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CHEMICAL BIODYNAMICS DIVISION

PHLOEM EXUDATION STUDIES IN SELECTED WOODY TREES

Laurence Raleigh Costello
(Ph.D. thesis)

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University of California
Berkeley, CA 94720

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ABSTRACT

Sugars transported in the phloem of actively growing trees were regarded as a potential feedstock for biomass fuels. It was postulated that phloem sugars could be harvested from mature trees on a yearly basis and then converted into ethanol or methane by microbial action.

Phloem sugars are not readily released from cut sieve elements. Shortly after wounding, sieve plate pores become plugged with a $\beta(1,3)$ glucan (callose) which effectively prevents the free exudation of sugars. Experiments were conducted, therefore, to investigate a means of inhibiting sieve element plugging reactions and thereby promote the exudation of phloem sugars.

Chelating agents, particularly EDTA, were tested for their effectiveness in enhancing the exudation of phloem sugars from selected woody tree species. Of the species selected, Fraxinus uhdei (Evergreen Ash) proved most useful for exudation studies. Experiments using ^{14}C labelled, excised leaflets of F. uhdei demonstrated that exudation was enhanced approximately 9-fold by a 2.0 mM EDTA treatment. An analysis of labelled products in the treatment solution showed that over 90% of label appeared in oligosaccharides and sucrose. These findings suggested that the sugars released into EDTA

treatment solutions were principally derived from phloem sieve elements. Electron microscope studies of the fine structure of sieve plate pores after treatment with EDTA or H₂O showed substantially less callose appearing in sieve plates of leaflets treated with EDTA. It was proposed that EDTA enhances exudation by inhibiting or reducing callose formation in sieve plate pores.

Subsequent experiments demonstrated that EDTA was effective in promoting exudation from whole plant specimens of *F. uhdei* as well as from excised leaflets. Using a bark flap method for collecting phloem sugars, exudation from young trees labelled with ¹⁴C was found to be enhanced approximately 1,000-fold by 10 mM EDTA. Over a 72 hr period, exudation rate was greatest after 24 hrs and then declined to minimum at 72 hrs. Exudation from H₂O treatments was negligible after 4 hrs. Similar to the excised leaflet experiments, over 90% of the label appeared in oligosaccharides and sucrose.

Experiments using 5 year old *F. uhdei* trees showed that a 20.0 mM EDTA treatment enhanced exudation 31-fold during a 72 hr period. Exudation could be maintained for up to 4 days in these experiments. By using detached bark flaps, it was found that sugars appearing in the H₂O treatments after 4 hrs were derived from "nonphloem" sources (nonspecific release), while over 90% of that in EDTA treatments could be specifically attributed to phloem exudation.

It was concluded that EDTA effectively enhances the exudation of phloem sugars from *F. uhdei* trees. These studies were thought to provide a strong indication that harvesting sugars from actively growing trees for an extended period of time is possible. Further studies are suggested before such a system becomes practical.

M. Calvin

TABLE OF CONTENTS

	Page
ACKNOWLEDGEMENTS.	vi
INTRODUCTION.	1
LITERATURE REVIEW	4
I. Sieve Element Structure and Function in Angiosperms. .	4
A. Sieve Element Size and Longevity.	4
B. Sieve Element Differentiation	4
C. Structure of Sieve Elements and Translocation . .	5
II. Callose Physiology.	6
A. Occurrence.	6
B. Metabolism.	6
C. Factors Affecting Formation	7
D. Function.	8
III. Exudation	9
A. The Incision Method	9
a. Dicots.	9
b. Monocots.	10
B. The Aphid Stylet Technique.	11
C. Chemical Promotion of Exudation	11

	Page
CHAPTER 1. Preliminary Experiments	13
Methods and Materials.	13
A. Plant Materials	13
B. Treatments.	14
C. Analysis of Exudate	21
Results and Discussion	24
Summary	30
CHAPTER 2. Exudation Studies Using Excised Leaflets of <u>Fraxinus uhdei</u>	31
I. EDTA Effects on Exudation Amount, Duration, and Rate	31
Methods and Materials	32
A. Plant Materials	32
B. Photosynthetic ¹⁴ C Incorporation.	32
C. Leaf Excision and Treatment	35
Results and Discussion	36
A. Exudation Enhancement	36
B. Concentration Effects	38
C. Exudation Duration and Rate	40

II. Analysis of Exudate	52
Methods and Materials.	52
Results and Discussion	53
III. EDTA Effects on Sieve Plate Fine Structure.	57
Methods and Materials.	57
A. Light Microscope Studies	57
B. Electron Microscope Studies	58
a. Plant Materials.	58
b. Fixation	58
c. Dehydration.	58
d. Embedding	59
e. Sectioning and Staining	59
Results and Discussion.	59
A. Light Microscope Studies	59
B. Electron Microscope Studies	62
IV. Mechanism of Action.	86
A. EDTA Treatment Time.	86
Methods and Materials	86
Results and Discussion.	87
B. Callose Synthesis Factor and EDTA Action	87
Methods and Materials	89
Results and Discussion	89
V. Summary	91

	Page
CHAPTER 3. Exudation Studies using Whole Plants.	93
I. Bark Flap Exudation Studies.	93
Methods and Materials.	94
A. Plant Materials	94
B. ¹⁴ C Labelling	94
C. Bark Flap Excision and Treatment.	94
D. Sampling and Analysis of Exudate.	97
Results and Discussion	100
A. Experimental Procedure and Assimilate Flow.	100
B. Effect of 10 mM EDTA treatment on Exudation	102
C. Concentration Effects	102
D. Exudation Rate and Duration.	105
E. Analysis of Exudate	111
II. Summary.	115
CHAPTER 4. Exudation Studies Using 5 Year Old <u>F. uhdei</u> Trees	117
Materials and Methods	117
A. Plant Materials.	117
B. Bark Flap Excision Technique	117
C. Treatments	122
i. Exudation Rate and Duration.	122
ii. Excised Bark Flaps	122

	Page
D. Carbohydrate Analysis	123
Results and Discussion	123
A. Exudation and Concentration Effects.	123
B. Excised Bark Flaps	127
Summary	133
CONCLUSIONS	136
LITERATURE CITED.	138

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INTRODUCTION

Sugars manufactured in green plants represent a product of the conversion of solar energy into chemical energy. The chemical energy stored in these sugars can be utilized by the plant for growth and development or stored in the form of large molecules such as starch or cellulose. For years man has used this stored chemical energy (biomass) to generate heat through the combustion of wood. Recent projects have been concerned with the conversion of biomass into fuels such as alcohol or gas. This project seeks to investigate a means of harvesting sugars from actively growing trees for use in gas or alcohol production. Specifically, it is proposed that mobile sugars can be tapped from woody trees on a yearly basis and then converted to methane or ethanol by the action of heterotrophic microorganisms. Methane or ethanol generated in this fashion could be collected, stored, and subsequently used as a fuel source. Such a system would utilize the chemical energy generated in photosynthetically active trees without harvesting or appreciably reducing the vigor of the tree.

Historically, the proposition of harvesting sugars from mature plants is not new. The tapping of sugar rich sap from palms has been practiced for many years. Darwin (1854) described sap collection from Jubaea palms in Chile. He reported that 409 liters (90 gallons) of sap could be obtained from one plant during a tapping period of several months. Gibbs (1911) wrote that a Corypha palm could produce 2,699 liters of sap in 132 days. In 1910, over 90 million liters of palm exudate were distilled in the Phillipines. Similar production figures are reported for Indonesia and India (Heyne, 1950; Morris, 1920; Pate, 1938).

Sugars have also been harvested from the sugar maple (Acer saccharinum), a temperate zone tree. Each year thousands of gallons of sugary sap are collected for use in maple syrup production. The sugar solution collected from the sugar maple is, however, of xylem origin and therefore very dilute. Moreover, the tapping period lasts for only a few weeks in the spring of each year. Nonetheless, few other examples of "nonpalm" woody trees being used for sugar tapping purposes can be cited.

The principal difficulty with using woody trees for sugar harvesting purposes is basically physiological in nature. For sugars to be collected from woody trees over an extended period of time, they must be harvested from the sugar transport system, the phloem. The phloem contains specialized cells (sieve elements) which form continuous tubes from the leaves to the roots (sieve tubes) and function specifically in sugar transport. To harvest phloem sugars, the sieve tube must be cut at some point along the trunk, allowing sugars to leak from the cut into a collecting vessel. However, when the sieve tube is injured, a wound reaction ensues whereby the sieve tube seals rapidly preventing the exudation of sugars. Sugar leakage from phloem sieve elements is effectively stopped shortly after wounding. Thus, unlike the sugar maple in which xylem sugars exude freely each spring, phloem sugars are not readily tapped.

It was postulated, nevertheless, that if the wounding reactions which seal cut sieve elements could be inhibited, then it may be possible to tap phloem sugars from woody trees. A consideration of the biochemistry of sieve tube sealing reactions provides some insight into the feasibility of this postulate.

The compound which seals or plugs the phloem sieve elements is a β -(1,3)-glucan known as "callose" (Currier, 1957). Callose is synthesized from UDPG (uridine diphosphate glucose) in the presence of a callose synthetase enzyme (Tsai and Hassid, 1963), which is thought to require calcium as an activator (Eschrich, 1965; Chu, 1973). It has been suggested that the removal of calcium from the site of callose synthesis may prevent its formation. King and Zeevaart (1974) reported that certain chelating agents enhance phloem sugar exudation and minimize callose deposition in excised leaves of a small herbaceous plant, Perilla crispa. They proposed that the chelating agents prevented callose formation by binding free calcium at the wound site. Based on these results, it was hypothesized that callose synthesis may be inhibited and sugar exudation enhanced in mature trees by treatment with chelating agents. Furthermore, if sugar exudation can be maintained over an extended period of time, then actively growing trees may prove to be a valuable source of biomass fuels.

Experiments designed to test this hypothesis were conducted from July, 1977 to June, 1980. The objectives of these experiments are outlined as follows:

1. To select a woody tree species with growth characteristics suitable for phloem exudation experiments.
2. To assess the effectiveness of chelating agents on the enhancement of sugar exudation from woody trees.
3. To investigate the physiology of the plants response to chelating agents and consider a possible mechanism of action.

LITERATURE REVIEW

I. Sieve Element Structure and Function in Angiosperms

Several excellent reviews of sieve element structure have been published (Esau, 1965; Fahn, 1967; Crafts and Crisp, 1971). A thorough discussion of papers concerned with sieve element structure is beyond the scope of this review. Mention will be made only of those papers helpful to understanding structure as it relates to function.

A. Sieve element size and longevity.

The size of secondary phloem sieve elements varies greatly among angiosperms (Esau, 1969). Length ranges from 100 μ to 500 μ (Fahn, 1959). Width varies from 10 μ to 70 μ (Parthasarathy, 1975). The functional life of most dicot sieve elements is one or two growing seasons (Esau, 1969). Sieve elements in Tilia, however, have been reported to function for 10 years (Evert, 1962). Perennial monocots which do not renew vascular tissue have sieve elements which function throughout the plant's lifetime (Parthasarathy and Tomlinson, 1967).

B. Sieve element differentiation

Immature sieve elements contain all organelles normally present in meristematic cells of the vascular cambium. Upon differentiation and maturation the sieve element nucleus, tonoplast, mitochondria, dictyosomes, and ribosomes disappear (Bouck and Cronshaw, 1965). The mature sieve element possesses a functional plasmalemma, some parietal endoplasmic reticulum, and a fibrillar protein known as P-protein. This P-protein (phloem protein) has been thought to result from the breakdown of cell organelles in differentiating sieve elements (Crafts and Crisp, 1971). Recent investigations have suggested a cytoplasmic origin for P-protein (Eschrich, 1975).

Pronounced changes in the sieve element wall occur concurrent with organelle disappearance. The primary wall differentiates into a sieve plate containing sieve pores lined with the plasmalemma of contiguous sieve elements (Esau and Cronshaw, 1965). Encircling the sieve plate pore is a thin layer of callose remaining from initial state of sieve plate differentiation (Esau and Cheadle, 1965). Diameter of the sieve plate pores varies from 1 to 5μ in functional sieve elements (Nobel, 1974). Northcote and Wooding (1966) and Tucker and Evert (1969) present complete descriptions of sieve element differentiation for tree species.

C. Structure of sieve elements and translocation

The structure of sieve elements in relation to function has been the subject of several reviews (Northcote and Wooding, 1968; Esau, 1969; MacRobbie, 1971). Several theories have been proposed to account for the mechanism of phloem translocation. Münch (1930) proposed a pressure flow mechanism suggesting that an osmotically generated hydrostatic pressure gradient exists in individual sieve tubes causing mass flow of water and solutes from areas of high pressure to areas of low pressure. Spanner (1958) suggested that an electro-osmotic pump located at the sieve plates accounts for translocation. An activated mass flow or streaming theory invoking cytoplasmic strands and peristaltic action was proposed by Thaine (1969).

The majority of workers in phloem physiology favor Münch's hypothesis. Recent structural and physiological data supports the pressure flow mechanism (Fischer, 1974). An evaluation of the Münch hypothesis for phloem transport is presented by Fischer (1978).

II. Callose Physiology

A. Occurrence

Callose, a β -(1,3)-glucose polysaccharide, is found in the sieve elements of both angiosperms and gymnosperms. During initial stages of angiosperm sieve plate pore development, callose platelets form at the future pore site (Esau, 1969). After pore differentiation is complete a thin layer of callose remains forming a collar around the pore (Anderson and Cronshaw, 1969). Plasmodesmata connecting the sieve tube member and its adjacent companion cell are also encircled by callose (Eschrich, 1975). Conifers exhibit callose in the sieve areas of phloem sieve cells (Kollmann and Schmacher, 1962; Evert et al. 1973). In both angiosperms and gymnosperms callose is deposited between the plasmalemma and cell wall (Esau, 1969) and is thought to be attached to the cell wall (Eschrich, 1975). These deposits exhibit a yellow fluorescence under UV light when stained with alkaline aniline blue (Eschrich and Currier, 1964).

Callose has also been found in the gametophytes of phanaerograms and cryptograms (Galouzot, 1970; Gorska-Bulass, 1968) and in the pollen mother cells of Cucurbita ficifolia flowers (Eschrich, 1965).

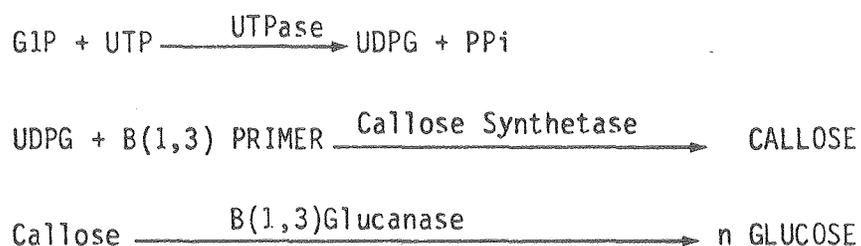
B. Metabolism

The chemical structure of callose was first resolved by Kessler (1958). Isolating dormancy callose from Vitus vinifera sieve plates, he reported it to consist of β -(1,3)-linked glucose units and small amounts of uronic acids. Feingold (1958) proposed that the glucose units were transferred by uridine diphosphate glucose (UDPG) to a β -(1,3)-linked primer, probably laminaribiose. A UDPG: β -(1,3)-glucan glucosyl

transferase was isolated by Hassid et al. (1959) and found to be present in sieve tube exudates (Eschrich et al., 1964).

Callose is easily degraded by β -(1,3)-glucanase found in sieve tube exudates of many plants (Eschrich, 1961). Both endo- and exo- β -(1,3)-glucanases have been isolated.

The enzyme systems for callose synthesis and degradation are thought to be located in plasmalemma (Crafts and Currier, 1963; Chu, 1975). A scheme for callose synthesis and degradation may be outlined as follows:



C. Factors Affecting Formation

Callose synthesis can occur as a result of various stimuli. Wounding from mechanical injury or infectious disease organisms has been shown to cause large callose depositions (Crafts and Crisp, 1971). Ultrasound (Currier and Webster, 1964), heat (McNairn and Currier, 1968), TIBA (Lerch, 1960), boron (McNairn and Currier, 1965; Currier, 1957), endothal (Dunning, 1959) and eosin Y (Schumacher, 1930) have all been reported to stimulate callose synthesis in sieve plate pores. Attempts to correlate low temperature treatment and callose deposition have not been successful (Giaquinta and Geiger, 1973). Eschrich (1975)

claims that the callose deposition response probably occurs within one minute after an appropriate stimulus.

Callose synthesis may also result from physiological factors. Masses of callose can accumulate in the sieve pores of temperate zone deciduous tree species in the autumn before dormancy commences (Evert and Murmanis, 1965; Kollmann and Schumacher, 1962). This "dormancy" callose may be redissolved in the spring if sieve tubes are reactivated. Datko and MacLachan (1968) report that indole acetic acid (IAA) stimulates β -(1,3)-glucanase activity and suggest that this may be a means of reactivating secondary phloem in the spring. However, Abeles and Forrence (1970) suggest IAA, GA₃ (gibberellin), and CK (cytokinin) inhibit β -(1,3) glucanase activity while ethylene enhances it in bean leaves. Control of dormancy callose synthesis and breakdown is apparently unclear. Crafts and Crisp (1971) report extensively on dormancy callose in grapes.

D. Function

Most investigators agree that callose serves a sealing function in phloem sieve tubes (Crafts and Crisp, 1971). Callose deposits in sieve plate pores effectively block the transport of phloem sap and thereby prevent "bleeding" when the system is injured. Various workers have reported that translocation is severely restricted or completely stopped as a result of callose deposition (McNairn and Currier, 1968; Shih and Currier, 1969; Currier, 1957, McNairn, 1972). Eschrich (1975) suggests that convincing evidence for this function is still wanting.

Functional sieve elements are reported to contain little or no callose. Zimmermann (1960) indicated that conducting sieve tubes of trees had no detectable callose during summer months when fresh samples

of bark were sectioned. Evert and Derr (1964) showed that functional sieve tubes of six species of deciduous trees were devoid of sieve plate callose. More recently, Fisher (1975), using radioactive tracers to identify functional sieve tubes and electron microscopy to study sieve plate pores, concluded that callose was not present in functional sieve elements of soybean. Thus far, evidence strongly suggests that callose is not functional in the translocation process. Eschrich (1975) suggests callose may function to control water balance in sieve tubes.

III. Exudation

Since the sieve plate pores of most angiosperms plug rapidly upon injury, it has been difficult to collect substantial amounts of phloem sap. Nonetheless, attempts have been made to do this on a few species. The techniques used can be classified into 3 categories: the incision method, the aphid stylet method, and chemical promotion of exudation.

A. The Incision Method

Plants which do not have a rapid plugging response may exude phloem sap over a limited period of time.

a. Dicots

Hartig (1860) was first to observe short term phloem exudation from Cucurbita species. His technique simply involved cutting a section of the stem and collecting droplets of sap which surfaced. Subsequent experiments have used Hartig's technique to study phloem exudation and translocation in various Cucurbita species (Zacharias, 1884; Crafts, 1936). Currently, Cucurbita maxima, pepo, and ficifolia are used for these studies (Eschrich, 1975). Huber (1953) found that the incision method could produce substantial quantities of phloem sap from Fraxinus ornus, a deciduous tree. Huber noted that in Sicily, F. ornus phloem

sap had been collected for commercial purposes. (The sap is harvested, dried, and sold as a product called "manna"). Zimmermann (1957a) found that Fraxinus americana, a deciduous tree native to the eastern United States, would produce exudate when the phloem was interrupted. He used this technique to study phloem translocation (1951b, 1958a). Evert et al. (1968) conducted similar experiments with Tilia cordata. The castor bean, Ricinus communis, is noted as a good source of phloem sap (Milburn, 1970). Interestingly, Milburn (1972) found that massaging the Ricinus stem prior to sap collection enhanced the amount of exudate produced.

The incision method has recently been used to collect phloem sap from legume fruits (Pate et al., 1974). This technique was later used to study fruit nutrition, xylem to phloem transfer of solutes, and translocation from leaves to fruits (Pate et al., 1975; Sharkey and Pate, 1976).

b. Monocots

Perhaps the greatest quantities of phloem sap have been obtained from monocots. Several genera of Asian, African, and South American palms have been used to produce thousands of tons of palm sugar (Child, 1964). Before sugar was produced commercially from sugar cane and sugar beets, people of these tropical regions used the palm exudate extensively as a sugar source. Tammes (1933) found that several Agave species exude copious amounts of phloem sap. Tammes and Van Die (1964) excised part of the male inflorescence of Yucca flaccida and collected exudate for several days in a beaker of water. They noted that the stalk had to be recut periodically. Their extensive studies of Yucca

flaccida exudation produced substantial data relevant to phloem physiology.

B. The Aphid Stylet Technique

A very interesting and more elaborate method of collecting phloem sap was described by Kennedy and Mittler (1953). Certain aphid species feed exclusively on phloem sap (Auclair, 1963; Evert et al., 1968; Zimmermann, 1963). If the aphid is anesthetized while feeding and the stylets are severed from the body, exudation from the cut stylet continues. Exudation may continue for several hours and produce up to 5 mm³/hr (Zimmermann, 1971). This technique has been used to collect phloem sap from Tilia americana (Zimmermann, 1963; Evert et al., 1968), Juniperus communis (Kollmann and Dörr, 1966) and various Salix species (Mittler, 1954). Weatherley et al., (1959) and Zimmermann (1971) have studied phloem translocation using the aphid stylet technique.

C. Chemical Promotion of Exudation

Most recent attempts to obtain phloem sap involve the use of chemical agents to promote exudation. King and Zeevaart (1974) tested various compounds to assess their effectiveness in enhancing exudation from Perilla crispa leaves. They demonstrated that Ethylenediamine-tetraacetic acid (EDTA), Ethyleneglycoltetraacetic acid (EGTA), and citric acid were all effective in increasing the exudation of labelled assimilates from excised Perilla leaves. Of these 3 chelators, a 20 mM EDTA solution proved most effective. Anatomical studies indicated that callose deposits in the sieve plate pores of EDTA treated leaves were substantially less than control leaves. They theorize that EDTA acts to chelate calcium at the cut surface which may be required for sealing reactions.

Various workers have since used EDTA to enhance exudation. Tully and Hanson (1979) studied amino acid translocation from turgid and water stressed barley leaves using EDTA to promote exudation. Fellows et al. (1978) used this technique for phloem translocation studies in soybean. By excising the stylar tip of soybean pods under a 20mM EDTA solution they were able to detect substantial quantities of labelled assimilates in the collecting solution for 5 hours. They concluded that this method provides "an uncomplicated and reproducible means of collecting phloem sap flowing into the soybean pod". Hanson and Cohen (1979) report that 0.5 mM EDTA buffered to pH 7.5 with 10 mM HEPES will promote exudation from pea seedlings without damaging the tissue.

In addition to chelating agents, phloem exudation has been promoted in Cucurbita maxima, Cucumis sativus, and Cucumis maxima using a solution of dithiothreitol and 2-mercaptoethanol (Sabnis and Hart, 1969; Sloan et al., 1976; Sabnis and Hart, 1978). Exudates in these experiments were analyzed for phloem proteins.

CHAPTER 1. Preliminary Experiments

The objectives of the preliminary experiments were twofold: first, to select plant species appropriate for the proposed exudation studies; and second, to test the effectiveness of EDTA and citric acid in promoting phloem exudation.

METHODS AND MATERIALS

A. Plant Materials

Species selection was limited to evergreen trees which exhibit fast growth and are reasonably tolerant of environmental extremes. Based on these criteria, three species were selected: Eucalyptus sideroxylon var. rosea, Pinus pinea L., and Fraxinus uhdei. A fourth, Ricinus communis L., was included because of its frequent use in phloem exudation studies. A brief description of each species follows:

Ricinus communis L. - the common castor bean is a low growing, annual shrub known principally for its poisonous seeds. It has been used extensively in phloem transport and exudation studies. See Smith and Milburn (1980) and Figs. 1a, 2.

