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Cytochemical Localization of ATPase Activity in Phloem Tissues of *Ricinus communis*

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Summary

Standard lead precipitation procedures have been used to examine the localization of ATPase activity in phloem tissues of *Ricinus communis*. Reaction product was localized on the plasma membrane of the companion cells associated with sieve elements and of parenchyma cells in phloem tissues from the leaf, petiole, stem and root. ATPase activity was also present on the plasma membrane and dispersed P-protein of sieve elements in petiole, stem and root tissue, but was absent from the plasma membrane of these cells in the leaf minor veins. Substitution of β-glycerophosphate for ATP produced no change in the localization of reaction product in leaf tissue. These findings are discussed in relation to current theories on the mechanism of sugar transport and phloem loading.

Keywords: ATPase cytochemistry; Phloem; *Ricinus communis*.

1. Introduction

Recent reports on the mechanism of sugar transport across plant cell membranes implicate a proton/sugar cotransport system driven by a gradient of electrochemical potential for protons created by a proton-pumping ATPase (for recent review, see BAKER 1978). The association of ATPase activity with membranes believed to be active in sugar transport has been demonstrated using cytochemical procedures (CATTESSON 1973, GILDER and CRONSHAW 1973 a, b, 1974, YAPA and SPANNER 1974, BENTWOOD and CRONSHAW 1978), but correlated physiological and biochemical/cytochemical studies are lacking. One system in which sugar/proton co-transport has been demonstrated is the sucrose loading mechanism present in the hollow petioles of *Ricinus* (MALEK and BAKER 1977, 1978). The present cytochemical investigation leads on from

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these last mentioned studies and examines the localization of ATPase activity in phloem tissues from *Ricinus* petiole, leaf, stem, and root.

Previous cytochemical investigations of ATPase activity in phloem have been confined to leaf and petiolar tissues and have indicated differences in localization of reaction product between different plant species (see BENTWOOD and CRONSHAW 1978). It is of interest to note whether the same discrepancies may also exist between different regions of the same plant. This study represents the first attempt to examine the presence of ATPase activity in phloem tissues of the root and also the first to compare the pattern of localization of ATPase activity in phloem tissues in sugar source (*i.e.*, leaf) and sugar sink (*i.e.*, root) regions of the same plant. The cytochemical method adopted for this study is the conventional lead capture technique of WACHSTEIN and MEISEL (1957) as applied to plant tissues by BENTWOOD and CRONSHAW (1978).

2. Materials and Methods

Samples were taken from 3–6-month-old specimens of *Ricinus communis* which had been cultivated in a mixture of peat and soil and grown in conditions of high temperature and high humidity. The samples consisted of segments of leaf lamina from fully expanded leaves, slices of the central region of the petiole, pieces of bark peeled from internode regions of the same plants, and segments of root tissue, taken about 5–10 mm from the root tip. Tissues were normally fixed in a mixture of 1% glutaraldehyde and 4% formaldehyde in 100 mM cacodylate buffer (pH 7.2) for 2 hours at 0 °C. After fixation, tissues were washed in 50 mM tris-maleate buffer (pH 7.2) for 2 hours at 23 °C, during which time transverse sections, 100 and 200 µm thick, were cut from leaf, petiole and stem tissue, using an Oxford Vibratome. Tissue slices and root segments were incubated in a medium containing 2 mM ATP, 2 mM $Pb(NO_3)_2$, 2 mM $Mg(NO_3)_2$ and 100 mM tris-maleate buffer (pH 7.2) at 23 °C for 2 hours. Controls involved incubation in media which lacked ATP or lead, or to which 10 mM NaF was added, or in which 2 mM β-glycerophosphate was substituted for ATP (used for leaf tissue only).

