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Adenosine Triphosphatase in the Phloem of *Cucurbita*

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Summary. The distribution of adenosine triphosphatase (ATPase) activity in the phloem of petioles and minor veins of *Cucurbita maxima* has been studied using a lead phosphate precipitation procedure. ATPase activity was localized in sieve elements, companion cells and parenchyma cells. Activity was found at the cell surfaces, associated with the dispersed P-protein of mature sieve elements, in mitochondria, sieve-element reticulum, and at specific regions of the cell walls. It is suggested that the ATPase at the phloem cell surfaces may function in intercellular transport of assimilates or ions, and that the ATPase activity associated with the P-protein may function in the translocation process or in callose deposition.

Introduction

It is known that an exogenous energy source in the form of adenosine-triphosphate (ATP) will stimulate an increase in the rate of movement of sugars from sites of synthesis in the mesophyll to sites of "loading" in the phloem (Kursanov, 1963). Also, it has been well documented that the subsequent transport of organic material within the phloem is dependent upon metabolic activity (see Eschrich, 1970; Crafts and Crisp, 1971; Zimmermann and Brown, 1971). In recent years the location of the sites of this activity, particularly the sites of ATP consumption, has become an important but so far unanswered question.

Although numerous studies have been made of the distribution of adenosine triphosphatase (ATPase) activities in animal cells, there have been few critical studies of the sites of ATPase activity in higher plants. Poux (1966, 1967) observed ATPase activity in the plasma membrane, vacuoles, endoplasmic reticulum (ER), mitochondria and dictyosomes of protoderm cells in *Cucumis* roots. Robards and Kidwai (1969) studied the distribution of ATPase in the cambium and differentiating vascular cells of beech where localization was found in the nucleoli, nucleoplasm, nuclear envelope, endoplasmic reticulum, plastids, mitochondria and at the plasma membrane. Hall (1969, 1970, 1971) has demonstrated ATPase activity in root tip cells of *Zea mays* finding high surface activity largely associated with the plasma membrane and plasmodesmata. Hall suggested that this ATPase activity was possibly functional in ion transport processes. ATPase activity was also demonstrated in mitochondria, dictyosomes, endoplasmic reticulum, plastids, and in developing vacuoles.

The ATPase associated with developing vacuoles was also thought to be associated with ion transport processes (Hall, 1971).

A number of the ATPases which have been detected in plant tissues (see Sexton and Sutcliffe, 1969) are stimulated by divalent cations as well as by sodium and potassium; suggesting that in plant cells, as in animal cells, these enzymes may be involved in cation transport. In addition to cation transport, other processes driven by the hydrolysis of ATP occur in phloem cells; many of these are related to the transport function of these cells.

With a view toward locating these energy-requiring processes, and of determining the mechanisms of short- and long-distance transport in the phloem and contiguous cells, studies have been initiated on the cytochemical localization of ATPase in phloem. Cronshaw and Gilder (1972) have reported preliminary observations on the distribution of ATPase in *Nicotiana* phloem. ATPase activity was localized mainly at the cell surfaces and in association with the dispersed P-protein of mature sieve elements. The present paper describes the distribution of ATPase activity in the phloem of *Cucurbita*.

Materials and Methods

Plants of *Cucurbita maxima* Duchesne were grown from seed in vermiculite under greenhouse conditions. 1-cm segments of petiole and small pieces of leaf were fixed for 2 h in formaldehyde-glutaraldehyde or acrolein in 50 mM sodium-cacodylate buffer, pH 7.2, at room temperature. After washing in 2 changes of the above buffer for 2 h, 50-μ sections were cut with a Sorvall TC2 Tissue Sectioner and washed further in 50 mM Tris-maleate buffer, pH 7.1, for 1–2 h. The sections were incubated from 15 min to 3 h at room temperature in a staining medium which contained 2 mM ATP, 2 mM magnesium nitrate, and 3.6 mM lead nitrate buffered to pH 7.1 with 50 mM Tris-maleate. Control sections were incubated in an identical manner except that no ATP was added to the staining medium. Following incubation the sections were washed 3–4 h in water at room temperature and postfixed overnight in 2% osmium tetroxide buffered at pH 7.2 with 50 mM sodium cacodylate at 4° C. The material was dehydrated through a graded acetone series and embedded in Epon 812. Thin sections were cut on a diamond knife with a Porter Blum MT2B microtome, viewed, and photographed with a Philips 300 electron microscope.

Results

When phloem tissue was incubated using the above conditions in medium containing ATP, a reaction product was consistently deposited at specific sites in the cytoplasm. With longer incubation times there was increased deposition of the reaction product, although there was some variability in the amount of deposit between samples. Sections incubated with ATP for 15 minutes showed slight deposits of reaction product, while in those incubated for 3 h the deposits were quite heavy. Control sections incubated in the absence of ATP showed little deposition of

