

# Enhancement of Phloem Exudation from *Fraxinus uhdei* Wenz. (Evergreen Ash) using Ethylenediaminetetraacetic Acid<sup>1</sup>

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L. R. COSTELLO, JAMES A. BASSHAM, AND MELVIN CALVIN  
*Laboratory of Chemical Biodynamics and Department of Chemistry, Lawrence Berkeley Laboratory,  
University of California, Berkeley, California 94720*

## ABSTRACT

Ethylenediaminetetraacetic acid (EDTA) enhanced the exudation of <sup>14</sup>C-labeled assimilates from excised leaflets and whole plant specimens of *Fraxinus uhdei* Wenz. A 2 millimolar EDTA concentration was found to be most effective in promoting exudation from excised leaflets, while 10 millimolar EDTA was most effective in whole plant experiments. Exudation rate reached a maximum after 24 hours in both experiments. The continuous presence of EDTA throughout the treatment period was required for maximum exudation from excised leaflets. Stachyose, raffinose, verbascose, and sucrose were the principal compounds found to occur in exudate samples. These compounds are typically transported in sieve elements of various *Fraxinus* species suggesting the exudate was of phloem origin. Electron microscope studies of petiole sieve plate pores from excised leaflets showed substantially less callose appearing after treatment with EDTA than after H<sub>2</sub>O treatment. It is suggested that EDTA enhances phloem exudation by inhibiting or reducing callose formation in sieve plate pores. The exudation enhancement technique described for whole plant specimens is suggested as a useful means of collecting phloem sap and studying translocation in woody plants.

Various techniques have been employed to collect phloem sap. The aphid stylet technique (7), the incision method (17), and excision of monocotyledonous axes (13) are most notable. Recent investigations have demonstrated that chelating agents, particularly EDTA, are effective in promoting exudation from selected species. King and Zeevaert (8) collected and analyzed phloem sap from EDTA-treated leaves of *Perilla crista*. Fellows *et al.* (5) measured translocation into and through soybean pods and collected phloem sap for analysis using EDTA enhancement techniques. Tully and Hanson (14) investigated amino acid translocation in water-stressed and turgid barley leaves using EDTA.

Thus far, however, applications of EDTA enhancement techniques have been limited to excised leaves and fruits of herbaceous species. With the exception of Dickson (2), who was unable to obtain exudate from excised leaves of *Populus deltoides*, no studies using this technique in woody plant species have been reported. Furthermore, attempts to enhance exudation from whole plant specimens have not been reported.

Here, the effectiveness of EDTA in enhancing exudation from excised leaflets and whole plant specimens of a woody tree species, *Fraxinus uhdei*, is examined. Exudation rates, sap analysis, and structural changes in sieve plate pores resulting from EDTA

treatments are reported. In addition, a simple means of collecting phloem sap from whole plant specimens of *F. uhdei* using a "bark flap" method is described.

## MATERIALS AND METHODS

### Excised Leaflets.

*Plant Materials.* Leaves of 10-year-old *F. uhdei* Wenz. trees were selected. Each pinnately compound leaf consisted of four leaflet pairs and one terminal leaflet. Only fully expanded, disease-free, sun leaves were selected.

The pinnately compound leaf of *F. uhdei* was considered particularly well suited for these studies. Since source-sink relationships may vary among leaves on the same shoot (6, 15), two simple leaves of similar size may not act as equal sources or sinks. As a result, it would be difficult to attribute exudation differences solely to experimental treatments when using leaves which are equivalent only in size. However, leaflet pairs of pinnately compound leaves, which are equivalent in size, age, and development, might be expected to exhibit only minimal differences in source-sink strengths. Consequently, exudation differences may be attributed more specifically to treatments.