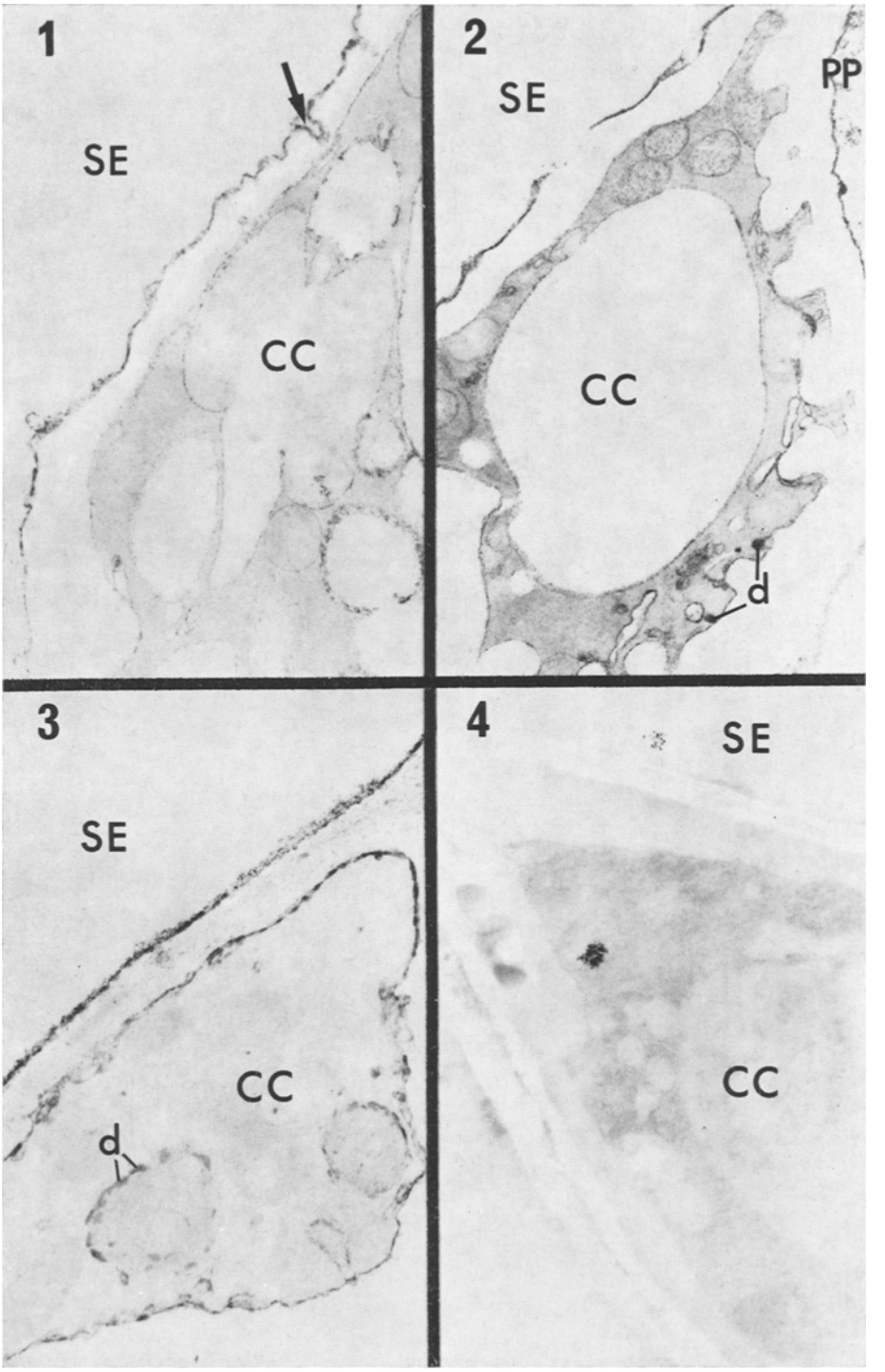
Figs. 1–3. Transverse sections of phloem sieve elements (*SE*) and companion cells (*CC*) incubated in the complete medium

Fig. 1. Phloem tissue from petiole. Staining of the sieve element plasma membrane is especially apparent within a plasmodesmata (arrow). Membranes of companion cells are stained to a lesser extent. ×17,000