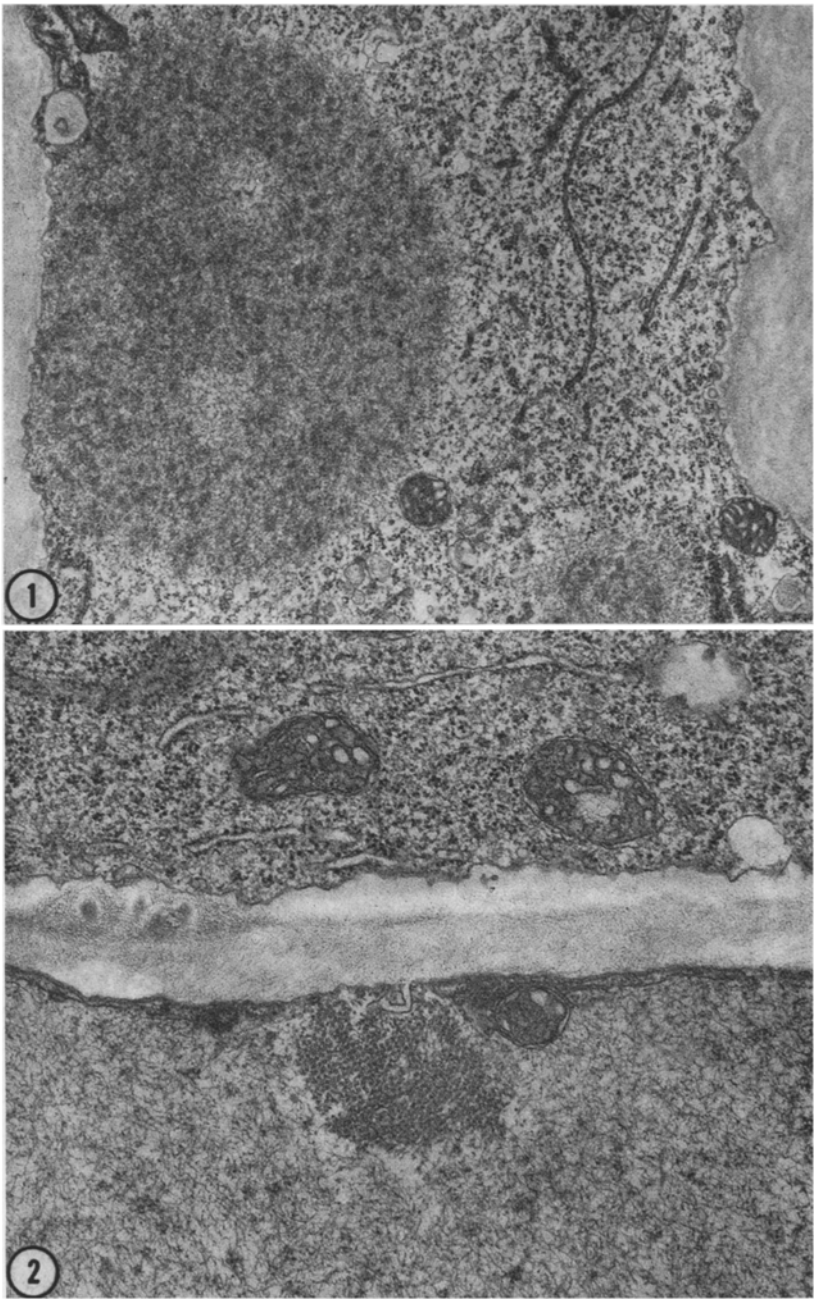
reaction product in differentiating (Fig. 1) or in mature (Fig. 2) phloem cells.

Acrolein fixation has been shown to substantially reduce enzyme activity (Sabatini *et al.*, 1963; Sexton *et al.*, 1971). In acrolein fixed *Cucurbita* phloem used as a control and incubated with ATP we found that there was usually no deposition of reaction product.

Phloem tissue of petiole and minor leaf veins is comprised of three distinct cell types: sieve elements, companion cells, and parenchyma cells. At maturity, the sieve element is characterized by a thickened nacreous wall, few parietally positioned organelles, and a lumen containing dispersed P-protein. Nuclei, dictyosomes, ribosomes, and vacuoles are lost and the endoplasmic reticulum is reorganized during sieve element differentiation (Cronshaw and Esau, 1968). Companion cells have a very dense cytoplasm which is abundant in ribosomes, and have characteristic, branched plasmodesmatal connections with the sieve elements. Phloem parenchyma cells can be identified by the appearance of their plastids, which contain typical grana and starch deposits. Parenchyma cells also have large vacuoles.

When the material was incubated in the presence of ATP each of the phloem cell types showed localization of ATPase activity. In the sieve elements, activity was associated with the cell surface (Figs. 3, 4), the dispersed P-protein (Figs. 3, 5, 6, 11, 14), and certain regions of the cell wall (Figs. 4, 14). Reaction product in the companion cells was seen mainly at the cell surface, plasmodesmatal regions and mitochondria (Figs. 4, 7, 10, 14). In the minor veins, however, a heavy deposit was often seen in the wall between the sieve element and companion cell (Fig. 14). ATPase activity in the parenchyma cells was observed primarily at the cell surface and within some nuclei (Fig. 14). In addition, there was frequently a light deposit in the wall between the sieve element and parenchyma cell of the minor veins (Fig. 14).

