A NATURALLY OCCURRING CHELATE OF IRON IN XYLEM EXUDATE[1,2]

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At the normal pH of plant tissues, iron forms extremely insoluble hydroxide and phosphate precipitates. The K_{sp} of iron phosphate has been reported to be of the order of 10^{-35} over a relatively wide pH range (5). The form in which iron is transported under these conditions becomes of particular interest, for if inorganic iron and phosphate are both present in the xylem stream, it would seem that iron would be present in such insoluble precipitates that the amounts moving from the roots to the shoots would be extremely small, and iron deficiency would develop in the leaves and stems.

Biddulph and Woodbridge (1) detected the presence of a precipitate of iron-phosphate on the root surfaces of plants grown in nutrient cultures with high phosphate content. They proposed that iron also may be precipitated in this way in the xylem channels thus causing a deficiency of iron in the plant. Olsen (9) suggested that iron-phosphate precipitation may lead to deficiency symptoms in plants which seem to have a normal iron content. Since under normal growing conditions plants do not show iron deficiency, the occurrence of such blocking precipitates seems doubtful.

Deficiency symptoms attributable to genetic variation have been reported in grapes (22) and in soybeans (23). In these cases, some variants developed deficiency symptoms in nutrient media in which other variants made normal growth. Brown and Tiffin (3) found differences in the iron content of exudate from the soybeans, and proposed that certain genetic variants are not able to absorb sufficient iron from the nutrient medium.

The present investigation was carried out to determine if a natural complex or chelate of iron which can prevent formation of the inorganic precipitates exists in plants and serves as a transport form for iron. Several workers in recent years have suggested this possibility. Burstrom and Tullin (4) proposed that some of the many organic materials found in xylem fluid may function as chelaters of metals and Stewart and Leonard (20) suggested that chelates are the natural form in which iron is absorbed from the soil by higher plants. The classes of organic compounds present in grape vine exudate have been studied by Priestley and Wormall (11), and in a variety of other plants by other workers.

Because of the difficulty of obtaining xylem sap in the required quantities directly from plant stems, the work to be reported here will include only studies on tobacco plant exudate.

MATERIALS & METHODS

Exudate was collected from the stumps of tobacco plants (*Nicotiana tabacum* L. var. Wisconsin 38) according to the method described by Grossenbacher (7). It normally was collected for approximately six hours after cutting and used immediately. When not used immediately, the exudate was kept under refrigeration.

Total iron content of the exudate was determined by a potassium thiocyanate method using amyl alcohol as an extracting medium and hydrochloric acid for acidification (15). For certain analyses, other reagents also were used. These included Tiron (24), chromotropic acid (3,6-disulfo-1,8-dihydroxynaphthalene), Bathophenanthroline (19), and acetylacetone (12). In the latter case, it was found that the iron-acetylacetone colored complex could be extracted into amyl alcohol. This considerably increased the sensitivity of the test.

Several time-color development tests were carried out employing reagents which form colored complexes with iron (Tiron & chromotropic acid). Because of the relatively low sensitivity of these reagents, color intensities were determined in a Klett-Summerson Colorimeter using a cell which provided a 4 cm light path.

Phosphorus was determined by a stannous chloride-reduced phosphomolybdate method (8) and nitrogen by a semi-micro Kjeldahl procedure. Ascorbic acid was analysed by the method of Roe et al (14).

Cation and anion exchange resins were used in both column and batch tests. Dowex-1 was prepared in the acetate form by treatment with sodium acetate and acetic acid; Amberlite IR-120, in the sodium form by treatment with sodium chloride and hydrochloric acid. Small resin columns were prepared by attaching a 1.0 cm I.D. glass tube (14 cm long) to the center of the base of a 125 ml Erlenmeyer flask. A constriction in the tube about three centimeters from the lower end served as a support for a wad of glass wool on which the resin bed was supported. The flask served as a reservoir for eluent.