Fig. 2. Phloem tissue from stem. Stain on plasma membranes of sieve element (*SE*), phloem parenchyma cell (*PP*) and companion cell modified in the form of transfer cell (*CC*). Membranes of endoplasmic reticulum, central vacuole, and other less easily recognisable organelles are also stained. Large rounded deposits (*d*) are present in minus-substrate controls. ×11,000

Fig. 3. Phloem tissue from root. Reaction product is present on plasma membrane of sieve element (*SE*) and companion cell (*CC*). Deposits (*d*) are present here and also in minus-substrate controls. ×34,000

Fig. 4. Transverse section of sieve element and companion cell from phloem tissue of stem. Incubated in the absence of substrate. ×31,000



Figs. 1–4

All tissues were subjected to a 2–3 hours post-incubation rinse in distilled water, followed by an overnight post-fixation in 1% OsO_4 in 20 mM cacodylate/acetate buffer (pH 7.2) at 2–3 °C. After post-fixation, tissues were rinsed (3 × 10 minutes) in cacodylate buffer, dehydrated in a graded acetone series, and infiltrated with Spurr resin. Gold coloured sections were cut for electron microscopy and viewed, without post-staining, in a Jeol 100S microscope.

3. Results

The pattern of deposits due to ATP-hydrolysing activity was essentially similar in the phloem tissues of the petiole, stem, and root. In the sieve-elements of these tissues, reaction product was present along the plasma membrane and in association with parietal P-protein filaments (Figs. 1–3). In the vicinity of the sieve plate, highly condensed accumulations of P-protein were less heavily stained than dispersed filaments (Fig. 5). In the sieve-element companion cells, reaction product was associated with the plasma membrane, endoplasmic reticulum and with certain vesicular components. The larger, more globular deposits, sometimes seen associated with membranes in these cells (Figs. 2 and 3), were often present in minus-substrate controls and were therefore not considered to result from enzymic activity. The distribution of reaction product in the companion cells of the stem that were not modified in the form of transfer cells, was identical to that seen in similar, modified cells. The pattern of staining in phloem parenchyma cells, as illustrated for root tissue (Fig. 6) was identical to that in companion cells, although where cytoplasmic content was obviously disrupted (not shown), deposits were also present in the cytoplasm. The plasma membrane of cambial cells stained positively for ATPase activity (Fig. 7), but reaction product was absent from cortical cells.

The incidence of reaction product due to ATP-hydrolysing activity was less consistent in phloem tissue of the leaf minor vein than in phloem from the other locations. When staining was visible, the pattern of distribution was similar in some respect to that previously described. Deposits were associated