*Photosynthetic <sup>14</sup>C Incorporation.* An attached fully expanded leaf was labeled with <sup>14</sup>CO<sub>2</sub> (13.9 mCi/mm) for 30 min. The leaf was enclosed in an air-tight Plexiglas chamber (30.5 × 20 × 2.5 cm) and connected to a steady-state gas-recirculating apparatus designed to pass <sup>14</sup>CO<sub>2</sub> across the leaf surface (10). Chamber temperature was maintained between 25 and 29°C and illumination provided by General Electric Cool White high output fluorescence lamps (600 μE m<sup>-2</sup> s<sup>-1</sup>).

*Leaflet Excision and Treatment.* Leaflet pairs were excised immediately after <sup>14</sup>C labeling. One leaflet of each pair was placed in a 10-ml tube containing 6.5 ml EDTA solution (adjusted to pH 7.0 with 6 N KOH). The matching leaflet was placed in 6.5 ml distilled H<sub>2</sub>O. The leaflets remained in solution for 48 or 72 h with their petioles immersed to a depth of 2 cm. All leaflets were kept in darkness during the treatment period.

Periodically, 1.0-ml samples of treatment solutions were taken and analyzed for total <sup>14</sup>C content. The amount of <sup>14</sup>C appearing in the treatment solutions over a timed period was used as a measure of treatment effectiveness.

*Analysis of Exudates.* <sup>14</sup>C-Labeled exudates were counted on a Packard Tri-Carb liquid scintillation spectrophotometer. A 1.0-ml sample was added to 6.0 ml Research Products International (Elk Grove Village, IL) 3a70B complete counting cocktail. Another 0.1-ml aliquot of exudate was spotted on Whatman No. 1 chromatography paper. The chromatograms were run in phenol-H<sub>2</sub>O for 24 h and in butanol-propionic acid-water for 24 h (9). Radioactive compounds were located on Kodak medical X-ray film after a 1-week exposure period. The amount of label appearing in specific compounds was determined by eluting chromatogram spots in 2 ml distilled H<sub>2</sub>O for 8 h and counting <sup>14</sup>C activity of

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each spot.

**Analysis of Sieve Plate Pore Structure.** After a 24-hr treatment period, 2-mm discs were sectioned from the proximal end of EDTA- or H<sub>2</sub>O-treated petiolules. The discs were cut longitudinally into four equal sections and fixed in 4% glutaraldehyde and 2% aqueous osmium tetroxide. Following dehydration in ethanol, the discs were embedded in Spurr's low viscosity media (12). After a 24-h curing period at 70°C, the tissue was sectioned on a Porter-Blum MT-2 ultramicrotome equipped with a Dupont diamond knife. Thin sections exhibiting gold or silver interference colors were expanded with chloroform vapor, mounted on Formvar-coated grids, and stained with 5% aqueous uranyl acetate for 30 min and Reynold's lead citrate for 5 min. Sections were viewed and photographed on a Zeiss 9A electron microscope.

#### Whole Plants.

**Plant Materials.** Two-year-old, container-grown *F. uhdei* trees measuring 90 to 120 cm in height were used in these experiments. Specimens selected were vigorous, disease-free plants exhibiting a uniform distribution of shoots and leaves around a single trunk. All plants were grown in full sun.

**Photosynthetic <sup>14</sup>C Incorporation.** The apical two pair of fully expanded leaves were enclosed in an air-tight chamber and exposed to <sup>14</sup>CO<sub>2</sub>, as described previously. After a 30-min labeling period, the chamber was sealed and the lights turned off. The leaves remained in the chamber throughout the exudation treatment period.

**Bark Flap Excision and Treatment.** Prior to <sup>14</sup>C-labeling, bark flaps were cut as shown in Figure 1. Two flaps, located on opposite sides of the trunk and measuring 3.5 cm long by 0.5 cm wide, were cut. Each flap was carefully lifted and separated from underlying xylem tissue. Microscopic examination of the bark flap showed that it contained secondary phloem as well as periderm tissue. Each flap was wrapped with a thin layer of Parafilm (American Can Corp.), recut at its distal end (1 mm), and immersed in 3.5 ml treatment solution contained in 5-ml vials. The vials were taped to the trunk as shown in Figure 1. Three pairs of vials were evenly spaced along the trunk, each pair rotated 90° relative to adjacent pairs down the central axis (Fig. 1). One vial of each pair contained distilled H<sub>2</sub>O and the other contained an EDTA solution (adjusted to pH 7.0 using 6 N KOH).

