Iron Translocation
I. Plant Culture, Exudate Sampling, Iron-Citrate Analysis

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Summary. Plant culture, exudate sampling, and analytical methods designed to ascertain the form of iron translocated are presented.

Restoration of iron to sunflower plants precultured at different Fe levels resulted in exudate iron concentrations ranging from 0.2 to 31 $\times$ 10^{-5} M. Citrate was from 3 to 89 $\times$ 10^{-5} M. Iron and citrate were highest in exudates from iron-deficient plants. Citrate/Fe ratios were between 1 and 3 for exudates of deficient plants. Exudate from normal plants gave a citrate/Fe ratio of 15.

Malate, iron, and a fraction of the citrate in stem exudates migrated electrophoretically to similar positions in acetate buffer. Extracts of narrow bands from the iron-containing areas gave curves suggesting that citrate bound the iron. Citrate that was not combined with iron migrated in a slower band. The effect of iron on citrate migration was confirmed in several related experiments.

The stability of Fe-citrate was demonstrated electrophoretically in malate buffer. Citrate retained iron against malate.

Data given in this paper indicate that citrate binds iron in sunflower exudate. The data suggest that citrate carries iron in intact plants.

It is generally assumed that metals supplied to plant roots may be intercepted in stem exudates to provide information about their translocation (2). In the present study, iron was supplied to sunflower roots, and exudates were used to sample translocated iron. The quantities of iron involved in other phases (root absorption and accumulation) have been considered mostly in relation to translocation.

Restoration of iron to plants precultured at different iron levels results in wide variations of this metal in the leaves (8, 14) and stem exudates (3, 14, 15). When iron is supplied as a synthetic chelate, the roots of sunflower (13) and soybean (14) absorb the metal but leave most of the chelating agent in the nutrient medium.

In the present study, sunflower plants precultured at different iron levels were given similar treatments at decapitation. Assays were carried out to determine quantities of citrate and iron in the exudates. The primary objective was to determine the form of iron translocated. A brief report of this work is published (11).

In later experiments (12) the iron treatments were reversed. Plants were precultured similarly but given different iron levels at decapitation. The purpose was to determine the effect of iron supplied in the exudation period on the citrate and iron content of the exudates. Attention has also been given to the changes in concentration of citrate and iron that occur during exudation.

Materials and Methods

Nutrient Solution. The standard nutrient was a modified Steinberg solution (10, 14). Elements were approximately one-fifth the Steinberg concentration, except for lower phosphorus. The nutrient contained (mg/liter): 51 Ca, 6.6 Mg, 59 N, 3 P, 44 K, 5 S, 0.130 Mn, 0.070 B, 0.040 Zn, 0.010 Cu, 0.010 Mo, and 0.056 Fe. The iron was supplied as ferric ethylenediamine di(*o*-hydroxyphenylacetate) (Fe EDDHA).

After germination, 8 liters of this nutrient were used in all phases of plant culture except when specified otherwise. After growth periods of 10 days, the nutrient usually was changed. Solutions were replaced more often when fast-growing species or large numbers of plants were grown in the 8-liter volumes.

General Culture and Sampling. Plants were grown in a controlled light chamber under 8 hours dark, 16 hours at 1500 ft-c, and 22 $\pm$ 2°. Harvests of plants (i.e. decapitations) were made after plants had received 6 to 8 hours of light. The exudate was collected at 22° in the dark. The general cultural and exudate sampling procedures are given in figure 1. Sunflower plants, *Helianthus annuus* L. var. Greystripe, were used in experiments reported here. Details concerning plant age and treatments are given in the legend of table I.

Stem Elongation. Hypocotyls of plants often are too short to withstand the bending necessary for

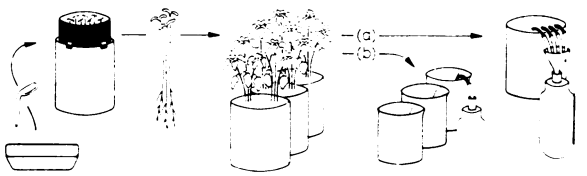

FIG. 1. Diagram of plant culture and exudate sampling methods. Plants are carried through 3 periods: I. Seeds are germinated 2 to 5 days on wet muslin in covered glass trays. The muslin, supported on a stainless frame, extends into water about 1 cm below the seeds. Seedlings are transferred and grown 2 to 8 days under partial shading in an enclosed frame to elongate the stems. Plants with stems 10 to 15 cm long are grouped for transfer. II. This period extends from one to several weeks. In the earlier part plants are cultured to obtain healthy root and top growth; in the latter part they are conditioned for specific tests in the exudation period. III. Plants are decapitated and exudate is delivered by plastic tubing into vials held in an icepack. Two methods are suggested: A) Exudate is pooled from several groups of plants (precultured similarly) to obtain a large volume of exudate or smaller volumes at time intervals, and B) exudate is collected from single groups of plants.

exudate to drip from the cut stems. Seedlings are elongated (fig 1) to prevent stem injury and facilitate exudate collection.