Eucalyptus sideroxylon var. rosea - selected for its rapid growth habit, the Pink Ironbark is an evergreen eucalyptus native to Australia. At maturity it stands 60-80 ft high and 20-30 ft wide. It is quite tolerant of heat and drought but is sensitive to freezing temperatures (Figs. 1b, 2).

Pinus pinea - a native of the Mediterranean region, the Italian Stone Pine is one of the hardiest pines. It tolerates high and low temperatures as well as drought and strong winds. It is a moderately fast growing evergreen tree standing 40-80 ft high and equally wide (Figs. 1c, 2).

Fraxinus uhdei - the Evergreen Ash is well noted for its rapid growth and strongly upright habit. Being a native of Mexico it is particularly tolerant of high temperatures and drought, but will drop most of its leaves in cold winter areas. At maturity, it can reach 80 ft in height and 40 ft in width. Of particular interest is its pinnately compound leaf of 5-9 leaflets (Fig. 3a,b).

B. Treatments

Either shoots or leaves of the selected species were excised and treated as follows:

<u>Species</u>	<u>Plant Part</u>	<u>Treatment</u>	<u>Trt. Time</u>
<u>E. sideroxylon</u>	Shoots (5 leaves)	20 mM EDTA	24 hr
		H ₂ O	24 hr
<u>P. pinea</u>	Shoots (12 cm)	40 mM EDTA	24 hr
		20 mM EDTA	24 hr
		H ₂ O	24 hr
<u>R. communis</u>	Leaf	20 mM EDTA	48 hr
		20 mM Citrate	48 hr
		H ₂ O	48 hr
<u>F. uhdei</u>	Leaflets	20 mM EDTA	48 hr
		H ₂ O	48 hr

Fig. 1a. Ricinus communis - Castor Bean

Fig. 1b. Eucalyptus sideroxylon var. rosea - Pink Ironbark

Fig. 1c. Pinus pinea - Italian Stone Pine

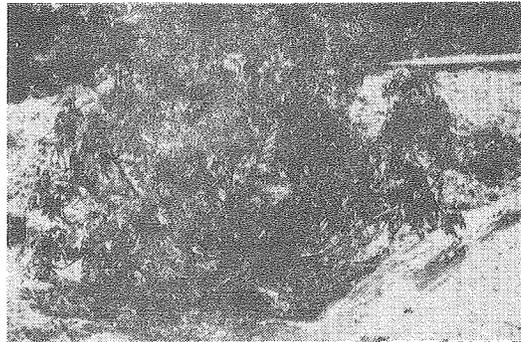


FIG. 1a

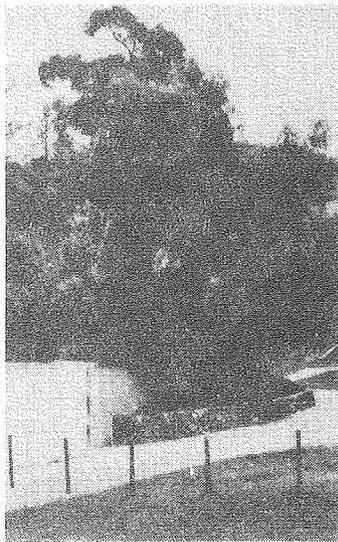


FIG. 1b

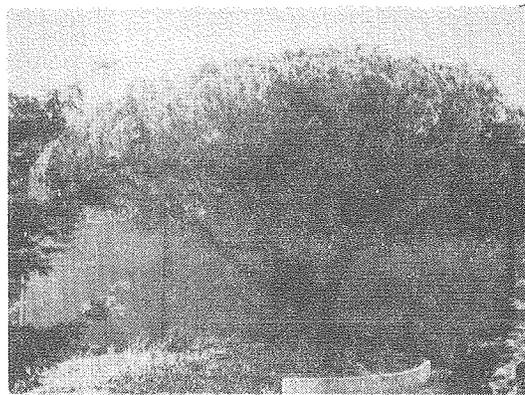


FIG. 1c

XBB 805-6856

Fig. 2. Ricinus communis leaf (left),
 Eucalyptus sideroxylon shoot (middle),
 and Pinus pinea shoot (right)



FIG. 2

XBB 806-9990

Fig. 3a. Fraxinus uhdei - Evergreen Ash

Fig. 3b. Pinnately compound leaf of F. uhdei

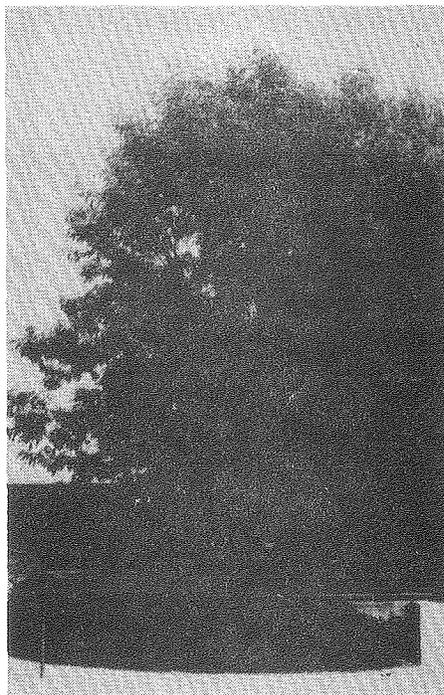


FIG. 3a

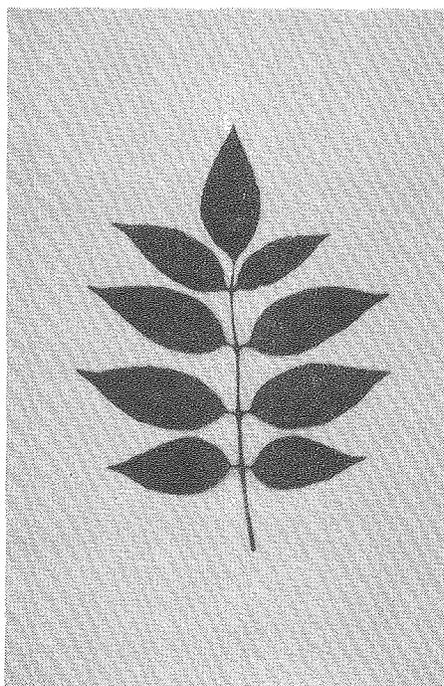


FIG. 3b

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Both EDTA and citrate were adjusted to pH 7.0 using 6N KOH. After excision of the shoot or leaf, the cut end was immersed to a depth of 2 cm in a treatment solution contained in 25 ml test tubes. All shoots and leaves were held in darkness throughout the treatment period. At the end of each treatment the leaf or shoot was removed and the treatment solution was analyzed for total carbohydrate content.

C. Analysis of Exudate

Treatment solutions were analyzed for total carbohydrate content using the anthrone method described by Yemm and Willis (1958) and amended by Jermyn (1975). Briefly, this assay involves combining a 1.0 ml sample of exudate with 1.0 ml hydrochloric acid, 0.1 ml 90% formic acid, and 8 ml freshly prepared anthrone reagent (20 mg anthrone in 100 ml 80% sulfuric acid). The mixture is heated for 12 min in a boiling water bath and then cooled for 5 min in ice water. After thorough mixing on a Vortex mixer the absorbance of each solution was read at 630 nm on a Gilford Spectrophotometer (Model 2000). Sucrose standards and blanks were prepared and read prior to analysis of treatments. Standard curves are shown in Fig. 4. After absorbance of each treatment solution was measured, sugar concentration was read from standard curves.

Fig. 4. Published and experimental standard curves for sucrose absorption at 630 nm using the anthrone assay of Yemn and Willis (1954).

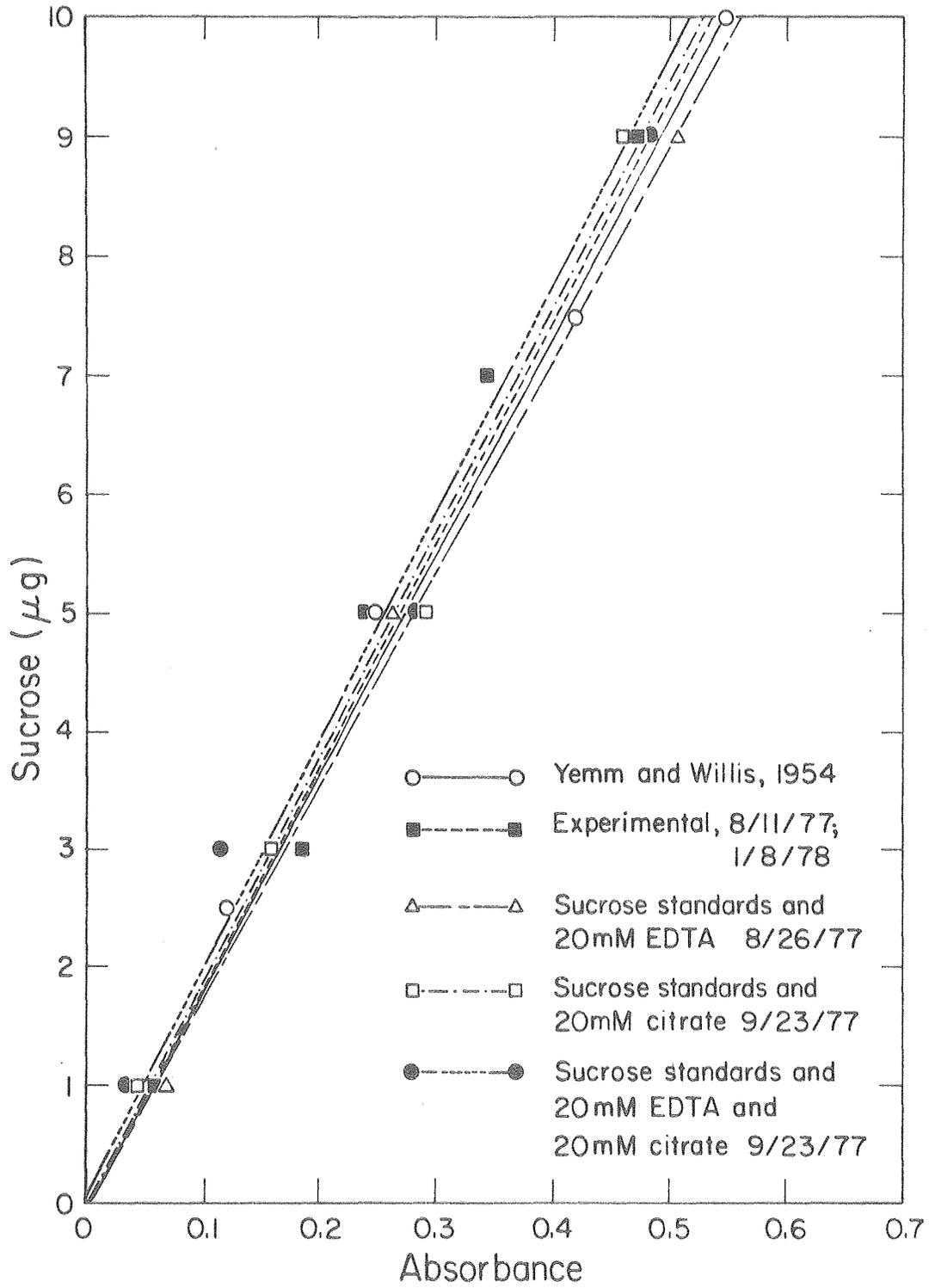


FIG. 4

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RESULTS AND DISCUSSION

Results presented in Tables 1 and 2 indicate a two-fold increase in exudation of sugars from E. sideroxylon and P. pinea shoots after a 24 hr treatment with 20 mM EDTA. A 40 mM EDTA treatment did not enhance exudation substantially more than the 20 mM treatment (Table 2).

Although an average increase in exudation was noted for the 20 mM EDTA treatment, a significant variation in the amount of exudation existed among replicates in both EDTA and H₂O treatments. Exudation from E. sideroxylon shoots treated with EDTA ranged from 7.5 to 27.5 µg, while H₂O treatments ranged from 6.25 to 12.5 µg. Similar differences were found among EDTA and H₂O replicates for P. pinea.

Such variations in exudation may have resulted from a number of factors. First, small differences in leaf area among replicates may have resulted in large variations in exudation. Even though shoot and leaf size among replicates was ostensibly minimized, it was difficult to ensure. As a result, differences in assimilation, phloem loading, and transport among leaves may have occurred and lead to some of the noted variability in exudation. Second, undetected physiological differences within shoots may have existed at the beginning of treatment periods and resulted in exudation differences. For instance, differences in leaf or shoot age may lead to physiological variations in source and sink strength among replicates. An attempt to minimize physiological differences was made by selecting shoots at roughly similar positions along a branch; however, no assurance of their equivalence could be guaranteed. Finally, since both Eucalyptus and Pinus species contain substantial amounts of oil and resins, it was

TABLE 1. Exudation of carbohydrate (CHO) from excised Eucalyptus sideroxylon shoots treated with 20 mM EDTA or H₂O for a 24 hr. period.

Treatment	Absorbance (630)	CHO in Sample ($\mu\text{g}/\text{ml}$)	Total CHO Exuded ($\mu\text{g}/\text{ml}/25\text{ml}$)
20 mM EDTA	.056	1.0	25
20 mM EDTA	.059	1.1	27.5
20 mM EDTA	.015	0.3	7.5
AVE.	.043	0.8	20.0
H ₂ O	.010	0.25	6.25
H ₂ O	.018	0.3	8.35
H ₂ O	.024	0.5	12.5
AVE.	.017	0.35	9.02

TABLE 2. Exudation from excised Pinus pinea shoots treated with 20 mM EDTA, 40 mM EDTA, or H₂O for a 24 hr. period.

Treatment	1 Hr.		24 Hr.		Total CHO Exuded ($\mu\text{g}/25\text{ml}$)
	Absorbance (630)	CHO in Sample ($\mu\text{g}/\text{ml}$)	Absorbance (630)	CHO in Sample ($\mu\text{g}/\text{ml}$)	
20 mM EDTA	.064	1.5	.024	0.53	50.75
20 MM EDTA	.082	1.9	.00	.00	14.75
AVE.	.073	1.7	.012	.265	37.75
40 mM EDTA	.021	0.45	.034	0.75	30.0
40 mM EDTA	.061	1.4	.027	0.58	49.8
AVE.	.041	0.92	.030	0.66	39.9
H ₂ O	.043	1.0	.00	.00	25.0
H ₂ O	.025	0.55	.00	.00	13.75
AVE.	.034	0.77	.00	.00	19.37

suspected that some variability among replicates was due to interference by these long chain hydrocarbons with chromophores in the carbohydrate assay. A cloudy precipitate formed in some treatment solutions after boiling with anthrone, resulting in those replicates being discarded. Although no direct evidence of such an interference was established, a duplicate experiment was conducted using a species lacking oil cells or resin canals.

Table 3 shows that exudation from R. communis leaves was substantially enhanced by a 20 mM EDTA treatment. Approximately twice as much carbohydrate was found in EDTA treatments than in H₂O treatments. Of more immediate concern, however, was the observation that no cloudy precipitates were found in any of the treatment solutions after boiling with anthrone. This result suggested that oils and resins contained in E. sideroxylon and P. pinea may have interfered with the carbohydrate assay in the previous experiments.

It may also be noted in Table 3 that a 20 mM citrate treatment did not appear to enhance exudation. Aside from two replicates, the citrate and H₂O treatments produced quite similar amounts of exudation. It was decided, therefore, that EDTA would be used in future experiments.

Also apparent in Table 3 is a notable amount of variability among replicates similar to that of previous experiments. Again, it was thought that much of this variability was due to differences in leaf size and physiology. In an effort to minimize these differences, a further experiment was conducted using the pinnately compound leaf F. uhdei. It was reasoned that variability may be reduced when leaflet pairs of equivalent size, age, and development are used.

TABLE 3. Exudation from excised *Ricinus communis* leaves treated with 20 mM Citrate, 20 mM EDTA or H₂O for a 48 hr. period.

Treatment	Absorbance (630)	CHO in Sample ($\mu\text{g}/\text{ml}$)	Absorbance (630)	CHO in Sample ($\mu\text{g}/\text{ml}$)	Total CHO Exuded ($\mu\text{g}/\text{ml}/48 \text{ hr}$)
20 mM EDTA	.344	6.49	.178	3.31	9.8
20 mM EDTA	.496	9.41	.544	9.90	19.31
20 mM EDTA	.079	1.41	.086	1.60	
20 mM EDTA	.209	3.90	.472	8.99	12.89
AVE	.372	5.30	.320	5.95	11.25
20 MM CITRATE	.126	2.36	.141	2.65	5.01
20 mM CITRATE	.082	1.57	.127	2.39	3.96
20 mM CITRATE	.089	1.60	.099	1.80	3.40
20 MM CITRATE	.503	9.50	.094	1.75	11.25
AVE	.300	3.75	.115	2.15	5.90
H ₂ O	.143	2.70	.090	1.71	4.41
H ₂ O	.00	.00	.031	0.63	0.63
H ₂ O	.059	1.0	.130	2.41	3.41
H ₂ O	.489	9.21	.210	3.95	13.16
AVE	.275	3.22	.091	2.18	5.40

TABLE 4. Exudation from excised *Fraxinus uhdei* leaves treated with 20 mM EDTA or H₂O for a 48 hr. period.

Treatment	1 hr		24 hr		48 hr		Total CHO Exuded ($\mu\text{g/ml}$)
	Asorbance	CHO in Sample ($\mu\text{g/ml}$)	Asorbance	CHO in Sample ($\mu\text{g/ml}$)	Absorbance	CHO in Sample ($\mu\text{g/ml}$)	
20 mM EDTA	.134	2.5	.151	2.82	.098	1.80	7.12
20 mM EDTA	.150	2.8	.125	2.35	.071	1.30	6.45
20 mM EDTA	.109	2.0	.486	9.2	.936	18.5	29.7
20 mM EDTA	.129	2.38	.152	2.85	.075	1.35	6.58
20 mM EDTA	.148	2.75	.121	2.25	.090	1.68	6.68
20 mM EDTA	.120	2.2	.190	3.55	.160	3.0	8.75
AVE	.132	2.43	.204	3.83	.238	4.4	10.88
H ₂ O	.206	3.83	.156	2.90	.079	1.40	8.13
H ₂ O	.172	3.21	.126	2.35	.096	1.75	7.31
H ₂ O	.156	2.90	.096	1.75	.130	2.40	7.05
H ₂ O	.162	3.05	.118	2.12	.108	2.00	7.17
H ₂ O	.092	1.70	.123	2.30	.096	1.75	5.75
H ₂ O	.156	2.90	.153	2.90	.068	1.20	7.00
AVE	.157	2.93	.129	2.38	.096	1.75	7.06

Results presented in Table 4 show that exudation was approximately 30% greater in EDTA treatments than H₂O treatments. Of greater importance, however, was the notable degree of agreement among replicates in both EDTA and H₂O treatments. Aside from one EDTA replicate, all exudation values were within 1.18 µg of the average value. This agreement among replicates was thought to reflect the close equivalence in size and physiological condition of leaflet pairs. Furthermore, even though only one EDTA replicate showed a significant increase in exudation, it was considered to be an indication that EDTA may be effective in promoting exudation in F. uhdei. Therefore, considering the reduction in variability among replicates and the possibility of exudation enhancement by EDTA, the pinnately compound leaf of F. uhdei was selected for use in further exudation studies.

SUMMARY

A 20 mM EDTA treatment enhanced exudation in all species tested. Exudation was not further enhanced by treating P. pinea shoots with 40 mM EDTA. No apparent effect was noted in R. communis using 20 mM citrate. Exudation variability among E. sideroxylon, P. pinea, and R. communis replicates was thought to be primarily due to differences in leaf size and physiological condition. Since variability was substantially reduced by using the pinnately compound leaf of F. uhdei, it was selected for use in future exudation studies.

CHAPTER 2. Exudation Studies Using Excised Leaflets of F. uhdei

Evidence presented in the preliminary experiments indicated that the pinnately compound leaf of F. uhdei was particularly well suited for exudation studies and that EDTA may be effective in promoting exudation from excised leaflets of F. uhdei. It was decided that further studies with F. uhdei may provide useful information concerning phloem exudation enhancement. The objectives of these experiments were: first, to develop a clear estimation of the effectiveness of EDTA in promoting exudation; second, to determine conditions for maximum exudation; and third, to characterize EDTA effects on sieve element structure and physiology.

Experiments designed to meet these objectives were divided into four sections:

- I. Analysis of EDTA effects on exudation amount, duration, and rate.
- II. Analysis of exudate composition.
- III. EDTA effects on sieve plate fine structure and callose synthesis.
- IV. Mechanism of action.

I. EDTA Effects on Exudation Amount, Duration, and Rate.

Procedures to determine exudation amount, duration, and rate from F. uhdei leaflets were essentially similar to those of the preliminary experiments. In these experiments, however, radioactive carbon, ^{14}C , was used to monitor exudation. Briefly, this method entailed

labelling leaves with $^{14}\text{CO}_2$ for a short period prior to excision. Leaflets were then excised, immersed in a treatment solution for a day or more, and then removed. The amount of label appearing in the treatment solution at the end of the treatment period was used as a measure of total exudation. Since ^{14}C can be measured quickly and precisely, this method was considered superior to the carbohydrate assay method used in the preliminary experiments.

METHODS AND MATERIALS

A. Plant Materials

Leaves were obtained from 10 year old F. uhdei trees grown on the Berkeley campus. Each leaf consisted of 4 leaflet pairs and one terminal leaflet (Fig. 3b). Only fully expanded disease-free, sun leaves were selected.

B. Photosynthetic ^{14}C Incorporation.

An attached, fully expanded leaf was labelled with $^{14}\text{CO}_2$ (specific activity 13.9 mCi/mM) for 20-30 min. The leaf was enclosed in an air tight plexiglass chamber measuring 30.5x20x2.5 cm (Fig. 5), and connected to a steady state gas recirculating apparatus designed to pass $^{14}\text{CO}_2$ across the leaf surface (as described in Platt, 1976; Fig. 15). $^{12}\text{CO}_2$ concentration was monitored with a Beckman Infrared CO Analyzer and $^{14}\text{CO}_2$ levels were detected with a Cary 401 vibrating reed electrometer. Chamber temperature was maintained between 25 and 29°C and illumination provided by GE Cool White high output fluorescent lamps ($600 \mu\text{Em}^{-2}\text{sec}^{-1}$).

Fig. 5. Pinnately compound leaf of F. uhdei enclosed in plexiglass chamber for ^{14}C -labelling. The leaf remained attached to the shoot throughout the labelling period.

Fig. 6. Excised leaflet of F. uhdei with petiolules immersed in treatment solutions. One leaflet of each matching pair was treated with EDTA and the other with distilled H_2O .