The pattern of ATPase activity associated with the P-protein of sieve element appears to be a function of maturity and dispersal. In mature sieve elements where the P-protein has dispersed in the cell lumen, the reaction product is commonly deposited along the P-protein filaments and at the cell surface (Figs. 3, 5, 11, 14). However, in some sieve elements the P-protein does not disperse and in these cells there was no indication of ATPase activity in the P-protein bodies, although localization was usually found at the surface (Figs. 4, 7). Also, the developing P-protein bodies of differentiating sieve elements lack ATPase activity although deposit is observed at the cell surface (Fig. 10). These observations indicate that the P-protein does not gain ATPase activity until its dispersal in the mature sieve element occurs. It was not possible to determine whether the activity was located in the P-protein itself or in an associated component. Occasionally, a mature sieve element with



Figs. 1 and 2. *Cucurbita maxima*. Longitudinal sections of differentiating (Fig. 1) and mature (Fig. 2) sieve elements from the petiole which were incubated as controls, in the absence of enzyme substrate. There is very little deposition of reaction product. Fig. 1: ×15000; Fig. 2: ×30000

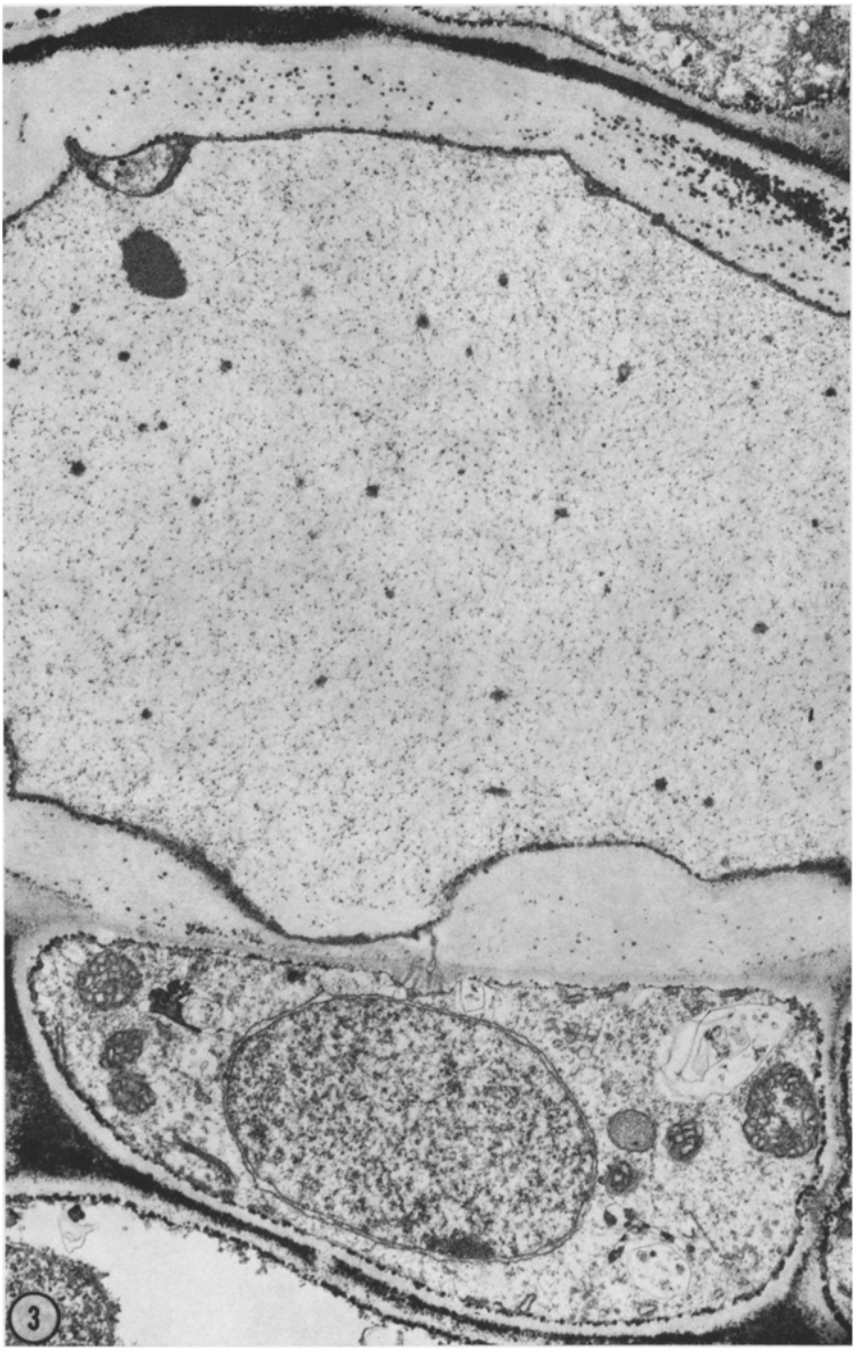


Fig. 3. *Cucurbita maxima.* Cross section of a sieve element and its associated companion cell from a minor vein. ATPase activity is localized at the cell surfaces, the intercellular spaces, and throughout the dispersed P-protein. $\times 15000$