Electrophoresis Apparatus and Buffers. An apparatus was constructed to accommodate large papers and volumes of buffer. The system included two 46 × 46 cm plexiglass sheets[1] to hold the treated papers, a plate glass support of the same dimensions, and 2 electrode compartments of 4-liter capacity. The use of two 20 × 57 cm sheets of Whatman No. 3 paper in the apparatus permitted fractionation of up to 12 ml of exudate.

The buffer used most was sodium acetate (2 liters per compartment) pH 5.4 (6). Other buffers were citrate and malate, both at 10^{-2} M and pH 5.4 (by NaOH). Generally, buffer was used at pH near that of the plant exudates. Exudate pH ranged between 5.2 and 5.6.

Electrophoresis, Isotope Detection, Elution. The red chelate, FeEDDHA, was used as the electrophoretic marker. This chelate moved compactly and was stable in all buffers tested. About 2 μl of a 0.1 M solution was applied per spot. After loading of exudate and marker, the papers were clamped between the plastic sheets and subjected to electrophoresis at 450 v for 2.3 hours, or until FeEDDHA migrated a prescribed distance. The papers were dried on the plastic support in a forced draft oven at 20°. The FeEDDHA spots were marked, prior to drying, to indicate solute migration in the drying period. Radioiron and ^{14}C-labeled acids were located after electrophoresis by X-ray film. The distribution patterns in the figures were obtained by direct photoprinting of the X-ray film positives. Bands were cut from the electropapers and eluted with water to recover exudate compounds.

Analysis of Solutions. Radioiron in the nutrient solutions, raw exudates, and extracts of electropapers was assayed by counting dried samples in a proportional counter. Specific activity of nutrient iron was used to estimate iron quantitatively in stem exudate. The values obtained were compared wtih total iron determined by orthophenanthroline (9). Citric acid was determined by the pentabromoacetone method (5).

The L-malate assay described by Hohorst (7) was modified slightly and used to determine L-malic acid in extracts from electrophoresis papers. The reaction mixture (3 ml) contained 1.5 ml glycine buffer pH 9.5, 0.5 ml of 5 × 10^{-3} M β-DPN, 0.2 ml (200 units) of L-malic dehydrogenase (Sigma Chemical Company), and standard L-malic acid or sample and water, if necessary to obtain volume. OD change at 340 mμ and 25° was measured by a DU spectrophotometer in a 10 minute period, beginning approximately 3 minutes after addition of standard or sample. The standard curve was obtained with zero to 2 μmoles of L-malic acid.

Experiments and Results

Effects of Preculture. The effects of preculture on the iron and citrate levels in sunflower exudate are given in table I. The 4 plant groups were treated alike for 25 days and then were transferred to conditioning nutrients. After growth in these nutrients for 6 days, groups 1 and 2 were chlorotic, and groups 3 and 4 were green. The chlorotic plants absorbed and translocated about the same amounts of iron. The citrate concentrations and also the citrate/iron ratios for groups 1 and 2 were similar. Plants in group 3 were under iron deficiency stress (although green), and thus absorbed iron in quantity similar to that absorbed by the chlorotic plants. But the concentration of citrate was only about one-third and the citrate/iron ratio about one-half that of chlorotic plants. The exudate from group 4 was quite different. Compared to the other exudates, the citrate and iron were low and the citrate/Fe relatively high.

Electrophoresis of Stem Exudates. Electrophoresis of 6-ml volumes of exudates 1, 2, and 3 (table I) was carried out in acetate buffer, and the dried papers were exposed to X-ray film (fig 2). The components from bands A, B, and C for each exudate are shown in table II. Extracts for D bands were carried through all analyses, but no components were found. This indicates a distinct break in the migrating fronts of iron, citrate, and malate. Table II shows the following distribution: Most of the iron was in band C; all of the malate was in band C; sufficient citrate to bind all the iron (1:1) was in band C; and most of the total citrate (without iron) was in band B.

