup>4</sup> The usual result is that media containing chelating agents give higher yields than do those without chelating agents, although they have equal amounts of micronutrients. A most interesting effect of EDTA on algal growth was noted by Walker.<sup>5</sup> EDTA relative to no EDTA decreased yields when levels approaching a deficiency of zinc and manganese were supplied; it had no depressing effect, however, when levels approaching a deficiency of iron were used. Either the zinc and manganese EDTA were absorbed less readily by the algae

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The abbreviations used in this report are: EDTA, ethylenediaminetetraacetic acid; ED-DHA, ethylenediaminedi-*o*-hydroxyphenylacetic acid; HEEDTA, hydroxyethylethylenediaminetriacetic acid; R5P, ribose-5-phosphate; TPN, triphosphopyridine nucleotide; PEP, phosphoenolpyruvate; RDP, ribulose diphosphate; PGA, 3-phosphoglyceric acid; GSH, reduced glutathione; *p*-CMB, parachloromercuribenzoate; ATP, adenosine triphosphate; OAA, oxaloacetic acid;  $\mu$ mole, micromole ( $10^{-6}$  mole).

than was the iron EDTA or the iron was separated more readily from the EDTA inside the cells than were zinc and manganese. The possible relationship of this to higher plants will be seen below.

Following the use of EDTA in nutrient solutions to maintain iron in a soluble form,<sup>6</sup> the iron chelates came into widespread use for correcting iron deficiency in plants by their application to soil<sup>7</sup> (there is a literature of several hundred references on the subject). Foliage application is also made, but less extensively and somewhat less successfully than soil application. Economic considerations, however, dictate low application rates and limit the use of chelates.

TABLE 1\*  
DRY WEIGHT YIELD AND FE CONTENT OF SOYBEAN SEEDLINGS AS INFLUENCED  
BY EDDHA IN SAND CULTURE†

Iron level and source (ppm)	Dry weight yield/plant (gm.)	Fe content of dry weight (ppm)
None	1.26	133
5 FeSO <sub>4</sub>	1.36	223
50 FeSO <sub>4</sub>	1.21	389
5 FeEDDHA	1.64	169
50 FeEDDHA	1.76	370
Equivalent 5 Fe as EDDHA	1.56	174
Equivalent 50 Fe as EDDHA	1.63	127

\* Part of TABLE 6, Wallace *et al.*<sup>4</sup>

† None of the plants had an iron deficiency.

TABLE 2\*  
YIELDS OF BEAN PLANTS GROWN IN A CALCAREOUS SOIL WITH IRON CHELATES†

Fe chelate‡	Fresh weight of plants (gm.)
None	18.7
EDTA	12.3
DTPA§	15.8
HEEDTA	11.0
EDDHA	24.5

\* Part of TABLE 4, Wallace *et al.*<sup>17</sup>

† None of the plants was iron-chlorotic.

‡ The iron supplied was 175 mg. for a 500-gm. quantity of soil.

§ Diethylenetriaminepentaacetate.

In some cases zinc and manganese deficiencies in higher plants have been corrected successfully with appropriate chelates.<sup>8</sup> Instability in soil is a problem in the use of zinc and manganese chelates.

Examples of improved plant yields from the use of metal chelates under conditions in which no micronutrient deficiencies exist are given in TABLES 1 and 2. The fact that chelating agents behave like auxins<sup>8</sup> has been shown to be doubtful as an explanation.<sup>9</sup> That the depressing effect of some iron chelating agents on absorption of such micronutrients as manganese sometimes results in an improved balance of micronutrients<sup>10</sup> is still a possible explanation. This phenomenon will be described in detail. The principal reasons for suspecting

that chelating agents have a net beneficial effect on enzyme reactions in plants, as another possible explanation of the yield increase, is the evidence that some reactions have increased activity in the presence of chelating agents and that the chelating agents are absorbed by plant roots and translocated to leaves when used in plant nutrition.

Wallace and North<sup>11</sup> first showed that both metal and chelate are absorbed by plant roots and translocated to the foliage, although Wallace *et al.*<sup>4</sup> later indicated that iron and chelating agents were present in leaves in different amounts after root application. Most of the data from this laboratory indicate that more chelating agent than metal, when both were tagged with isotopes,

TABLE 3  
EDTA AND IRON RATIOS IN PLANT PARTS FOLLOWING ROOT APPLICATION  
OF C<sup>14</sup>- AND Fe<sup>59</sup>-LABELED MATERIAL

Plant	EDTA/Fe ratios*		
	Leaf	Stem	Root
Soybean	3.6	2.5	0.9
Pyracantha	6.8	5.0	0.4
Rough lemon	4.1	3.5	1.1

\* Calculated from specific activities. Single salt solutions containing 5 ppm iron were supplied for 5 days in sand culture.