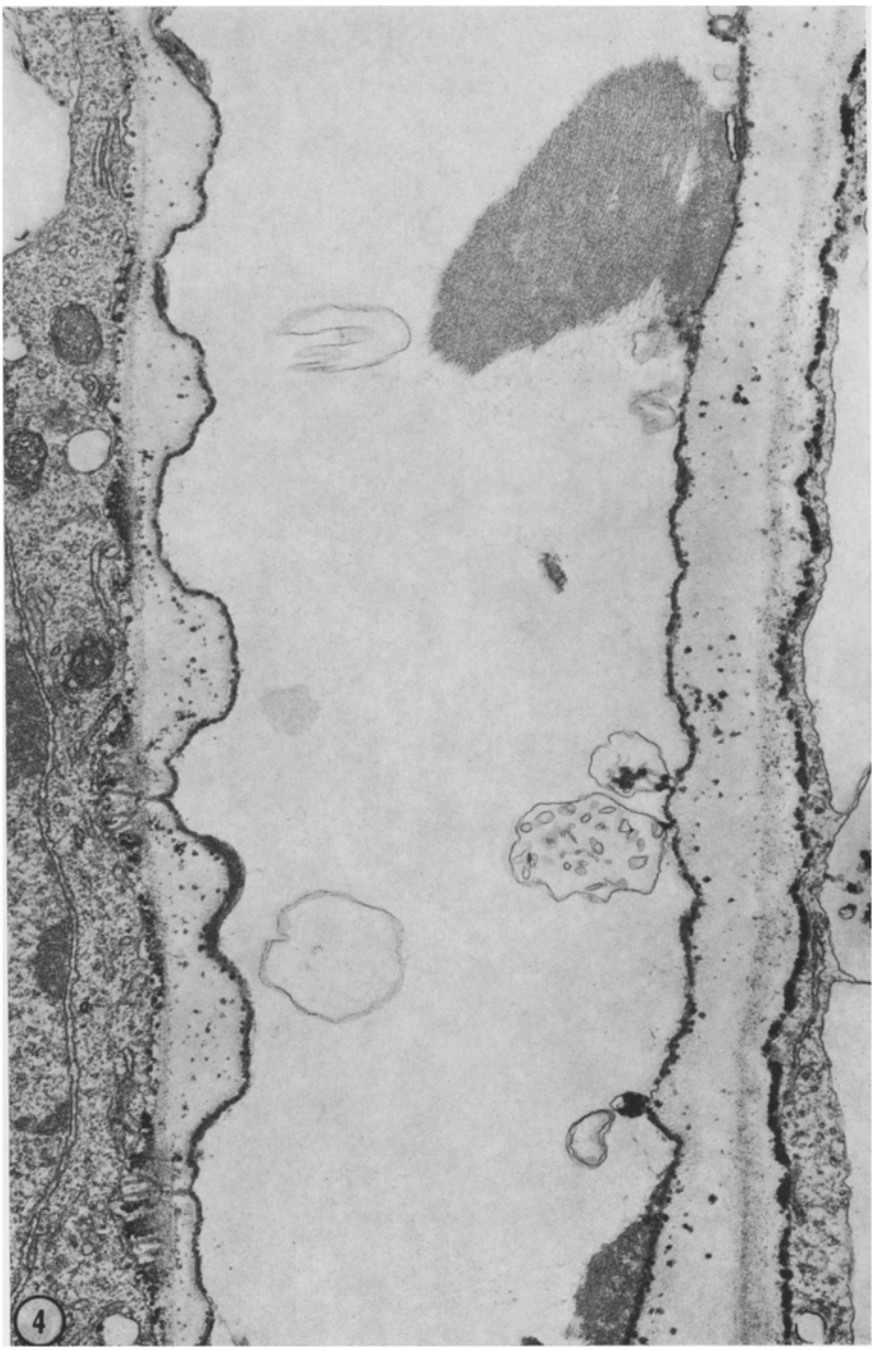
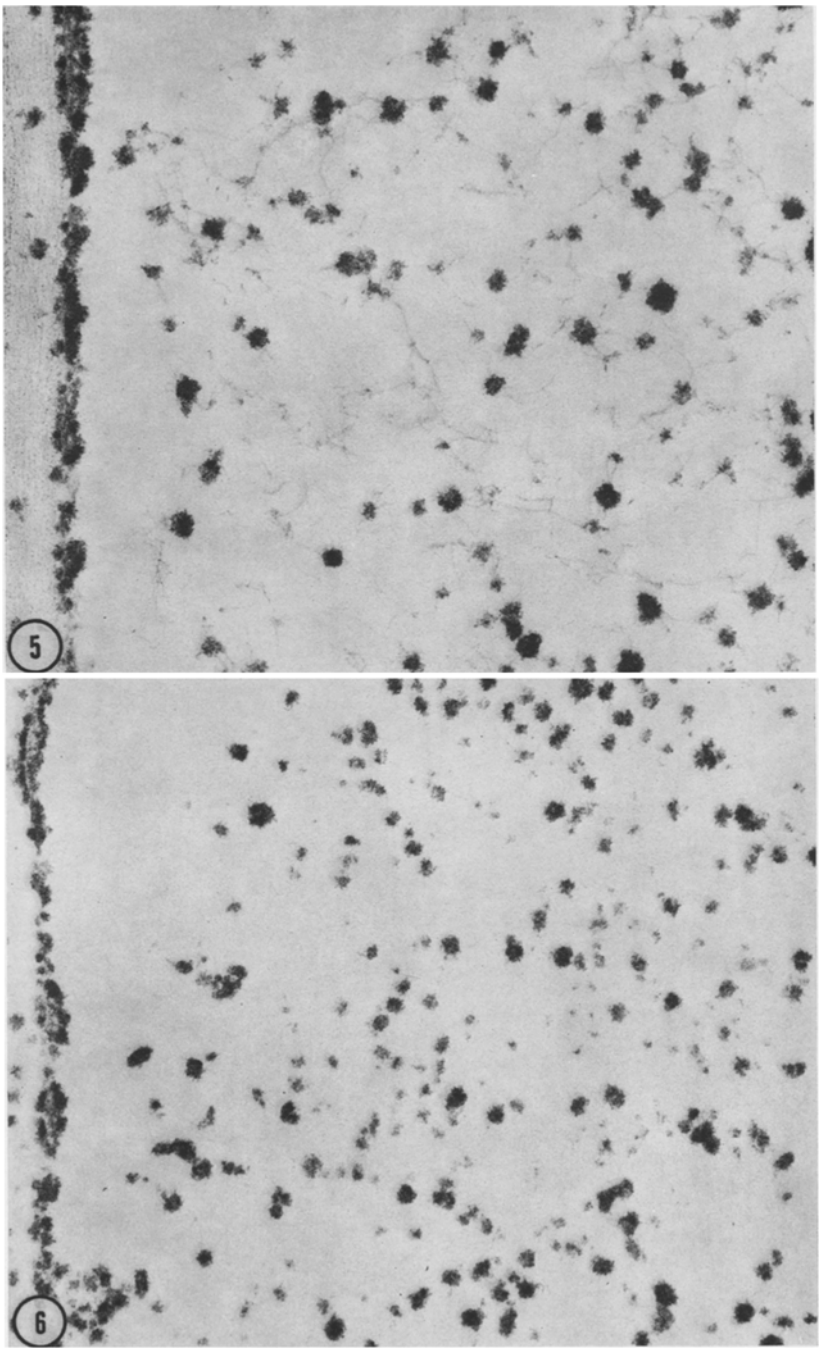