Results in table II indicate that malic acid in the exudates applied at the origin ranged between 0.2 and 0.9 mM. If citric acid had not been present in band C it would be reasonable to conclude that malate bound the iron. A point in support of citrate binding

Table I. *Effect of Preculture on Fe and Citrate Levels in Sunflower Exudate*

Plant age (days) and treatments: 0, germinated seed; 3, seedlings into standard nutrient and shaded to elongate stems; 6, groups into full light and standard nutrient; 13 and 20, renewed nutrient; 25, groups into conditioning nutrients; 31, transferred each group (3 plants) to 1.5 liters of absorption nutrient (1 μc ^{59}Fe/μmole Fe), decapitated, and collected exudate 18 hours. The conditioning and absorption nutrients are standard nutrients with iron changes specified.

Plant group	Conditioning nutrient FeEDDHA	Absorption nutrient FeEDDHA	Post** absorption nutrient FeEDDHA	Iron in exudate	Citrate in exudate	Ratio: citrate / iron
	Molar	M $\times$ 10^{-5}	M $\times$ 10^{-5}	M $\times$ 10^{-5}	M $\times$ 10^{-5}	
1*	10^{-8}	1	0.08	31	89	2.9
2*	10^{-7}	1	0.04	28	80	2.9
3	10^{-6}	1	0.15	20	32	1.6
4	10^{-5}	1	0.84	0.2	3	15.0

* Plants were chlorotic.
** After containing plants 18 hours.

Table II. *Sunflower Exudate Components in Wide Electrophoretic Bands*

Band widths are shown in figure 2.

Exudate number	Extract component	Quantities in band extracts (mμmoles)* A	B	C
1	Iron	1	2	130
	Citrate	140	540	150
	Malate	0	0	860
2	Iron	0	1	101
	Citrate	80	410	130
	Malate	0	0	470
3	Iron	0	2	49
	Citrate	40	80	60
	Malate	0	0	280

* Values given indicate the migration of components originally in 1 ml of exudate applied at the origin.

is suggested by the distributions of this acid in bands B and C for exudates 1 and 3. Comparing the ratios, it is obvious that 540/150 does not equal 80/60. A similar inequality can be noted for exudates 2 and 3. These comparisons indicate that a disproportionate amount of the citrate of exudate 3 moved into band C. This distribution suggests that iron was bound to citrate and thus regulated the amount of citrate that migrated in the faster band. The effect of iron on citrate migration was confirmed in later experiments in which different ratios of iron:citrate were run in acetate buffer.

Figure 3 shows the electrophoretic migration of ^{59}Fe in an experiment similar to that given in figure 2. Conditions were the same except that the X-ray film was exposed 3 hours. The short exposure was used to locate the most concentrated ^{59}Fe band. The purpose was to establish over a narrow width the iron distribution curve and compare it with the curves for citrate and malate. The narrow band c was cut out to obtain the peak for ^{59}Fe activity, and the other bands were taken on each side as shown in the figure. No components were detected in extracts of band e. The quantities of iron, citrate, and malate in the other extracts are given in table III. The results show the malate peak in band b and considerable overlapping of malate in band c. The peaks of citrate and iron were in band c.

Taken together, tables II and III show 2 peaks for citrate. The first and largest is in band B, table

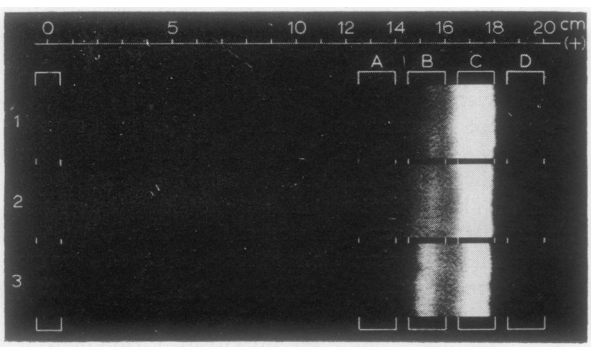

FIG. 2. Electrophoretic distribution of ^{59}Fe in sunflower stem exudate. X-ray film exposure was 30 hours. The 3 exudates are characterized in table I. Brackets at zero cm indicate areas of exudate loading. Bands A to D show areas eluted (elution data, table II). Electrophoresis conditions: Whatman No. 3 paper, 0.05 M acetate buffer, pH 5.4, 450 v, 2.3 hours.