**Sampling and Analysis of Exudate.** EDTA and H<sub>2</sub>O vials were removed after 4, 24, and 48 h, and replaced with fresh preparations. A 1.0-ml aliquot from each of the used solutions was analyzed for <sup>14</sup>C content as described previously. The amount of label appearing in each treatment solution was used as a measure of exudation. Exudation rates were calculated from periodic samplings. A 0.25-ml sample of EDTA and H<sub>2</sub>O treatments was analyzed for soluble exudates by paper chromatography methods, as described previously.

## RESULTS AND DISCUSSION

#### Excised Leaflets.

**Exudation.** Treatment of excised leaflets with EDTA greatly enhanced the exudation of <sup>14</sup>C (Table I). Preliminary experiments indicated that a 2 mM EDTA concentration was most effective in promoting exudation over a 72-h period. Short term exudation enhancement resulted from 10 mM and 20 mM treatments, but after 24 h, exudation rate declined rapidly and marginal necrosis and distortion of the leaf blade were observed. EDTA concentrations lower than 2 mM were less effective in promoting long or short term exudation. The 2 mM treatment resulted in 4- to 10-fold increases in exudation among replicates (Table I).

Of the small amount of label exuded from H<sub>2</sub>O treatments, over 50% appeared during the 1st h (Fig. 2). Thereafter, exudation rate declined rapidly, reaching a low level at 72 h. The brief release of label during the 1st h may have resulted from either nonspecific loss of cytoplasm from injured cells at the cut surface or short