TABLE 4  
EDTA AND ZINC RATIOS IN PLANT PARTS FOLLOWING ROOT APPLICATION OF  
C<sup>14</sup>- AND Zn<sup>65</sup>-LABELED MATERIAL

Plant	EDTA/Zn ratios*		
	Leaf	Stem	Root
Soybean	4.2	2.2	1.2
Pyracantha	6.0	2.5	1.3
Rough lemon	5.0	3.0	0.2

\* Calculated from specific activities. Single salt solutions with 5 ppm zinc were supplied for 5 days in sand culture.

reached the leaves after soil application (TABLES 3 and 4). Recent unpublished studies with Fe<sup>59</sup>- and C<sup>14</sup>-labeled EDDHA, which forms an extremely stable iron chelate, have indicated sometimes equimolar amounts of the chelate and iron in leaves. Tiffin and Brown,<sup>12</sup> however, reported recently that the chelating agent remained in the nutrient solution and that only the metal was absorbed. In cases in which a free chelating agent without a chelated metal has been transported to leaves (sodium salts of chelating agents supplied to roots appear to translocate readily to leaves) or in which such are separated from metal chelates in roots or in leaves after translocation, it is expected that they will have important effects on plant metabolism through chelation of metals present in leaves, if the chelating agents themselves are not metabolized.

There is little evidence that synthetic chelating agents are metabolized in

plants. Although iron chelating agents undergo oxidation in the presence of sunlight,<sup>13</sup> and leaves are exposed to sunlight, there is no direct evidence that this oxidation is the manner in which chelating agents decompose in plants.

Metal chelates appear to be absorbed slowly or not at all by the roots of some plant species.<sup>14</sup> These plants, of course, do not respond to the applications. What is involved in the absorption failure is not known.

Moderate to high levels of heavy metals are toxic to plants. The addition of certain chelating agents to the media containing such levels of heavy metals often overcomes some or all of the toxicity. This is illustrated in TABLE 5. Heavy metals may be detoxicated either by preventing their absorption by

TABLE 5\*

EFFECT OF EDDHA WITH DIFFERENT MICRONUTRIENT LEVELS ON SOYBEAN YIELDS

Nutrient variables	Without EDDHA (gm. dry weight)	With 0.0015 M EDDHA (gm. dry weight)
No Fe	1.39	1.77
No Fe + $\text{HCO}_3^-$	1.77	2.22
High Mn and Zn	1.00	2.16
High Cu and P	0.76	1.50

L.S.D. (0.05)†

0.57

\* Part of TABLE 5, Wallace *et al.*<sup>4</sup>

† Least significant difference at the 5 per cent possibility of error.

TABLE 6

FeEDDHA EFFECT ON YIELD AND MICRONUTRIENT CONTENT OF SOYBEANS GROWN IN A CALCAREOUS SOIL

Lbs./acre equivalent Fe as EDDHA	Yield (gm.)	Fe (ppm)	Mn (ppm)	Zn (ppm)
0	1.39	40	88	77
5	1.64	41	11	86
200	1.54	359	7	97
L.S.D. (0.05)*	0.21	20	13	18

\* Least significant difference at the 5 per cent possibility of error.

plants or by chelation after they are in plants. The relative importance of each is not known.

Iron chelates applied to plants often induce manganese deficiencies.<sup>15</sup> Iron chelates actually hinder the absorption of manganese by plants (TABLE 6). This can be a practical means of overcoming toxicity such as that caused by manganese, as was successfully done in coffee.<sup>16</sup> The nature of this inhibition of absorption of manganese thus far has resisted investigation.

Some chelating agents are toxic to plants, and often the line between adequacy to correct a nutrient deficiency and that producing toxicity is narrow. EDDHA has been the least toxic of the synthetic chelating agents,<sup>17</sup> plants being able to grow well in the presence of large quantities of it. Under iron deficiency conditions, however, a point is reached at which this chelating agent

is quite lethal (TABLE 7). This indicates the metabolic effects of the chelating agent within a plant.

Iron chelating agents have masked the symptoms of some virus diseases in plants.<sup>18</sup> In camellia, symptoms of chlorophyll deficiency disappeared from the leaves, and virus symptoms also disappeared from flowers that were red and white under the virus condition but a solid red when large applications of iron chelates were made. Whether such effects are due solely to iron or in part to the chelating agent is not known.

*Review of Literature on Effect of Chelating Agents on  
Carboxylating Enzymes in Plants*

Weissbach *et al.*<sup>1</sup> showed that a chelating agent or an agent with one of the —SH groups, such as GSH, was necessary for maximal activity of the carboxy-dismutase enzyme. They implied that both agents used in inactivating heavy metals served to protect the —SH groups on the enzyme.

Huffaker *et al.*<sup>19</sup> found that chelating agents enhanced activity for the CO<sub>2</sub> fixation catalyzed by the PEP carboxylase enzyme. They strengthened the

TABLE 7\*  
YIELDS OF BUSH BEAN SEEDLINGS GROWN AT IRON LEVELS AND WITH  
0.0015 M CHELATING AGENTS

Fe in nutrient solution (ppm)	No chelate (gm.)	EDDHA (gm.)	HEEDTA (gm.)
0	4.8†	3.0	5.7
5	4.9	13.3	2.8

\* Part of TABLE 5, Wallace *et al.*<sup>4</sup>

† Very chlorotic plants.

case for the protection against heavy metals by showing that preparations from plants pregrown with slight iron deficiencies resulted in higher amounts of CO<sub>2</sub> fixation in cell-free preparations with either a PEP or an R5P (R5P → RDP) reaction system than did those from plants pregrown with adequate iron. A chelating agent added to reaction systems further increased CO<sub>2</sub> fixation. The chelating agent also overcame the inhibition for both systems that was caused by the *in vitro* addition of iron to the preparations.