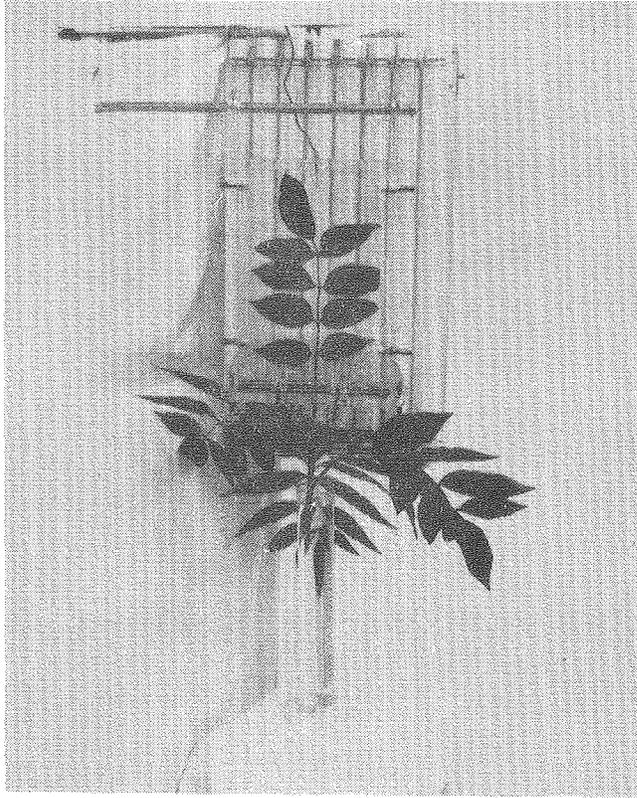


FIG.5

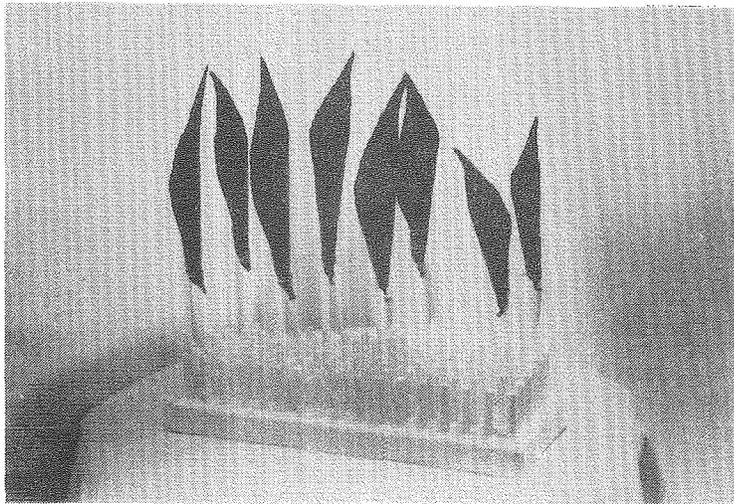


FIG.6

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C. Leaf Excision and Treatment.

Leaflet pairs were excised immediately after ^{14}C labelling. One leaflet of each pair was placed in a 10 ml tube containing 6.5 ml of an EDTA solution (adjusted to pH 7.0 with 6N KOH). The matching leaflet was placed in 6.5 ml distilled H_2O (Fig. 6). The leaflets remained in the solutions from 21 to 72 hrs with their petiolules immersed to a depth of 2 cm. Selection of leaflet pairs for particular treatments was randomized in order to minimize any labelling differences due to unequal distribution or assimilation of $^{14}\text{CO}_2$. All leaflets were kept in darkness during the treatment period. The amount of ^{14}C label appearing in the treatment solutions over a timed period was used as a measure of the effectiveness of various EDTA treatments. Periodically, leaflets were transferred to fresh preparations while 1.0 ml samples were taken from used solutions and analyzed for ^{14}C content. The 1.0 ml sample was added to 6.0 ml of Research Products International 3a70B complete counting cocktail and counted on a Packard Tri-Carb liquid scintillation spectrometer. Total counts in each sample were recorded in disintegrations per min (dpm).

Treatments varied in each experiment according to the following schedule:

<u>Exp.</u>	<u>Title</u>	<u>Trt</u>	<u>Trt Time</u>
1.	Total exudation	2.0 mM EDTA	21 hrs
2.	Exudation time course	2.0 mM EDTA	21 hrs
3.	EDTA concentration effects	0.5, 2.0, 10.0, 20.0 EDTA	24 hrs
4.	Exudation time course	2.0 mM EDTA	72 hrs.
5.	Exudation time course	2.0 mM EDTA	72 hrs.

RESULTS AND DISCUSSION

A. Exudation Enhancement

Results presented in Table 5 indicate a substantial increase in ^{14}C exudation resulting from a 21 hr treatment with 2 mM EDTA. In each of the two independent experiments shown in Table 5, a 6 to 11-fold average increase in exudation was found using EDTA. Individual replicates or leaflet pairs exhibited an exudation enhancement of 2- to 20-fold in EDTA treatments.

These differences in total exudation within EDTA replicates are not unexpected since difference in leaf size among leaflet pairs were apparent. This size difference is clearly illustrated in Fig. 3b where the top leaflet pair is smaller than the middle or bottom leaflet pairs. Differences in leaf area might be expected to lead to differences in assimilation, loading, and exudation of label among leaflet pairs. The observed variation in exudation among replicates may reflect such differences among leaflet pairs. Therefore, exudation values for EDTA and H_2O replicates were averaged when assessing treatment effectiveness.

Furthermore, since the absolute amount of exudation varied considerably between the duplicate experiments shown in Table 5, the ratio of exudation of EDTA treatments to H_2O treatments was considered a

TABLE 5. Exudation of ^{14}C From Excised leaflets of Fraxinus uhdei after treatment with 2 mM EDTA or H_2O for 21 hrs.

Leaflet Number	Treatment	^{14}C 9/8/78 in exudate (dpm/ml)	^{14}C 9/19/78 in exudate (dpm/ml)
1 a	EDTA	51,950	463,001
b	H_2O	6,044	122,333
2 b	EDTA	42,700	797,068
a	H_2O	1,971	27,269
3 a	EDTA	47,821	303,403
b	H_2O	2,605	
4 b	EDTA	38,206	255,803
a	H_2O	15,483	15,038
	EDTA ave.	45,094	458,708
	EDTA/ H_2O	6,525	42,824
	EDTA/ H_2O	6.91	10.71

more useful measure of EDTA effectiveness. For instance, between the two experiments there is an approximate ten-fold difference in average exudation. This difference was anticipated since differences in leaf size and age, rate of label assimilation, and length of labelling period were evident in each experiment. However, when the ratio of EDTA exudation to H_2O exudation is used to measure treatment effectiveness, variations between experiments in absolute amounts of exudation are less significant. Examination of these ratios in Table 5 shows only a 3.8-fold difference between the two experiments in EDTA exudation enhancement. The EDTA/ H_2O ratio was considered, therefore, as a good measure of treatment effectiveness.

B. Concentration Effects

It was noted from the preliminary experiments that a 20 mM EDTA concentration was moderately effective in enhancing exudation from F. uhdei leaflets. However, it was observed that localized necrosis and distortion of the leaf blade resulted from treatments lasting longer than 24 hrs. As a result a 2 mM concentration was selected for the first two experiments. Although a 2 mM treatment appears effective, the effectiveness of other EDTA concentrations on exudation enhancement had not been determined. Therefore, an experiment to determine the EDTA concentration most effective in enhancing exudation was conducted.

Results presented in Table 6 indicate that exudation was enhanced 10.39 times in 10 mM EDTA treatments while only 7.38, 6.20, and 4.43 times respectively in 2 mM, 20 mM and 0.5 mM EDTA treatments. It appears from these results that a 2 mM concentration was less effective than a 10 mM concentration, but more effective than either a 0.5 mM or 20.0 mM concentration. However, leaf distortion and necrosis was noted

TABLE 6. Effect of EDTA Concentration on ^{14}C Exudation Over a 48 hr. Period

Leaflet Number	Treatment	^{14}C in Sample (dpm/ml)	$\frac{\text{EDTA (dpm/ml)}}{\text{H}_2\text{O (dpm/ml)}}$
1a	0.5 mM EDTA	17,461	4.34
b	H ₂ O	4,021	
2a	2.0 mM EDTA	35,438	7.38
b	H ₂ O	4,801	
3a	10.0 mM EDTA	40,267	10.39
b	H ₂ O	3,874	
4a	20.0 mM EDTA	28,668	6.20
b	H ₂ O	4,624	
	H ₂ O Ave.	4,330	

in the 10 mM treatment as well as in the 20 mM treatment. Considering that the 2.0 mM treatment could effectively enhance exudation without causing apparent leaf damage, it was selected for use in future experiments.

C. Exudation Duration and Rate

The rate of ^{14}C exudation from 2 mM EDTA treatments increased steadily over a 21 hr period, while exudation from H_2O treatments declined after 4 hrs (Table 7, Fig. 7). Exudation rate was approximately 3 times greater in EDTA treatments than H_2O treatments after 4 hrs, while after 21 hrs exudation rate was approximately 13 times greater in EDTA treatments. The average total exudation from EDTA replicates was 9.12 times greater than from H_2O treatments.

Most of the label recovered in H_2O treatments appeared to be released during the first 4 hours after excision. The decline in exudation rate after 4 hrs suggests that the sieve plate pores may have become blocked during this period. Conversely, the increase in exudation rate from 0-21 hrs in EDTA treatments suggests that the sieve plate pores remain open up to 21 hrs after excision. Exudation beyond 21 hrs would be a function of not only the condition of the sieve plate pores but also of the amount of label remaining in the leaflet. Since a limited amount of label was initially fed to the leaf, exudation of ^{14}C would eventually be expected to decline regardless of the functional condition of the sieve plate pores.

Results from a 72 hr experiment (Table 8, Fig. 8) show a similar increase in exudation rate for 21 hrs in EDTA treatments and decline in exudation after 3 hrs in H_2O treatments. By 21 hrs, exudation rate was approximately 10 times greater in EDTA treatments. Again, most of the

TABLE 7. 21 Hr. Time Course of ^{14}C Exudation from Excised Fraxinus uhdei Leaflets Treated with 2 mM EDTA (9/21/78)

Leaflet number	Treatment	^{14}C in exudate (dpm)				Total ^{14}C in exudate (dpm) $\times 10^5$
		0-1 hr.	1-2 hr.	2-4 hr.	4-21 hr.	
1a	EDTA	58,579	81,697	99,242	1,463,414	17.02
b	H ₂ O	9,357	9,501	43,476	398,756	4.61
2a	EDTA	7,293	11,084	46,478	3,010,033	30.74
b	H ₂ O	6,416	6,561	20,522	250,522	2.83
3a	EDTA	9,993	16,161	47,606	3,146,853	32.20
b	H ₂ O	28,251	8,884	22,099	66,182	1.25
4a	EDTA	18,888	20,938	78,547	1,337,295	14.55
b	H ₂ O	9,706	11,827	38,543	105,815	1.65
	EDTA Ave.	23,688	32,470	67,968	2,239,398	23.63
	H ₂ O Ave.	13,432	9,193	31,087	205,318	2.59
	EDTA/H ₂ O	1.7	3.5	2.5	10.90	9.12

Fig. 7. ^{14}C exudation rate from excised F. uhdei leaflets treated with 2 mM EDTA or distilled H_2O . Exudation was monitored for 21 hrs.

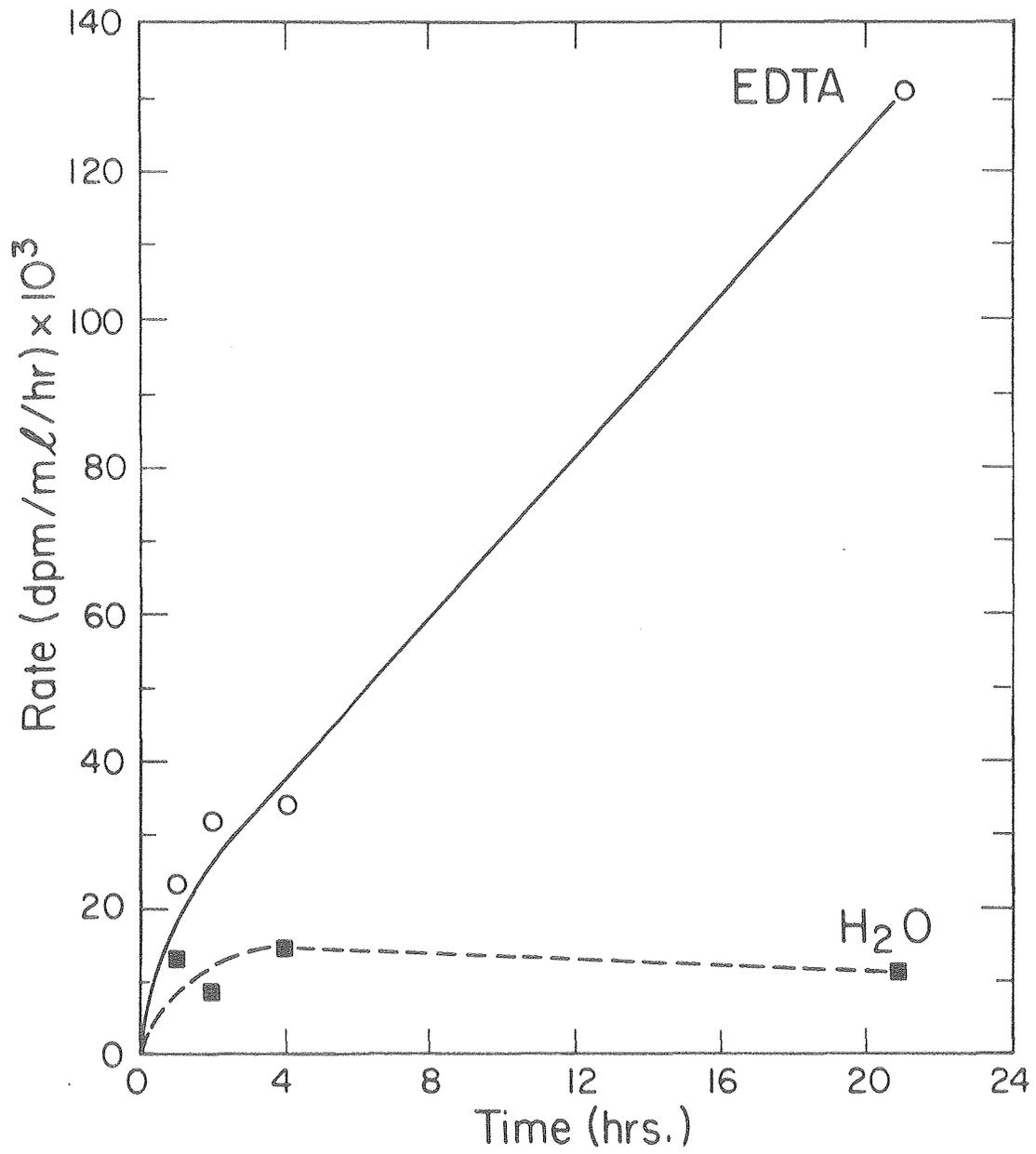


FIG. 7

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label released into H₂O treatments apparently occurred shortly after excision.

The exudation rate in EDTA treatments declined after 21 hrs. Exudation rate declined to 70% of the 21 hr maximum rate after 48 hrs and to only 24% of maximum after 72 hrs. Again, this decline in exudation after 21 hrs may have resulted from either a depletion of label or partial closure of sieve plate pores. Evidence presented in a following section indicates that sieve plate pores at the cut surface of EDTA treated leaflets appear to remain open at least 24 hrs after excision. It would seem, therefore, that at least some of the apparent decline in exudation rate after 21 hrs resulted from a depletion of label in the leaflet.

It may be noted in Fig. 8 that between 3 and 8 hrs after excision, exudation rate declined in both EDTA and H₂O treatments. Thereafter, exudation rate increased in EDTA treatments and continued to decline in H₂O treatments. This momentary decline in exudation in EDTA treatments after 3 hrs was thought to result from pressure differences between phloem sieve elements and the atmosphere. Since pressures within phloem sieve elements are greater than 1 atm (Hammel, 1968), severance of the sieve tube might be expected to cause a rapid release or surging of its contents. This response would dissipate quickly, however, as pressure differences between the sieve tube and the atmosphere diminished. Such a pressure release immediately after excision may be responsible for the initial large release of label (0-1 hrs) noted in both EDTA and H₂O treatments. The subsequent momentary decline in exudation in EDTA treatments (3-8 hrs) may likewise reflect an equilibrium between pressures in exposed sieve elements and the atmosphere. Phloem loading

TABLE 8 . 72 Hr. Time Course of ^{14}C Exudation from Excised Fraxinus uhdei
 Leaflets Treated With 2 mM EDTA or Distilled H_2O (9/29/78)

Leaflet number	Treatment	^{14}C in exudate (dpm/ml)							Total ^{14}C exudate (dpm) $\times 10^5$
		0-1 hr.	1-2 hr.	3-5 hr.	5-8 hr.	8-21 hr.	21-48	48-72 hr.	
1A	EDTA	37,306	47,109	46,073	89,286	1,120,797	1,859,504	396,443	35.96
b	H_2O	69,526	137,164	60,746	38,107	170,605	107,434	168,833	7.52
2a	EDTA	85,777	29,619	19,643	30,716	1,139,240	2,284,263	706,370	42.95
b	H_2O	16,304	9,350	13,038	1,483	44,820	103,214	195,188	3.83
3a	EDTA	61,587	65,430	15,621	14,308	324,615	304,635	198,402	9.84
b	H_2O	14,965	22,755	36,597	19,757	33,782	100,185	56,514	2.84
4a	EDTA	36,675	63,378	98,343	105,029	488,554	622,941	181,651	15.96
b	H_2O	42,220	33,527	24,279	87,750	98,077	77,552	38,227	4.00
	EDTA Ave.	55,336	51,361	44,920	59,834	768,301	1,267,835	370,716	26.18
	H_2O Ave.	35,476	50,699	33,665	36,774	86,821	97,096	114,705	4.55
	EDTA/ H_2O	1.56	1.01	1.33	1.62	8.8	13.05	3.23	5.75

Fig. 8. 72 hr time course of ^{14}C exudation rate from excised F. uhdei leaflets treated with 2 mM EDTA or distilled H_2O . Values represent average rates for a sampling period.

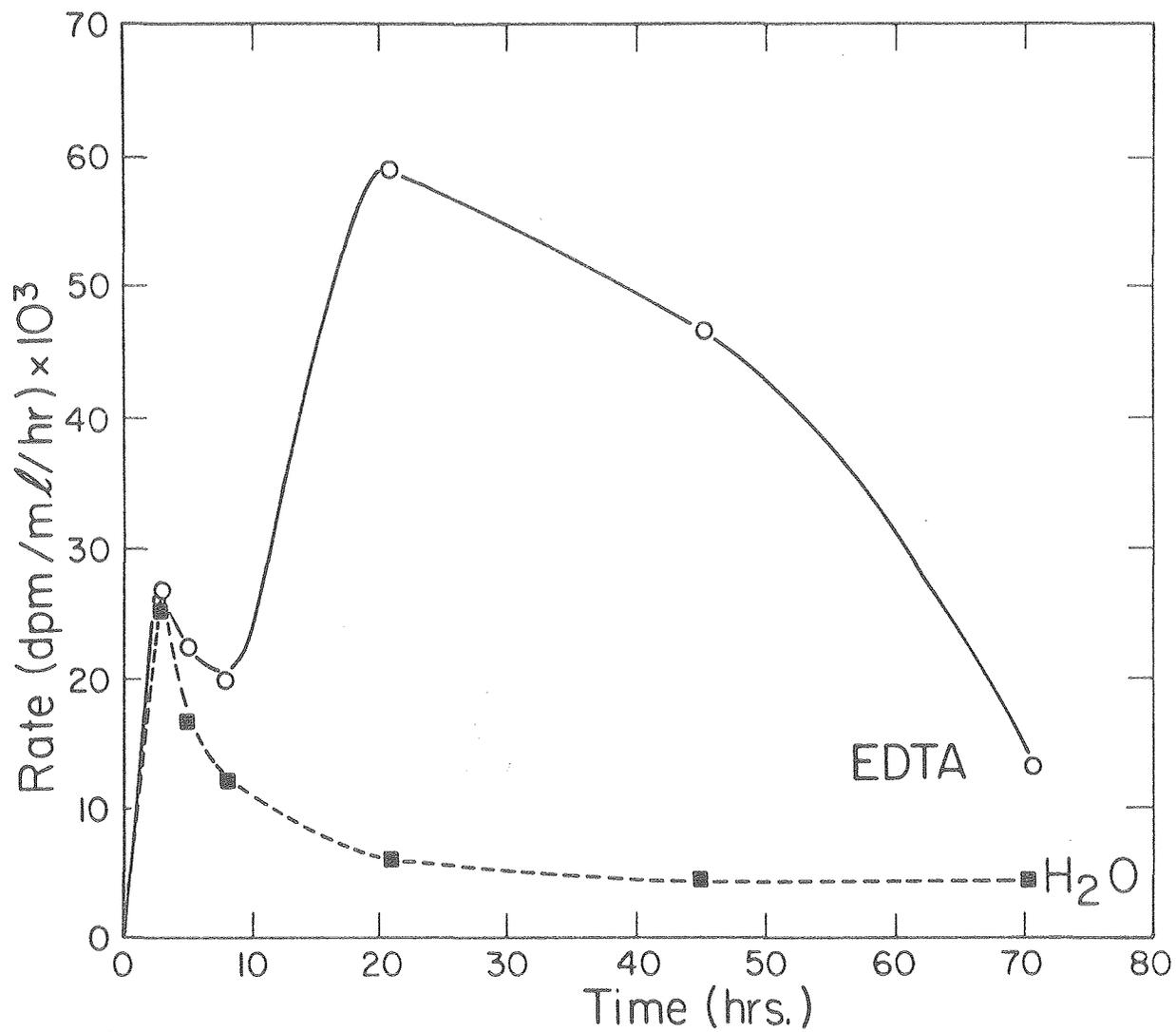


FIG. 8

XBL 801-4015

in sieve elements distal to the cut surface would later restore the pressure gradient needed to drive phloem transport and exudation. The decline in exudation in H₂O treatments was thought to reflect a change in the functional condition of sieve plate pores rather than be a consequence of such a pressure release.

The rate values in Figs. 7 and 8 are average values for exudation between sampling times. Concern as to whether these values accurately represent the time course of exudation prompted a duplicate experiment using one hour sampling periods. Results presented in Table 9 and Fig. 9 agree closely with those of previous experiments. Rate curves for one hour sampling periods (Fig. 9) and averaged sampling periods (Fig. 8) exhibit a close correspondence. In both experiments, the exudation rate for EDTA treatments rose to a maximum 21 to 24 hrs after excision, and then declined to a minimum after 72 hrs. After an initial release of label, exudation in H₂O treatments declined after 3 hrs in both experiments.

Of incidental interest in Fig. 9 is the absence of the momentary decline in exudation in EDTA treatments as noted in Fig. 8. This effect may have occurred but gone unnoticed, however, since samples were not taken during the 3-8 hr time period it was found to occur.

Nonetheless, the close correlation between rate data of the two experiments suggests that average values satisfactorily represent the time course of exudation. As a result, average rate values were used in subsequent experiments.

Table 9. 72 Hr Time Course of ^{14}C Exudation from Excised *Fraxinus uhdei* Leaflets Treated with 2 mM EDTA or Distilled H_2O (7/31/79)

Leaflet Number	Treatment	^{14}C in Exudate (dpm/ml)							Total ^{14}C in Exudate
		0-1 hr	1-2 hr	2-24 hr	24-25 hr	25-48 hr	48-49 hr	49-72 hr	
1 a	EDTA	1,189	1,666	47,617	2,067	34,058	946	9,122	96,665
b	H_2O	2,444	1,362	8,733	687	6,043	564	1,420	21,253
2 a	EDTA	711	1,916	76,688	3,967	33,115	888	8,699	125,984
b	H_2O	1,454	813	9,369	484	5,499	267	2,305	20,191
3 a	EDTA	854	840	73,560	3,008	42,595	1,145	7,171	129,173
b	H_2O	626	665	6,386	195	945	208	8,721	17,746
4 a	EDTA	88	837	32,494	2,139	20,313	550	3,579	60,000
b	H_2O	275	314	3,349	170	1,312	120	1,421	6,961
EDTA Ave.		710.5	1,314	57,589	2,795	32,520	882	7,193	14,714
H_2O Ave.		1,199	788	6,959	384	3,449	289	3,466	2,362
EDTA/ H_2O Ave.		0.59	1.66	8.72	7.27	9.42	3.05	2.07	6.22

Fig. 9. ^{14}C exudation rate over 72 hr period from excised leaflets of F. uhdei. Leaflets were treated with 2 mM EDTA or distilled H_2O . Values represent actual hourly rates, not averaged rates.

