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Studies on the release of sugar into the vessels of sugar maple (*Acer saccharum*)

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The release of sucrose into the vessel water of sugar maple (*Acer saccharum* Marsh.) has been investigated with biochemical and enzyme-cytochemical methods. The amount of sucrose released into the vessels in isolated stem segments was found to be temperature-dependent. The release of sucrose also proved to be inhibited when *p*-chloromercuribenzoate as an inhibitor of respiratory enzymes was present in the vessels. This suggests that the sucrose release is dependent upon the respiratory activity of some cells. In the cytochemical studies much increased activity of respiratory enzymes (succinate, NAD-dependent isocitrate, and alcohol dehydrogenases) and of acid phosphatases could be demonstrated in the contact cells of the ray and axial parenchyma. Phosphatase activity was concentrated on the large pits between contact cells and vessels. These increased enzyme activities in contact cells were restricted to the time when sucrose appeared in the vessels. Since contact cells are the only cells of the ray and axial parenchyma that show peculiar pit connections with the vessels and also show increased respiratory and phosphatase activity, these cells are considered to be the specific sites of a metabolically controlled sucrose release.

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La libération du sucrose dans l'eau des trachées de l'érable à sucre (*Acer saccharum* Marsh.) a été étudiée avec des méthodes biochimiques et par enzymologie cytochimique. La quantité de sucrose libérée dans les trachées chez des segments de tige isolés s'est montrée liée à la température. La libération du sucrose a été inhibée quand le poison respiratoire *p*-chloromercuribenzoate était présent dans les trachées. Ceci suggère que la libération du sucrose dépend de l'activité respiratoire de certaines cellules. Les études cytochimiques ont permis de démontrer une activité beaucoup plus grande des enzymes respiratoires (succinate, isocitrate tributaire du NAD et les déshydrogénases de l'alcool) et des phosphatases acides dans les cellules de contact du parenchyme des rayons et du parenchyme axial. L'activité phosphatasique était concentrée sur les grandes ponctuations entre les cellules de contact et les trachées. Ces activités enzymatiques accrues dans les cellules de contact étaient restreintes aux périodes où le sucrose apparaissait dans les trachées. Puisque les cellules de contact sont les seules cellules du parenchyme des rayons et du parenchyme axial qui montrent des ponctuations particulières en relation avec les trachées et qu'elles montrent aussi une activité respiratoire et phosphatasique accrue, les auteurs considèrent ces cellules comme étant le siège spécifique de la libération du sucrose sous contrôle métabolique.

[Traduit par le journal]

Introduction

In late winter, the xylem sap of sugar maple contains a relatively high concentration of sugars, mostly sucrose, minute amounts of organic acids, some nitrogen compounds, and inorganic salts (12, 18, 34). The sugar content has been observed to range from 0.5 to 7%, with an average of about 3% (12, 13). With regard to this high sugar content, sugar maple differs greatly from all the other trees known to contain sugars in their xylem sap during early

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spring (10, 15, 35, 41). Although this phenomenon has been known for a long time and is still of some local importance for the production of maple syrup, physiologically it is not understood. For the selection and breeding of "good sap trees," however, the understanding of the physiology of the release of sugar into the xylem sap is of basic interest.

The appearance of sugars in the xylem sap could be regarded as the result of mere leakage of sugars from the parenchymatous cells (16). They might thus enter the xylem passively during the long winter period. However, cell membranes are known to be highly impermeable to sugars. It is therefore more likely that sugars are actively secreted into the vessel sap by the living cells and that metabolic energy is involved in the process. So far there is no direct experimental support for this second view, neither is it known where such processes might take place nor which cells could be involved. The present studies try to answer some of these questions.

Our previous work with other tree species had concerned the "contact cells" of rays and paratracheal parenchyma and indicated that they are most likely the site of solute exchange between parenchyma and vessels (3, 24, 25, 26, 27, 28, 31). In the present investigation of sugar maple we therefore also focused our attention on the contact cells. The phenomenon of sugar release was studied with enzyme-cytochemical and biochemical methods simultaneously.

Material and Methods

Five- to ten-year-old trees of *Acer saccharum* Marsh., naturally grown at the Harvard Forest in Petersham, Massachusetts, were used. For the enzyme-cytochemical studies trees were collected monthly, from February 1970 until September 1971. The intervals were shorter, however, during the spring of both years. For all the enzyme-cytochemical tests fresh, radial sections (35-μ-thick) were prepared from stem disks which were normally taken at 1.3-m height of the stems. Material, fixed in 70% ethanol, was used for starch determination. For the biochemical investigations stem segments of 3 to