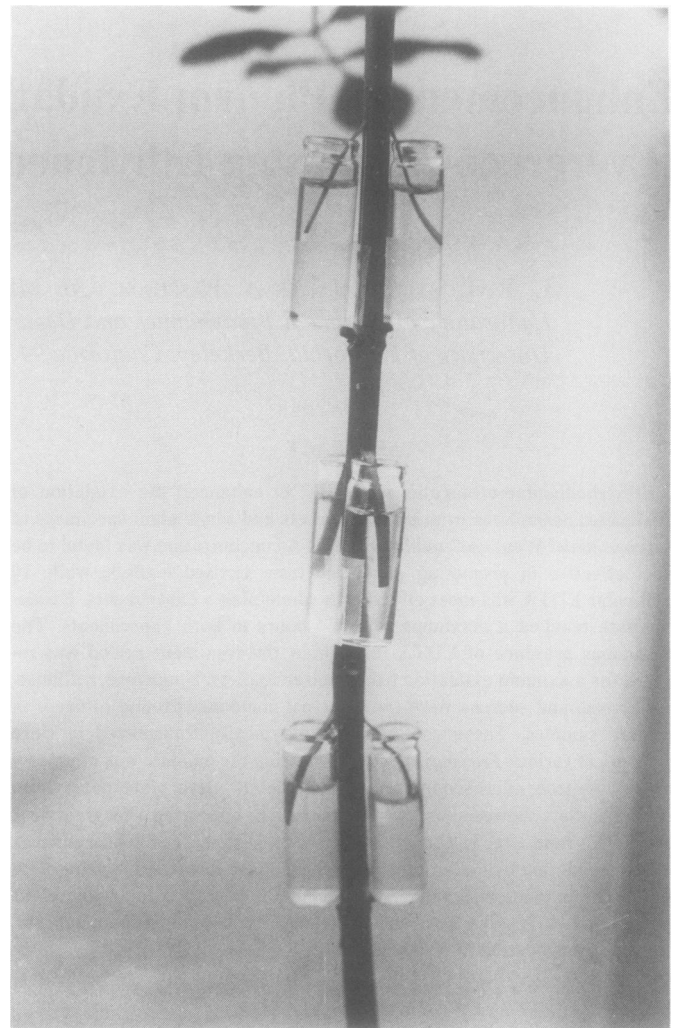


FIG. 1. Three pair of bark flaps are shown immersed in treatment solutions. One collection vial of each pair contains 10 mM EDTA and the other contains distilled H<sub>2</sub>O. Bark flaps and collection vial pairs were rotated 90° relative to one another down the central axis of the trunk.

Table I. Exudation of <sup>14</sup>C from Excised Leaflets  
The leaflets were treated with 2 mM EDTA or H<sub>2</sub>O for 72 h.

Leaflet No.	Treatment	<sup>14</sup> C in Exudate dpm/ml · 72 h
1 a	EDTA	96,700
b	H <sub>2</sub> O	21,300
2 a	EDTA	126,000
b	H <sub>2</sub> O	20,200
3 a	EDTA	129,000
b	H <sub>2</sub> O	17,700
4 a	EDTA	60,000
b	H <sub>2</sub> O	7,000

term phloem sap exudation prior to sieve tube sealing.

Volume losses of less than 0.5 ml were noted from EDTA and H<sub>2</sub>O treatment solutions after 72 h. Such losses appeared evenly distributed between treatments and among replicates. Concentration changes due to volume losses were considered to be very small.

Exudation rate from leaflets treated with EDTA was initially 40% less than those treated with H<sub>2</sub>O (Fig. 2). After 24 h, however, exudation from EDTA-treated leaflets increased to a maximum rate, approximately 8 times greater than that in H<sub>2</sub>O treatments.

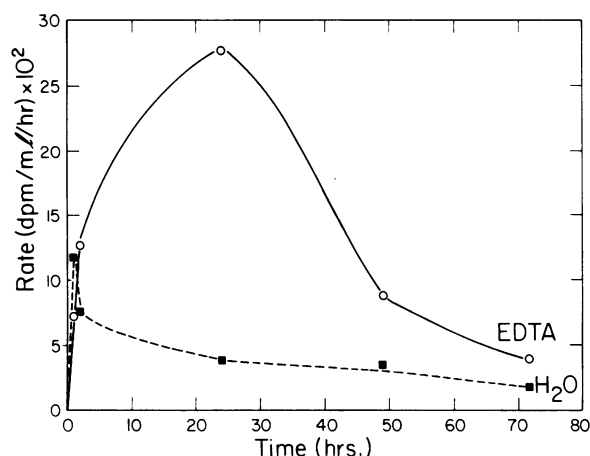


FIG. 2. <sup>14</sup>C exudation rate during 72-h treatment period. After excision, leaflets were placed in H<sub>2</sub>O or a 2 mM EDTA solution. Values represent the average of four replicates.

Table II. Effect of 2 mM EDTA Treatment Time on <sup>14</sup>C Exudation from Excised *F. uhdei* Leaflets over a 48-Hour Period

One leaflet of each pair remained in 2 mM EDTA continuously for 48 h, while the other leaflet was transferred to distilled H<sub>2</sub>O after a 0.5, 1, 2, or 4 h EDTA treatment.

Leaflet No.	Treatment	<sup>14</sup> C in Exudate dpm/ml
1 a	EDTA (0.5 h), H <sub>2</sub> O (47.5 h)	11,200
1 b	EDTA (48 h)	28,000
2 a	EDTA (1 h), H <sub>2</sub> O (47 h)	10,700
2 b	EDTA (48 h)	28,800
3 a	EDTA (2 h), H <sub>2</sub> O (46 h)	8,800
3 b	EDTA (48 h)	122,600
4 a	EDTA (4 h), H <sub>2</sub> O (44 h)	5,400
4 b	EDTA (48 h)	43,200

By 48 h, exudation had been reduced to 34% of maximum, and eventually to 15% at 72 h in EDTA-treated leaflets. This apparent decline in exudation rate may be attributed to various factors: reduced phloem loading and translocation, dilution and loss of label, or partial sieve tube sealing.