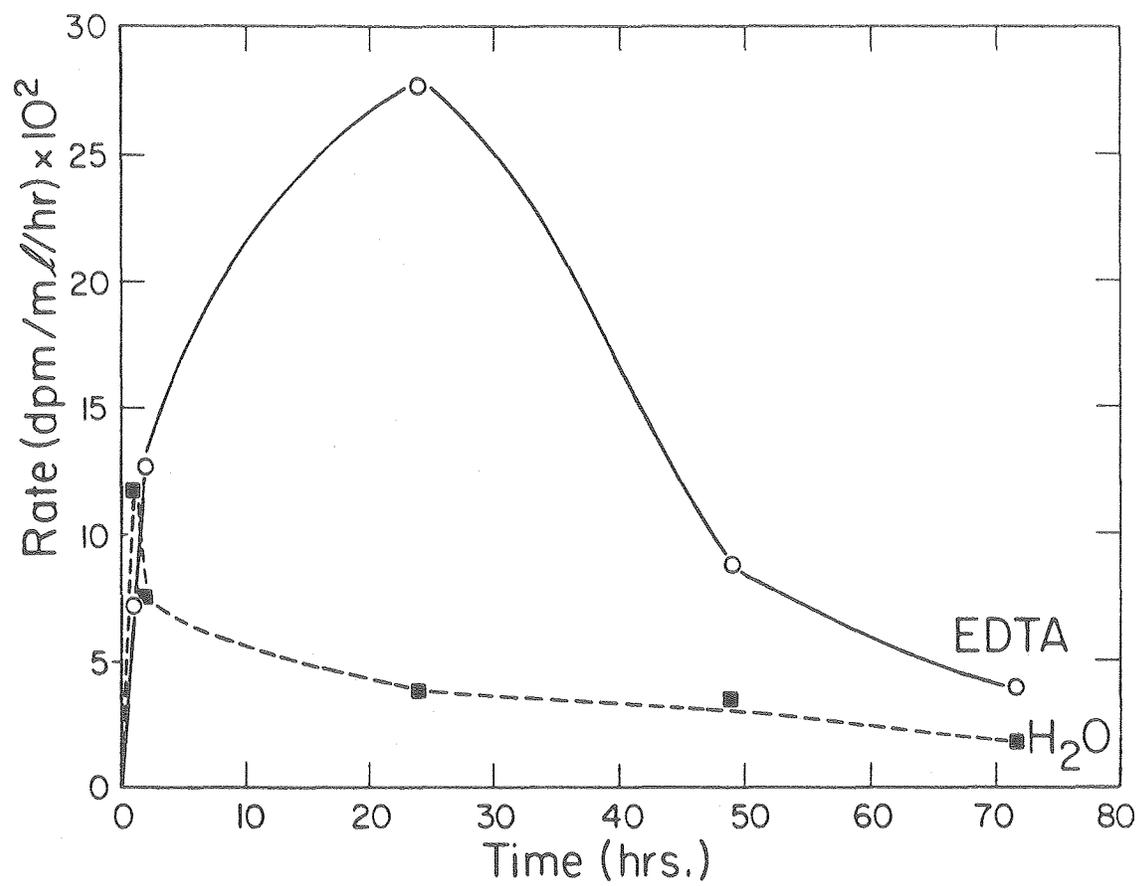


FIG. 9

XBL 80I-4014A

II. Analysis of Exudate

^{14}C recovered in the treatment solutions of the previous experiments was thought to represent materials principally of phloem origin. Since $^{14}\text{CO}_2$ assimilated into organic compounds in the leaf is transported to other parts of the plant via the phloem, it seems reasonable that labelled materials released from excised leaflets would be derived principally from phloem sieve elements. However, some label may have been released from injured cells at the cut surface. Additional label may have diffused across cells located close to the cut surface. Therefore, it seemed necessary to identify labelled materials in the exudate. It was thought that if the label appeared principally in compounds specific to phloem sieve elements, then it would be reasonable to conclude that the label represented materials of phloem origin.

METHODS AND MATERIALS

Soluble metabolites in exudate samples were separated using a two-dimensional paper chromatography system. Five 0.1 ml samples of EDTA and H_2O treatment solutions were spotted on Whatman No. 1 chromatography paper. The chromatograms were run in phenol : acetic acid : H_2O for 24 hrs and in butanol : propionic acid : H_2O for 24 hrs (Platt, 1976). Radioactive compounds were located on Kodak X-ray film after a 1-week exposure. Identification of labelled metabolites was made by co-chromatography with unlabelled standards. Radioactivity in each compound was determined using a Packard Tri-Carb liquid scintillation spectrophotometer. Chromatogram spots were eluted in distilled H_2O for 8 hrs and then transferred to scintillation vials containing 12 ml RPI

3a70B complete counting cocktail. Counts were made for 1 min and recorded in disintegrations per min (dpm).

RESULTS AND DISCUSSION

Approximately 94% of the label appeared in sucrose and oligosaccharides - raffinose, stachyose, and verbascose (fig. 10, Table 10). Small amounts of glucose and fructose were identified and lesser amounts of glutamate, malate, and citrate.

Analyses of phloem sap from various Fraxinus species closely parallel the analysis presented in Table 10. Zimmermann (1975) found phloem sap from Fraxinus americana to contain principally stachyose, raffinose, sucrose, mannitol, and trace amounts of verbascose. Kluge (1965) found similar results upon analyzing phloem sap of F. excelsior, F. oregona, F. ornus, and F. oxycarpa.

The correspondence between published analyses and the results presented in Table 10 suggests that the label appearing in treatment solutions represents compounds of phloem origin. The presence of large amounts of sucrose is characteristic of phloem sap, and, in this case, the high oligosaccharide content is distinctive of phloem sap from Fraxinus species. Although glucose, fructose, glutamate, malate, and citrate are not exclusively found in phloem sap, they all have been reported to occur in the phloem of various species (Ziegler, 1975). Therefore, it seems reasonable to conclude labelled exudates from excised F. uhdei leaflets are principally derived from phloem sieve elements.

Fig. 10. Autoradiograph of 0.1 ml sample of exudate from excised F. uhdei leaflets treated with 2 mM EDTA.

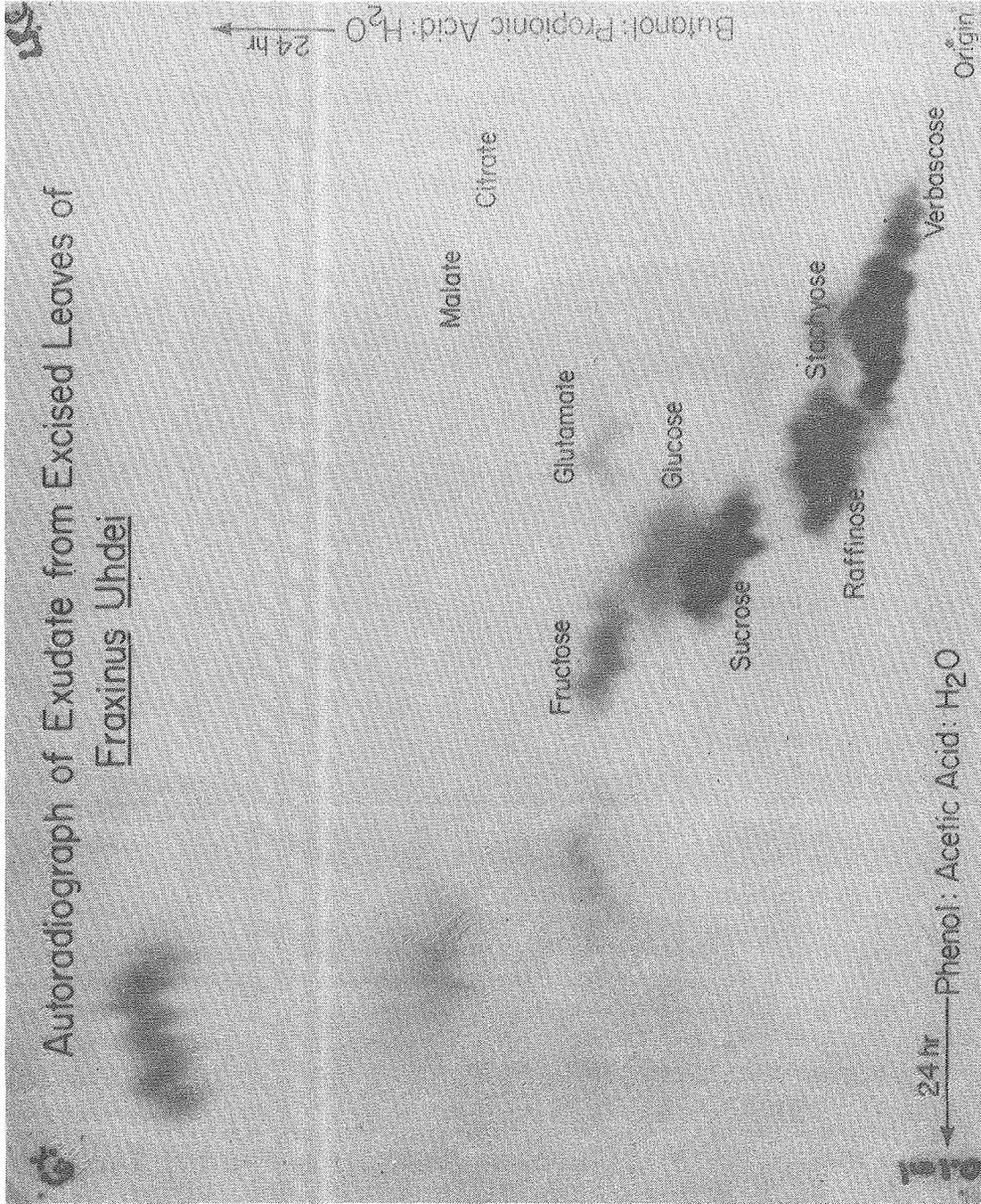


FIG. 10

XBB 806-7211

TABLE 10. Paper Chromatography and Autoradiography Analysis of 0.1 ml Exudate from 2.0 mM EDTA Treatment. Relative amounts of each compound given in dpm/ml

Compound	^{14}C in spot (dpm)	% of total
Citrate	480	<1.0
Fructose	18,215	2.9
Glucose	15,016	2.4
Glutamate	2,671	<1.0
Malate	876	<1.0
Raffinose	56,805	9.2
Stachyose	343,018	55.7
Sucrose	108,434	17.6
Verbascose	70,036	11.4

III. EDTA Effects on Sieve Plate Fine Structure

Experimental evidence thus far indicates that EDTA effectively enhances phloem exudation from excised leaflets of *F. uhdei*. It has been suggested by King and Zeevaart (1974) that EDTA enhances exudation by inhibiting callose formation in sieve plate pores. Using a callose fluorescence technique, they examined callose deposits in sieve plates of *Perilla crispa* leaves after treatment with EDTA or H₂O. Although they observed less callose in EDTA treated sieve plates, they also found a great deal of variability among replicates because tissue sections could not always be properly oriented. As a result they could not quantify precisely the number of fluorescent sieve plates per unit area of the phloem.

It was thought that electron microscopy may provide a more precise means of evaluating callose occurrence in sieve plate pores. The resolution of the electron microscope is sufficient to clearly identify phloem sieve plate pores and callose deposits contained therein. Such an analysis could provide direct evidence for a correlation between callose formation and EDTA enhanced exudation.

METHODS AND MATERIALS

A. Light Microscope Studies

Light microscope studies were conducted to locate phloem bundles in *F. uhdei* petiolule cross-sections. Fresh 0.5 mm sections were cut from the proximal end of excised leaflets. Sections were stained for 30 min with phloroglucinol and then with aniline blue for another 30 min. Phloroglucinol stained fibers, tracheids, and vessels red, while

aniline blue stained phloem cells blue. After staining, sections were viewed and photographed in a Zeiss light microscope.

B. Electron Microscope Studies

a. Plant Materials

F. uhdei leaflet pairs were excised and placed in test tubes containing either 2 mM EDTA or H₂O (fig. 6). After a 24 hr treatment period in darkness, the leaves were removed and prepared for fixation.

b. Fixation

2 mm discs were sectioned from the proximal end of EDTA and H₂O treated petiolules. The discs were cut longitudinally into four equal sections and fixed in 4% glutaraldehyde and 0.05 M Sorensen's phosphate buffer, pH 7.2, for 2 hrs. The sections were washed in 0.05 M Sorensen's buffer for 60 min (3 changes, 20 mins each) and then fixed in 1% osmium tetroxide for 2 hrs. The sections were washed again in Sorensen's buffer for 10 mins.

c. Dehydration

The fixed sections were dehydrated in an ethanol series - 30%, 50%, 70%, 85%, 90%, 95% for 10 mins each, and 100% for 2 changes at 20 mins each. While making ethanol changes, a thin film of alcohol was left over the tissue to prevent desiccation. After dehydration, propylene oxide (room temp.) was added to the tissue (2 changes, 20 min each). The propylene oxide was then discarded and a 1-1 propylene oxide-resin mix was added. The tissues remained in the propylene oxide-resin mix overnight on a slowly rotating carousel tilted at a 45° angle.

d. Embedding

Tissues were embedded in Spurr's low viscosity media (Spurr, 1969). The media consisted of: vinylcyclohexene dioxide (VCD), diglycidyl ether of polypropyleneglycol (DER 736), nonenyl succinic anhydride (NSA), and dimethylaminoethanol (DMAE) in a proportion of 10:6:26:0.4 (wt basis). The tissues remained in 100% media for 12 hrs and then were transferred to BEEM capsules containing fresh media. Blocks were cured in a 70°C oven for 24 hrs.

e. Sectioning and Staining

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 (100 mesh) and stained with 5% aqueous uranyl acetate for 30 min and Reynold's lead citrate for 5 mins. Sections were viewed and photographed on a Zeiss 9A electron microscope.

RESULTS AND DISCUSSION

A. Light Microscope Studies.

Phloem bundles are found radially dispersed in petiolule cross sections between the cortex and xylem (figs. 11, 12). Each bundle consists of sieve elements, companion cells, phloem parenchyma, and phloem fibers (stained red). Sieve elements are identified by their angular side walls and clear lumen, while companion cells appear very dense and are located adjacent to the sieve elements. Phloem parenchyma cells are larger in cross section than sieve elements and are interspersed throughout the phloem. Phloem fibers appear principally on the cortical side of the phloem bundle and have thick, rounded side walls.

Fig. 11. Cross-section of F. uhdei petiolule (top left). x 25.

Fig. 12. Cross-section of F. uhdei petiolule (top right) x 200.

Fig. 13. Thin cross-section of phloem bundle (bottom). x 250.

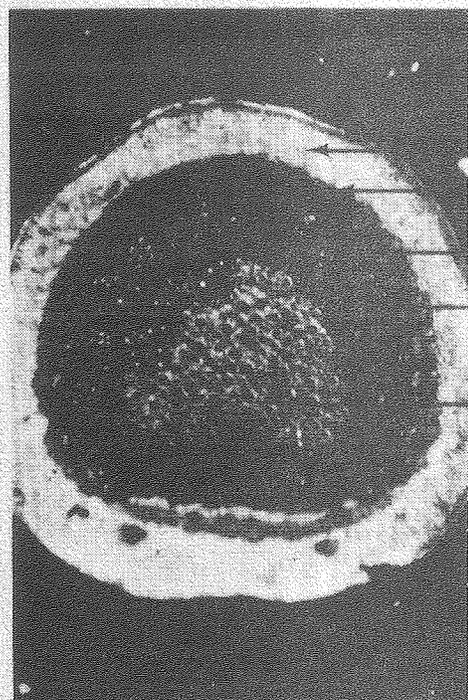


FIG. 11

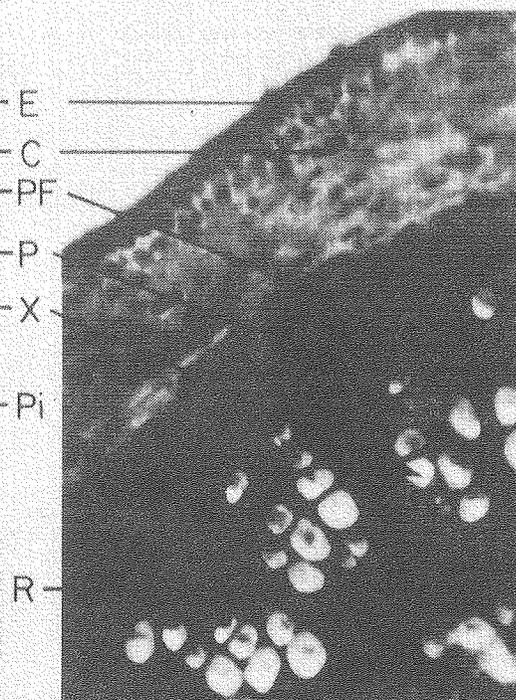


FIG. 12

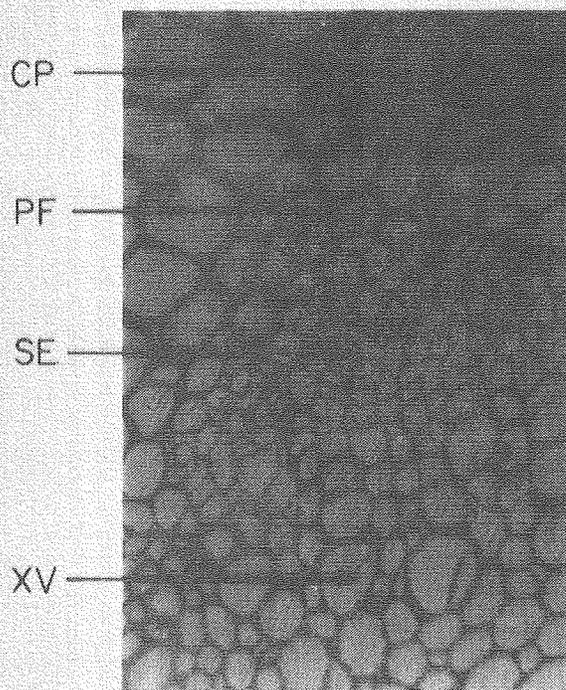


FIG. 13

- E = Epidermis
- C = Cortex
- PF = Phloem Fibers
- P = Phloem
- X = Xylem
- Pi = Pith
- CP = Cortex Parenchyma
- SE = Sieve Element
- XV = Xylem Vessel
- R = Ray

These various cell types are clearly seen in thin section (fig. 13).

Xylem vessels appear translucent in Figs. 11 and 12, while xylem fibers are stained a very dark red. Xylem and phloem rays project radially from the pith and appear black in these figures.

B. Electron Microscope Studies

Electron micrographs (Plates 1-9) of F. uhdei sieve elements exhibit features characteristic of angiosperms. Sieve tube members (STM) are seen connected end to end by sieve plates (SP) which contain sieve plate pores (SPP). Seen also in Plate 1 is P protein (PP) and callose (C).

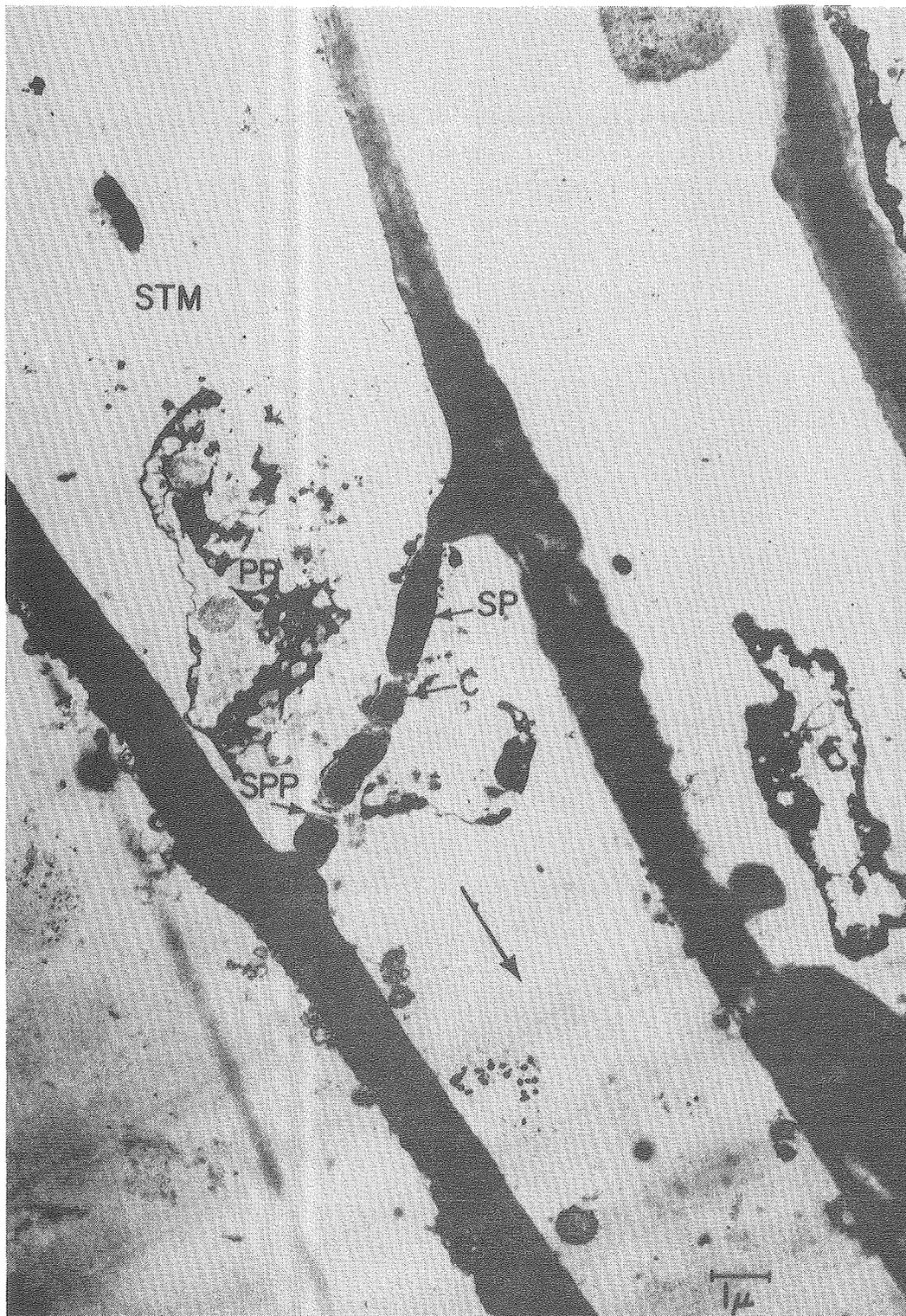
Callose appears as a less dense material lining the pores of the more dense sieve plates. Substantial amounts of callose were found in sieve plate pores of leaflets treated with H₂O (Plates 1-3b). Callose appears to virtually occlude the sieve plate pore in each of these sections. Little or no callose was seen in sieve plate pores of leaflets treated with 2 mM EDTA (Plates 4-8). The pores appear open in most cases, except when filled with P protein (Plate 8).

Quantities of P protein within sieve elements varied among replicates and between treatments. Generally, greater amounts of P protein were found in EDTA treatments, while lesser amounts were found in H₂O treatments. Some EDTA replicates, however, exhibited little P protein (e.g., Plate 4.). P Protein located in sieve plate pores may have caused a reduction in phloem exudation.