FIG. 1. Radial section of the wood of sugar maple (*Acer saccharum* Marsh.) showing starch (*s*) in all the living cells (ray cells, *rc*; living fibers, *lf*; paratracheal parenchyma, *pp*) except the contact cells (*cc*) at the end of the starch resynthesis period in late April. × 180. FIG. 2. Contact cells (*cc*) of a wood ray showing no starch (*s*) in contrast to the other ray cells (*rc*) which are not connected with the vessels (March 31, 1971). × 540. FIG. 3. Contact cells (*cc*) of the paratracheal parenchyma (*pp*) showing no starch in contrast to the other paratracheal parenchyma and fiber cells (March 31, 1971). × 540. FIG. 4. Cytochemical demonstration of acid phosphatase activity in contact cells (*cc*) of a wood ray. Their large vessel-facing pits (*p*) are sites of much increased enzyme activity. Although this activity can be detected during most of the time from November through April, it is most prominent during early sap flow season and during the period of bud swell when the picture was taken (April 24, 1971). *v* = vessel. × 1200. FIG. 5. Shows the same as Fig. 4 for the contact cells (*cc*) of the paratracheal parenchyma. × 1200.

FIG. 6. Cytochemical demonstration of increased phosphatase activity in the contact cells (*cc*) of the ray and the paratracheal parenchyma (April 27, 1970). *v* = vessel. × 180. FIG. 7. Cytochemical demonstration of phosphatase activity in the wood during the vegetation period (July 6, 1971). The contact cells (*cc*) of the wood rays show no increased enzyme activity. × 180. FIG. 8. Cytochemical demonstration of isocitrate dehydrogenase activity in the ray cells at the beginning of the sap flow season (February 26, 1971). The contact cells (*cc*) exhibit much increased isocitrate dehydrogenase activity. × 180. FIG. 9. Cytochemical demonstration of increased isocitrate dehydrogenase activity in contact cells (*cc*) of the ray and paratracheal parenchyma during the period of bud swell (April 27, 1970). *v* = vessel. × 180. FIG. 10. Cytochemical demonstration of increased alcohol dehydrogenase activity in contact cells of the paratracheal parenchyma (*cc*) during sap flow season (March 31, 1971). × 180. FIG. 11. Cytochemical demonstration of isocitrate dehydrogenase activity in the wood during the vegetation period (July 6, 1971). The contact cells (*cc*) of the ray and paratracheal parenchyma show no increased enzyme activity. × 180.

FIG. 12. Control to the cytochemical demonstration of dehydrogenases. No activity appears when the specific substrate is omitted from the incubation medium. *cc* = contact cells. × 540. FIG. 13. High activity of isocitrate dehydrogenase in contact cells (*cc*) of a wood ray in late winter (February 26, 1971). The enzyme activity is located at particles of the size and shape of mitochondria (arrows). × 1200. FIG. 14. High activity of alcohol dehydrogenase in contact cells (*cc*) of the paratracheal parenchyma in late winter (February 26, 1971). The enzyme activity appears mostly in the cytoplasm. × 540. FIG. 15. Cytochemical demonstration of isocitrate dehydrogenase activity in the cells surrounding the vessels during sap flow season (March 16, 1970). The contact cells (*cc*) of the paratracheal parenchyma show much increased enzyme activity while the starch (*s*) storing living fiber (*lf*) is almost inactive at the given incubation time (30 min). The enzyme activity is clearly located at particles of the size and shape of mitochondria (*m*). × 1200. FIG. 16. Cytochemical demonstration of succinate dehydrogenase activity during sap flow season (March 31, 1971). A contact cell (*cc*) of a wood ray which faces a vessel (*v*) shows much increased enzyme activity while ordinary ray cells (*rc*) not connected with the vessel stay quite inactive at the given incubation time (30 min). This enzyme, too, is located in mitochondria (*m*). × 1200.