Maximum exudation was noted when leaflets remained in EDTA solutions continuously over a 48-h period. Transferring leaflets to H<sub>2</sub>O after a short EDTA treatment resulted in a 60 to 93% reduction in exudation (Table II). Fellows *et al.* (5) noted that soy bean pods required a similar uninterrupted EDTA exposure for maximum exudation. However, King and Zeevaart (8) reported that a short EDTA exposure was sufficient for continued exudation from *P. crisper* leaves.

**Analysis of Exudate.** Approximately 94% label appeared in oligosaccharides (stachyose, raffinose, verbascose) and sucrose (Table III). Small amounts of glucose and fructose and lesser amounts of malate and citrate were identified. Analysis of phloem sap from various *Fraxinus* species (18) closely parallels the analysis presented in Table III. The correspondence between analyses suggests that label appearing in exudate samples represents compounds of phloem origin.

**Structural Changes in Sieve Plate Pores.** Marked callose depositions were observed in sieve plate pores of petiolules placed in H<sub>2</sub>O for 24 h (Fig. 3). By contrast, EDTA-treated petiolules exhibited essentially callose-free sieve plate pores (Fig. 4). In 10 replicate samples, callose deposits were found in approximately 90% H<sub>2</sub>O treatments and 10% EDTA treatments.

Although it was not possible to estimate precisely the callose per unit phloem area (due to variability among tissue section

Table III. Paper Chromatography and Autoradiography Analysis of Exudate from EDTA Treatment Solutions

Relative amounts of each compound given in dpm/ml. Values included for excised leaflets and whole plant experiments.

Compound	Excised Leaflets		Bark Flaps	
	<sup>14</sup> C in spot	% total	<sup>14</sup> C in spot	% of total
	dpm/0.1 ml		dpm/0.25 ml	
Aspartate	419	<1.0	4,100	<1.0
Citrate	480	<1.0	4,300	<1.0
Fructose	18,200	2.9	110,000	4.8
Glucose	15,000	2.4	91,000	3.8
Glutamate	2,700	<1.0	22,000	<1.0
Malate	876	<1.0	3,100	<1.0
Raffinose	56,800	9.2	432,000	18.2
Stachyose	343,000	55.7	1,260,000	53.4
Sucrose	108,000	11.6	298,000	12.6
Verbascose	70,000	11.4	140,000	5.9

replicates), it seems, from the above observations, that callose deposition is substantially reduced as a result of EDTA treatment. Using a fluorescence technique for locating callose in sieve tubes (4), King and Zeevaart (8) reported a similar reduction in callose deposits in *P. crisper* sieve plates resulting from an EDTA treatment. Such evidence indicates that EDTA may enhance exudation by inhibiting callose formation in sieve plate pores.

**Whole Plants.** Exudation of <sup>14</sup>C-labeled sugars from bark flaps of whole plant specimens was negligible over a 48-h period when the flaps were immersed in water, but exudation was enormously enhanced when the flaps were immersed in 10 mM EDTA solution (Table IV). Exudation was enhanced from 3,000- to 18,000-fold among EDTA-treated replicates. Preliminary experiments found that EDTA concentrations greater or less than 10 mM were less effective in enhancing exudation.

Exudation of label from either EDTA or H<sub>2</sub>O treatments was negligible for 4 h after <sup>14</sup>CO<sub>2</sub> incorporation (Fig. 5). This exudation lag may represent the time required for assimilation and loading of label in the leaves and its subsequent translocation to the bark flaps. From 4 to 24 h, exudation into EDTA solutions increased rapidly, while exudation into H<sub>2</sub>O treatments remained slight. After reaching a maximum rate at 24 h, exudation into EDTA solutions declined slowly. The decline in rate after 24 h may have resulted from dilution or loss of label, reduced assimilation and phloem loading, or partial sealing of sieve plate pores.