Large resin columns were made from glass tubing

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2.5 cm I.D. and 37 cm long. At the lower end, a fritted glass plate was inserted to serve as a base for the resin bed. For ease in collecting fractions, the lower end of the column was constricted to a small spout. After passing exudate through the large resin columns, they were eluted with 0.25 N HCl and fractions of 10 ml volume were collected with a Gilson automatic fraction collector. Every third fraction was tested for iron content.

In batch work with resins, exudate samples were in contact with the resin in an Erlenmeyer flask for 24 hours under refrigeration and with occasional stirring.

Exudate was also fractionated with Sephadex G-25, a gel filter (10). The large column and the fraction collector used were as described for the ion exchange resin.

Paper chromatograms were prepared with Whatman no. 1 paper cut into rectangles 7.5 by 9 inches. Before use, the paper was washed in dilute hydrochloric acid followed by at least five washings in distilled water. This treatment significantly reduced iron contamination. Separation of amino acids was by the method of Sisakyan et al (17) with *n*-butanol, methyl ethyl ketone, water (2/2/1) as the solvent system. Diethylamine, which they also used, was not included in the solvent for the present work. Amino acid spots were detected by ninhydrin spray reagent.

In further attempts to isolate the iron containing fraction in the exudate, a mixture similar to the solvent system published by Chu and Chu (6) for separation of iron containing porphyrins was employed. The developing solvent consisted of isopropanol, water, pyridine (0.1/5.5/0.4).

Bathophenanthroline, an extremely sensitive iron reagent, was employed to locate iron-containing spots on the chromatograms. The paper was dried at room temperature after development, sprayed with 10 % hydroxylamine hydrochloride, dried at 70° C, and sprayed with 0.03 % bathophenanthroline reagent prepared according to Smith et al (19). On further drying at 70° C, the presence of iron was indicated by the appearance of pink spots.

All filtration of iron containing exudates or solutions in the present work was through a Millipore filter, size HA (0.45 μ pore size.)

RESULTS

TOTAL IRON, NITROGEN, & PHOSPHORUS CONTENTS OF EXUDATE: In most cases the iron content of the exudate was in the range of 1.0 to 1.3 μg/ml (ppm), although some exudates contained as little as 0.4 μg/ml and the maximum was 1.7. Exudation occurred in a diurnally periodic pattern similar to that reported by Skoog et al (18) in sunflowers, but the iron content of the exudate gradually fell with time after cutting and showed no periodicity.

Phosphorus content varied from 50 to 70 μg/ml with one determination showing over 100 μg/ml. Nitrogen was present in concentrations of 60 to 75 μg/ml. The pH of the exudate was approximately 5.5.

REMOVAL OF IRON FROM EXUDATE BY MILLIPORE FILTRATION: To ascertain whether or not the exudate iron was present in a precipitated form, exudate was passed through a Millipore filter and the iron content of the filtrate was compared with that of the original exudate. In no case was the filtrate iron content reduced by this treatment.

At various times, batches of exudate were collected, held in the refrigerator for different lengths of time up to 3 days, and then tested for iron before and after Millipore filtration. The results are presented in figure 1 in which per cent of total iron remaining after Millipore filtration is plotted as a function of time from collection. It may be seen from the upper curve that the iron remains quite soluble even after 3 days. The slight removal with time may be due to bacterial action leading to breakdown of an iron complex. Within 2 days, the exudate becomes quite turbid and a precipitate settles to the bottom, but even then the amount of iron still complexed in solution has changed only slightly.