In further studies, Huffaker and Wallace<sup>20</sup> showed that, for the same two reaction systems, inhibition was caused by the *in vitro* addition of molybdenum, copper, zinc, and manganese. The addition of EDDHA to the preparations overcame part of the inhibition caused by zinc, copper, and manganese. Pre-growing plants with high levels of micronutrients resulted in decreased activities, which could be increased somewhat by the *in vitro* addition of EDDHA to the reaction systems. Plants pregrown with EDDHA as well as with high levels of micronutrients resulted in higher amounts of CO<sub>2</sub> fixation, particularly that catalyzed by the carboxydismutase system, than similar plants not pregrown with EDDHA. This effect may be of great importance and may relate to the phenomenon, mentioned above, of unmetabolized chelating agents accumulating in parts of plants.

An interesting report that may relate to  $\text{CO}_2$  fixation in photosynthesis concerns a very large stimulation by EDTA of the glyceraldehyde-3-phosphate dehydrogenase-TPN-requiring enzyme, which catalyzes the reduction of PGA to the aldehyde.<sup>21</sup> This is the carbon reduction step in photosynthesis, the source of the reduced TPN being photoreduction of oxidized TPN. This PGA reduction follows the formation of 2 molecules of PGA from the enzymatic combination of  $\text{CO}_2$  with RDP. A possible relationship of this EDTA effect to equilibrium conditions in this reaction sequence may have important implications in growth effects.

As mentioned above, certain chelating agents have proved toxic to plants. The chelating agent HEEDTA showed more toxicity than any other (TABLE 7) and also was found to inhibit  $\text{CO}_2$  fixation with both PEP and R5P as substrates more than any other.<sup>22</sup> Lineweaver-Burke double-reciprocal plots with varying R5P as a substrate indicated a noncompetitive type of inhibition. Additional studies of this effect are reported below.

#### *Purpose of Study*

Since some chelating agents, especially EDDHA, often result in yield increases beyond the effect of micronutrient supply, since the chelating agent itself can accumulate in plants, and since chelating agents have been shown to have pronounced effects on activities of several enzyme reactions including those of carboxylating enzymes, more information is needed concerning the nature of the effects of synthetic chelating agents on reactions catalyzed by specific enzymes.

#### *Experimental Methods*

The reagents were prepared as follows:

A stock solution of 0.01 *M* of the cyclohexylamine salt of PEP was prepared in 0.2 *M* Tris [tris(hydroxymethyl)aminomethane] buffer, pH 8.0. The K salt of R5P was prepared from the Ba salt by precipitating the Ba with an equivalent amount of  $\text{K}_2\text{SO}_4$ . The reagent was centrifuged to remove the precipitate, and stock solutions of 0.02 *M* were prepared in Tris buffer as above. Stock solutions of 0.01 *M* Na salts of chelating agents were prepared and made to pH 8.0.

Bush bean seeds and corn were germinated in sand and allowed to grow for about 10 days to about 2 inches in length. Recently matured sweet orange leaves, used in some studies, were obtained from trees in the orchard at the University of California.

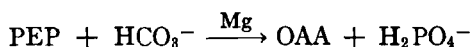
The assays were made as follows:

Leaf homogenates were produced by grinding 1 weight of fresh leaves with 3 vol. 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 used, this storage period never exceeding one-half hour. All the reaction mixtures received 140  $\mu\text{moles}$   $\text{KHC}^{14}\text{O}_3$  containing  $1.2 \times 10^6$  counts per minute (cpm) as the  $\text{BaCO}_3$  precipitate with a Q-gas counter, and 0.1 ml. enzyme preparation, which was added last. The total volume of each reaction mixture was 1.0 ml. before addition of acid. Except where noted oth-

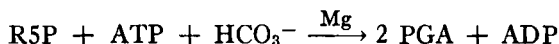
erwise, the reaction mixtures contained the following, where appropriate, for the 1.0 ml. of reaction mixture (in micromoles); 1 PEP, 2 R5P, 20 Mg, and 4 ATP. The mixtures were incubated for 10 min. at 37° C., and the enzyme was killed with 0.1 ml. 1 *N* HCl, which contained 2,4-dinitrophenylhydrazine to convert any OAA present to the hydrazone form. Such a system was found to prevent almost completely the decarboxylation of labeled products during the period when the planchets were dried prior to counting. This also expelled unreacted  $\text{HCO}_3^-$ . The mixtures were centrifuged, and aliquots of 0.2 ml. of the supernatant then were dried in forced air at room temperature in Pyrex planchets and were counted. The results were reported in counts per minute in this aliquot. This method was described by Jackson and Coleman<sup>23</sup> as a modification of that employed by Bandurski and Greiner<sup>24</sup> and by Saltman *et al.*<sup>25</sup>

The coefficient of variability of this method was found to be less than 4 per cent, 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.