A precise quantitative correlation between callose occurrence and H₂O or EDTA treatments was not possible from these photomicrographs. Tissue section replicates were highly variable in each treatment sample and estimates of callose deposited per unit phloem area could not be

made. Based on general observations, however, it can be surmised that EDTA treatment causes a reduction in callose formation in sieve plate pores. In the absence of EDTA, callose appears to frequently restrict or occlude sieve plate pores. It seems reasonable to conject that EDTA acts to enhance exudation by suppressing callose formation. The mechanism by which EDTA suppresses callose formation is considered in the following section.

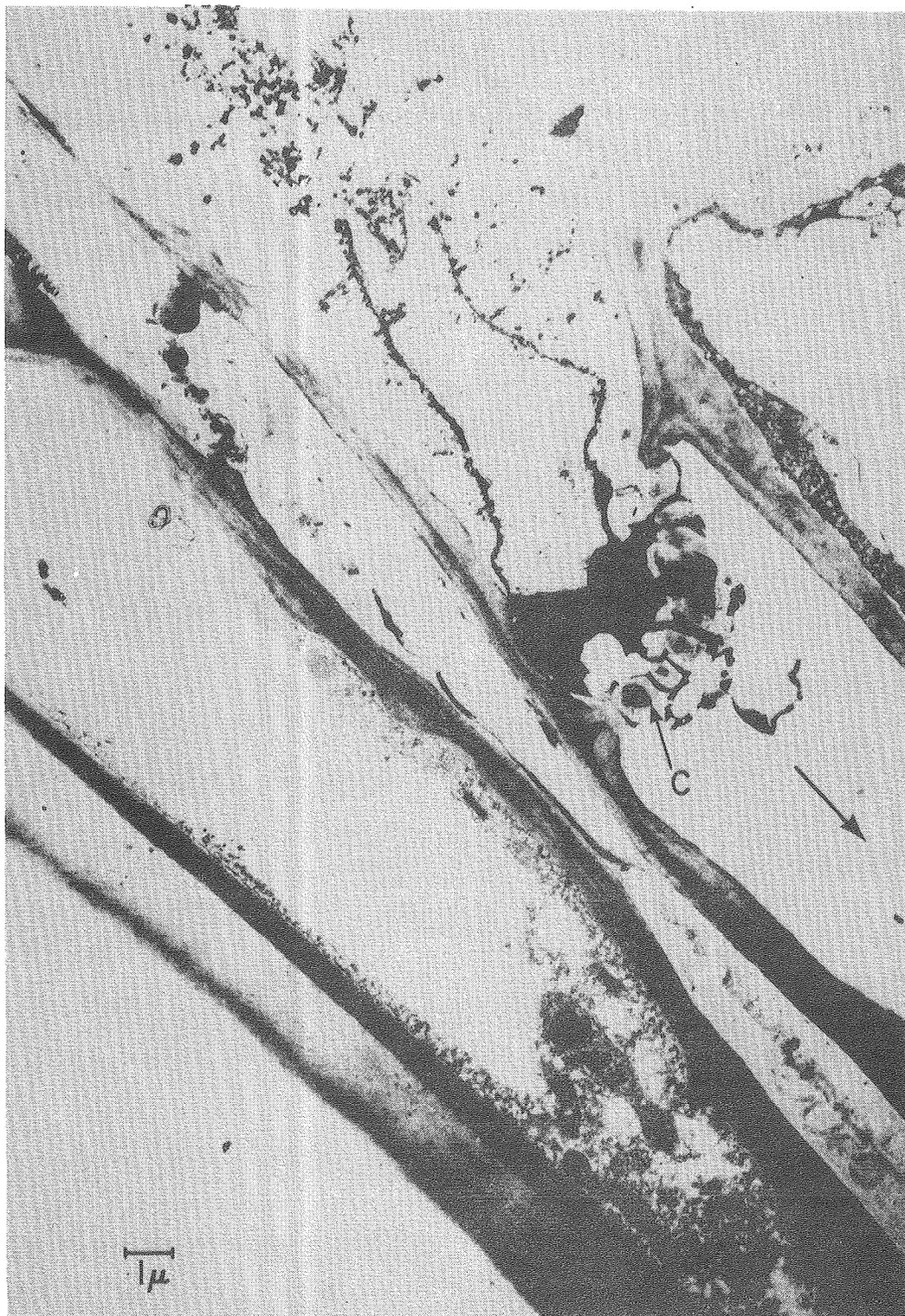
Plate 1. Longitudinal section of F. uhdei petiolule sieve element after 24 hr treatment with distilled H₂O. STM = sieve tube member, SPP = sieve plate pore, SP = sieve plate, C = callose, PP = P protein. Note callose deposits in sieve plate pores. Arrow indicates probable direction of phloem transport. x 10,000. (Note: Plastids and starch granules may be included in material designated as P-protein).



PL.1

XBB 802-1612

Plate 2a. Longitudinal section of sieve element showing callose deposits in sieve plate pores after 24 treatment in distilled H₂O. C = callose. Arrow indicates probable direction of phloem transport. x8,750.



PL. 2a

XBB 802-1604

Plate 2b. Higher magnification of plate 2a. Note sieve plate pores containing callose (C) and darkly stained P protein. Arrow indicates probable direction of phloem transport. x 10,200.



PL.2b

XBB 802-1603

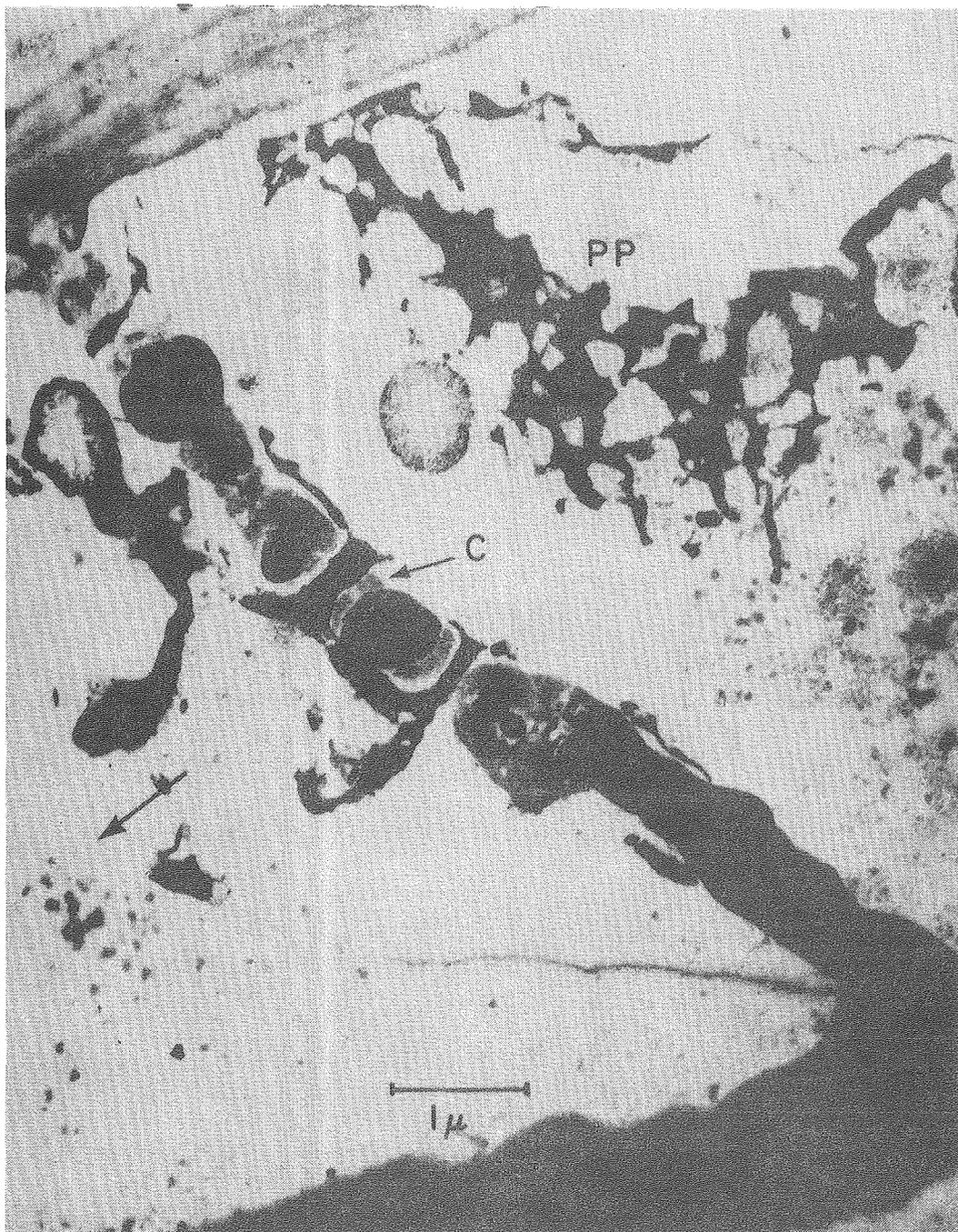
Plate 3a. Longitudinal section of sieve element showing sieve plate pore containing callose (C) after 24 hr treatment in distilled H₂O. Arrow indicates probable direction of phloem transport. x 11,400.



PL. 3a

XBB 802-1613

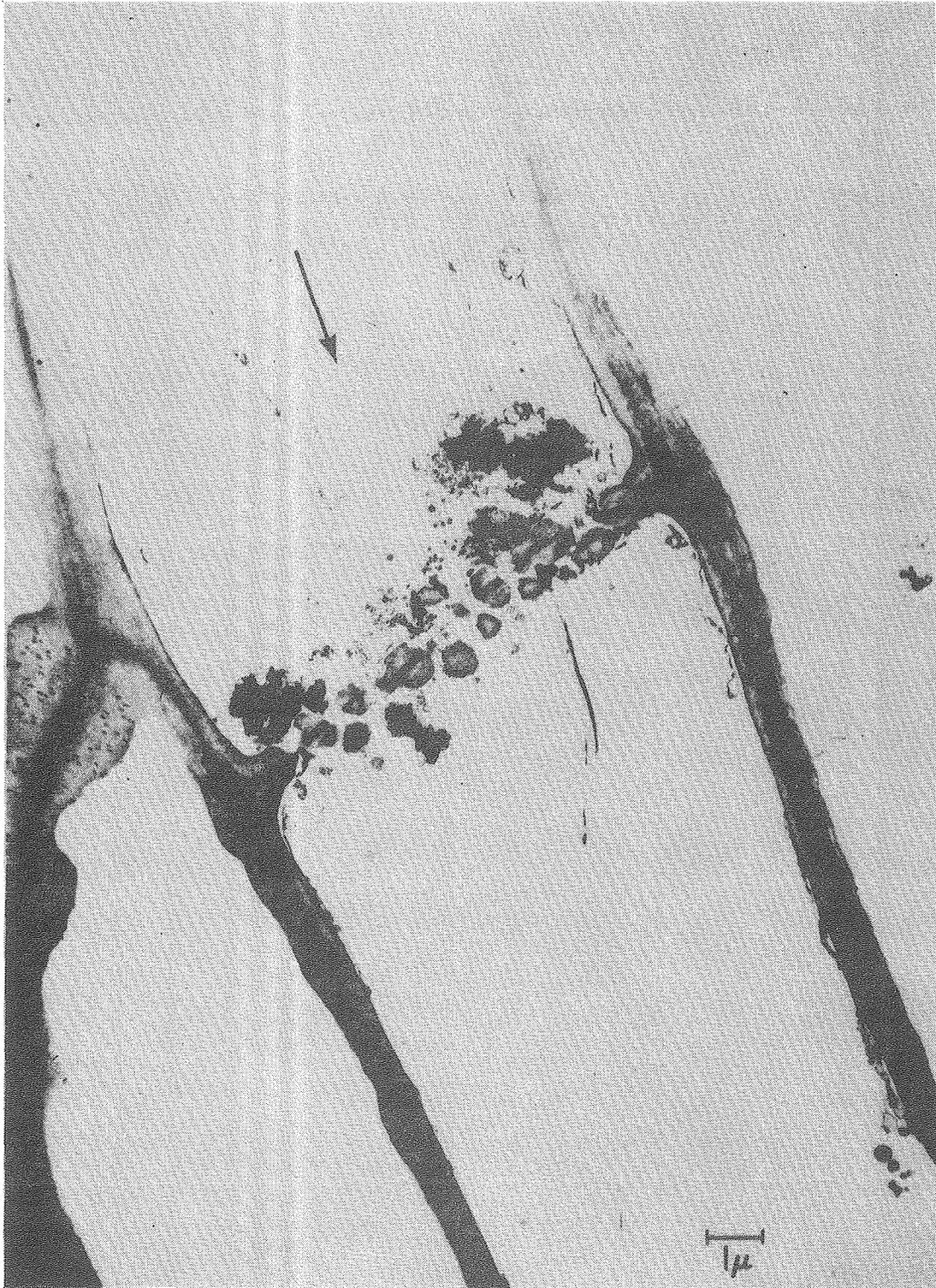
Plate 3b. Higher magnification of plate 3a. Note substantial deposits of callose (C) in sieve plate pore. Arrow indicates probable direction of phloem transport.
x 22,500.



PL. 3b

XBB 802-1614

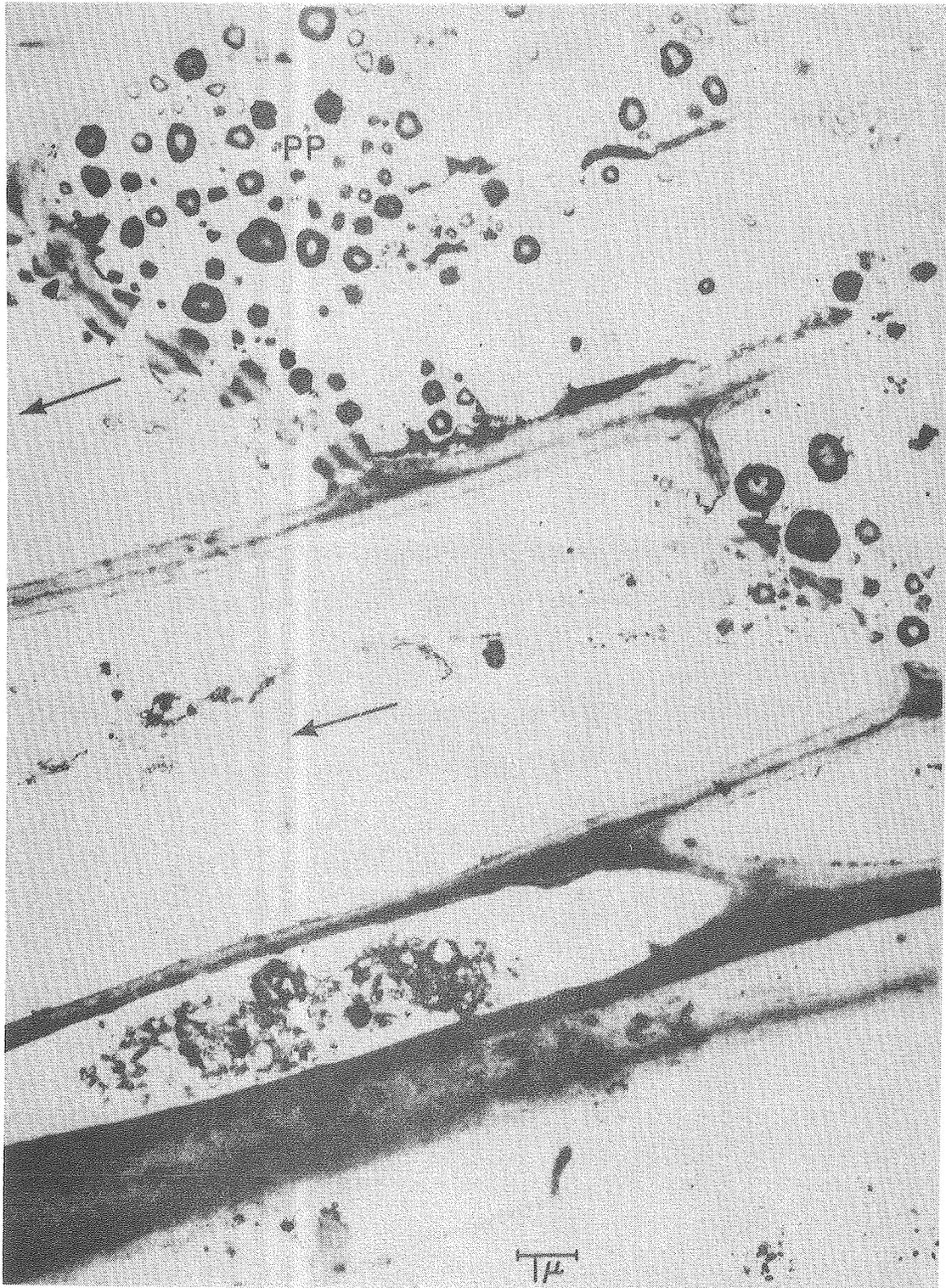
Plate 4. Longitudinal section F. uhdei sieve element after 24 treatment in 2 mM EDTA. Note sieve plate pores essentially free of callose. Arrow indicates probable direction of phloem transport. x 10,200.



PL. 4

XBB 802-1609

Plate 5. Longitudinal section of adjacent sieve elements from F. uhdei petiolule. Sieve plate pores appear open after 24 hr treatment with 2 mM EDTA. Arrow indicates probable direction of phloem transport. x 10,200.

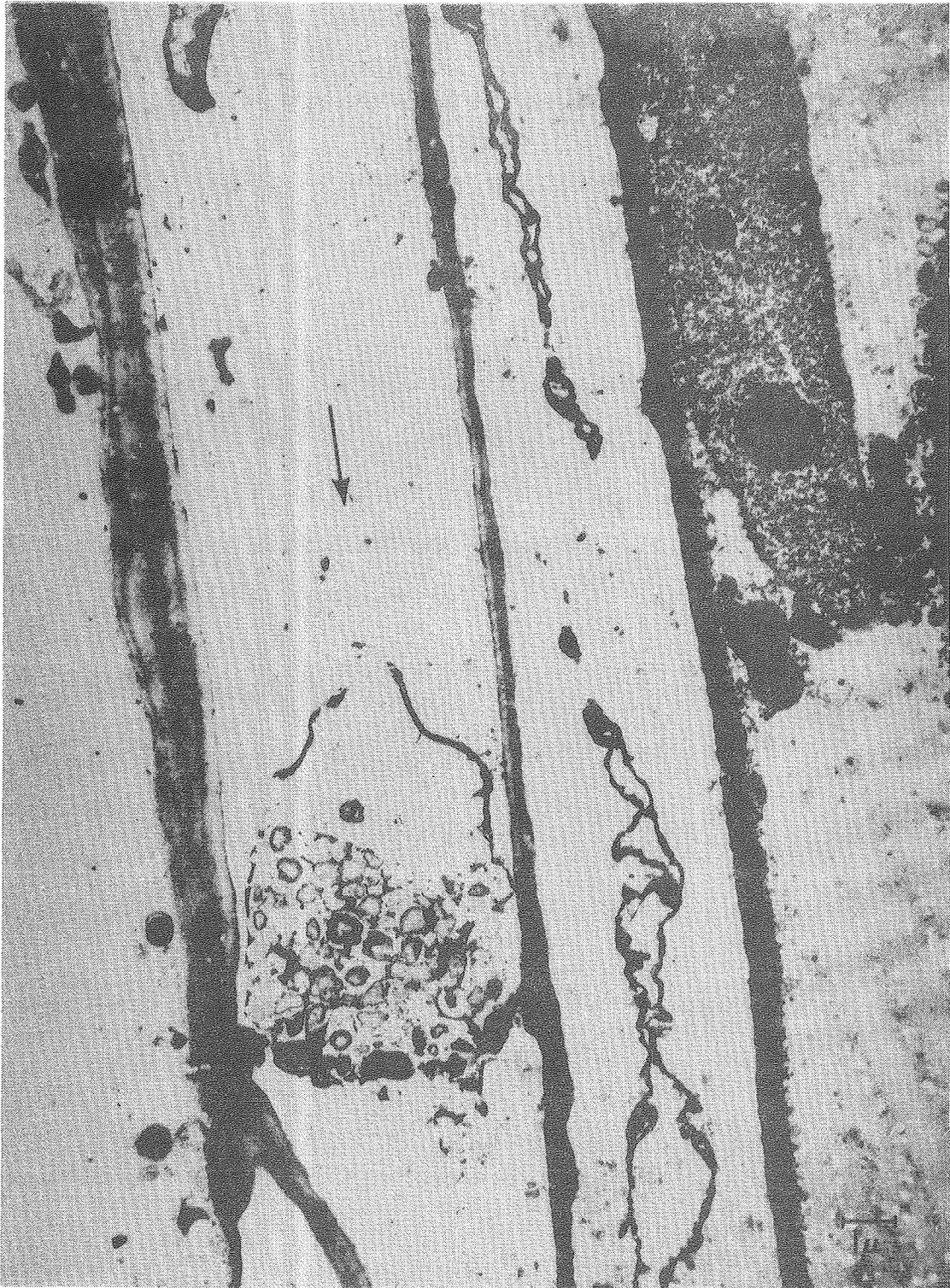


PL. 5

XBB 802-1608

Plate 6. Longitudinal section of sieve element showing sieve plate pores essentially free of callose after 24 hr treatment with 2 mM EDTA. Arrow indicates probable direction of phloem transport. x 1,750.

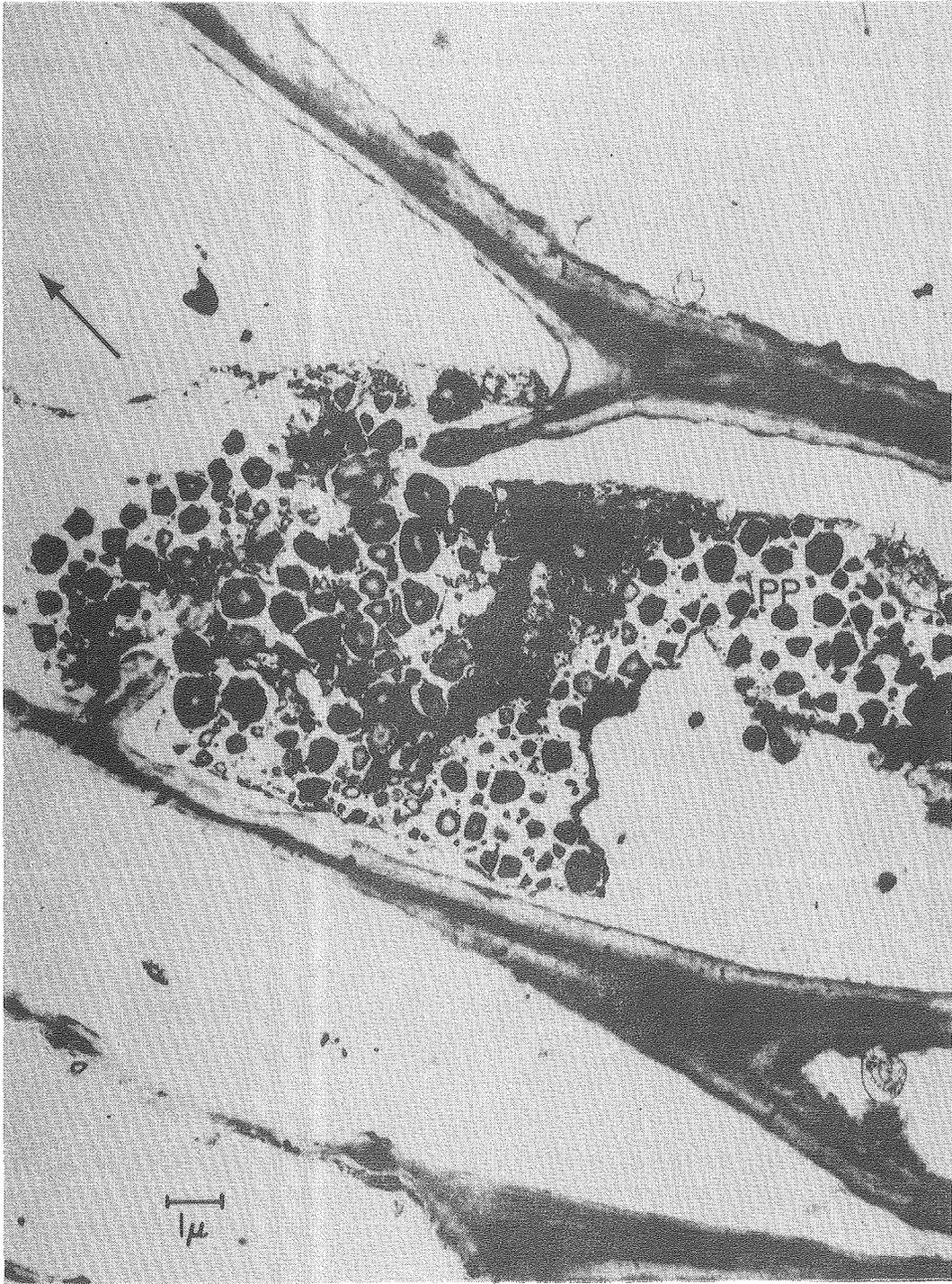
(Note: Material designated as P-protein (PP) may include plastids and starch grains. These materials are shown lodged against sieve plate probably as a result of turgor release.