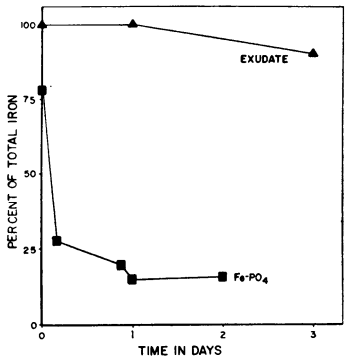
For purposes of comparison, a pH 5.0, acetate-buffered solution containing 1 μg of inorganic iron and 50 μg of inorganic phosphorus per milliliter was prepared and aliquots were passed through a Millipore filter at the time intervals indicated in figure 1. It may be seen (lower curve) that after only 4 hours, Millipore filtration removed a large percentage of the iron from solution. These results are in agreement with those of Rediske and Biddulph (13) and in contrast with the behavior of iron in exudate.

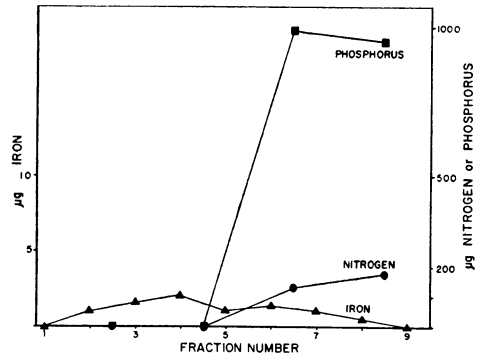
STUDIES WITH ION EXCHANGE RESINS: The possible presence of iron as a free cation in the exudate was checked through the use of a cation exchange resin. A batch test was made with Amberlite IR-120 and the supernate was analysed for iron. In the several tests made, there was no reduction in iron content of the supernatant liquid indicating that exudate iron is not in a form which can be removed by a cation exchange resin.

Amberlite IR-120 was capable of removing ionic iron from an acetate-buffered solution containing ferric nitrate. After iron had been fixed onto the resin from the acetate-buffered solution, it then could be removed by chromotropic acid, indicating that the resin's affinity constant (log K) for iron is less than 17.0 (that of chromotropic acid).

In further investigations, all the iron was removed from samples of exudate passed through a small column of an anion exchange resin, Dowex-1. This indicated that exudate iron was present in an anionic form. To eliminate the possibility of a filtering action by the resin bed, a batch test also was carried out with this resin. After 24 hours all iron again had been removed from the supernate.

It also was found that after exudate had been passed through a Dowex-1 column and the column





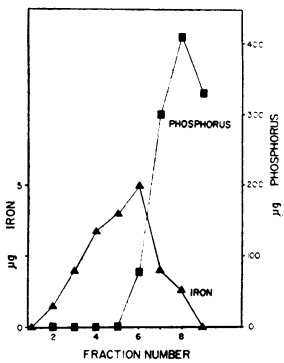


FIG. 1. Iron present in Millipore filtrates of tobacco plant exudate and in a synthetic solution containing iron and phosphate filtered at various intervals after exudate collection or solution preparation.

FIG. 2a. Iron, phosphorus, and nitrogen contents of successive samples from a Sephadex fractionation of tobacco exudate.

FIG. 2b. Iron and phosphorus contents of successive samples from a Sephadex fractionation of tobacco exudate.

had been washed with distilled water, it was capable of removing additional iron (1 µg/ml) from an acetate-buffered solution. This suggested that a compound which had become bound to the anion column was capable of tying up more iron than was originally present in the exudate.

After trials with various eluting agents, 0.25 N HCl solution was found suitable for washing the exudate iron from a Dowex-1 column on which it had been fixed. The fractions having a high iron content were pooled, neutralized to pH 5.0 with dilute ammonia, mixed with Dowex-1 in a test tube, and allowed to sit with occasional stirring in the refrigerator for about 48 hours. A test for iron revealed that all the iron again had been taken up by the resin.

PAPER CHROMATOGRAPHY OF EXUDATE: The possible existence of an iron complex also was investigated by chromatographic separation of the organic materials in exudate. Chelation with an amino acid was suggested by the work of Bollard (2) on the amino acids in xylem sap of apple trees. Tobacco exudate was chromatographed with the solvent of Sisakyan et al (17) and following spraying with Ninhydrin several amino acid spots were observed. The largest spot was shown to be glutamine; the smaller spots were not identified. No movement of exudate iron occurred although the Ninhydrin spots were all away from the origin. No Ninhydrin color was visible at the origin. A spot of ferric nitrate standard on the same chromatogram also failed to move from the origin.