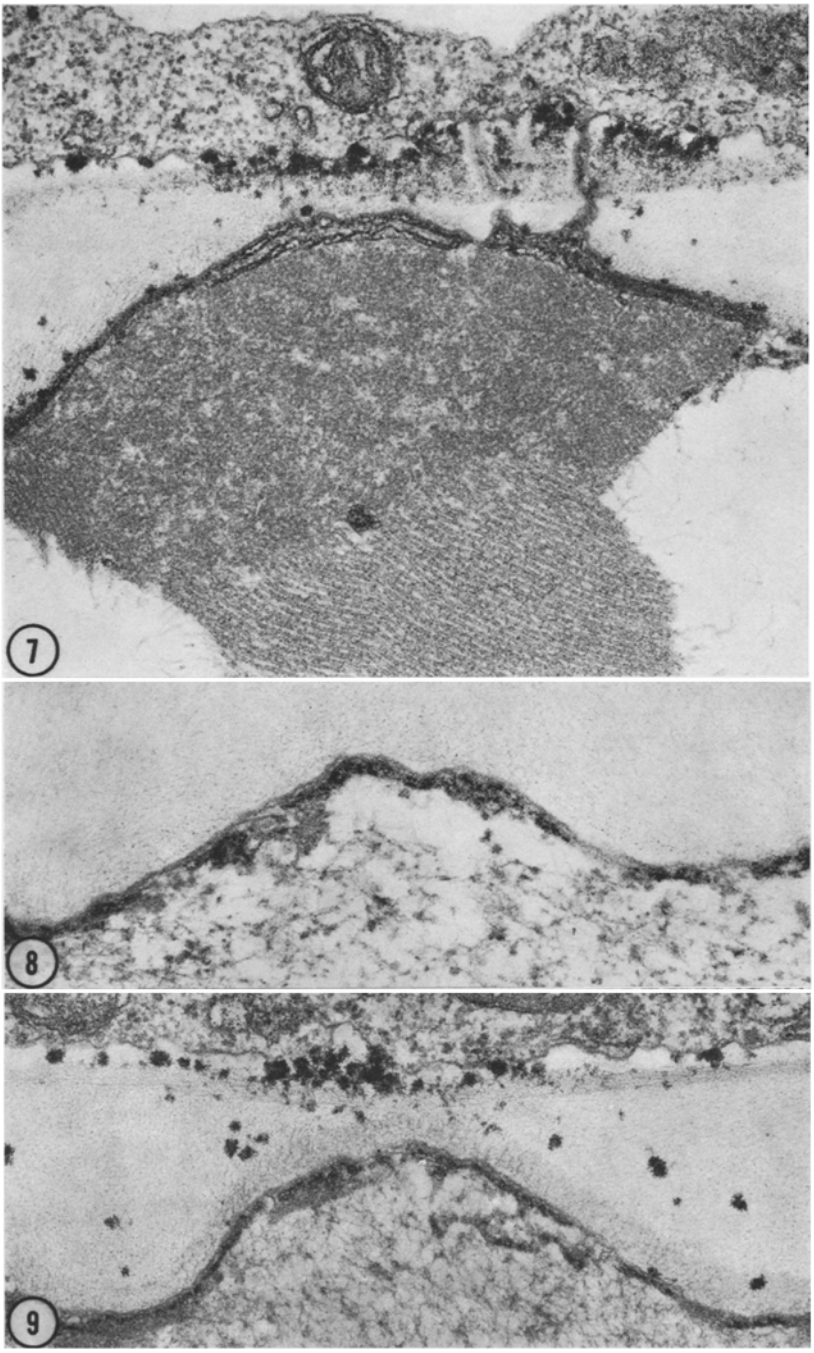