Two carboxylating systems were studied. The first system catalyzed by PEP carboxylase was represented by the following reaction systems:



The second system catalyzed by carboxydismutase (carboxylation enzyme) was represented by the following reaction system:



Actually, the latter is a 3-enzyme step that requires also the phosphoriboisomerase and phosphoribulokinase enzymes.

Although the general trends observed were consistent, there was considerable day-to-day variation in the magnitude of the responses, which is evidently in part the result of the method.

EDDHA was used in most of the studies because it was the most successful iron chelate in plant nutrition, although it was not the agent that effected the most positive response with the PEP system.<sup>22</sup> Unless otherwise noted, the quantity of EDDHA used in 1.0 ml. of reaction mixture was 1  $\mu\text{mole}$ .

#### *Effect of EDDHA on Heavy-Metal Inhibition of Carboxylating Enzymes*

Iron and other heavy metals supposedly are inhibitors of the 2 carboxylating reactions.<sup>19,26</sup> It is not known whether, when the chelating agent is added, the increased  $\text{C}^{14}$  in fixation products—always observed when R5P was the substrate for  $\text{CO}_2$  fixation and often observed when PEP is the substrate—is the result of chelation of endogenous heavy metals. When certain heavy metals were added, however, EDDHA did restore some and in some cases all of the decrease in  $\text{C}^{14}$  in fixation products for both R5P (TABLE 8) and PEP (TABLE 9) systems. GSH had an effect similar to EDDHA, except that it had more effect than EDDHA on overcoming Cu and Zn inhibition of the R5P system. GSH did not overcome Cu inhibition of the PEP system.

A series of studies was made to determine the following: whether the effect

of the EDDHA was to prevent metal inhibition of the enzyme reaction or whether the chelate merely prevented decarboxylation of  $C^{14}$  acids after they had been synthesized and, particularly, during the drying of them on planchets; and why decarboxylation of fixed products should occur when the hydrazone of OAA was formed in the PEP reactions to prevent such losses.

In TABLE 10 the data for the R5P reaction, and under the experimental conditions, with sweet orange leaf preparations, indicate that EDDHA, when present in the reaction mixture, essentially prevented zinc inhibition. In contrast, the addition of the EDDHA after the reaction but before drying had a slight effect when only zinc but no chelate had been added to the reaction mixture.

TABLE 8

EFFECT OF EDDHA IN OVERCOMING INHIBITION OF SOME HEAVY METAL SALTS ON  $C^{14}O_2$  FIXATION WHEN R5P WAS USED AS A SUBSTRATE IN CELL-FREE PREPARATIONS OF BUSH BEAN LEAVES\*

Heavy metal salts added (1 $\mu$ mole)	EDDHA (cpm/aliquot)		
	None	1 $\mu$ mole	2 $\mu$ moles
None	4680	5760	3940
Cu	62	154	2920
Mo	2790	3400	2900
Zn	35	90	2360

\* See under *Experimental Methods* for procedure.

TABLE 9

EFFECT OF EDDHA AND GSH ON HEAVY METAL INHIBITION OF THE PEP CARBOXYLASE REACTION IN PREPARATIONS FROM SWEET ORANGE LEAVES\*

Heavy metal salts	( $\mu$ mole)	Nothing used (cpm/aliquot)	EDDHA (cpm/aliquot)		GSH (cpm/aliquot)
			1 $\mu$ mole	3 $\mu$ moles	1 $\mu$ mole
None	—	2500	2550	2850	2310
Cu	1.0	1210	2230	3140	600
Hg	0.1	370	410	1420	2660
Mn	1.0	2310	2450	3310	2450

\* See under *Experimental Methods* for procedure.

EDDHA added after the reaction and before the drying did increase considerably the  $C^{14}$  count in each case. This indicates the presence of unstable products that decompose in the presence of nonchelated zinc and other metals. In part, the effect of the chelating agent is to prevent such decomposition.

The data for PEP with sweet orange (TABLE 10) indicate that EDDHA, at least in part, does not necessarily overcome a zinc inhibition of the enzyme, but rather that it overcomes a zinc-induced decarboxylation of fixed product. With corn, however, the data indicated an effect of zinc on the enzyme reaction.

Our studies never have indicated an EDDHA stimulation of the PEP reaction with preparations from roots. Data in TABLE 11 indicate an EDDHA inhibition of the reaction with preparations from bush bean roots and also a



constant degree of inhibition by the chelating agent obtained with and without manganese. Manganese also inhibited the reaction. This result is different from that obtained from the preparations from leaves.