Approximately 90% label recovered in exudate samples appeared in sucrose and the oligosaccharides stachyose, raffinose, and verbascose (Table III). Only 8% appeared in glucose and fructose (possibly occurring as breakdown products of sucrose), while less than 2% was found in glutamate, aspartate, citrate, and malate, collectively. This analysis corresponds closely with that found for exudate collected from excised leaflets of *F. uhdei*, and various *Fraxinus* species (18), suggesting that the exudation of <sup>14</sup>C from *F. uhdei* bark flaps into EDTA treatment solutions occurs principally from phloem sieve elements.

It may be argued that exudation differences noted in Table IV may have resulted in part from *in situ* differences in assimilate flow down the tree trunk. For instance, if labeled assimilates were preferentially loaded into axial sieve elements on one side of the trunk (perhaps due to an uneven leaf area distribution), then assimilate flow, and consequently, exudation potential, may not be equal at apparently equivalent exudate collection sites. Exudation differences at these sites may, therefore, not solely reflect differences due to treatments, but may also represent differences in assimilate flow.

Two measures were taken to avoid possible exudation effects due to differential assimilate flow. First, only the terminal two



FIG. 3. Longitudinal section of sieve tube member after 24-h  $H_2O$  treatment. Callose (C) deposits are visible in sieve plate pores ( $\times 10,200$ ). Arrow indicates probable direction of translocation.

pair of fully expanded leaves were labeled with  $^{14}C$ . Since *F. uhdei* leaves are initiated in opposite pairs which alternate in two distinct planes down the central axis of the trunk (decussate phyllotaxis), the terminal two pairs of leaves formed a uniform leaf area distribution around the trunk. As such, potential differences in assimilate flow due to a nonuniform leaf area distribution were ostensibly minimized.

Second, both EDTA and  $H_2O$  treatments were placed in an alternating sequence down the trunk, and treatment pairs were rotated  $90^\circ$  relative to one another along the central axis (Fig. 1). These procedures ensured that both EDTA and  $H_2O$  treatment vials were positioned at three different points around the trunk rather than in a vertical file on each side of the trunk. As a result, differences in exudation which may result from possible differences in assimilate flow down one side of the trunk would be apportioned evenly between both EDTA and  $H_2O$  treatment vials and among their replicates.

A further consideration concerning procedures should be noted. After bark flaps were cut and lifted from the underlying tissue, they were wrapped with a thin layer of Parafilm. Then, before being immersed in treatment solutions, a 1-mm section of the distal end of each bark flap was removed, exposing fresh tissue. These steps were necessary to ensure that the cut ends of the flaps were the only portions directly exposed to the treatment solutions. The Parafilm layer served as a barrier between the treatment solution and tissue exposed on the interior surface of the bark flap. (It was wrapped sufficiently tightly so treatment solutions did not rise up the flap.) Label appearing in the treatment solutions

could therefore be considered to be released principally from sieve elements at the cut end of the bark flap rather than from cells located on its interior surface.

The results of these experiments indicate, therefore, that EDTA is very effective in enhancing exudation from whole plant specimens of *F. uhdei*. This bark flap method of collecting phloem sap may be a useful technique for studying translocation in other woody species, particularly trees. For instance, the translocation of some phloem mobile compounds such as herbicides, pesticides, or growth regulators may be studied using this technique. Furthermore, translocation kinetics (11), distribution patterns of translocate, and the composition of phloem sap in many woody species could also be investigated.