Fig. 4. *Cucurbita maxima*. Longitudinal section through a petiole companion cell, sieve element, and parenchyma cell. ATP-ase activity is located at the cell surfaces, and at the plasmodesmatal regions of the cell wall. Note the lack of activity in the non-dispersed P-protein. $\times 20000$



Figs. 5 and 6. *Cucurbita maxima*. Higher magnification view of ATPase activity associated with the dispersed P-protein and the cell surface of a sieve element from a minor vein. Fig. 5: stained with uranium and lead; ×57000. Fig. 6: serial section, unstained; ×57000



Figs. 7—9

dispersed P-protein was encountered where little or no reaction product was visible (Fig. 10). These were interpreted as cells which were non-functional, or possibly where dispersal had just previously occurred. Higher magnification views of the sieve element lumen demonstrate the presence of reaction product in unstained preparations (Figs. 6, 11). Its association with the dispersed P-protein can be seen in a serial section which has been stained (Fig. 5). Although the intensity of deposit associated with the P-protein was variable, there was generally a lowered level of activity in the P-protein with respect to the surrounding material. Consequently, the longer incubation times were necessary to develop intense deposits along the P-protein fibrils. This could have been due to differential inhibition of the non-membrane associated ATPase, an inherently slower turnover rate, or a more weakly-bound reaction product (which would be more susceptible to washing out during preparation of the specimen).

Strong localizations of ATPase activity were observed at cell surfaces of sieve elements where P-protein was dispersed (Figs. 3, 6). Reaction product was also strong at the surface of sieve elements where P-protein did not disperse (Fig. 4). Higher magnifications of mature sieve elements indicate that a large part of this surface activity is contained specifically within the sieve element reticulum (Figs. 7–9). However, some activity is seen at the wall surface of the plasma membrane (Figs. 5, 6), especially in differentiating sieve elements (Fig. 10). Further indications of membrane associated activity are seen at the sieve plates, both in cells where P-protein has (Fig. 12) and has not (Fig. 13) dispersed.

In companion cells and parenchyma cells ATPase activity was located at the cell surface, mitochondria, dictyosomes and nuclei (Figs. 3, 14). Sometimes there appeared to be an activity gradient with heavier deposit at the surface of the companion cell than the opposing sieve element (Fig. 11). In contrast to the sieve element reticulum, the

Fig. 7. *Cucurbita maxima*. Higher magnification view of a petiole sieve element and companion cell in longitudinal section. ATPase reaction product is deposited within the plasmodesmata and the surrounding wall surface. At this magnification, the activity at the surface of the sieve element is seen to be localized within the sieve element reticulum. Note the lack of activity in the undispersed P-protein. ×39000

Fig. 8. *Cucurbita maxima*. Higher magnification view of sieve element reticulum. ATPase activity is specifically located within the cisternae of the sieve element reticulum. The plasma membrane shows no activity in this cell. ×65000

Fig. 9. *Cucurbita maxima*. Higher magnification view of a petiole sieve element reticulum and companion cell surface. Activity in the sieve element is contained in cisternae of the sieve element reticulum, while the companion cell activity is located at the wall surface and outer surface of the plasma membrane. ×45000