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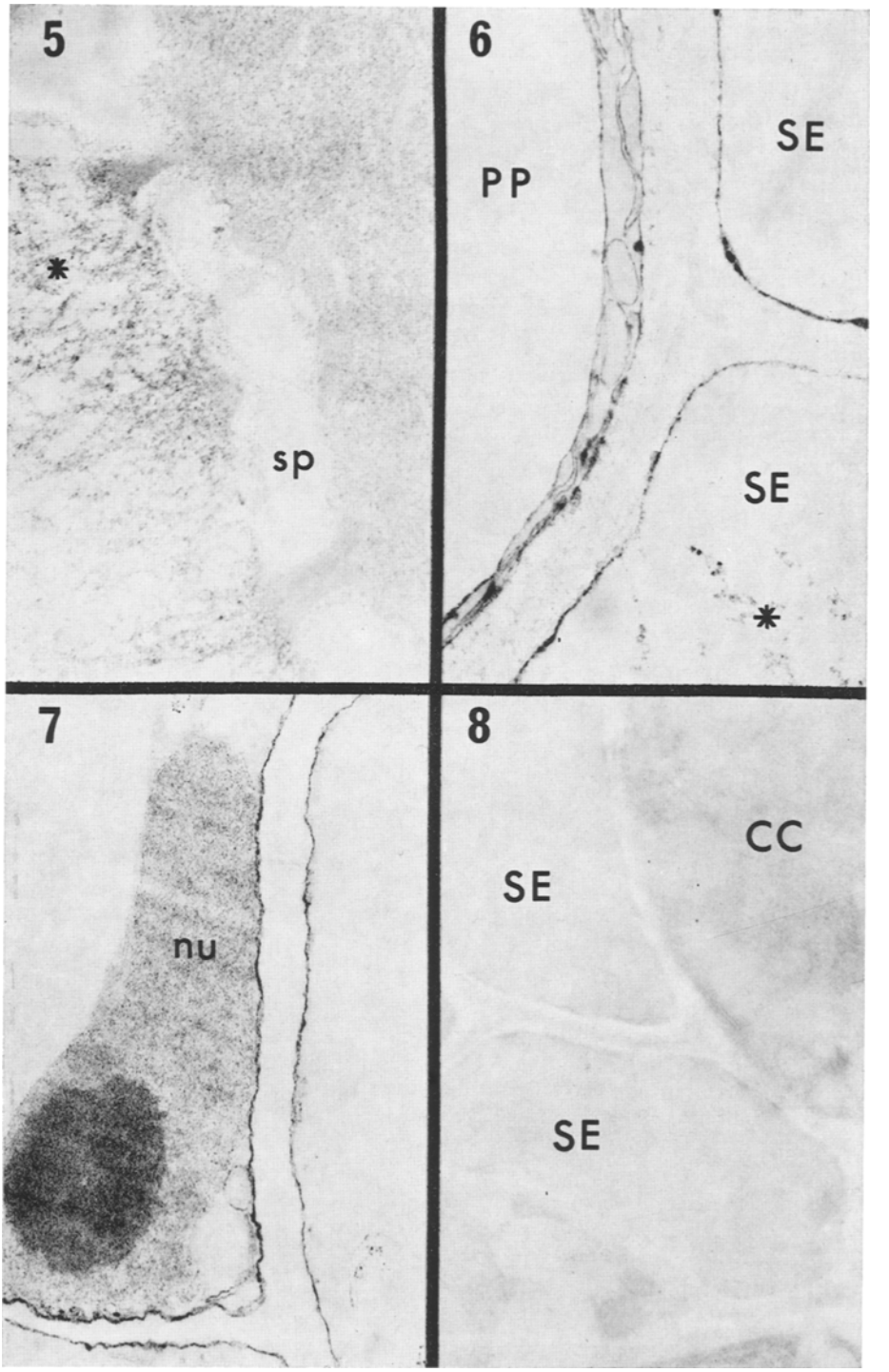
Fig. 5. Longitudinal section through sieve plate (*sp*) from stem incubated in the complete medium. Dispersed filaments of P-protein (*) stain more strongly than compacted P-protein on the opposite side of the sieve plate. The plasma membrane is unstained. ×30,000

Fig. 6. Transverse section across a phloem parenchyma cell (*PP*) and two sieve elements (*SE*) from root incubated in the complete medium. Plasma membrane of all cells, and tonoplast and organelle membranes of parenchyma cell, are stained. Reaction product is also present on dispersed P-protein filaments (*). ×21,000

Fig. 7. Transverse section of cambial layer from stem incubated in the complete medium. Plasma membranes stain strongly; nucleus (*nu*) and nucleolus stain here and also in medium lacking substrate. ×12,000

Fig. 8. Transverse section across phloem from leaf minor vein. Control section incubated in the absence of substrate. No reaction product visible. ×16,000



Figs. 5–8

with dispersed fibrils of P-protein in the lumen of the sieve element and around its perimeter (Figs. 9–11). The plasma membrane of the sieve element was often obscured by deposits of reaction product on parietal P-protein but did not itself appear to be stained (Fig. 11). The endoplasmic reticulum of the sieve element also remained unstained (Fig. 11). Reaction product was usually associated with the plasma membrane of the companion cells (Figs. 9 and 10) but not invariably so (Fig. 11). The extent to which the other cellular membranes were associated with reaction product was very variable.

Apart from the previously mentioned "globular" deposits on membranes, minus-substrate controls were negative for all the tissues used (*e.g.*, stem tissue, Fig. 4, leaf tissue, Fig. 8). No reaction product was present in the absence of lead in the incubation medium. In treatments where deposits normally occurred, their presence could be averted when 10 mM NaF was present in the incubation solution (*e.g.*, leaf tissue, Fig. 12). When β-glycerophosphate replaced ATP, the distribution of reaction product in the phloem tissues of leaf minor vein remained essentially unchanged (Fig. 13).