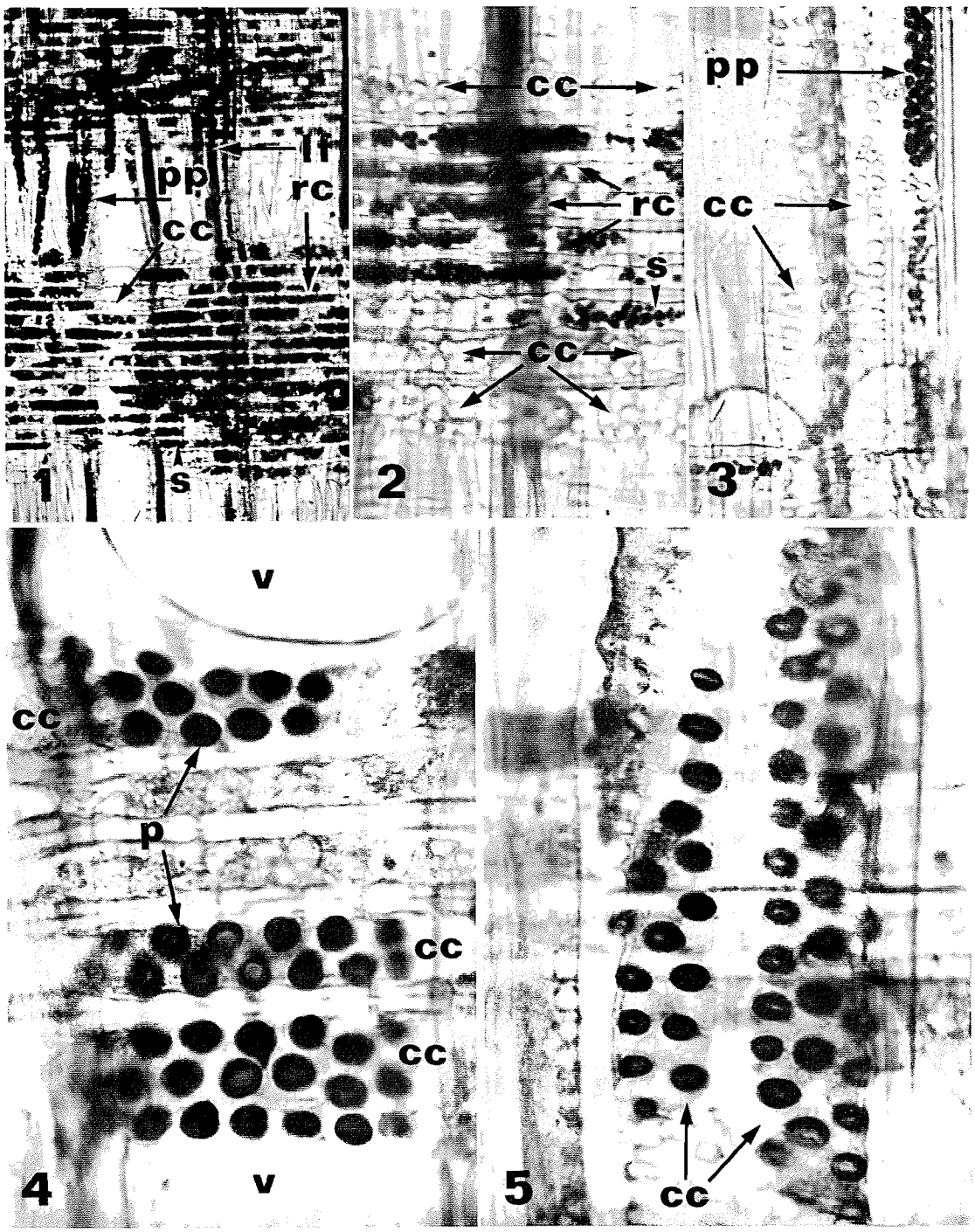
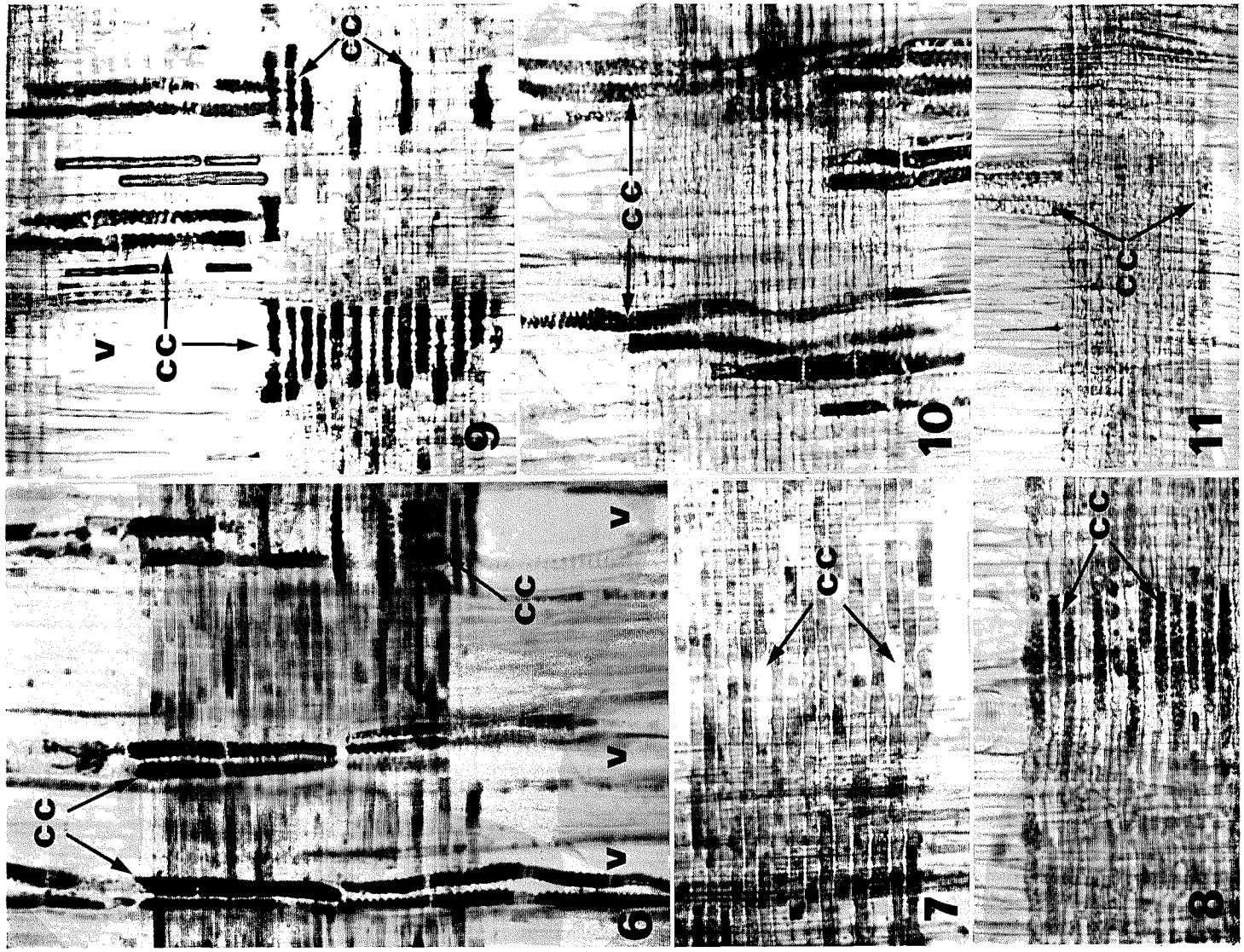
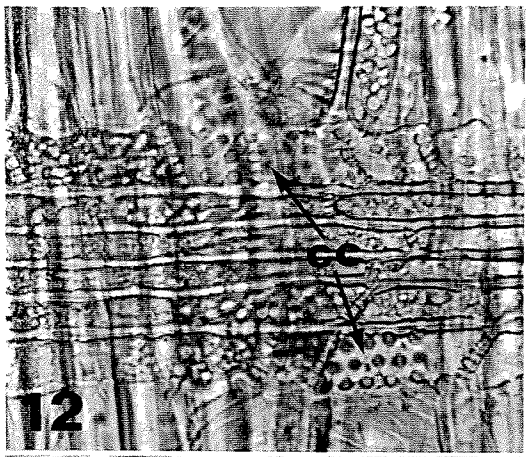