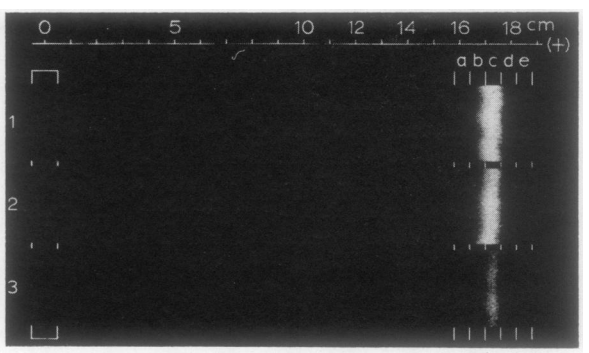

FIG. 3. Electrophoretic distribution of ^{59}Fe in sunflower stem exudate. X-ray film was exposed 3 hours. The 3 exudates are characterized in table I. Bands a to e show areas eluted (elution data, table III). Electrophoresis conditions: Whatman No. 3 paper, 0.05 M acetate buffer, pH 5.4, 450 v, 2.3 hours.

Table III. *Sunflower Exudate Components in Narrow Electrophoretic Bands*

Band widths are shown in figure 3.

Exudate number	Extract component	Quantities in band extracts (mμmoles)* a	b	c	d
1	Iron	10	53	136	12
	Citrate	15	33	85	28
	Malate	216	507	291	0
2	Iron	8	39	121	12
	Citrate	19	30	77	29
	Malate	40	240	144	0
3	Iron	5	23	60	2
	Citrate	11	15	29	0
	Malate	0	240	140	0

* Values given indicate the migration of components originally in 1 ml of exudate applied at the origin.

II. This peak centers at about 15 cm in figure 2. The second peak is smaller and is in band c, table III; its center is at about 17 cm, figure 3. The overall pattern indicates that total citrate migrates as 2 fractions. The faster one appears to be associated with iron. This association is inferred from the symmetry of the iron and citrate curves in table III. It is necessary to recognize, however that citrate in table III is not equimolar with iron. To be consistent with table II, more citrate should have been found in bands b and c.

Several points are noted in summary of the exudate electrophoresis. First, the data do not show unambiguously that citrate carried iron. The malate overlapped the citrate and iron in the acetate system. However, there were indications of citrate-iron complexing. They are as follows: 1) Sufficient citrate to bind Fe (1:1) was in band C for all exudates (table II). 2) Proportionally more of the citrate of exudate 3 was in band C than was noted for the other exudates (table II); this suggested an iron function in citrate distribution. 3) Tables II and III, together, show 2 citrate fractions: one without iron, and the other in the area of concentrated iron. Implicit in this distribution was the suggestion that some factor was causing part of the citrate to migrate at a faster rate. From the symmetry of the citrate and iron curves, the factor appeared to be iron.

Experiments, based on the above findings, were designed to answer the following questions: Does Fe-citrate migrate faster than citrate? Will a strong competitor take Fe from Fe-citrate? Does malate compete strongly with citrate for Fe?

Electrophoresis of Organic Acids and Fe-Acid Complexes. Electrophoresis of ^{14}C citrate and $Fe^{14}C$ citrate was carried out in acetate and citrate buffers. Figure 4 shows distributions of citrate in acetate buffer. Except for slight streaking toward the anode, the ^{14}C citrate migrated compactly to 1 position. The increases of iron (paths 2, 3, 4) caused increasing quantities of citrate to migrate as the faster Fe-citrate fraction. It is evident from this experiment that citrate can be separated into 2 bands and that the relative amounts in each band can be varied appreciably by iron.

The electrophoresis in citrate buffer (not shown) also demonstrated an effect of iron on the migration of citrate. But there was a striking difference in the ^{14}C distribution. On path 4 in the acetate buffer (fig 4), most of the $Fe^{14}C$ citrate migrated about 15 cm. In contrast, the electrophoresis of this sample in citrate buffer gave a heavy ^{14}C streak from 14.5 to 17 cm. The interpretation is that during electrophoresis an exchange of Fe was taking place between labeled citrate and citrate buffer. As the labeled molecules lost Fe to the buffer they migrated more slowly. This would account for the streaking. This result illustrates the effect of a strong competitor. It also recalls the results in tables II and III where malate overlapped citrate and iron. The crucial question is whether malate successfully competed for iron.

The following experiment was designed to test malate competition. Malate buffer was the supporting electrolyte. It was assumed that citrate would migrate at a slower rate if it lost iron to malate. Figure 5 shows that malic acid and its iron complexes (paths 1, 2, 3) migrated to similar positions. The free citrate (path 4) moved behind malate, but the iron compounds of citrate (paths 5, 6) moved well

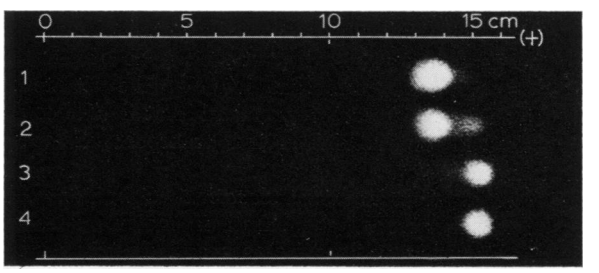

FIG. 4. Radiograph showing the effect of iron on the electromigration of ^{14}C citrate in acetate buffer. ^{14}C citrate (10^{-2} M) was separated at citrate/iron ratios of 1:0, 1:0.1, 1:0.5, 1:1 on paths 1 to 4 respectively.