A mixture of iron and glutamine was chromatographed with the above solvent. The iron again failed to move, thus apparently discounting the possible function of this amino acid as the iron complexing agent in exudate.

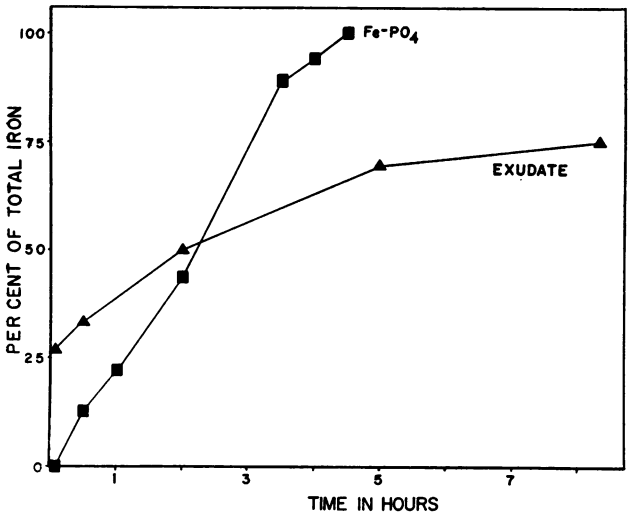
Further attempts were made to find a solvent which could move exudate iron. Development of chromatograms with butanol-acetic acid-water solvent, a standard method for separation of organic acids which are known to be present in exudate, was not successful in moving iron. The solvent used by Chu and Chu (6) to separate iron-containing porphyrins moved exudate iron from the origin to a point near the solvent front. However, iron from a ferric nitrate standard as well as from an iron-phosphate mixture did not move.

During the course of investigations with Dowex-1 columns, it had been noted that on passing exudate through a column a dark-brown layer was deposited at the top of the column. This layer moved downward on elution with dilute hydrochloric acid. Furthermore, exudate always had a slight yellow color. Shannon (16), in the course of studies on iron chlorosis, employed ascorbic acid as a chelating agent, and this was considered as a possible explanation for the color in the resin column and as the natural chelating agent. Initial results were promising, for when oxidized ascorbic acid was run through a Dowex-1 column, a brown layer formed at the top and if acetate-buffered iron solution were then passed through the same column, the iron was retained.

Furthermore, a buffered solution of iron and oxidized ascorbic acid was successfully chromatographed with the isopropanol-pyridine-water solvent (6) and the iron was moved to the same degree as in exudate spots.

However, in a test of the dialysibility of exudate iron, it was found that very little iron moved out of the membrane whereas in the case of iron-ascorbate, iron was readily moved through the membrane. Exudate contained about 15 µg of ascorbic acid per ml.

GEL FILTRATION COLUMN: At this time, Sephadex (a gel filter used to separate relatively large molecular weight substances from solution) was introduced as an additional means of purifying the exudate fraction containing iron. Millipore-filtered exudate was passed through a column of Sephadex and the resulting fractions analysed for iron, nitrogen, and phosphorus. The results are presented in figures



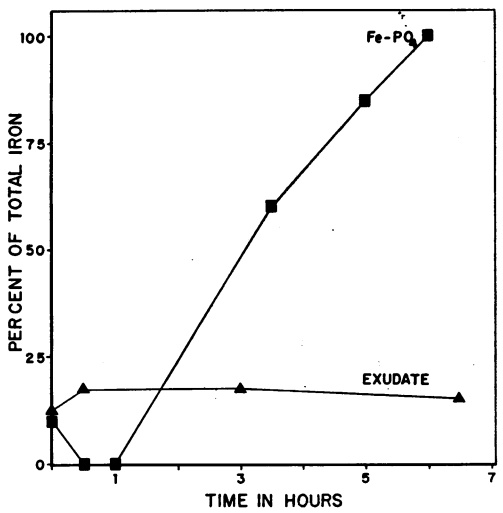


FIG. 3a. The capacity of Tiron (log K=20.7) to complex iron over a period of time from tobacco exudate and from a solution containing inorganic iron and phosphate.