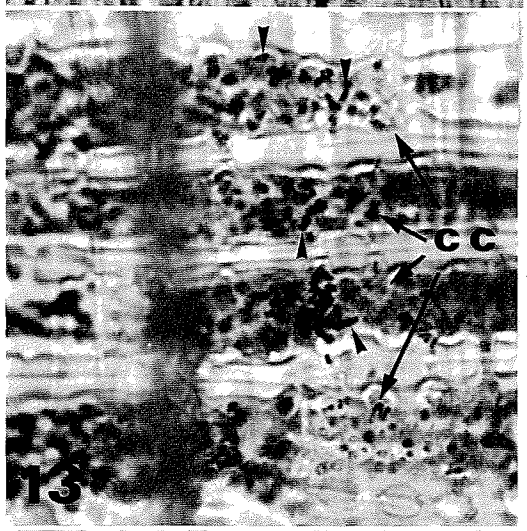
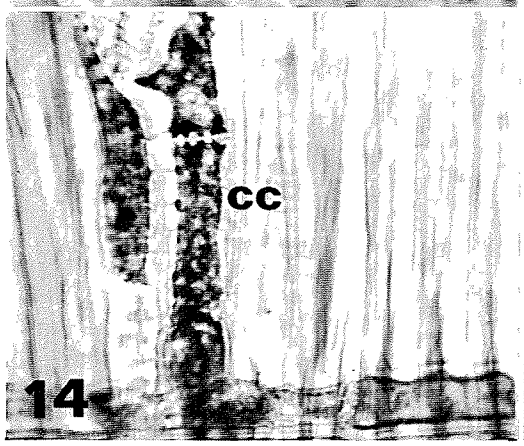