PL.6

XBB 802-1619

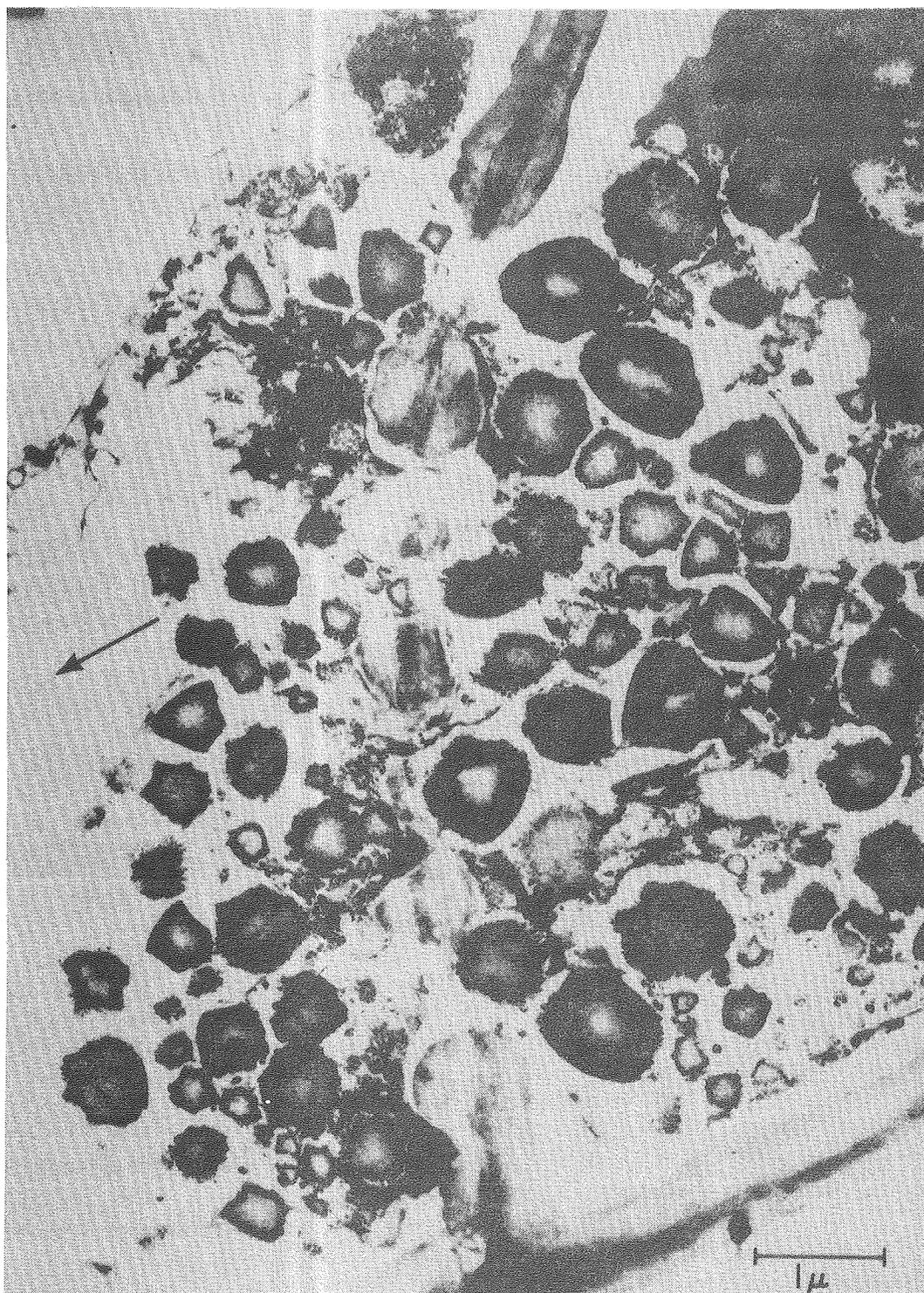
Plate 7a. Longitudinal section of sieve plate after 24 hr treatment with 2 mM EDTA. Sieve plate pores are essentially free of callose but contain P protein (some of which has passed through the pores). Arrow indicates probable direction of phloem transport. x 9,200.



PL.7a

XBB 802-1622

Plate 7b. Higher magnification of plate 7a. P protein appears to pass through callose free sieve plate pores. Arrow indicates probable direction of phloem transport. x 24,000. (Note: particles with fibrillar edges are forms of P-protein).



PL.7b

XBB 802-1610

Plate 8. Longitudinal section of sieve element after treatment with 2 mM EDTA for 24 hrs. Sieve plate pores appear devoid of callose but contain substantial amounts of P protein. Arrow indicates probable direction of phloem transport. x 10,800.



PL.8

XBB 802-1621

IV. Mechanism of Action

The following two experiments briefly consider how EDTA acts to suppress callose formation. The first experiment examines EDTA treatment time required for maximum exudation, while the second considers the nature of a callose synthesis factor with which EDTA may interact.

A. EDTA Treatment Time

In previous experiments, petiolules were immersed in treatment solutions for the duration of the exudation period (21-72 hrs). It was questioned whether this continual exposure to EDTA was necessary for maximum exudation enhancement or whether a short term exposure would be equally as effective. It was reasoned that if a short term exposure was equally as effective in promoting exudation, then EDTA may act by binding and sequestering a callose synthesis factor which is immobile in the leaflet and localized at the cut surface. Such a factor may not be readily resupplied to the site of callose synthesis by vascular or intercellular transport. Conversely, if the continued presence of EDTA is required for maximum exudation, then the callose synthesis factor may be mobile and readily transported to the cut surface immediately after an EDTA treatment. An experiment designed to test this proposal was conducted in a fashion similar to previous experiments.

METHODS AND MATERIALS

¹⁴C labelled leaflet pairs were excised and their petiolules immersed in 2 mM EDTA. One leaflet of each pair was removed from the EDTA after a short period (1/2, 1, 2, or 4 hrs) and placed in distilled H₂O. Matching leaflets remained in the EDTA solution for 48 hrs. After

48 hrs, all leaflets were removed and treatment solutions were analyzed for ^{14}C content.

RESULTS AND DISCUSSION

Results presented in Table 11 show that short term EDTA exposures were not as effective in promoting exudation as continual exposure. Exudation was reduced from 60-90% among replicates which were transferred to H_2O after a short EDTA treatment. Exudation was greatest in those leaflets exposed to EDTA for the entire 48 hr treatment period.

The continual presence of EDTA for maximum exudation suggests that the factor which interacts with EDTA in preventing callose formation becomes available at the site of callose synthesis immediately after an EDTA treatment. This callose synthesis factor may have been transported via phloem sieve elements or across adjacent cells perhaps from the xylem. Perhaps then upon removal of EDTA from the treatment solution callose synthesis may have been initiated and thereby causing a reduction in exudation. Identification of the factor which interacts with EDTA is considered in the following section.

B. Callose Synthesis Factor and EDTA Action

Exudation of label was found to be substantially reduced upon transferring excised leaflets from EDTA to H_2O in the previous experiment. It was suggested that in the absence of EDTA a callose synthesis factor may become available for participation in callose formation. Since calcium has been reported to be involved in callose synthesis (Chu, 1973; Eschrich, 1975) and considering that EDTA forms stable complexes with divalent ions, it was postulated that calcium may be

TABLE 11. Effect of EDTA Treatment Time on ^{14}C Exudation from Excised Fraxinus uhdei Leaflets Over a 48 Hr. Period

Leaflet Number	Treatment		Total ^{14}C in exudate for 48 hrs. (dpm)
1a	1/2 hr EDTA	47-1/2 hr H ₂ O	11,161
b	48 hr EDTA		27,960
2a	1 hr EDTA	47 hr H ₂ O	10,702
b	48 hr EDTA		28,749
3a	2 hr EDTA	46 hr H ₂ O	8,750
b	48 hr EDTA		122,608
4a	4 hr EDTA	44 hr H ₂ O	5,400
b	48 hr EDTA		43,206

sequestered by EDTA and thus prevented from participating in callose synthesis.

If calcium is required for callose formation, then the synthesis of callose may be limited by the availability of calcium. Likewise, exudation may be expected to fluctuate relative to calcium concentration at the cut surface. High calcium concentrations might be expected to suppress exudation while low concentrations may enhance it. It was reasoned, therefore, that if calcium were added to the treatment solution after a brief EDTA treatment, then exudation might be sharply reduced or suppressed. More precisely, exudation would be expected to be reduced to a greater extent in a calcium containing solution than in a calcium free solution after an equivalent EDTA treatment. If such were the case, then calcium would be implicated as a factor involved in EDTA suppression of callose synthesis and promotion of exudation.

METHODS AND MATERIALS

^{14}C labelled leaflets were placed in 2 mM EDTA solutions. After 2 hrs, one leaflet of each pair was transferred to either distilled H_2O , 10 mM CaCl_2 , or 10 mM MgCl_2 . Matching leaflets remained in EDTA or were transferred to distilled H_2O . After 48 hrs, all leaflets were removed and the treatment solutions were analyzed for ^{14}C content.

RESULTS AND DISCUSSION

Exudation was effectively suppressed by transferring EDTA treated leaflets to either H_2O , CaCl_2 , or MgCl_2 solutions (Table 12). The CaCl_2 and MgCl_2 solutions reduced exudation by approximately 50%, while the

TABLE 12. Effect of Ca^{++} and Mg^{++} on ^{14}C Exudation from *F. uhdei* Leaflets
After a 2 hr EDTA Treatment. (10 mM CaCl_2 , 10 mM MgCl_2 , 2 mM EDTA)

Leaflet number	Treatment		^{14}C in Sample (dpm/ml)				Total ^{14}C in exudate	
			0-2 hr	2-4 hr	4-21 hr	21-24 hr		
1a	2 hr EDTA	46 hr H_2O	7,553	4,067	9,378	3,500	1,466	25,964
b	48 hr EDTA		9,018	12,973	159,600	35,884	55,166	272,641
2a	2 hr EDTA	46 hr CaCl_2	4,644	2,271	3,737	1,362	2,410	14,424
b	2 hr EDTA	46 hr H_2O	8,426	3,430	11,528	2,339	10,546	36,269
3a	2 hr EDTA	46 hr MgCl_2	4,123	2,294	4,471	2,174	2,315	15,377
b	2 hr EDTA	46 hr H_2O	4,428	2,087	4,384	2,351	19,657	32,907

H₂O treatment suppressed exudation by 90%. The rate of exudation in all three treatments declined steadily after being transferred from EDTA.

It is apparent from the results that a post-EDTA treatment with Ca⁺⁺ did not further reduce exudation as postulated. In fact, both the MgCl₂ and H₂O treatments were more effective in suppressing exudation than CaCl₂. These results may be interpreted to mean that Ca⁺⁺ is either not directly involved in callose synthesis, or if it is involved then it must become readily available at the cut surface immediately after EDTA treatment. This latter case would imply a rapid internal redistribution of calcium in the excised leaflet. Sufficient calcium must be transported to the cut surface to saturate the callose synthesis reaction in which it may participate. If such were the case then additional Ca⁺⁺ in the treatment solution would not be expected to directly affect callose formation or exudation. However, further biochemical and physiological evidence would be needed to demonstrate that Ca⁺⁺ could be readily transported within or across the phloem to the site of callose synthesis. The results of this experiment are, therefore, inconclusive regarding a role for calcium in callose synthesis.

V. Summary

Phloem exudation studies were conducted using excised F. uhdei leaflets labelled with ¹⁴C. Experimental results showed that a 2 mM EDTA solution was effective in enhancing exudation of label from leaflets over a 72 hr period. EDTA concentrations greater than 2 mM enhanced exudation but leaf damage was noted. Concentrations less than 2 mM were less effective in promoting exudation. Exudation rate reached

a maximum between 21 and 24 hrs and then declined to a minimum at 72 hrs. Exudation into H_2O treatments occurred principally during the first 4 hrs after excision.

Paper chromatography and autoradiography of the labelled exudate showed that label appeared primarily in oligosaccharides and sucrose. Analyses of phloem sap from various Fraxinus species closely paralleled the exudate analysis in this study. The correspondence between analyses suggested that label appearing in treatment solutions represents compounds principally of phloem origin. Precise quantitative correlations between EDTA treatment and callose occurrence in sieve plate pores were not possible from photomicrographs. However, observations of EDTA treated and H_2O treated sieve plate pores indicated that greater amounts of callose were found in H_2O treatments while lesser amounts were noted in EDTA treatments. These results suggested that EDTA acts to promote exudation by inhibiting callose formation.

Exudation was found to be substantially reduced by transferring leaflets to pure H_2O after a brief exposure to 2 mM EDTA. A continuous EDTA exposure was required for maximum exudation. These results were thought to imply that if EDTA acts by sequestering a callose synthesis factor at the cut surface, then that factor must become available to participate in callose synthesis reactions after a short EDTA treatment period.

Since calcium has been reported to be required for callose synthesis, experiments were conducted to determine whether it was involved in EDTA inhibition of callose formation. These experiments, however, were inconclusive in confirming or denying a role for calcium in callose synthesis.

CHAPTER 3. Exudation Studies Using Whole Plants

As noted in the introduction, a primary objective of this study was to assess the effectiveness of chelating agents in enhancing phloem exudation from mature trees. Exudation studies thus far have been limited to experiments using excised shoots and leaves of various species, and in particular, to excised leaflets of F. uhdei. This study proposes to investigate a means of collecting phloem sap from whole plants. Specifically, the effectiveness of EDTA in promoting exudation from F. uhdei trees will be considered.

The objectives of this study were, first, to devise a simple technique for exposing phloem bundles for EDTA treatment, and second, to characterize exudation in response to EDTA treatment.

I. Bark Flap Exudation Studies

In these experiments a "bark flap" technique was used to study EDTA effects on phloem exudation. Briefly, this method involved making two parallel incisions along the trunk of a F. uhdei tree, and then a third incision across the parallel cuts and distal to the tree apex (see Fig. 16). The incisions were made through the bark and into the xylem. The cut bark was carefully lifted and separated from the underlying xylem, forming a flap. The bark flap consisted of phloem and bark tissue with one end exposed and the other remaining attached to the trunk. The exposed end was then immersed in a treatment solution and exudation monitored using ^{14}C tracer techniques. A series of experiments were conducted to evaluate exudation amount, duration, and rate from F. uhdei trees. An analysis of the exudate was conducted using chromatography and autoradiography techniques.

METHODS AND MATERIALS

a. Plant Materials

Two year old, container grown Fraxinus uhdei trees measuring 3-4 ft in height were used in these experiments. Specimens selected were vigorous, disease-free plants exhibiting a uniform distribution of shoots and leaves around a central trunk. All plants were fertilized with Osmocote (18-6-12) timed release fertilizer and a half strength Hoagland's micronutrient supplement. Plants were watered on a regular basis and kept in full sun until used in exudation experiments.

B. ^{14}C Labelling

The terminal two pairs of fully expanded leaves of a selected specimen were enclosed in an air tight plexiglass chamber (Fig. 14). The chamber was connected to a steady state gas recirculating apparatus designed to pass $^{14}\text{CO}_2$ (specific activity 13.9 mCi/mM) across the leaf surface (Fig. 15). The chamber temperature was maintained between 25 and 29°C and illumination provided by General Electric Cool White high output fluorescent lamps ($600 \mu\text{Em}^{-2}\text{sec}^{-1}$). After a 20-30 min labelling period the lights were turned off and the chamber was disconnected from the $^{14}\text{CO}_2$ source. The leaves remained sealed in the chamber throughout the treatment period.

C. Bark Flap Excision and Treatment

Immediately prior to ^{14}C labelling, bark flaps were cut as shown in Fig. 16. Two flaps, measuring 3.5 cm long by 0.5 cm wide and located on opposite sides of the trunk, were cut. Each flap was tightly wrapped with a layer of Parafilm and then the distal end was recut (1 mm section), exposing fresh tissue. The bark flap was immersed in 3.5 ml

Figure 14. F. uhdei tree in 1 gal. container positioned for ^{14}C exudation study. Upper two leaf pairs are enclosed in chamber for $^{14}\text{CO}_2$ assimilation. Bark flaps and collecting vials are located on the trunk below the chamber. The two fluorescent light banks provide a uniform light intensity.

Figure 15. ^{14}C gas recirculating apparatus with labelling chamber connected to system. $^{14}\text{CO}_2$ contained in a pressurized cylinder is slowly released into circulating system and mixed with $^{12}\text{CO}_2$. ^{14}C and ^{12}C gas passes through the labelling chamber where it is assimilated by the leaves.



FIG. 14



FIG. 15

XBB 805-6860

treatment solution contained in 5 ml vials. The vials were taped to the trunk as shown in Fig. 17. Three pairs of bark flaps and vials were evenly spaced along the trunk, each pair oriented at right angles to one another down the central axis (Fig. 17). One vial of each pair contained distilled H₂O and the other an EDTA solution.

Treatments varied in duration and in EDTA concentration. Experiments lasting 24, 48, and 72 hrs were conducted using 10 mM EDTA. Concentration effects were investigated using 0.5 mM, 2.0 mM, 10.0 mM, and 20.0 mM EDTA treatments.

D. Sampling and Analysis of Exudate

EDTA and H₂O treatment solutions were removed periodically and replaced with fresh preparations. 1.0 ml aliquots of used solutions were pipetted into 6.0 ml of Research Products International 3a70B complete counting cocktail and counted on a Packard Tri-Carb liquid scintillation spectrometer. ¹⁴C counts were read in disintegrations per min (dpm).

Soluble exudates in the treatment solutions were analyzed using paper chromatography and autoradiography techniques. These methods were the same as those outlined in Chapter 2. Sample aliquots of 0.25 ml were used in this analysis, however. Radioactivity of chromatogram spots were measured by eluting each spot in 2.0 ml H₂O for 8 hrs, adding 12.0 ml RPI 3a70B complete counting cocktail, and then counting dpm on a Packard Tri-Carb liquid scintillation counter.

Figure 16. Bark flaps on trunk of young F. uhdei tree. Flaps measured 0.5 cm wide by 3.5 cm long and consisted of phloem and bark tissue. The flaps were wrapped with a thin layer of Parafilm and recut at their distal end before being treated.

Figure 17. Bark flap immersed in treatment solutions placed along the trunk of a young F. uhdei tree. One vial of each pair contained distilled H₂O and the other an EDTA solution. Treatment pairs were rotated at right angles to one another down the central axis of the tree.

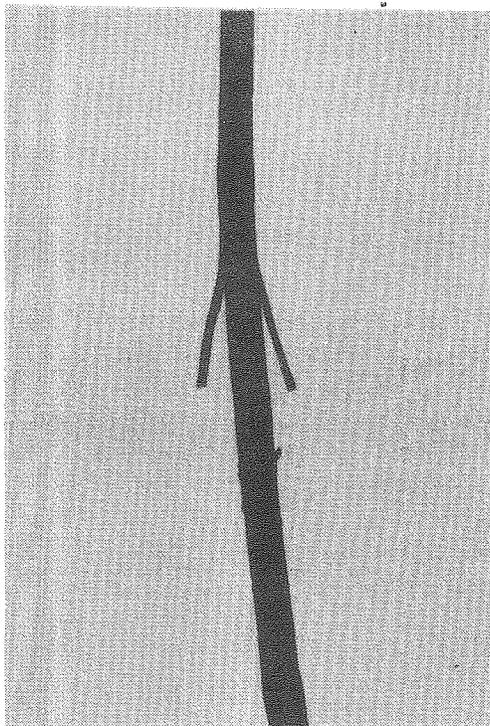


FIG. 16

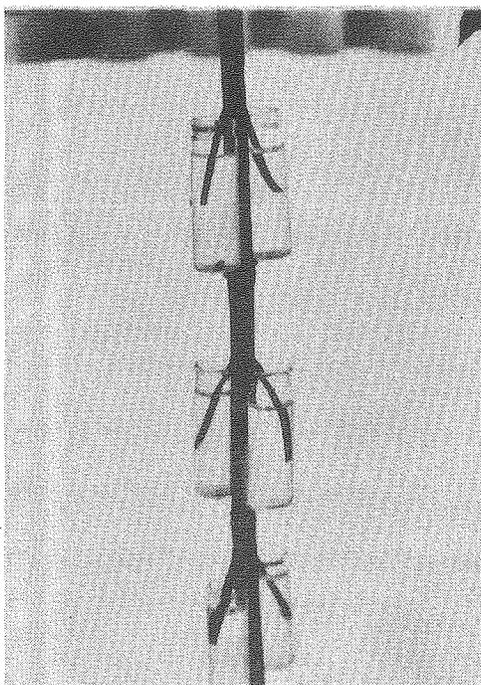


FIG. 17

XBB 805 6859

RESULTS AND DISCUSSION

A. Experimental Procedure and Assimilate Flow

Since H₂O treatments and EDTA treatments were placed on opposite sides of the trunk, it was important to ensure that assimilate flow was equivalent down both sides of the trunk. Otherwise, differences in the exudation thought to be due to specific treatments may actually be the result of an increased assimilate flow on one side of the trunk or a restricted flow on the other. For instance, if leaf area was greater on one side of the trunk than the other, then assimilate flow might be expected to be greater immediately below the region of greater leaf area. Even though this effect may dissipate down the trunk due to the helical orientation of phloem sieve elements along the central axis, localized differences in assimilate flow may exist. Conversely, if a restriction within phloem sieve elements occurred at any point along the stem, then assimilate flow may also be diminished. For example, the presence of phloem-specific pathogens may reduce the number of functional sieve elements and thus limit phloem transport at certain points along the trunk. As a result, assimilate flow may not be equal at apparently equivalent sites.

Regardless of the cause, exudation potential would not be expected to be equivalent at points along the trunk where assimilate flow was unequal. Therefore, measures were taken to eliminate or minimize differences in assimilate flow at points where exudation measurements were made.

First, only plants with a uniform distribution of leaves around the trunk were selected for these experiments. In addition, since F. uhdei leaves are initiated in opposite pairs which alternate in two

distinct planes down the central axis (decussate phyllotaxis), only the terminal two pair of fully expanded leaves were selected for ^{14}C labelling. These leaf pairs formed a uniform leaf area distribution around the trunk apex. As a result, preferential flow due to leaf area distribution was considered to be minimal.