The cytochemical procedures adopted in the present study have been carried out at pH 7.2 to enable comparison with other studies and to minimize interference from acid phosphatase activity, which often occurs in phloem tissue (ESCHRICH and HEYSER 1975).

4. Discussion

Before attempting to comment upon the present findings, it is necessary to make a brief reference to the validity of the cytochemical approach in the light of a recent report claiming that reaction product described in many previous studies of ATPase localization might contain no lead and therefore

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Figs. 9–11. Transverse sections showing sieve elements (*SE*) and companion cells (*CC*) from leaf minor vein. Complete medium

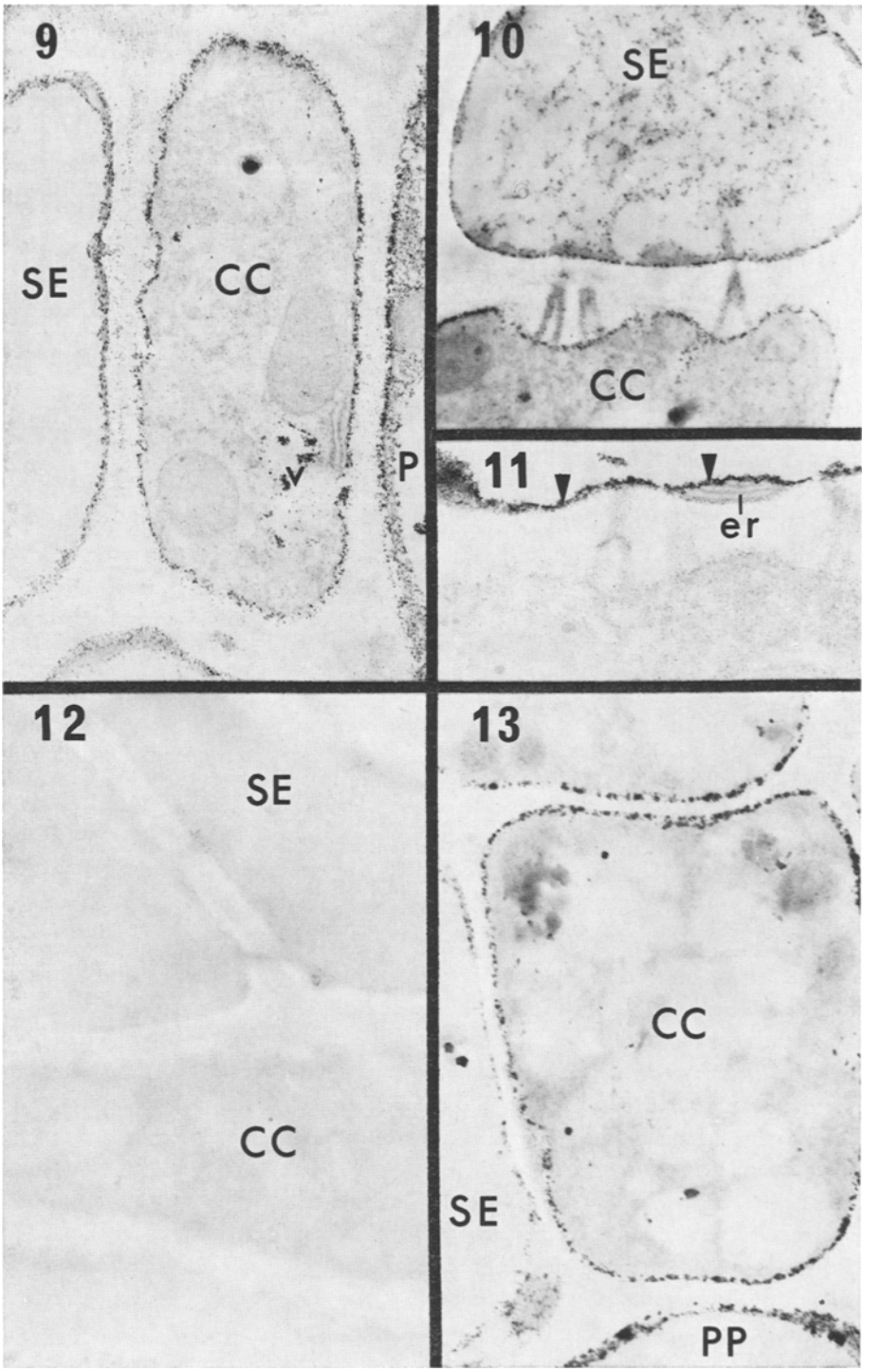
Fig. 9. Reaction product visible around perimeter of sieve element and companion cell. Stain present in some regions of the cell wall and in association with vesicles (*v*) within the companion cell. Cytoplasm in an adjacent, poorly preserved, parenchyma cell (*P*) is also stained. ×26,000