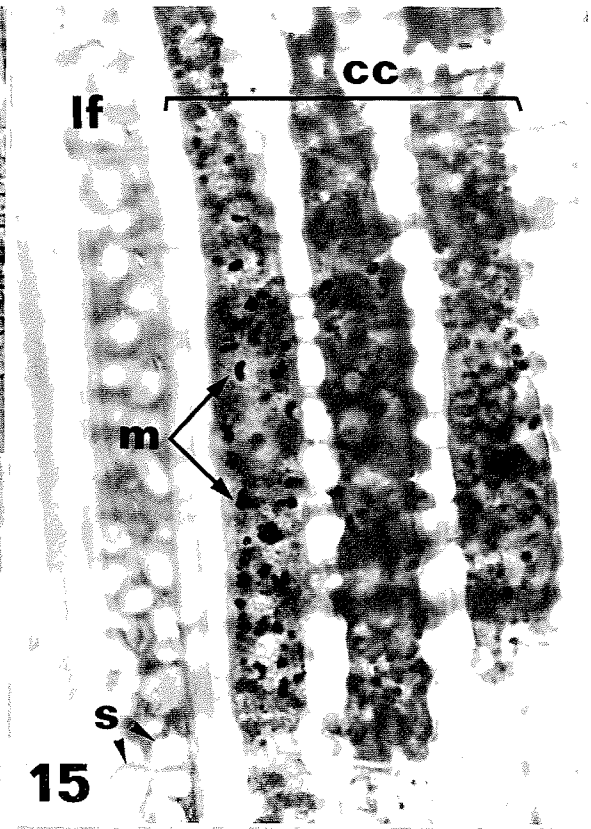
PLATE II

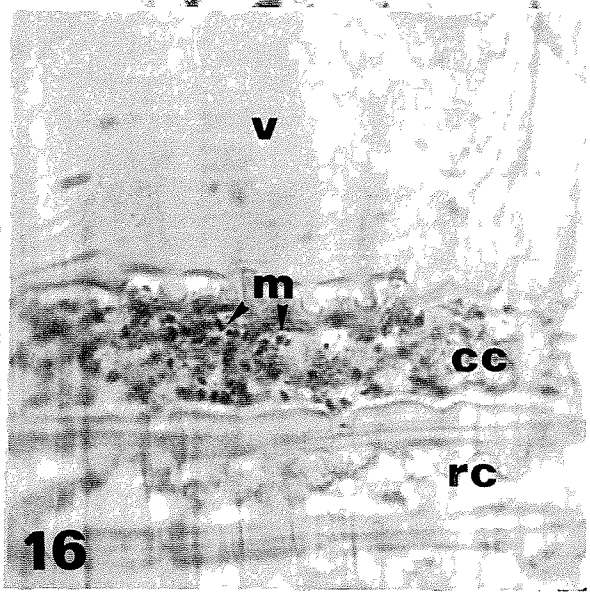












5 cm in length were cut from 5-year-old stems. The diameter of these stem segments was 1.5 cm.

Enzyme-Cytochemical Methods

Acid Phosphatases

The activity of acid phosphatases was investigated with the Gomori method in the form described by Sauter (24, 26). Fresh sections were incubated for 5 and 16 h at 37°C in a substrate medium containing β-glycerophosphate and in a control medium without this substrate (for details see 24, 26).

Dehydrogenases

The activity of succinate dehydrogenase (EC. 1.3.99.1), nicotinamide adenine dinucleotide (NAD) dependent isocitrate dehydrogenase (EC. 1.1.1.41), and of alcohol dehydrogenase (EC. 1.1.1.1) was followed with the nitrobluetetrazolium method in the form described by Sauter and Braun (30). The incubation media consisted of 0.2 ml substrate solution (1 *M*), 0.25 ml phosphate buffer (1/15 *M*; pH 7.0), 0.25 ml Nitro-BT solution (2 mg/ml distilled water; Sigma Chemical Company), and 0.3 ml distilled water. Five milligrams NAD (β-nicotinamide adenine dinucleotide; Sigma) was added when the isocitrate and alcohol dehydrogenases were tested. As substrates we used the disodium salt of succinic acid (Sigma), the trisodium salt of DL-isocitric acid (Sigma), and ethanol (1 *M*). Media without substrate or without the coenzyme NAD were used as controls. The incubation times for the fresh sections usually were 30 and 60 min at 37°C. Normally, after that time, enough diformazan had been produced in the cells by the enzyme-specific dehydrogenation of the individual substrates. Without the specific substrates almost no staining appeared at the same incubation times.

Biochemical Methods

The amount of sucrose released into the vessel water at a given temperature and at a given time of storage was determined biochemically in perfusates of short segments of 5-year-old maple stems. After the bark had been removed the segments were thoroughly washed and perfused with tap water with a suction pump (at 15 in. Hg). Fifty milliliters of water per segment was found to be sufficient to deplete the vessels of sucrose almost entirely. For the pretreatment of the segments with the inhibitor *p*-chloromercuribenzoate (PCMB), this compound was added to the perfusion water in a concentration of 10^{-4} *M*. The segments were then stored for different periods of time up to 48 h (see Figs. 17–19), or in another set of experiments, at different temperatures (see Fig. 19). Thereafter they were perfused a second time with 50 ml tap water. An aliquot of this perfusate was used for the sucrose assay with thiobarbituric acid according to the method of Percheron (17).

Results

The Contact Cells

In sugar maple, as in many other trees, specialized parenchyma cells, called "contact cells" (cf. 1, 2, 3, 25, 26), connect the axial and radial parenchyma systems with the vessels. Anatomically, these contact cells are distinguished in sugar maple by having numerous large elliptical pits of 3 to 5 μ in diameter which face the vessels like windows (Figs. 1–4). They occur in both the wood rays and the paratracheal parenchyma strands (Figs. 2 and 3). In small wood rays all of the horizontal cell rows can be connected with the vessels via the contact cells; in broader ones only the upper and the lower margins show such connections.

Cytochemical Results in Contact Cells

Starch

In the fall, most contact cells showed relatively little starch accumulation in contrast to other ray and axial parenchyma cells which were filled with starch at that time. Starch disappearance in late fall began first in the contact cells, later in the other parenchyma cells. In winter, all contact cells were distinct from other parenchyma cells in that they had no starch. Even in late winter when the sap-flow season started and starch began to be resynthesized in all the living cells (including rays, axial parenchyma, and living wood fibers), contact cells usually stayed free of starch (Figs. 1–3).