TABLE 10

EFFECT OF ADDING EDDHA TO REACTION MIXTURES AFTER THE REACTION PERIOD AND JUST BEFORE ADDITION OF THE ACID TO STOP CO<sub>2</sub>-FIXING REACTIONS WITH R5P AND PEP SYSTEMS WITH PREPARATIONS FROM LEAVES\*

Additions during reaction	Amounts of additions (μmole)	Regular procedure (cpm/aliquot)	EDDHA just before acid (cpm/aliquot)
			1 μmole
Sweet orange leaves			
R5P reactions			
—	—	7050	7940
EDDHA	1	9080	9790
ZnSO <sub>4</sub>	0.2	255	446
EDDHA + ZnSO <sub>4</sub>	1 + 0.2	7970	9000
PEP reactions			
—	—	2340	2780
EDDHA	1	2360	—
ZnSO <sub>4</sub>	0.2	1970	2270
EDDHA + ZnSO <sub>4</sub>	1 + 0.2	2450	—
			10 μmoles
Corn leaves			
PEP reactions			
—	—	1100	2500
EDDHA	1	998	1195
ZnSO <sub>4</sub>	0.5	404	520
EDDHA + ZnSO <sub>4</sub>	1 + 0.5	874	910

\* See under *Experimental Methods* for procedure.

TABLE 11

MANGANESE AND EDDHA INHIBITION OF C<sup>14</sup>O<sub>2</sub> FIXATION WITH PEP AS A SUBSTRATE IN PREPARATIONS FROM BUSH BEAN ROOTS\*

Mn (μmole)	EDDHA (cpm/aliquot)		
	None	0.5 μmole	1 μmole
0	3940	2720	2610
1	2550	1940	1800
2	2300	1910	1620
5	1620	1200	1050

\* No Mg was used. See under *Experimental Methods* for procedure with Mg.

### *The Nature of PEP Carboxylase and Carboxydismutase*

Carboxydismutase has been shown to be a sulfhydryl enzyme,<sup>1</sup> as have been the isomerase and kinase enzymes that also are involved when R5P is used as a substrate.<sup>21</sup> PEP carboxylase is also suspected of being a sulfhydryl enzyme; metal inhibitors (TABLE 12) do not contradict this. The cyanide inhibition of the R5P reaction may be attributed to the formation of the cyanohydrin<sup>27</sup> with

the ribulose diphosphate rather than to metal inhibition, since azide failed to inhibit the reaction. With azide for both reactions there appeared to be stimulation. Although several possibilities exist, the stimulation could occur, at least for cyanide with PEP, through a combination with reaction products and thus prevent decarboxylation or, alternatively, it could be caused by cyanide combination with heavy metals, thus rendering them nontoxic.

Studies were made to determine whether EDDHA would be helpful in characterizing the 2 reaction systems. Under the study conditions used, GSH was able to overcome the inhibition resulting from  $10^{-4}$  M *p*-CMB for both the PEP and R5P reactions, but EDDHA was not able to do so (TABLE 13); *p*-CMB

TABLE 12  
EFFECT OF INHIBITORS ON CARBOXYLATING REACTIONS IN PREPARATIONS FROM SWEET ORANGE LEAVES

Inhibitor*	PEP (cpm/aliquot)	R5P (cpm/aliquot)
Control	3350	4210
Azide	3500	5270
Fluoride	3660	3880
Cyanide	5890	274

\* Inhibitor concentration was 10  $\mu$ moles. See under *Experimental Methods* for procedure.

TABLE 13  
ABILITY OF GSH BUT NOT EDDHA TO OVERCOME INHIBITION OF *p*-CMB  
ON CARBOXYLATION REACTIONS

<i>p</i> -CMB ( $\mu$ mole)	Control (cpm/aliquot)	EDDHA, 1 $\mu$ mole (cpm/aliquot)	GSH, 1 $\mu$ mole (cpm/aliquot)
Bush bean roots, PEP			
0	3820	3820	3820
0.1	1400	1190	3820
Bush bean leaves, PEP			
0	3310	3310	3310
0.1	900	874	3220
Sweet orange leaves, R5P			
0	910	7470	3600
0.1	41	22	3400

is an alkylating agent. These results indicate a similar nature of the enzymes involved.

That the 2 systems may be fundamentally different, however, is suggested by some of the data in TABLES 14 and 15. For example, EDDHA and GHS greatly stimulated the R5P system, but usually had less or no effect on the PEP system. Phosphate at  $3 \times 10^{-2}$  M stimulated the PEP reaction, but not the R5P. Ammonium sulfate at  $5 \times 10^{-2}$  M inhibited the R5P reaction, but not the PEP. The omission of Mg and the addition of *p*-CMB, HgCl<sub>2</sub>, and iodoacetate inhibited the R5P more than the PEP reaction. The heavy metal inhibition studies, some of which have been referred to, usually showed considerably greater inhibition of R5P than of PEP. Part of this effect, however, could be due to the fact that the R5P system is a 3-enzyme system.

$\text{Ca}(\text{NO}_3)_2$  resulted in greater inhibition of the R5P reaction than the PEP, but this is possibly<sup>28</sup> the effect of Ca on ATP.