### CONCLUSIONS

The exudation of phloem sap from excised leaflets and whole plant specimens of *F. uhdei* was appreciably enhanced by EDTA. A 2 mM EDTA solution was most effective in promoting exudation from excised leaflets while a 10 mM EDTA was most effective in whole plant experiments. The reason for these differences in concentration effects is unclear, however. Conceivably, more phloem surface area was exposed at the cut end of the bark flaps than that of the cut petiolules, thus requiring a greater number of EDTA molecules available for exudation enhancement reactions. Alternatively, EDTA is likely to have had secondary metabolic effects, *i.e.* effects on metabolism not specifically associated with phloem exudation. Any such secondary metabolic effects may be accentuated at high EDTA concentrations and may indirectly

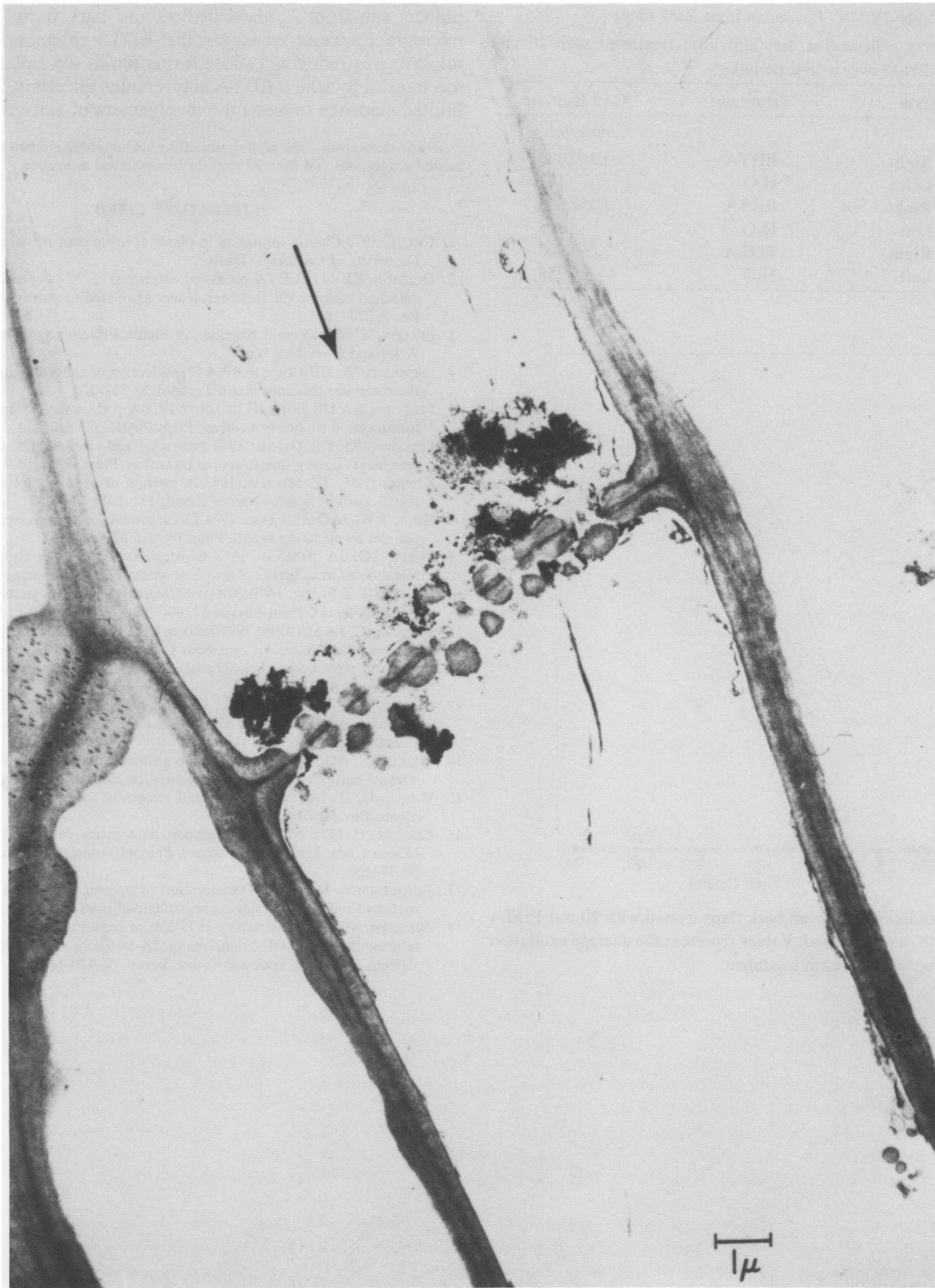


FIG. 4. Longitudinal section of sieve tube member after 24-h EDTA treatment (2 mM). Sieve plate pores are essentially callose free ( $\times 10,200$ ). Arrow indicates probable direction of translocation.

affect phloem transport and exudation. This may be particularly expressed in excised leaflet experiments where EDTA could move into the xylem and through the transpiration stream to the leaf blade. An accumulation of EDTA in the leaf blade may adversely interfere with leaf metabolism. Ostensibly, such was the cause of leaflet blade necrosis and distortion noted when excised leaflets were treated with 10 mM or 20 mM EDTA. In whole plant experiments, where no contact was made between the EDTA