Acid Phosphatases

Starting in late fall when starch disappeared in contact cells, phosphatase activity increased remarkably in these cells and stayed at a fairly high level during the whole winter. Thus, during the sap-flow season all contact cells were distinguished from the rest of the ray and axial parenchyma cells by a highly increased phosphatase activity (Fig. 6). During most of this period (November through April), phosphatase activity was concentrated on their large, vessel-facing pits (Figs. 4 and 5). This localized activity at the pits disappeared only temporarily when the starch was resynthesized towards the end of the sap-flow season (end of March until mid-April), but was again very distinct when the buds started to swell and the starch mobilization began (end of April). When the leaves had fully developed, increased phosphatase activity disappeared in contact cells and at their large pits (Fig. 7) and could not be detected anymore during the following summer months.

Activity of Respiratory Enzymes

The activity of three enzymes was studied cytochemically in the various parenchyma cells: the NAD-dependent isocitrate dehydrogenase and the succinate dehydrogenase, which both

are thought to give information about the aerobic respiration, and the alcohol dehydrogenase, which is thought to indicate anaerobic respiration in our tissues.

The first striking result was that all three enzymes were found to be very active in ray and axial parenchyma cells throughout the whole winter period (November through April). An incubation time of only 30 min was sufficient to produce a considerable amount of the reduced nitrobluetetrazolium salt (diformazan) by the activity of the tested enzymes within the cells (Figs. 8, 9, and 10). This contrasts considerably with the enzyme activity found during the following vegetation period (June through September) when the same tissues needed more than twice the incubation time to produce similar amounts of diformazan under exactly the same test conditions (Fig. 11). During the vegetation period, only the parenchyma cells of the newly formed annual ring showed high activities, whereas in the other annual rings of the sapwood little activity could be detected.

The second unexpected result was that all three enzymes proved to be much more active in all the contact cells when compared with the other living cells in the wood (Figs. 8–10 and 13–16). These greatly increased activities of the isocitrate, succinate, and alcohol dehydrogenases in contact cells could be demonstrated throughout winter and spring, but not during the following months of the vegetation period (Fig. 11). In summer, only a few contact cells of the newly formed annual ring showed increased dehydrogenase activities for reasons we still do not know.

Figures 15 and 16 show most clearly the difference in the activities between contact cells and ordinary ray or fiber cells for the isocitrate and the succinate dehydrogenases during the sap-flow season.

To exclude the possibility that these increased enzymatic activities of the contact cells are artificially caused by a faster penetration of the

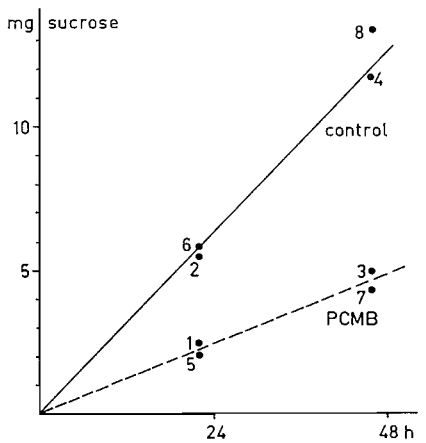


FIG. 17. Amount of sucrose released into the vessels of PCMB-treated (segments 1, 3, 5, 7) and untreated stem segments (control segments 2, 4, 6, 8) after 22 and 46 h of storage at 22°C. The amount of sucrose is given in milligrams per 10 grams dry weight of wood. Stem collected on March 2, 1971. The numbers indicate in conse-c utive order the positions of the segments along the axis of the stem (1 apical, 8 basal end).

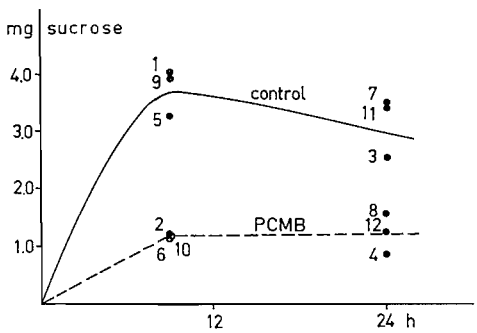


FIG. 18. Amount of sucrose released into the vessels of PCMB-treated and untreated stem segments after 9 and 24 h of storage at 22°C (in milligrams per 10 grams dry weight of wood). Stem collected on March 30, 1971.