Furthermore, differences in assimilate flow, perhaps the result of restrictions in phloem transport, were minimized by alternating the position of treatments and replicates down the stem. If all H_2O treatments were placed in a vertical file on one side of the stem and all EDTA treatments likewise on the other side, then a restriction in assimilate flow along one side may result in exudation differences which are not solely the result of experimental treatments. Therefore, treatments and replicates were positioned in an alternating sequence and oriented at right angles in a horizontal plane down the stem (Fig. 17). Effects of preferential or restricted assimilate flow would then be distributed equally between treatments and among replicates.

These measures, ensuring uniform leaf area distribution and alternating the position of treatments down the stem were collectively thought to minimize possible exudation differences caused by assimilate flow variations.

A further consideration concerning procedures should be noted. After bark flaps were cut and lifted from the underlying xylem 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 bark flaps were the only

portions directly exposed to the treatment solutions. Otherwise, cells located on the interior surface of the bark flap would be exposed to treatment solutions and leakage of label may be possible. The Parafilm layer was intended to serve as a barrier between the treatment solution and tissues located on the interior surface of the bark flap. Label appearing in treatment solution could therefore be considered to be released solely from exposed phloem sieve elements.

B. Effect of 10 mM EDTA Treatment on Exudation

From the data presented in Table 13, a 10 mM EDTA solution markedly enhanced exudation over a 24 hr period. Very small amounts of label appeared in H₂O treatments, while copious amounts were found in two of the three EDTA replicates. Since the EDTA replicate which received little label was located below the other two replicates, it is possible that labelled assimilates simply had not reached the lowest vials during the 24 hr treatment period. Nevertheless, in the upper two EDTA treatments exudation was significantly enhanced by 10 mM EDTA.

C. Concentration Effects

The selection of a 10 mM EDTA concentration for the previous experiment appeared fortuitous from the results presented in Table 14. When 0.5 mM, 2.0 mM, 10.0 mM, and 20.0 mM EDTA concentrations were tested for effectiveness in promoting exudation from bark flaps, only the 10 mM treatment was found to be effective. Relatively insignificant amounts of label were found in 0.5 mM, 2.0 mM, and 20.0 mM treatments and even lesser amounts appeared in H₂O treatments. Some discoloration of the bark flap was noted in the 20.0 mM treatment,

TABLE 13. Effect of 10 mM EDTA on the Exudation of ^{14}C from "Bark Flaps" of Fraxinus uhdei Trees.

Vial Position		Treatment	^{14}C in exudate (dpm/ml) (24 hrs)
Top	Right	EDTA	5,060,387
	Left	H ₂ O	60
Middle	Left	EDTA	9,260,802
	Right	H ₂ O	30
Bottom	Right	EDTA	158
	Left	H ₂ O	64

TABLE 14. Effect of EDTA Concentration on the Exudation of ^{14}C from F. uhdei Bark Flaps Over a 48 hr Period.

Treatment	^{14}C in Exudate (dpm/ml)			Total
	0-4	4-24 hr (hr)	24-48 hr	
0.5 mM EDTA	3	6	15	24
H ₂ O	2	22	3	27
2.0 mM EDTA	5	8	24	37
H ₂ O	6	8	3	17
10.0 mM EDTA	6	533,444	86,916	620,366
H ₂ O	4	141	6	151
20.0 mM EDTA	7	11	42	60
H ₂ O	5	3	5	13

suggesting tissue damage may have resulted at the higher EDTA concentration.

It is difficult to interpret these results in terms of the negligible exudation response from 0.5 mM, 2.0 mM, and 20.0 mM EDTA treatments. Ostensibly, the 0.5 mM and 2.0 mM treatments were too dilute to enhance exudation, while the 20.0 mM treatment was too concentrated and may have caused tissue damage. Regardless of the lack of response from other EDTA concentrations, however, the results are clear concerning the effectiveness of a 10 mM EDTA treatment in promoting exudation: exudation from bark flaps was significantly enhanced by a 10 mM treatment during the 48 hr treatment period.

D. Exudation Rate and Duration

Exudation of label into either EDTA or H₂O treatments was negligible for 4 hrs after ¹⁴CO₂ incorporation (Table 15, Fig. 18). However, from 4-24 hrs exudation into EDTA treatments increased markedly, while exudation in H₂O remained slight. After reaching a maximum rate after 24 hrs, exudation in EDTA treatments declined slowly for the following 24 hrs. After 48 hrs, exudation rate had declined to only 83% of the maximum rate.

Unlike the results of the previous experiment (Table 14), all EDTA replicates exuded relatively large amounts of label into treatment solutions. Although the EDTA replicate positioned in the middle exhibited exudation rates of less than half of those above and below, it was, nonetheless, very much greater than all H₂O treatments.

In a 72 hr experiment, exudation continued to decline after 48 hr and then virtually ceased after 72 hrs (Fig. 19). Between 0 and 24 hr exudation rate from EDTA treatments was similar to that in the

TABLE 15. 48 hr Time Course of ^{14}C Exudation from *F. uhdei*
 "Bark Flaps" After Treatment with 10 mM EDTA

Vial Position	Treatment	^{14}C in sample (dpm/ml)			Total ^{14}C in Exudate (dmp)
		0-4 hr	4-24 hr	24-48 hr	
Top	Right EDTA	178	1,543,739	1,812,500	3,356,417
	Left H_2O	117	31	34	182
Middle	Left EDTA	58	318,285	830,985	1,149,328
	Right H_2O	27	27	321	375
Bottom	Right EDTA	28	1,903,225	1,452,316	3,355,569
	Left H_2O	31	23	299	356

Figure 18. Rate of ^{14}C exudation from bark flaps over a 48 hr period. The flaps were treated with 10 mM EDTA or distilled H_2O .

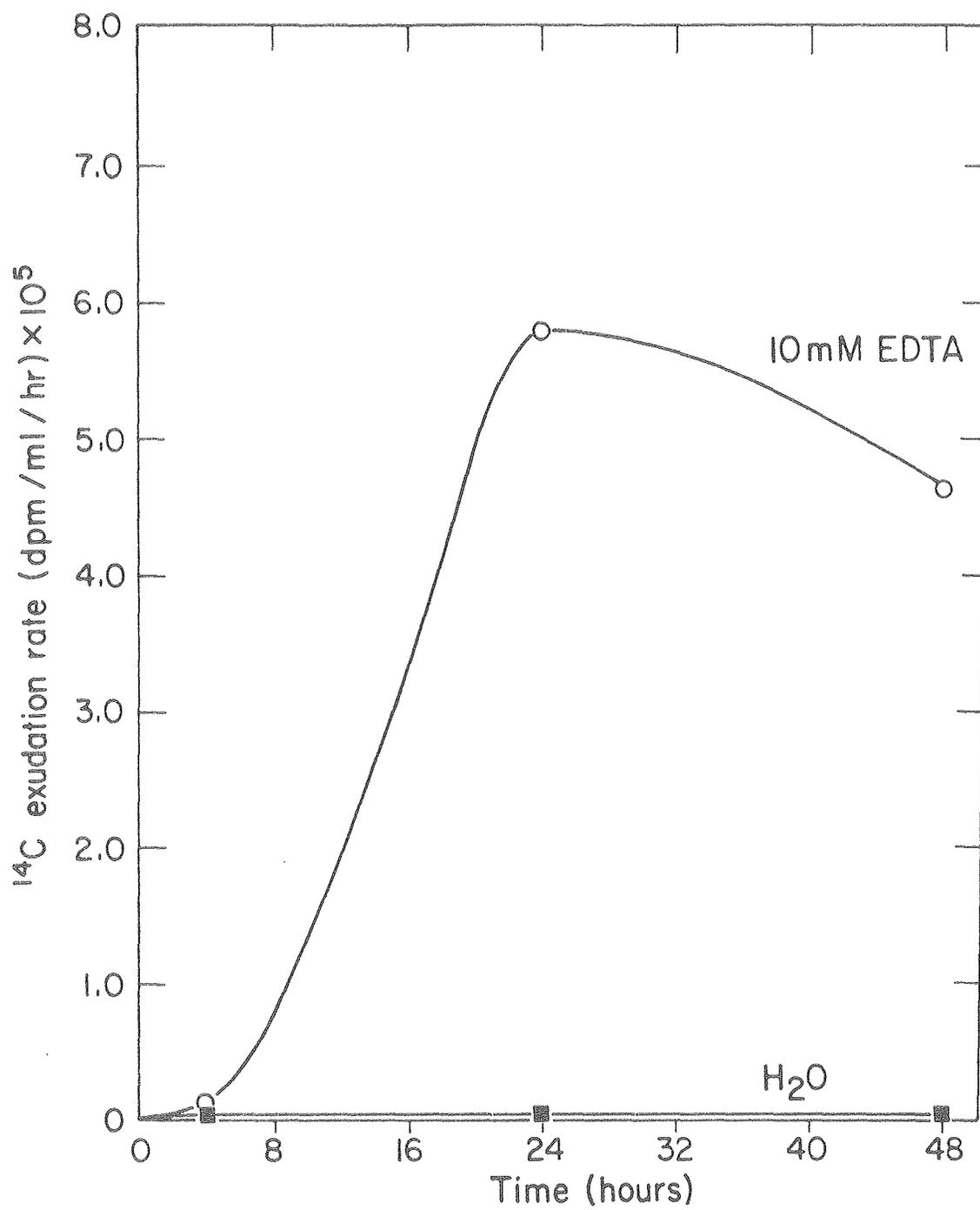


FIG. 18

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Figure 19. Rate of ^{14}C exudation from bark flaps over a 72 hr period. Bark flaps were treated with 10 mM EDTA or distilled H_2O .

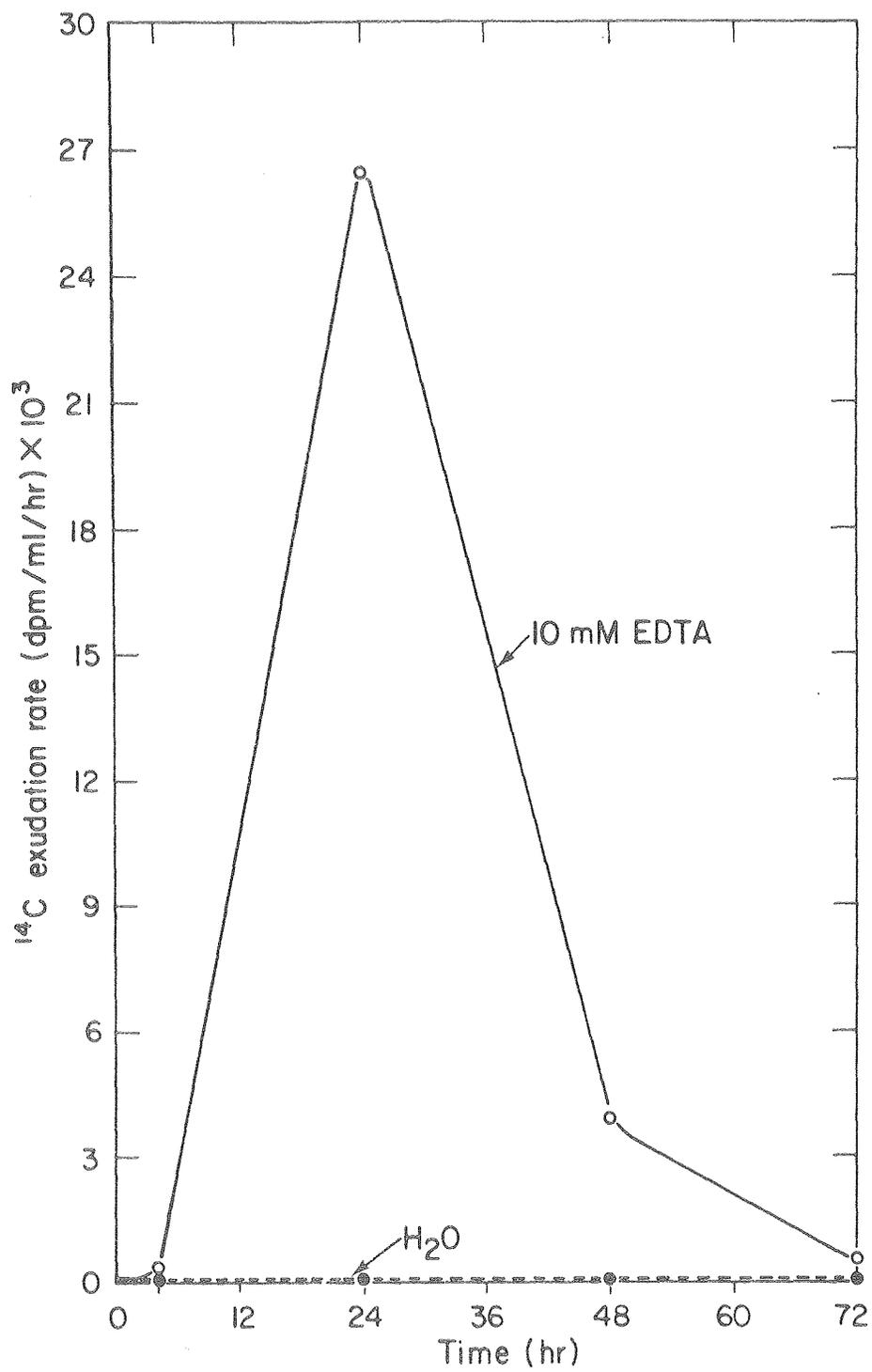


FIG. 19

XBL 806-4223

previous experiment. However, between 24 and 48 hrs exudation declined to 15% of the maximum rate, or 68 percentage points less than the previous experiment. Since different plants were used in the two experiments as well as different labelling periods, it might be expected that variations in exudation patterns may occur. Exudation rate decline after 24 hrs in Fig. 19 may represent a depletion in label within the plant as much as a decline in exudation.

Regardless of the relatively small differences in exudation rate between the two experiments, however, the exudation time course appears quite similar in Figs. 18 and 19. In both cases, little exudation was noted during the first 4 hrs, and then a sharp exudation increase in EDTA treatments occurs between 4 and 24 hrs. After reaching a maximum at 24 hrs in both experiments, exudation rate declines.

It appears then that EDTA effectively enhances phloem exudation from bark flaps as well as from excised leaves. Presumably, EDTA acts to inhibit callose formation in sieve plate pores as was suggested in the previous experiments. However, a study of sieve plate pore fine structure in the bark flap tissue would be needed to confirm a callose inhibiting role for EDTA in these experiments.

E. Analysis of Exudate

Evidence that label appearing in treatment solutions represents materials of phloem origin is presented in Fig. 20 and Table 16. As in experiments with excised leaves, most of the label appeared in oligosaccharides and sucrose. Approximately 90% of the label recovered appeared in raffinose, stachyose, verbascose, and sucrose; and 53% of that appeared in stachyose alone. Only 8% appeared in glucose and

Figure 20. Autoradiograph of 0.25 ml exudate sample from F.
uhdei bark flap treated with 10 mM EDTA.

Autoradiograph of Exudate from Back Flaps of
Fraxinus Uhdei

UIC

24 hr ← Butanol: Propionic Acid: H₂O

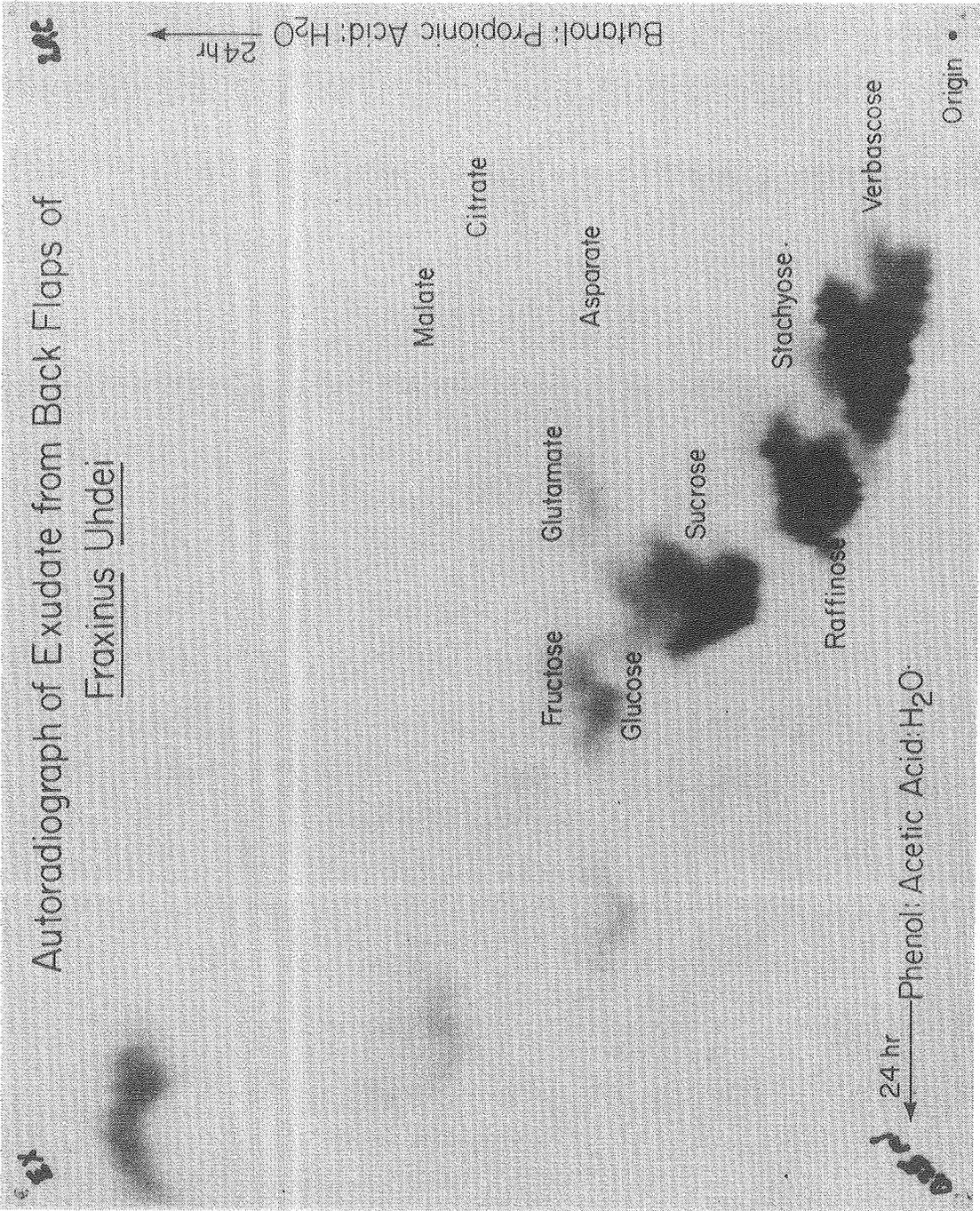


FIG. 20

XBB 806-7212

TABLE 16. Paper Chromatography and Radiography Analysis of 0.25 ml Exudate. Relative Amounts of Each Compound Given in dpm/ml. Sample Taken From 10 mM EDTA treatment

Compound	¹⁴ C in Spot (dpm/ml)	% of Total
Aspartate	4,056	0.17
Citrate	4,314	0.18
Fructose	109,642	4.8
Glucose	90,654	3.8
Glutamate	21,966	0.92
Malate	3,062	0.13
Raffinose	432,484	18.2
Stachyose	1,263,008	53.4
Sucrose	298,108	12.6
Verbascose	139,650	5.9

fructose, while less than 2% was found in glutamate, aspartate, citrate, and malate.

This analysis of labelled exudates agrees closely with that found for excised leaflets in the previous experiments. Likewise, it corresponds with published analyses of phloem exudate from various Fraxinus species, as cited in Chapter 2. Therefore, it seems reasonable to conclude that exudation of label from F. uhdei bark flaps into EDTA treatment solutions occurs principally from phloem sieve elements.

II. Summary

A "bark flap" method was used in these experiments to assess the effectiveness of EDTA in enhancing phloem exudation from whole plants. Container grown F. uhdei trees were labelled with ^{14}C and exudation from bark flaps located along the trunk was monitored for up to 72 hrs. At the outset of each experiment measures were taken to minimize effects on exudation due to preferential or restricted assimilate flow down one side of the trunk. Exudation rate was calculated from periodic sampling of treatment solutions.

A 10 mM EDTA treatment was found to be very effective in enhancing exudation from bark flaps. EDTA concentrations greater or less than 10.0 mM were ineffective in promoting exudation. Exudation rate was initially slow in both EDTA and H_2O treatments, but after 4 hrs, it increased rapidly in EDTA treatments and was negligible in H_2O treatments. Exudation was greatest after 24 hrs in EDTA treatments and then declined to a minimum at 72 hrs. An analysis of labelled

exudates showed most of the label appearing in oligosaccharides and sucrose, in agreement with experiments with excised leaves and published reports. It was concluded that label appearing in treatment solutions represented materials of phloem origin and, therefore, that EDTA promotes phloem exudation from whole plants as well as from excised leaves.

CHAPTER 4. Exudation Studies Using 5 Year Old F. uhdei Trees

Exudation studies using the bark flap method outlined in the preceeding section were conducted with 5 year old F. uhdei trees. These experiments were intended to serve as a practical application of methods described in previous experiments. The effectiveness of EDTA in enhancing exudation from large trees was considered, with particular interest in maintaining exudation over an extended period of time. To approximate field conditions, all experiments were conducted outdoors.

METHODS AND MATERIALS

A. Plant Materials

F. uhdei trees were grown in 15 gal. containers (Fig. 21). The trees were approximately 5 years old and measured 12 to 15 ft. in height and 2 to 2.5 inches in trunk diameter. (Trunk diameter measurements were taken 12" above the container soil level.) All specimens were fertilized with Osmocote (18-6-12) timed release fertilizer and half strength Hoagland's micronutrient supplement.

B. Bark Flap Excision Technique

Bark flaps measuring 1 cm wide and 5 cm long (Fig. 22) were cut in a fashion similar to that described in the preceding section. Flaps were located on vertical branches and oriented at right angles to one another down the branch (Fig. 23). Immediately after the flaps were cut, they were wrapped with a thin layer of Parafilm (Fig. 22), recut at their distal ends (1 mm section), and then immersed in treatment solutions. 8 ml of treatment solution was contained in 10 ml vials which were taped to the branch as shown in Fig. 23.

Figure 21. 5 year old F. uhdei tree used in exudation studies. Grown in 15 gal. containers, the trees measured 12 to 15 ft. in height and 2 to 2.5 inches in trunk diameter.



FIG. 21

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Figure 22. Bark flaps cut on vertical branch of 5 year old F. uhdei tree. Flaps measured 1 cm wide and 5 cm long and are wrapped with a layer of Parafilm.

Figure 23. Treatment vials positioned along vertical branches of 5 year old F. uhdei tree. One vial of each pair contained distilled H₂O and the other an EDTA solution. Treatment pairs were oriented at right angles to one another down the central axis of the tree.

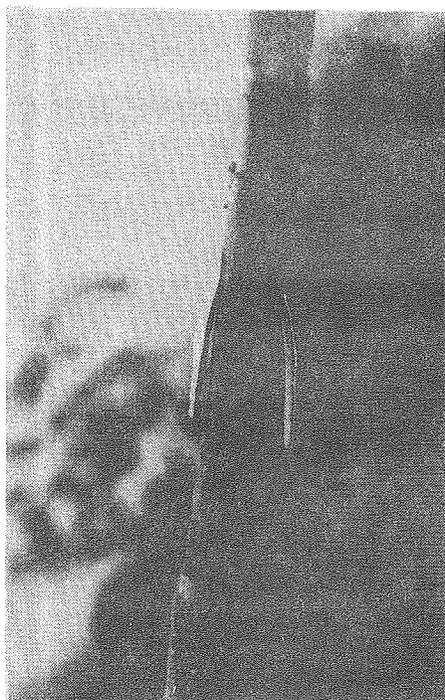


FIG.22



FIG. 23

XBB 805-6857

C. Treatments

i. Exudation Rate, Duration and Concentration Effects

Exudation rate, duration and concentration effects were investigated in a series of experiments. In the first two experiments, 0.5 mM, 2.0 mM, 10.0 mM, and 20.0 mM EDTA treatment were tested for exudation enhancement effects. In the later two experiments, only 20.0 mM EDTA was used.