solution and the transpiration stream, a greater tolerance for higher EDTA concentrations might be expected. No noticeable effects were found when 10 mM EDTA was used in the bark flap experiments. Thus, the variance in response to EDTA concentrations in the two experiments may possibly be attributed to transport and accumulation of EDTA in the excised leaflets and the absence of such movement in bark flaps.

In summary, EDTA specifically enhances the exudation of

Table IV.  $^{14}\text{C}$  Exudation from Bark Flaps

The exudate was collected at the flaps after treatment with 10 mM EDTA or distilled  $\text{H}_2\text{O}$  over a 48-h period.

Vial Position		Treatment	$^{14}\text{C}$ in Exudate dpm/ml
Top	Right	EDTA	3,360,000
	Left	$\text{H}_2\text{O}$	182
Middle	Right	EDTA	1,150,000
	Left	$\text{H}_2\text{O}$	375
Bottom	Right	EDTA	3,360,000
	Left	$\text{H}_2\text{O}$	356

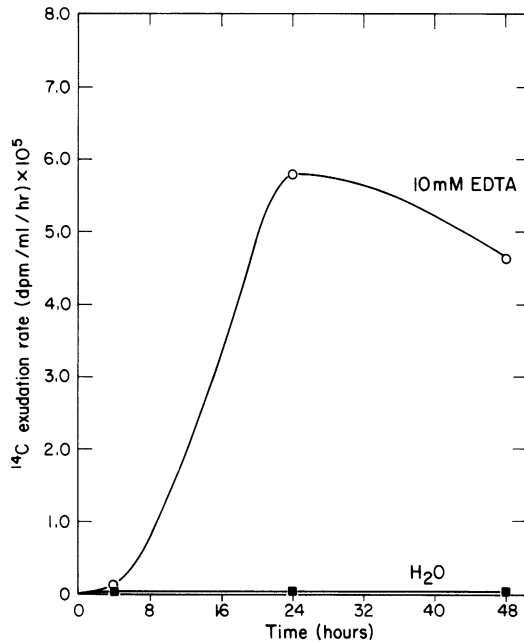


FIG. 5.  $^{14}\text{C}$  exudation rate from bark flaps treated with 10 mM EDTA or distilled  $\text{H}_2\text{O}$  for a 48-h period. Values represent the average exudation rate from three replicates of each treatment.

phloem sap from *F. uhdei* leaflets and bark flaps. The electron micrographs presented suggest that EDTA enhances exudation by inhibiting or reducing callose formation in sieve plate pores. The mechanism by which EDTA acts remains uncertain, however, but limited evidence suggests the involvement of calcium (1, 7).

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