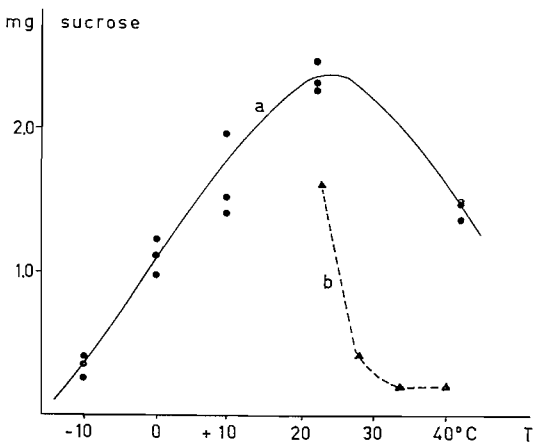


FIG. 19. Amount of sucrose released into the vessels of small stem segments in milligrams per segment after 23 h of storage at different temperatures (*a*: −10°, 0°, 10°, 22°, 40°, and *b*: 22°, 28°, 34°, 40°C, respectively). The stem segments for curve *a* were collected on March 18, 1971, for curve *b* on March 25, 1971.

chemicals into these cells during the cytochemical test, controls were run in which sections were preincubated for 3 h in the cold (4°C) before the actual incubation at higher temperature (37°C for 30 min) was done. In spite of this preincubation the same specific differences in enzyme activities between contact cells and ordinary parenchyma cells appeared.

Further controls emphasized the specificity of the reaction; when the specific substrate was missing in the incubation media almost no diformazan was produced (Fig. 12).

The intracellular localization of the diformazan produced by the action of the enzymes is interesting, too. In the case of the alcohol dehydrogenase most (but not all) of the staining was found in the cytoplasm (cf. Fig. 14); whereas, in the case of the isocitrate and succinate dehydrogenases most staining appeared on particles of the size and shape of mitochondria (cf. Figs. 15 and 16). As these latter two enzymes are known to be confined to these organelles there is little doubt that the particles revealed in Figs. 15 and 16 are indeed mitochondria. They show up so clearly (cf. Fig. 15) that we seem to have found, for the first time, an excellent method for the reliable demonstration of mitochondria in woody cells by light microscopy.

Biochemical Results on Sugar Secretion

The increased activities of respiratory enzymes and of acid phosphatases in contact cells may be taken as an indication that these cells are actively involved in the release of sugars into the vessels. To study this metabolic dependence further, sugar release into the vessel water was measured quantitatively in untreated stem sections and in stem sections treated with a respiratory inibitor, *p*-chloromercuribenzoate (PCMB).

Figure 17 shows the result of a typical experiment with eight segments of a maple stem. At the beginning of the experiment, all eight segments (numbers 1 to 8 in Fig. 17) were perfused until no sugar was left in the vessel water; four of them were perfused with tap water (controls), the other four with tap water to which PCMB (10^{-4} *M*) had been added. The segments were stored for 1 and 2 days at room temperature and the sucrose released into the vessel water during this time was then determined again in both sets. The upper line in Fig. 17 shows a nearly linear relationship between the amount of released sucrose and the time of storage in the control segments (numbers 2, 4, 6, and 8 in Fig. 17). Stems collected in February and early March always showed this linear relationship. Treatment with PCMB, however, resulted in a clear suppression of sucrose release down to 39% and 34% of that of the controls for 1 and 2 days of storage, respectively (lower line of Fig. 17).

When the same experiment was performed with stems collected in late March or early April, the inhibition of sucrose release into vessels with PCMB could be demonstrated in a similar way (Fig. 18). However, the amount of sucrose released into the vessels did not increase any longer in the control segments after 10 h of storage. This might indicate somewhat different physiological conditions of the sucrose release, e.g., a shortage of sucrose. This is also indicated by the fact that most of the starch had been resynthesized at this time in the living tissues.

In other experiments the temperature dependence of the sucrose release was studied. When stem segments were stored for 1 day at temperatures ranging from −10°C to 22°C (curve *a* in Fig. 19) and in a similar experiment from 22°C to 40°C (curve *b* in Fig. 19), the largest amount of sucrose in the vessel water was found between 10° and 22°C.

Discussion

In trees such as *Acer*, *Betula*, *Carpinus*, *Alnus*, and *Populus*, considerable amounts of sugars have been found in the vessel water during early spring (13, 15, 35, 41). The phenomenon itself had been studied already by the early physiologists (7, 10). The main question, however, whether these sugars enter the vessel water passively by diffusion, or whether metabolic activity of living cells is involved, is still open.

Münch (16) considered the appearance of sugars in the vessel water during spring to be due to exosmosis. Diffusion of sugars out of the parenchymatous cells of the wood was assumed to go on from late fall until spring, thus leading to the commonly observed sugar accumulation in xylem water. The rate of sugar diffusion per day and per square meter of surface area of parenchyma cells which would be necessary to explain the observed sugar concentration in the

vessels was found to be very low (1 to 4 mg sugar per day and per square meter of parenchyma surface). Münch's explanation, therefore, seemed to be quite reasonable.

There are, however, earlier results which are quite inconsistent with this view. Jones and Bradlee (12) found, in extracts from sugar maple wood, sucrose and hexoses in similar concentrations, while sucrose was the only sugar in the xylem sap (more than 99% of the sugar was sucrose). It would be difficult to explain how sucrose but not the smaller hexose molecules could diffuse into the vessel water. In other tree species in which hexoses *and* sucrose have been detected in the vessel water (41) the decision whether these sugars have accumulated by exosmosis is more difficult.