In experiment I, EDTA concentrations were placed in order of increasing concentration down the trunk and opposite vials containing distilled H₂O. In experiment II, an alternating sequence of EDTA concentrations was used. 20.0 mM EDTA was at the top followed by 2.0, 10.0, and 0.5 mM EDTA down the branch. Used treatment solutions were periodically replaced with fresh preparations for exudation rate determination. Solutions were analyzed for total carbohydrate content.

ii. Excised Bark Flaps

An attempt was made to estimate the amount of carbohydrate appearing in treatment solutions as the result of "nonspecific release", i.e. carbohydrate not specifically derived from phloem sieve elements. Freshly cut bark flaps were detached from the branch and quickly wrapped with a thin layer of Parafilm. The flaps were recut at their distal end and immersed in 20 mM EDTA or H₂O to a depth of 3 cm. Six replicates were used in each treatment. Bark flaps were periodically transferred to fresh solutions over a 72 hr period after which solutions were analyzed for carbohydrate content. All carbohydrate found in these treatment solutions was considered to result from nonspecific release.

D. Carbohydrate Analysis

Carbohydrate content in treatment solutions was determined using the anthrone assay outlined in the preliminary experiments. Values were expressed as g sugar detected in 8 ml of treatment solution.

RESULTS AND DISCUSSION

A. Exudation and Concentration Effects

A 20.0 mM EDTA treatment proved most effective in enhancing exudation in experiments I and II (Table 17). Little exudation enhancement was noted from 0.5 mM or 2.0 mM EDTA treatments. A 10.0 mM treatment enhanced exudation from 3 to 4 times greater than H₂O treatments, while the 20.0 mM treatment showed a 3 to 44-fold increase in exudation.

Exudation differences resulting from concentration sequence along the trunk were not apparent from these results. In both experiments, the 20.0 mM treatment was most effective regardless of whether it was placed at the top or the bottom of the treatment sequence. Likewise, the 0.5 mM treatment was least effective regardless of position in the concentration sequence. Therefore, exudation differences were considered to result from concentration differences and not from treatment position.

Exudation rate was initially high in both EDTA and H₂O treatments (Fig. 24). After 4 hrs, however, exudation from 0.5, 2.0, and 10.0 mM EDTA treatments as well as H₂O treatments declined rapidly, while exudation from the 20.0 mM EDTA treatment continued to increase.

TABLE 17. Effect of EDTA Concentration on the Exudation of Sugars from F. uhdei Bark Flaps

Treatment	g sugar in 8 ml Reservoir						Total	
	0-4	4-24	24-48	(Hours) 48-72	72-96	96-144		144-168
Exp. I								
0.5 m EDTA	360	216	16	4	0	4	4	604
H ₂ O	808	304	56	0	0	24	4	1196
2.0 m EDTA	664	192	72	0	76	4	0	1008
H ₂ O	1200	200	32	0	56	24	0	1512
10.0 mM EDTA	696	1696	664	240	24	88	24	3432
H ₂ O	688	312	0	0	0	24	0	1024
20.0 mM EDTA	528	520	1344	1088	24	4	24	3532
H ₂ O	608	288	64	4	88	120	0	1172
Exp. II								
20.0 mM EDTA	1400	11,200	9040	1200	720	736	184	24,480
H ₂ O	536	96	40	0	24	0	0	696
20.0 mM EDTA	1274	656	248	0	40	0	0	2118
H ₂ O	640	328	104	2	368	104	0	1546
10.0 mM EDTA	560	648	800	464	4	344	72	2892
H ₂ O	472	152	8	0	0	88	0	720
0.5 mM EDTA	960	448	16	0	0	0	0	1425
H ₂ O	984	168	8	0	24	0	0	1184

Figure 24. Average rate of sugar exudation from bark flaps over a 168 hr period. Bark flaps were treated with 20 mM, 10.0 mM, 2.0 mM, 0.5 mM EDTA and distilled water. Values represent averaged data from experiments I and II.

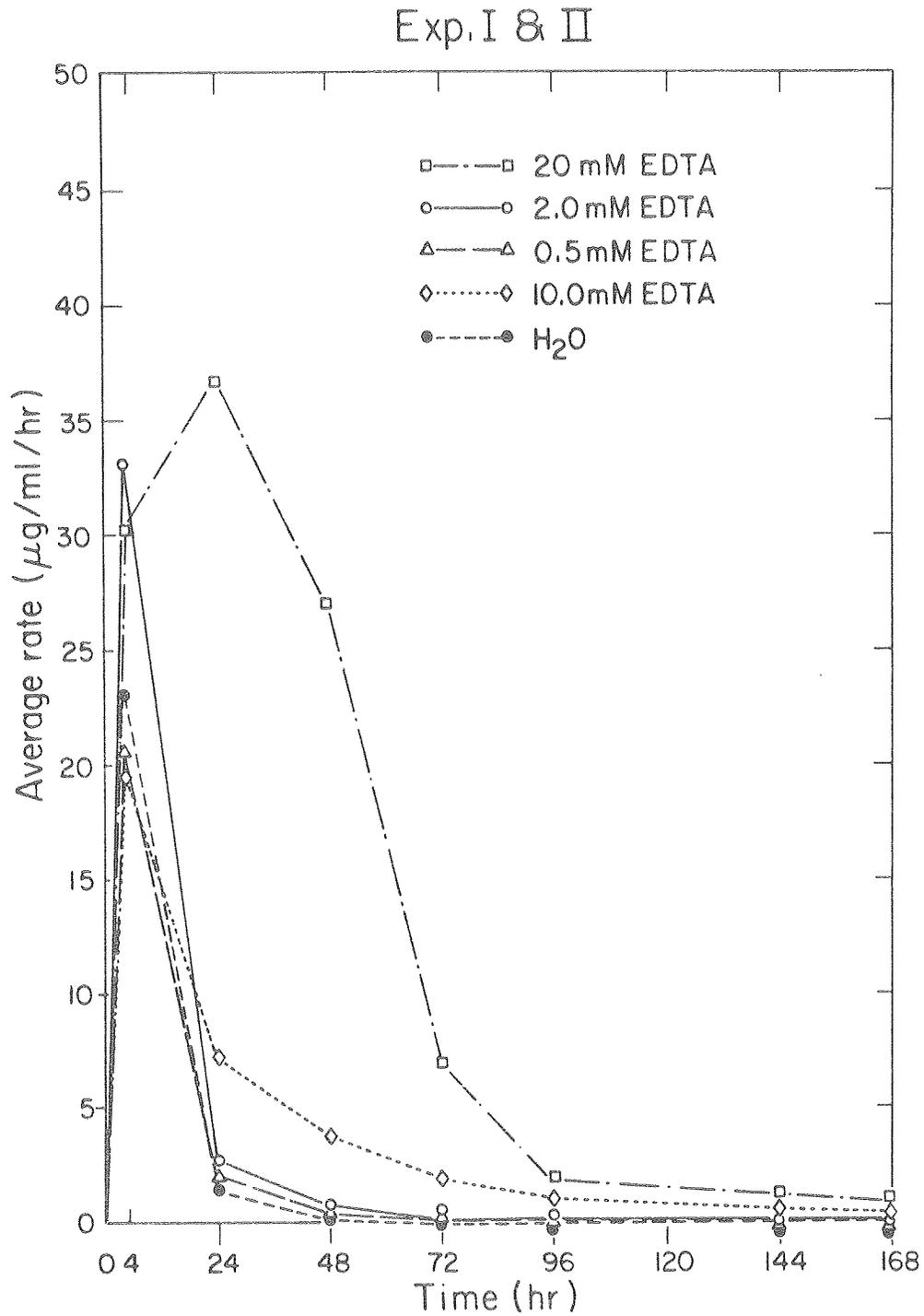


FIG. 24

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Maximum exudation was noted after 24 hrs in the 20.0 mM treatment. Exudation declined thereafter reaching 70% of the maximum rate after 48 hrs and 18% of maximum after 72 hrs. The 0.5 mM and 2.0 mM EDTA treatments and H₂O treatments had ceased exudation after 72 hrs. Between 72 and 168 hrs, exudation from 20.0 mM and 10.0 mM treatments continued at a slowly declining rate.

When the experiment was repeated using only 20.0 mM EDTA treatments (experiments III and IV), exudation was found to be enhanced between 28 and 39 times greater than H₂O treatments (Table 18). When exudation values from 20 mM EDTA treatments were averaged in experiments I through IV, exudation was an average 31.2 times greater than corresponding H₂O treatments (Table 18). It appears, therefore, that exudation can be effectively enhanced in large F. uhdei trees for 3 to 4 days using 20.0 mM EDTA.

B. Excised Bark Flaps

It was speculated that some of the sugar appearing in both EDTA and H₂O treatments was not derived from phloem sieve elements. Sugars may have been released by damaged cells at the cut surface of the bark flap; or EDTA itself may have had a injurious effect on cells adjacent to the phloem causing leakage of their contents. It was suspected that the initial large release of sugars (0-4 hrs) in both EDTA and H₂O treatments noted in Fig. 24 was derived partly from bark flap cells other than phloem sieve elements, or from so-called nonspecific release.

An experiment to estimate the amount of sugar derived from such nonspecific release was conducted. Detached bark flaps were held in 20 mM EDTA or H₂O treatment solutions for 72 hrs. Although some sugar is likely to have been released from phloem sieve elements in the detached

TABLE 18. Average Total Exudation from 20 mM EDTA and H₂O Treatments Over 72 hr Period.

Treatment	Total Sugar Release ($\mu\text{g}/8 \text{ ml}$)
20 mM EDTA	
Exp. I	3,480
II	22,840
III	46,440
IV	134,464
Ave.	51,806
H ₂ O	
Exp. I	964
II	672
III	1,656
IV	3,588
Ave.	1,720
EDTA/H ₂ O	31.2

bark flap, most was considered to be derived from "nonphloem" sources.

Results presented in Fig. 25 and Table 19 indicate that approximately 91% of the sugar released from excised bark flaps into H₂O treatments occurred from 0 to 5 hrs, while 83% of that released into EDTA treatments occurred during the same period. After 2 hrs the rate of sugar release into both treatments decreased markedly. Only small amounts of sugars were detected after 24 hrs. Approximately 10% more sugar was released from bark flaps immersed in 20 mM EDTA than those in H₂O.

The relatively large amount of sugar detected from 0 to 5 hr from the detached bark flaps suggests that much of the initial large sugar release from attached bark flaps may be attributed to nonspecific release. When values for sugar released from detached bark flaps are subtracted from those for attached bark flaps (experiments I to IV), the initial large release is no longer apparent (Fig. 26). It appears that from 76 to 78% of the sugar appearing in treatment solutions from 0 to 2 hrs in attached bark flap experiments can be attributed to nonspecific release. The remainder of sugar appearing during this period is presumably derived from phloem exudation.

After 4 hrs, 100% of the sugar released into H₂O treatments appears to result from nonspecific release. What was considered to be phloem exudation from H₂O treatments between 4 and 48 hrs in experiments I and II, was most likely due to nonspecific release. There appears to be no phloem exudation from H₂O treatments after 4 hrs.

In EDTA treatments, however, nonspecific release represents 66% of sugar detected from 2 to 5 hr, but only 8 and 3% of that detected after 24 and 72 hrs, respectively. Thus, only a small percentage of sugars

TABLE 19. Average and Corrected Exudation Rates for 20 mM EDTA and H₂O Treatments from Experiments I-IV.

(Attached-Excised Bark Flaps = Corrected Rate)

Treatment	Exudation rate ($\mu\text{g/ml/hr}$)				
	0-2 hr	2-5 hr	5-24 hr	24-48 hr	48-72 hr
20 mM EDTA					
Exp. I	8.25	8.25	3.25	7.0	5.66
II	21.8	21.8	70.0	47.1	6.25
III	54	139	90.5	114	333.8
IV	185	29.3	286	300	154
Ave.	67.26	49.58	112.43	117.03	49.92
Corrected	15.76	22.58	103.85	111.78	48.39
H ₂ O					
Exp. I	9.5	9.5	1.8	0.3	.02
II	8.3	8.3	0.6	0.2	0
III	30.5	28	3.15	0	0
IV	175	15.3	1.31	.83	0.3
Ave.	55.82	15.2	1.72	0.33	0.08
Corrected	12.32	-5.5	-3.91	-0.38	-0.43

Figure 25. Rate of sugar exudation from excised bark flaps over a 72 hr period. The detached bark flaps were held in 20 mM EDTA or distilled H₂O. Values represent average data from 6 replicates. Sugar released from detached bark flaps was considered to be nonspecific release.

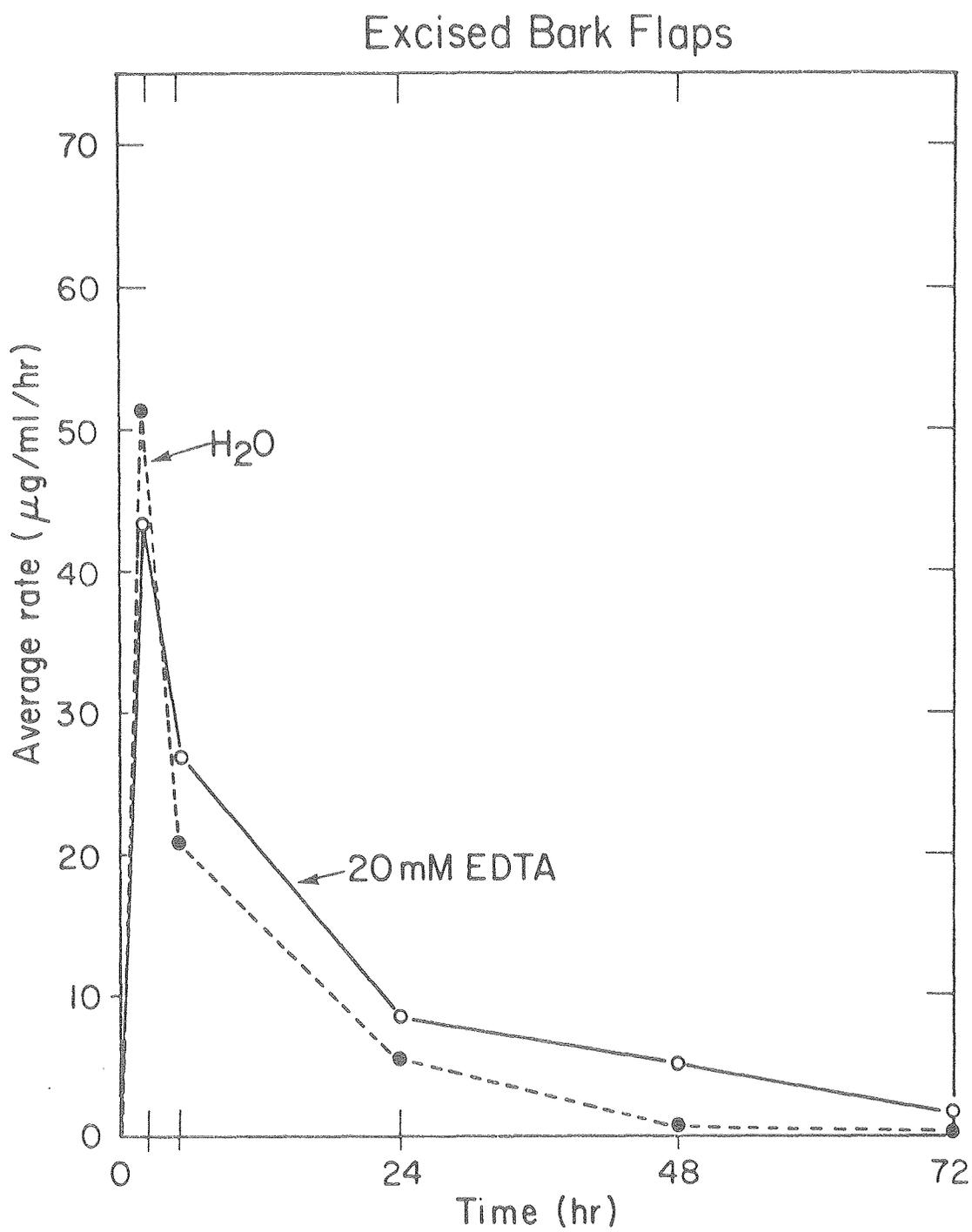


FIG. 25

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appearing in EDTA treatments after 5 hrs may be attributed to nonspecific release. Over 90% of the sugar detected after 5 hrs can be attributed directly to phloem exudation.

When exudation values are adjusted for nonspecific release, as in Fig. 26, the time course curve for exudation closely resembles that found in previous experiments (Figs. 18 and 19). The agreement in data between experiments adds further evidence that EDTA acts specifically to enhance phloem exudation rather than to stimulate a nonspecific sugar release. It was concluded, therefore, that EDTA can effectively promote the exudation of phloem sugars for 3 to 4 days from 5 year old F. uhdei trees.

SUMMARY

Experiments were conducted to investigate phloem exudation from 5 year old F. uhdei trees. A bark flap technique described in the preceding section was used to collect phloem sap. Exudation was found to be effectively enhanced for 3 to 4 days using a 20.0 mM EDTA treatment. Less concentrated EDTA solutions were not as effective in promoting exudation.

An experiment using detached bark flaps provided an estimate of the amount of sugar appearing in treatment solutions not specifically derived from phloem sieve elements (nonspecific release). After 4 hrs, all the sugar detected in H₂O treatments could be attributed to nonspecific release. Over 90% of the sugar appearing in EDTA treatments after 5 hrs, however, was considered to result directly from phloem exudation. It was concluded that EDTA specifically enhances the exudation of phloem sugars from F. uhdei trees.

Figure 26. Exudation of phloem sugars from attached bark flaps (experiments I to IV) after values were corrected for nonspecific release. Values were calculated by subtracting detached bark flap data (fig. 25) from attached bark flap data (Table 18).

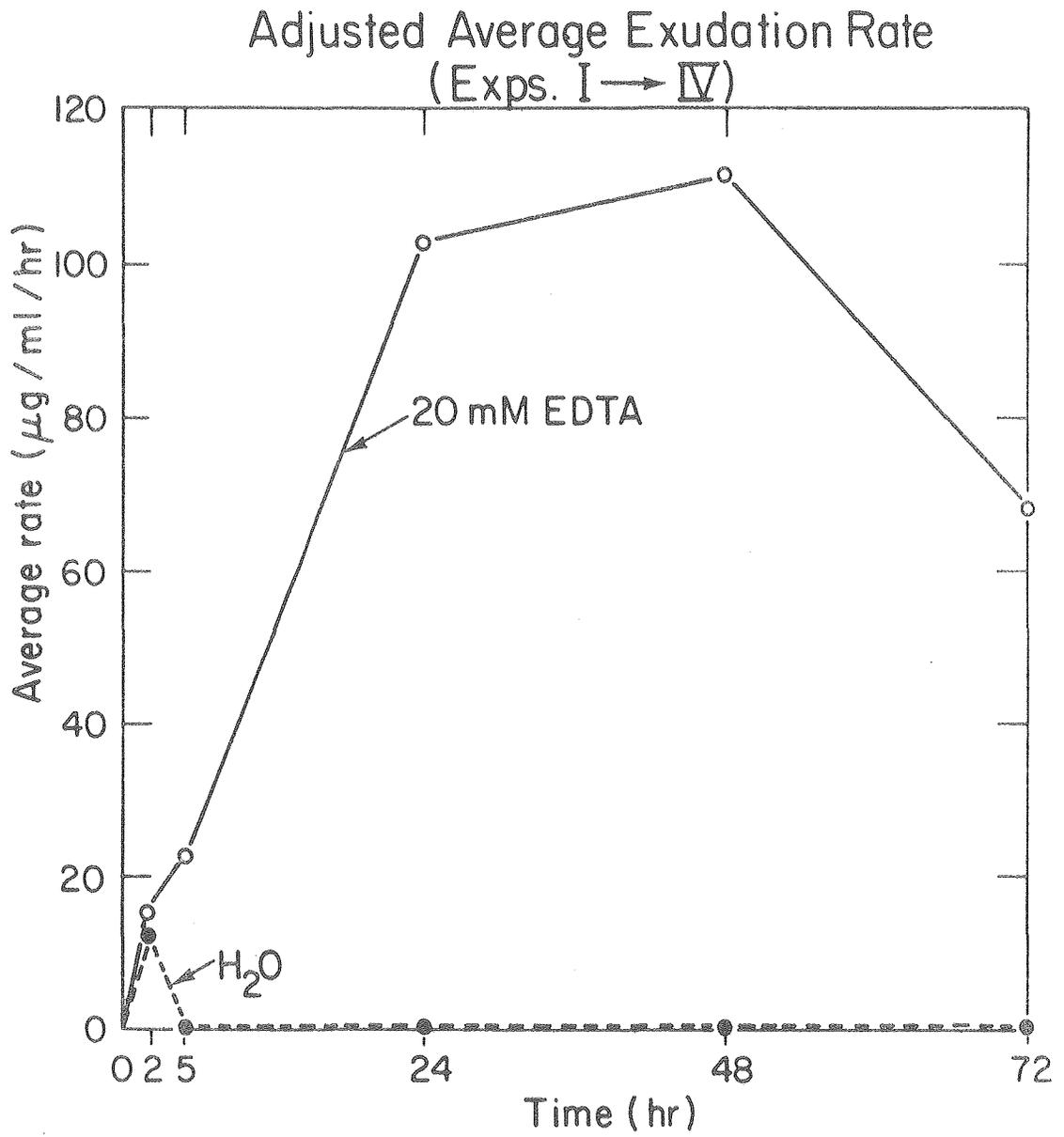


FIG. 26

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CONCLUSION

Phloem exudation studies of selected woody trees have shown:

1. Fraxinus uhdei is particularly useful for phloem exudation experiments.
2. EDTA effectively enhances the exudation of phloem sugars from excised leaflets and whole plant specimens of F. uhdei.
3. EDTA appears to enhance exudation by inhibiting or reducing callose formation in sieve plate pores.
4. EDTA exudation enhancement techniques can be used in the field as well as in the laboratory.

These studies have provided a strong indication that harvesting sugars from actively growing trees for an extended period of time is possible. However, it is apparent that before any system for collecting sugars from trees becomes practical, further studies need to be conducted. For instance, to collect sugars on a year round basis, a means of extending the exudation period beyond the four day period found in these studies must be sought. A consideration of anatomical changes in tissues adjacent to the phloem may prove to be significant in this regard. The design of an appropriate collecting vessel must also be considered. Such a vessel should include features for securely fastening it to a tree trunk and removing it for periodic maintenance. A system for replacing the treatment solutions and withdrawing sugar samples should be included. Vessel size and material of construction must also be considered.

It is evident, therefore, that these and other considerations must be made before the proposed sugar harvesting system becomes practical. Nevertheless, exudation studies conducted thus far have provided an

encouraging basis for which to develop and refine a practical means of harvesting sugars from actively growing trees.

In addition to applications in biomass associated systems, these exudation studies may also have important applications in phloem research. In particular, the bark flap method of harvesting phloem sugars may be a useful tool for studying translocation in trees. Currently, translocation studies in trees are limited to a very few species which exude small amounts of phloem sap over a short period of time (1-2 hrs). Using the bark flap method, however, translocation could be studied in many species and for much longer time periods (3-4 days). Such a research tool would be particularly useful for studying the transport of foliar applied herbicides, pesticides, and growth regulators which may be slowly incorporated and transported in the phloem. Furthermore, translocation kinetics, distribution patterns of translocate, and composition of phloem sap in various tree species may be determined using this method.

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