*Effect of EDDHA on Salt Inhibition of the R5P Reaction*

Salts at relatively high concentrations inhibit the R5P reaction system.<sup>29</sup> Calcium nitrate is particularly inhibiting, partly as a result of Ca reactions with

TABLE 14  
EFFECT OF EDDHA IN THE PRESENCE OF DIFFERENT CHEMICALS ON THE PEP CARBOXYLASE REACTION\*

Additions	Amount ( $\mu\text{mole}$ )	EDDHA (cpm/aliquot)					
		Bush bean roots			Sweet orange leaves		
		None	2 $\mu\text{moles}$	5 $\mu\text{moles}$	None	2 $\mu\text{moles}$	5 $\mu\text{moles}$
Control	—	1690	1430	854	3940	5060	4210
$\text{KH}_2\text{PO}_4$	10	1820	1410	841	4680	4210	3710
$\text{KH}_2\text{PO}_4$	30	2230	1670	990	3940	3310	2980
$\text{NaHAsO}_4$	20	2010	1740	1020	3400	4070	3390
$\text{HgCl}_2$	1	94	65	39	24	26	26
<i>p</i> -CMB	1	331	147	81	20	33	24
$\text{Ca}(\text{NO}_3)_2$	50	349	312	210	1220	2500	2350
$(\text{NH}_4)_2\text{SO}_4$	100	1340	1130	835	1670	2230	1800
Iodoacetate	10	1340	1060	735	1970	4200	3300

\* See under *Experimental Methods* for procedure.

TABLE 15  
COMPARISONS OF PEP AND R5P SYSTEMS IN CELL-FREE PREPARATIONS

Additions	Amount ( $\mu\text{mole}$ )	PEP + Mg + $\text{HCO}_3^-$ (cpm/aliquot)	R5P + ATP + Mg + $\text{HCO}_3^-$ (cpm/aliquot)	PEP + Mg + $\text{HCO}_3^-$ (% of base reaction)	R5P + ATP + Mg + $\text{HCO}_3^-$ (% of base reaction)
Soybean leaves					
Control	—	2720	319	100	100
EDDHA	1	2560	508	94	159
EDDHA	2	2660	525	98	164
GSH	1	2850	2140	105	670
$\text{KH}_2\text{PO}_4$	30	3500	339	128	106
$(\text{NH}_4)_2\text{SO}_4$	50	3060	108	112	34
$\text{Ca}(\text{NO}_3)_2$	25	453	93	17	29
Omit Mg	—	673	12	25	4
<i>p</i> -CMB	0.1	1400	20	51	6
$\text{HgCl}_2$	0.1	543	12	20	4
Iodoacetate	10	1800	111	66	35
Corn leaves					
Control	—	2150	7940	100	100
EDDHA	1	2560	10640	122	133
EDDHA	5	6040	14140	280	178
Sweet orange leaves					
Control	—	2360	5270	100	100
EDDHA	1	2450	6340	103	119
EDDHA	5	3400	10450	143	198

\* See under *Experimental Methods* for procedure.

ATP, as mentioned.<sup>28</sup> Although EDDHA more than proportionally overcame the inhibition (TABLE 16), a Lineweaver-Burke double-reciprocal plot of the  $\text{Ca}(\text{NO}_3)_2$  inhibition with and without the chelating agent (FIGURE 1) indicated a noncompetitive type of inhibition.

TABLE 16

$\text{C}^{14}\text{O}_2$  FIXATION IN CELL-FREE PREPARATIONS OF SWEET ORANGE LEAVES USING AN R5P + ATP +  $\text{HCO}_3^-$  REACTION SYSTEM WITH DIFFERENT LEVELS OF EDDHA AND WITH AND WITHOUT  $\text{Ca}(\text{NO}_3)_2$  AS AN INHIBITOR\*

Chelate level ( $\mu\text{mole}$ )	$\text{Ca}(\text{NO}_3)_2$			
	None (cpm/aliquot)	25 $\mu\text{moles}$ (cpm/aliquot)	None (%)	25 $\mu\text{moles}$ (%)
0	6870	1520	100	100
2	9130	3180	133	209
5	8610	3410	125	224

\* See under *Experimental Methods* for procedure.