Fig. 10. Reaction product present in the vicinity of the plasma membrane in sieve element (*SE*) and companion cell (*CC*). Stain also present on dispersed P-protein filaments. ×23,000

Fig. 11. Reaction product along the perimeter of the sieve element (arrowheads) is not continuous with the plasma membrane in the vicinity of cisternae of endoplasmic reticulum (*er*). No stain is present on plasma membrane of companion cell. ×30,000

Fig. 12. Longitudinal section of phloem from leaf minor vein. Control section incubated in complete medium with 10 mM NaF. No reaction product visible. ×23,000

Fig. 13. Transverse section of phloem from leaf minor vein. Incubated with β-glycerophosphate as substrate. Stain present along plasma membrane of companion cells (*CC*) and over cytoplasm of a damaged phloem parenchyma cell (*PP*). Stain also present around perimeter of sieve element (*SE*). ×21,000



Figs. 9–13

be misleading (van Steveninck 1979). This claim is refuted in depth in a forthcoming paper from the present authors (Hall *et al.* 1980) but notwithstanding this, it is clear that when, as in the present study, appropriate control treatments are performed (*i.e.*, minus ATP, minus $Pb(NO_3)_2$, and plus NaF), reaction product must be due to lead and must represent ATP-hydrolysing activity in the tissue. The lead content of the deposits described in the present study in *Ricinus* leaf tissue has been additionally confirmed using X-ray microanalysis (for details, see Hall *et al.* 1980).

The distribution of ATP-hydrolysing activity in phloem tissues of *Ricinus* described in the present study corresponds closely to results obtained from other plant species. The presence of reaction product at the sieve tube plasma membrane in petiole, stem, and root tissues of *Ricinus* agrees with results using *Cucurbita* petiole and leaf (Gilder and Cronshaw 1973 a), *Nicotiana* leaf (Gilder and Cronshaw 1973 b, 1974) and *Tetragonia* petiole (Yapa and Spanner 1974). Reaction product is not, however, always found at this location and the apparent absence of ATP-hydrolyzing activity along the plasma membrane of sieve elements in *Ricinus* leaf tissue corresponds with results obtained from leaf tissue of *Pisum* (Bentwood and Cronshaw 1978). This point of discrepancy between different sources of phloem might reflect a difference in functioning of these cells but further corroborative evidence is clearly required. The localization of ATP-hydrolyzing activity associated with dispersed fibrils of P-protein is another area in which results from different cytochemical studies are at variance. Deposits associated with dispersed P-protein are present in *Cucurbita* (Gilder and Cronshaw 1973 a), *Nicotiana* (Gilder and Cronshaw 1973 b, 1974), and *Ricinus* (present study), but absent in *Robinia* and *Acer* (Cattesson 1973), *Tetragonia* (Yapa and Spanner 1974), and *Pisum* (Bentwood and Cronshaw 1978). This discrepancy reflects the controversy associated with the biochemical characterization of phosphatase activity in phloem exudate (see Evert 1977). Biochemical approaches to this question appear preferable in that they allow superior control of incubation conditions, *i.e.*, pH, cation content, substrate concentration. If the cytochemical findings are confirmed, the lack of universal ATPase activity associated with P-proteins might indicate that this material is not fulfilling a major role in the active transport process.