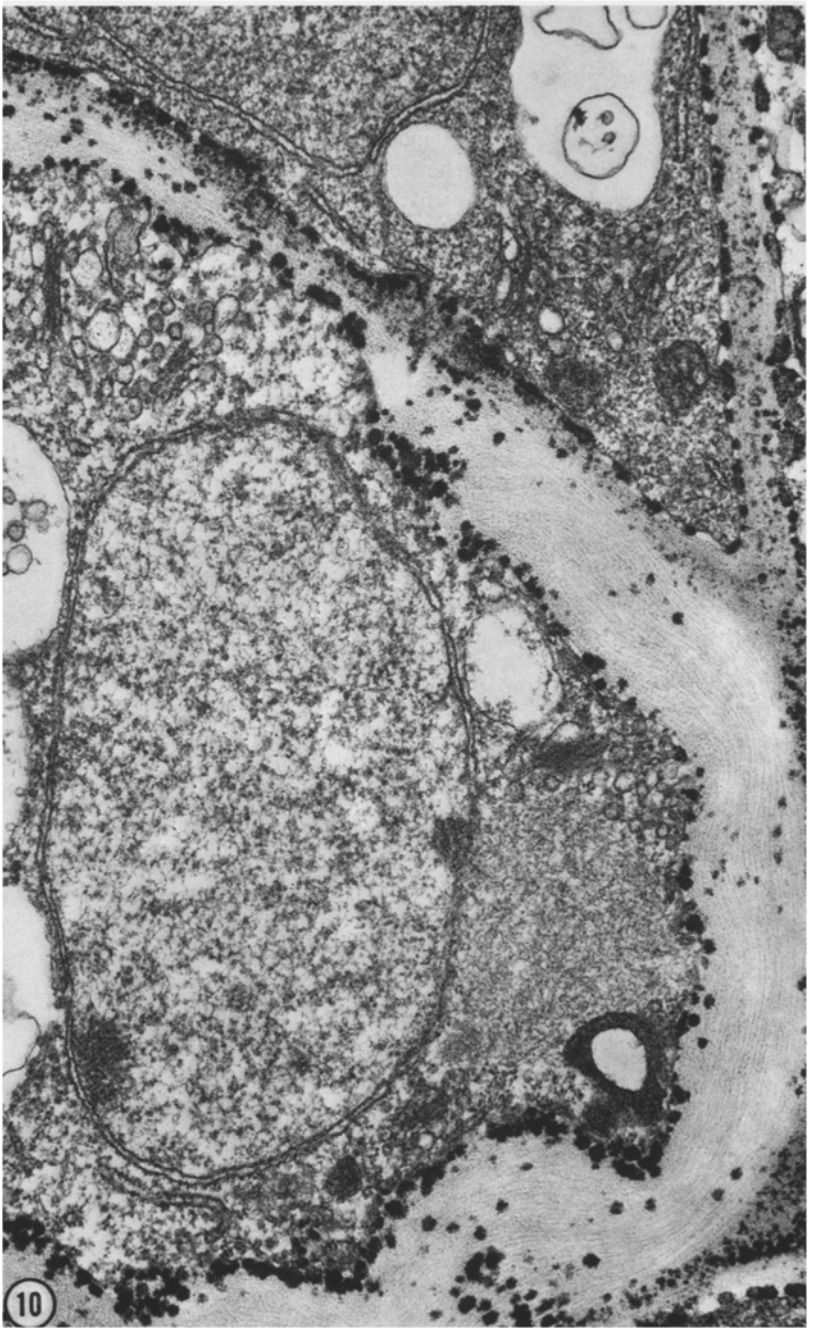


Fig. 10. *Cucurbita maxima.* Transection of a differentiating sieve element and companion cell from a minor vein. ATPase activity is present at the cell surfaces and in the dictyosomes. Note the absence of activity in the developing P-protein. ×27000