There are further arguments against Münch's hypothetical explanation. In sugar maple, for instance, sucrose is found in the vessels as early as late fall (33). This would necessitate "diffusion rates" at least 10 times higher than those considered by Münch (16). Furthermore, we have good reason to believe that it is not the total surface area of parenchyma which is contributing to the sucrose accumulation in the vessels as presumed by Münch but at the most only that part of it which is adjacent to the vessels. The permeation rate of sucrose must therefore be still higher. These considerations are supported by our perfusion experiments in which 6 to 9 mg of sucrose per day and per given stem segment was released into the vessel water. This is 10 to 30 times faster than Münch's calculated diffusion rates of 0.3 to 0.6 mg (3).

The results of our study suggest that the passage of sucrose from the living cells into the vessel water is not merely a diffusion process but dependent upon some cellular activity. The release of sucrose into the vessels is temperature-dependent and likely to be dependent upon the respiratory activity of cells. When the storage temperature of the stem segments was raised to 22°C, the sucrose release into the vessels was considerably enhanced, while still higher temperatures up to 40°C decreased it again. Introducing PCMB into the stem segments via the vessels resulted in a drastic reduction of sucrose release down to one third of that of the controls. Since PCMB completely inhibits most of the dehydrogenases that are essential for the aerobic and anaerobic respiration (19), this finding is taken as an indication that respiratory activity is necessary for the release of sucrose into the vessels of sugar maple.

Whether PCMB has an additional inhibitory effect on the sucrose release which might be caused by its interference with membrane-located permeases (8) cannot yet be decided.

A metabolically controlled sucrose release in sugar maple is also indicated by our cytological and enzyme-cytochemical results. The cytological studies clearly showed that the "contact cells" are the only cells of the ray and axial parenchyma that are connected with the vessels by means of unusually large and numerous pits (for the cytological and physiological peculiarities of contact cells see 1, 2, 3, 24, 25, 26, 28). With respect to their anatomy, at least, these specialized cells seem to be the specific sites of sucrose release. This is further emphasized by the enzyme activities which were detected in these cells. Contact cells proved to differ remarkably from all the other parenchyma cells by a much increased activity of respiratory enzymes during the times of sugar secretion. Both the aerobic respiration (as indicated by the succinate and the NAD-dependent isocitrate dehydrogenases) and the anaerobic respiration (indicated by the alcohol dehydrogenase) are obviously much higher in contact cells. These results are somewhat related to the results which have been obtained in other plant cells engaged in secretion or transport of sugars. Ziegler (40, 41) found increased respiratory activity in sugar secreting nectaries. High respiratory activity is also a characteristic feature of cells and tissues specialized for the transport of assimilates (14, 30, 32, 36, 37, 38, 42).

The activity of acid phosphatases, too, proved to be much higher in contact cells, at least at certain times. Similar results have been reported for poplar and birch (26, 28). This is of particular interest as, in all plant cells concerned in secretion or transport of sugars, such as companion cells, Strasburger cells (albuminous cells), bundle-sheath cells, and nectaries, high phosphatase activity could be demonstrated too (4, 5, 9, 29, 30, 39, 41).

A relationship between the observed respiratory and phosphatase activities in contact cells and the release of sucrose is further indicated by the fact that these increased activities were found only during the time when sucrose appeared in

the vessels (November through April). In the fall, when sucrose first appeared in the vessels, contact cells began to show much increased respiratory and phosphatase activity; in late spring, as soon as the leaves had fully expanded, increased activity disappeared in almost all contact cells.

The results also indicated that there might be temporary conditions during that period which are less favorable for the sucrose release. The phosphatase activity dropped remarkably in contact cells when starch was resynthesized (25, 27, 28) in the living cells of the wood, in late winter (end of March), and the availability of sucrose for secretion might have decreased. Phosphatase activity increased again immediately as soon as the mobilization of starch started during the period of bud swell.

Thus, the biochemical and cytochemical findings suggest that the contact cells are the specific sites of sucrose release and that their metabolism is involved in this process. Yet very little can be said about the mechanism of sucrose release in detail. The high phosphatase activity found at the large pits between contact cells and vessels seems to be part of the release mechanism as this enzyme activity is detectable only when sucrose has to pass these specific sites. The same observations were made in poplar and birch where this peculiar enzyme localization had been studied for several years (26, 28) and where a preliminary explanation had been suggested (28). In yeast and animal cells which do not have such peculiar pit formations, "surface-located" phosphatases have been reported and were also considered to be involved in sugar permeation (6, 11, 20, 21, 22, 23, 36). Although the results in both plant and animal cells are quite comparable in suggesting the participation of phosphatases in permeation of sugars through the plasmalemma, further information is needed before we know how these enzymes really act in the mechanism of permeation.

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