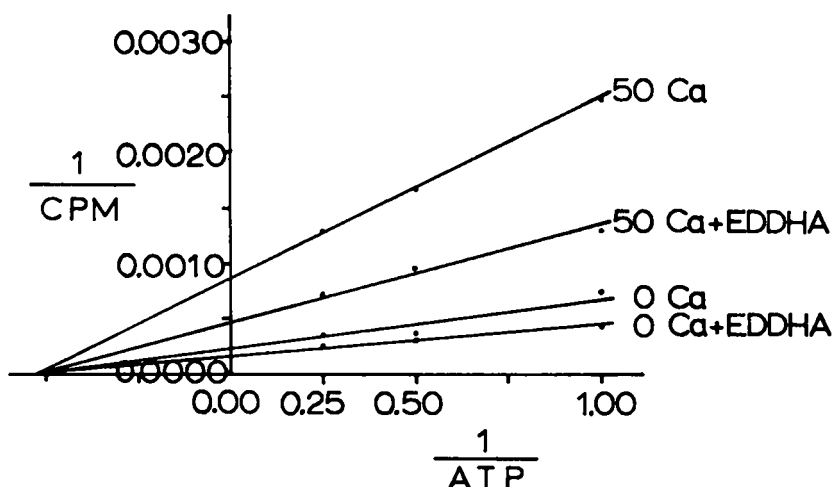


FIGURE 1. Double-reciprocal plot of  $\text{Ca}(\text{NO}_3)_2$  inhibition and reversal of inhibition with EDDHA of  $\text{C}^{14}\text{O}_2$  fixation with the R5P system with preparations from sweet orange leaves at varying levels of ATP. The concentration of EDDHA was 1  $\mu\text{mole}$  for the 1.0 ml. of reaction mixture. Except as noted in the figure, the concentration of reactants was similar to that described under *Experimental Methods*.

#### *Effects of HEEDTA on Carboxylation Enzyme Inhibition*

A study was made (TABLE 17) to determine whether metals would overcome any of the toxicity of the chelating agent in the R5P reaction system. To a slight extent only, and usually on the R5P more than on the PEP reaction system, was there effect. This indicates that both the metal chelate and the chelating agent inhibit the system.

In addition to the reports of noncompetitive inhibition already made for

HEEDTA with the R5P reaction, determined with R5P as a substrate,<sup>22</sup> similar double-reciprocal plots were made for  $\text{HCO}_3^-$  and ATP as substrates. In each of these cases the noncompetitive inhibition also was indicated (FIGURES 2 and 3).

TABLE 17  
EFFECT OF METALS ON TOXICITY OF CHELATING AGENT HEEDTA ON TWO  
DIFFERENT  $\text{CO}_2$  FIXATION REACTIONS\*

Salt*	Substrate (cpm/aliquot)	
	R5P	PEP
No chelate		
None	7470	2360
HEEDTA, 1 $\mu$ mole		
None	771	442
$\text{FeSO}_4$	940	364
$\text{ZnSO}_4$	1220	693
$\text{CuSO}_4$	900	514
$\text{CaCl}_2$	1250	603

\* One  $\mu$ mole of heavy metal salt and 50  $\mu$ moles of  $\text{Ca}(\text{NO}_3)_2$  were added to the reaction mixtures where applicable. See under *Experimental Methods* for procedure.

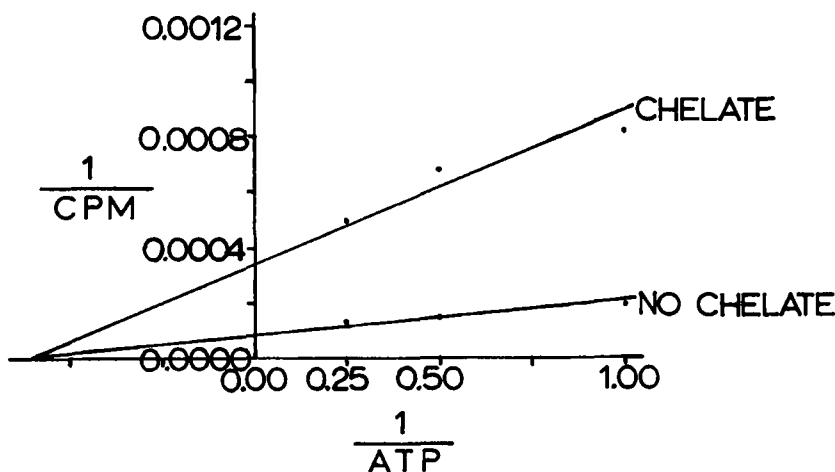


FIGURE 2. Double-reciprocal plot of HEEDTA inhibition of the R5P system in preparations from sweet orange leaves at varying levels of ATP. The concentration of chelating agent was one third  $\mu$ mole for the 1.0 ml. of reaction mixture. That of other reagents and enzymes was as indicated under *Experimental Methods*.

#### *Effect of EDDHA on PEP Reaction in Etiolated Plants*

Although EDDHA rather inconsistently increased  $\text{C}^{14}$  fixation with the PEP carboxylase system in preparations from green leaves, there was a consistent increase in activity with etiolated plants, as illustrated by the data in TABLE 18. The increase, however, was not great.

*Effect of EDDHA on CO<sub>2</sub> Fixation at Different Magnesium Levels*

Both reactions studied require Mg as a metal activator. Sometimes endogenous Mg is sufficient for maximal activity, especially in the PEP system. In the R5P system the relatively high level of ATP used may inactivate Mg. It was thought that the chelating agent might have inhibitory effects on the reactions under conditions of limiting Mg or that it might even render small levels of Mg more reactive. Data for the PEP reaction in a root preparation (TABLE 19) indicate that endogenous Mg was sufficient for maximal activity