FIG. 3b. The capacity of chromotropic acid (log K=17) to complex iron over a period of time from tobacco plant exudate and from a solution containing inorganic iron and phosphate.

2a and 2b. For the data of figure 2a, successive pairs of fractions were combined and analysed for phosphorus and nitrogen after individual fractions had been analysed for iron. The results with phosphorus led to a further test in which the phosphorus content of each sample from a Sephadex fractionation was determined and compared with the iron content (fig 2b). The separation of iron from both nitrogen and phosphorus strongly indicated that the iron complex was not a compound containing nitrogen or phosphorus and that the iron was associated with a relatively large molecule. Tests for the amino acid content of fractions from a smaller gel filter column had shown that no amino acids occur in the fractions containing iron, thus substantiating the results with paper chromatography of amino acids. The fact that phosphorus was separated from the iron also corroborates results with paper chromatography and Millipore filtration. A mixture of iron-ascorbate was retained by the gel filter against elution, thus further discounting this compound as a possible complexing agent.

COMPLEXING OF EXUDATE IRON: In order to more firmly establish the approximate affinity constant of exudate for iron, the capacity of several commercially available chelating agents which form colored complexes with ferric iron to complex with iron in exudate was determined. Results with three of these are presented in table I together with the percentage of the total iron which they indicated to be present in the exudate. These results were obtained with the colorimeter immediately after mixing the exudate and the complexing agents. It is obvious that in no case did these reagents indicate the true amount of iron present in the samples, thus strongly suggesting the presence of a material which was holding the exudate iron and not readily releasing it to the reagents used.

The capacities of Tiron and chromotropic acid to complex with iron in exudate and in a synthetic iron-phosphate mixture were then determined over a period of time. The time-color development curves for these studies are presented in figures 3a and 3b. The values on the ordinate axes are derived from determinations corrected for an exudate blank and for a reagent blank.

In figure 3a, the behavior of exudate and of the iron-phosphate mixture with Tiron is shown. While the removal of iron from the iron-phosphate mixture is linear with time, the curve for removal from exudate would have to be plotted in a logarithmic fashion in order to obtain a straight line. It is to be noted that the two curves indicate a very different behavior

TABLE I

COMPARISON OF CAPACITY OF VARIOUS COMPOUNDS WHICH FORM COLORED COMPLEXES WITH FERRIC IRON TO COMPLEX WITH IRON IN XYLEM EXUDATE*

REAGENT	IRON CONTENT, μg/ml	% OF TOTAL IRON COMPLEXED
KSCN	1.3	
Tiron (20.7)	0.3	23
KSCN	0.5	
Acetylacetone (9.0)	0.0	0
KSCN	1.0	
Chromotropic acid (17)	0.2	20

* Affinity constants (Log K) are given in parentheses.

of iron from the two sources, and that after eight hours Tiron had complexed 75 % of the total exudate iron.

Figure 3b presents results of a time-color development study with chromotropic acid. The reaction with the iron from the iron-phosphate mixture is again linear and occurs quickly while not more than 20 % of the exudate iron reacted with chromotropic acid. At 24 hours it had risen somewhat, but a spurious orange color had formed and the results are reported to 9 hours only.

The fact that these two reagents seem to bracket the affinity of exudate for iron suggests that the stability constant of the natural complexing agent and iron is between 17 and 20.7.