The pattern of distribution of ATP-hydrolyzing activity in companion cells and phloem parenchyma cells in *Ricinus*, agrees with that found in *Cucurbita*, *Nicotiana*, and *Pisum* (see above for references). However, the lack of substrate specificity observed in leaf tissue in the present study conflicts with results from cytochemical and biochemical studies on ATPase activity in the phloem of *Nicotiana* (Gilder and Cronshaw 1974), which demonstrated a specificity for nucleoside phosphates. In this latter study it was shown that formaldehyde/glutaraldehyde fixation of crude homogenates of vascular tissue caused a large (ca. 87%) reduction in enzyme activity and a drop in substrate

specificity. It would appear that substrate specificity should be determined biochemically rather than by cytochemical procedures involving fixed tissue. If confirmed, the presence of a relatively nonspecific phosphatase at the plasma membrane would fit with the concept of sugar transport by some form of vectorial group transfer reaction involving successive phosphorylation and dephosphorylation steps (for reviews see LÜTTGE 1978, BAKER 1978). There is however little evidence that sucrose is hydrolyzed prior to the loading process (see GEIGER 1975) and in the maize scutellum, a tissue for which this model was originally formulated, current results point more towards an electrogenically coupled uptake mechanism (*cf.*, HUMPHREYS 1973 to 1978).

The relationship between the presence of ATP-hydrolyzing activity in the phloem and possible mechanisms of phloem transport is, as mentioned above, open to speculation but its existence does support currently held ideas that phloem loading may be linked to ATP-hydrolysis by an ATP-energized proton efflux pump which provides a gradient for proton/sugar co-transport inwards across the loading membrane (see BAKER 1978). The diversity of sites within the phloem which have been reported in this and similar cytochemical studies (GILDER and CRONSHAW 1973 a, b, 1974, BENTWOOD and CRONSHAW 1978), to be associated with ATP-hydrolyzing activity, makes it impossible to identify the site of the hypothetical membrane transport step using this approach. If, however, a common mechanism is operating in all the plant species studied, the most plausible site of phloem loading is at the plasma membrane of companion cells and/or phloem parenchyma cells, since these membranes are stained in almost all preparations. This conclusion also fits with the findings of studies which have examined the site of sucrose loading using autoradiography, micro-densitometry, differential plasmolysis and differential cryopreservation (see FISHER 1978). The present finding that the companion cells and phloem parenchyma cells are associated with ATP-hydrolyzing activity regardless of whether they occur in source (*i.e.*, leaf) or sink (*i.e.*, root) tissues, requires explanation since it might be taken to imply that both the sugar loading and unloading steps are active processes. If the sugar/proton co-transport model is to be invoked, a reversal in the direction of the sugar pump requires a reversal in the direction of the proton gradient or a change in the electrical potential of the transport membrane. Again, in sink tissues, one might envisage a pumping of sugar together with co- or counter-transport of some other ion, such as potassium which appears at high concentration in phloem exudates (HALL and BAKER 1972). Alternatively, unloading in roots may well occur via the symplast without the need to cross a membrane; a proton-pumping ATPase might then provide a gradient for keeping sugars within the symplast. It should perhaps be emphasized at this point that the association between ATP-hydrolyzing activity and phloem transport established from cytochemical studies is at the best indirect and there is a need for corroborative biochemical evidence. This latter approach is complicated by the

difficulty of obtaining well defined membrane fractions (see Quail 1979), particularly when, as in the present case, these fractions need to be prepared from specified cell types (*i.e.*, sieve elements, companion cells, etc.) which are themselves difficult to isolate. An additional complication of the biochemical approach is that it destroys the barriers between different cellular and subcellular compartments and dissipates the very proton gradients which are suggested to form the driving force for the pumping of sugars. These problems can perhaps be overcome if biochemical determinations can be made on vesicles reformed from the appropriate membrane, an approach which has already proved successful in bacterial systems (Kaback 1974).

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