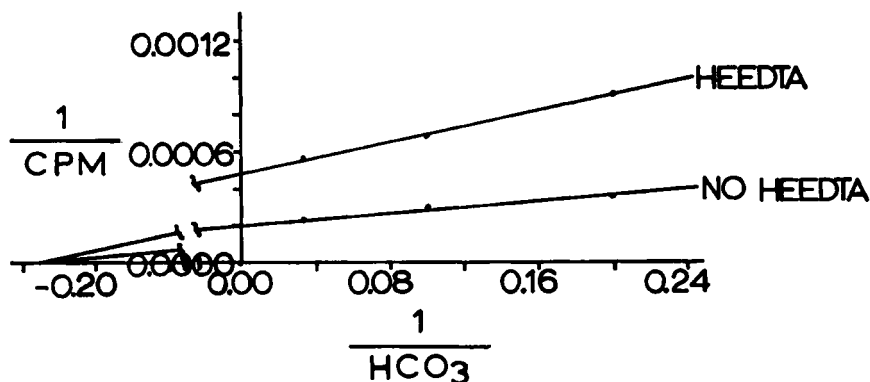


FIGURE 3. Double-reciprocal plot of HEEDTA inhibition of the R5P system in preparations from sweet orange leaves at varying levels of bicarbonate. The concentration of chelating agent was one third  $\mu$ mole for the 1.0 ml. of reaction mixture. That of other reagents and enzymes was as indicated under *Experimental Methods*.

TABLE 18  
EFFECT OF ETIOLATION ON PEP REACTION SYSTEMS\*

Reaction systems	Green (cpm/aliquot)		Etiolated (cpm/aliquot)	
	Bush bean	Corn	Bush bean	Corn
Minus substrates	387	135	82	194
PEP + Mg	4860	5760	6040	6050
PEP + Mg + EDDHA	4880	5720	6340	6400

\* Seeds were germinated and left in a dark closet for 1 week. See under *Experimental Methods* for procedure.

when EDDHA was omitted, but not when it was present. Chelated Mg, then, was a hindrance to the reaction.

Similar data for the R5P system with leaf preparations (TABLE 20) indicate that there was a slightly less proportional increase for the EDDHA with endogenous Mg than when Mg was added. For all levels of added Mg, however, the same ratio of reaction with EDDHA and without EDDHA was obtained. This again indicates that chelated Mg hinders the reaction.

*Summary*

Heavy-metal inhibition of two carboxylation reactions studied in cell-free preparations of plant material was overcome, sometimes completely, by the

chelating agent EDDHA. Part of the effect, in the case of the system requiring R5P, was the result of preventing product decomposition during the drying of samples on the planchets. Most of the effect in the PEP system could be in preventing product decomposition. EDDHA and GSH consistently resulted in relatively large increases in  $C^{14}O_2$  fixation in the R5P system, but in a less consistent influence on the PEP system. When heavy metals were added to the PEP system, the chelating agent usually was beneficial. Arsenate and phosphate stimulated the PEP, but not the R5P, reaction. Heavy metals, *p*-CMB, ammonium sulfate,  $HgCl_2$ , and the omission of Mg inhibited the

TABLE 19  
EFFECT OF EDDHA ON THE PEP CARBOXYLASE REACTION IN BUSH BEAN  
ROOT PREPARATIONS AT DIFFERENT LEVELS OF MAGNESIUM\*

Mg added ( $\mu$ mole)	EDDHA (cpm/aliquot)			Inhibition of EDDHA (%)	
	None	0.5 $\mu$ mole	1 $\mu$ mole	0.5 $\mu$ mole	1 $\mu$ mole
0 (endogenous only)	3940	2720	2600	31	34
2	4070	3500	3060	14	25
5	4680	3940	3220	16	31
20	4000	3710	3600	7	10

\* See under *Experimental Methods* for general procedure.

TABLE 20  
EFFECT OF MG ON CHELATING AGENT EFFECT ON  $CO_2$  FIXATION WITH THE R5P + ATP +  
 $HCO_3^-$  REACTION SYSTEM WITH HOMOGENATE FROM BUSH BEAN LEAVES\*

Mg level ( $\mu$ mole)	EDDHA (cpm/aliquot)		Chelate/no chelate ratio
	None	EDDHA, 1 $\mu$ mole	
0 (endogenous only)	161	224	1.4
1	204	680	3.3
2	311	917	3.0
5	956	2990	3.1
10	1050	3940	3.7
20	1110	3940	3.5
30	1460	4210	3.0

\* See under *Experimental Methods* for general procedure.

R5P systems more than the PEP. All these effects indicate differences in the nature of the 2 reactions for  $CO_2$  fixation.

EDDHA increased the PEP reaction more consistently in preparations from etiolated plants than in those from preparations with green plants.

Limiting Mg decreased the R5P system much more than the PEP system. It decreased  $C^{14}$  fixed with PEP when EDDHA was added and resulted in a smaller increase with R5P when EDDHA was added, as against the results of treatments with adequate Mg.

HEEDTA inhibition of both the carboxylation reactions was overcome only slightly by the addition of heavy metals to the reaction, indicating that HE-

EDTA toxicity probably is not the result of chelation. Double-reciprocal plots for the R5P system with R5P, ATP, and  $\text{HCO}_3^-$  each as substrates indicated noncompetitive inhibition of HEEDTA.

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