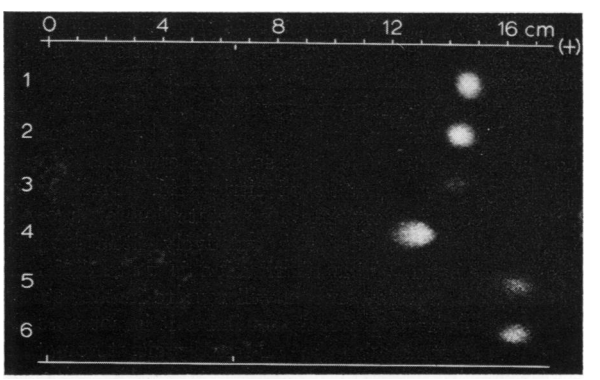

FIG. 5. Radiograph showing the effect of iron on the electromigration of organic acids in malate buffer. The following were separated on paths 1 to 6 respectively: ^{14}C-malate, $Fe^{14}C$ malate, ^{59}Fe malate, ^{14}C citrate, $Fe^{14}C$ citrate, ^{59}Fe citrate. Iron, organic acids, and malate buffer were 10^{-2} M.

ahead of all other compounds. It is noted that in malate buffer, as in other buffers, the Fe-citrate migrated faster than the iron-free acid. But the most significant fact was that the malate saturating the paper did not pull iron from citrate.

It is believed that the results given in this section help explain the exudate data in tables II and III and confirm that citrate was the agent principally involved in the iron transport.

Discussion

Responses of Normal and Chlorotic Plants. Exudate from green sunflower (group 4, table I) contained low concentrations of citrate and iron. Citrate was present at about 3 % of the concentration found in exudates of chlorotic plants, and iron was less than 1 %. A controlled uptake of iron is evident from the fact that most of the iron supplied to the roots was excluded. The chlorotic plants absorbed large quantities of iron and translocated it at concentrations considerably above the external medium.

In contrast to values of 2 or 3 it is noted that normal plants gave a citrate/iron ratio of 15. Although more work needs to be done, it is probable that values well above unity, i.e., citrate concentrations in considerable excess of iron in the exudate, are characteristic of plants grown with adequate iron.

If the combining quantities of citrate/iron are 1:1 in the exudate of normal plants, the ratio of citrate/iron = 15/1 suggests that 1/15 of the citrate is involved in transport of iron and that 14/15 could function in the solubility and transport of other metals.

Further Studies, Translocation Concepts. All exudates in table I gave citrate in molar excess of iron. By using various isotopes and electrophoresis it should be possible to determine whether citric acid binds other trace metals in stem exudates.

The citrate/iron ratios of 15 and 1.6 are far out of proportion. The question arises as to the possibility of getting ratios below unity. The following paper (12) gives evidence that iron can flood into roots and be translocated at concentrations greater than those of citrate.

It will be necessary to revaluate the soybean work (15) which emphasized iron binding by malate. It has been reported (3) that soybean exudates contain relatively large amounts of citric acid. Preliminary runs of soybean exudate in acetate buffer show 2 iron-containing bands with relatively little iron in the faster band. This distribution is quite different from that of sunflower, and will require further study.

With methods presently available it is not possible to demonstrate the binding of iron by citrate or other molecules in the xylem vessels. There is evidence, nevertheless, which supports the concept of acid-iron translocation in vivo. It is summarized as follows: A) Iron precipitates in aqueous media at physiological pH when solubilizing agents are not present. B) Iron binding in solutions of organic acids can be readily demonstrated. C) Stabilities of organic acid chelates of iron are relatively high (1, 4). D) Soluble iron and organic acids are usually present in stem exudates of plants. E) Citrate-iron complexing is indicated by exudate electrophoresis.

The role of citric acid is most often considered in relation to cellular metabolism. But released from metabolic sites and moving in the xylem, this acid would be expected to perform other functions. Iron binding is suggested as one of those functions. Further study should indicate whether citric acid participates in the translocation of other trace metals.

[1] Plexiglass pattern P-4, Rohm and Haas Company, Philadelphia 5, Pennsylvania. Trade names and company names are included for the benefit of the reader, and do not imply endorsement by the United States Department of Agriculture.

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