Discussion

Although recent literature on iron nutrition contains suggestions that a natural chelate functions in iron transport in plants (21), to the authors' knowledge there are no published results verifying this proposal or identifying the complex. In the present investigation, evidence has been presented for the existence of an iron-containing complex in exudate, primarily through demonstrations that in a variety of tests exudate iron behaves differently than if it were present as free iron or as an insoluble precipitate. Furthermore, treatment with ion exchange resins revealed that exudate iron is associated with a complex which behaves as an anion.

The existence in the xylem of a precipitate containing iron seems to have been eliminated by filtration studies. In no case was iron removed from exudate by Millipore filtration. Even in view of this, it might still be argued that exudate iron is present as a very finely divided phosphate precipitate which could pass through the filter. This seems to be negated by the fact that in a synthetic iron-phosphate mixture, prepared to approximate the phosphorus and iron contents of exudate, iron immediately began precipitating in a form which could be Millipore filtered. Furthermore, paper chromatographic evidence showed that, in contrast with exudate iron, iron in a synthetic mixture does not move with the solvents employed.

The data presented include evidence on the approximate affinity constant between iron and the exudate complexing agent. This was determined to be between 17 and 20.7 by the differential capacity of Tiron and chromotropic acid to complex with exudate iron. Both of the complexing agents were capable of removing iron from the iron-phosphate mixture but only Tiron could remove iron from exudate. The affinity of the complexer need not be as great as might be anticipated, since once iron has been complexed it apparently is not readily released even for the formation of very insoluble precipitates. For example, acetate ion is capable of holding iron in solution at pH 5, although it releases iron to Amberlite IR-120 cation exchange resin.

Identification of the iron complexing agent in plant exudate must await further studies. In the present investigation, amino acids, organic acids, and ascorbic acid have been demonstrated to be present in exudate. However, they seem to have been eliminated as the possible chelating agent on the basis of behavior in several tests. Paper chromatography eliminated amino acids and results with gel filtration showed that nitrogen was not associated with the iron. The possibility that ascorbic acid or a related compound may function as a complexer was discounted by the fact that in a gel filter iron in an iron-ascorbate complex behaves differently from exudate iron.

It is of course obvious that an iron complex present in xylem exudate may not be present in the xylem stream of a transpiring plant and may not be the transport form of iron under these conditions. As mentioned earlier, the difficulty of obtaining adequate quantities of xylem sap led to the use of tobacco exudate. Following identification of the iron complex in exudate, it is hoped that this work can be extended to studies on sap collected from transpiring plants.

Summary

Since iron forms extremely insoluble phosphate and hydroxide precipitates at the pHs encountered in plants, studies were carried out to determine if a natural complexing agent is present in plant sap which prevents iron precipitation and serves as a transport form of this element. This was investigated primarily through comparisons of the behavior of iron in tobacco plant exudate and in synthetic solution containing comparable quantities of inorganic iron and phosphate.

Millipore filtration did not reduce the iron content of exudate but almost completely removed iron from the synthetic iron-phosphate mixture. Exudate iron also moved on paper chromatograms; iron from the synthetic iron-phosphate mixture did not.

Exchange resins, chromatography, and gel filtration were employed to separate the proposed natural iron complex as an initial step in its identification. Iron was not removed from tobacco exudate by cation exchange resins, but was adsorbed onto an anion exchange resin from which it subsequently could be leached and reattached to the same resin. This suggests that the iron was associated with an anionic structure. Exudate iron moved directly through a Sephadex gel filter, suggesting it is associated with a relatively large molecule. A Sephadex column also separated the iron from nitrogen and phosphorus containing compounds. While these results strongly suggest the existence of a naturally occurring iron complexing agent which can prevent precipitation, its identity has not been established.

Paper chromatographic separations carried out thus far have indicated that neither amino acids, organic acids, nor ascorbic acid is the natural complexing agent in exudate.

The stability constant (log K) of the exudate iron complex was found to lie between 17.0 and 20.7 through a comparison of the capacities of synthetic chelating agents having various affinities for iron to remove iron from the exudate complex.

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