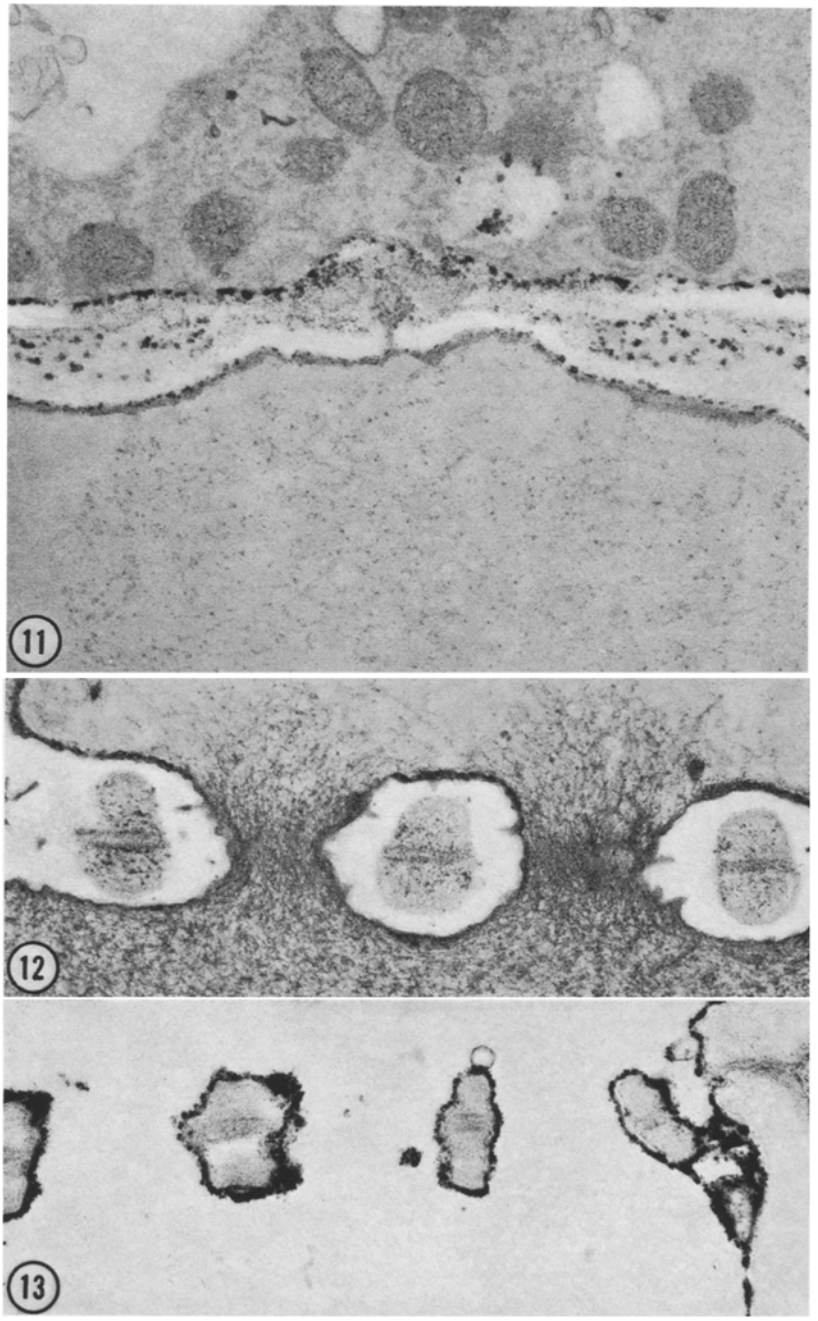


Fig. 11. *Cucurbita maxima.* Unstained section of a minor vein sieve element and companion cell showing activity in the cell surfaces, mitochondria, plasmodesmata, and dispersed P-protein. ×25000

Figs. 12 and 13. *Cucurbita maxima.* Higher magnification views of activity at petiole sieve plates, showing cells where the P-protein has (Fig. 12) and has not (Fig. 13) dispersed. Fig. 12: ×29000; Fig. 13: ×29000

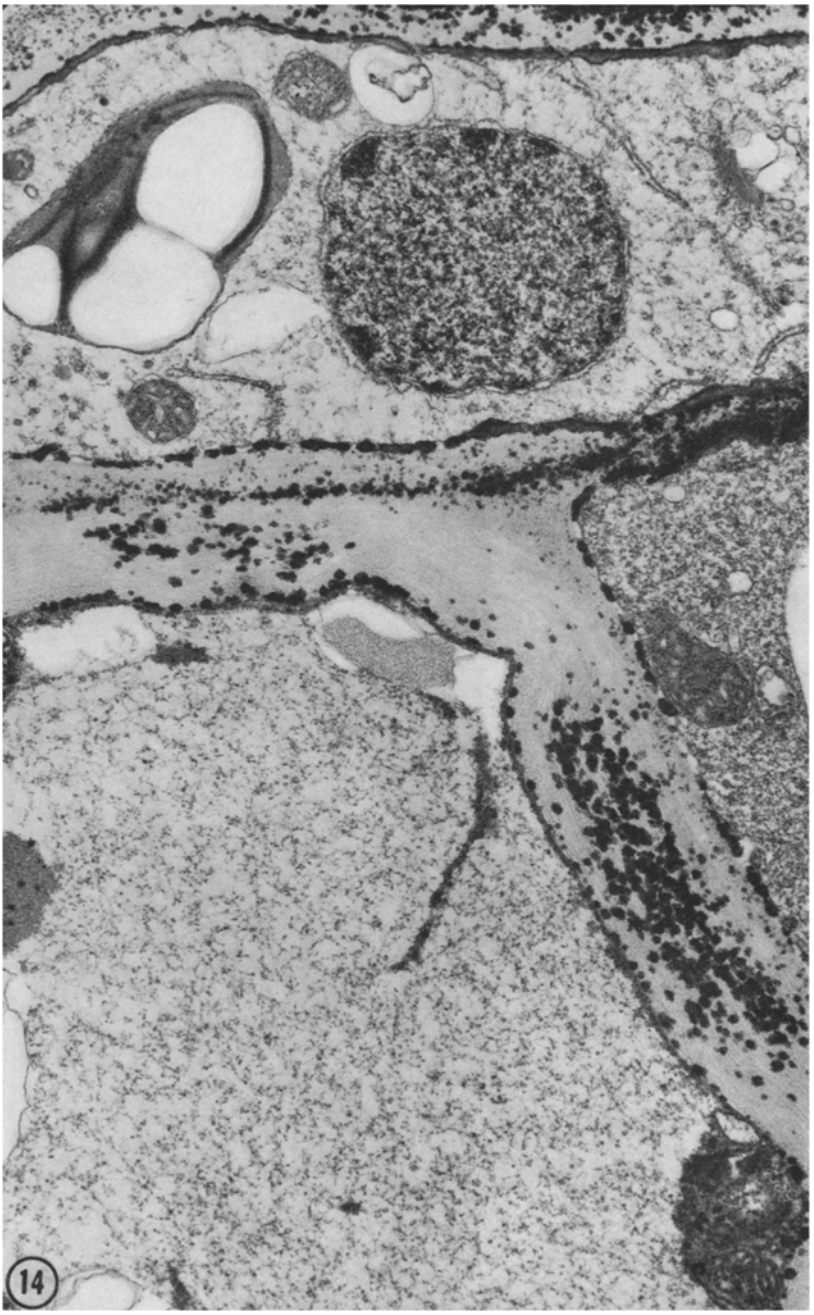


Fig. 14. *Cucurbita maxima.* Transection through sieve element, companion cell, and parenchyma cell in a minor vein. Activity is present at the cell surfaces, sieve element reticulum, dispersed P-protein and parenchyma cell nucleus. Note the heavy deposit in the wall between the companion cell and sieve element, as well as the lighter deposit between the parenchyma cell and the sieve element. $\times 19000$

ER of companion cells and parenchyma cells usually showed little or no indication of ATPase activity. Localization in the companion cell mitochondria was especially evident in the unstained material (Fig. 11).

The cell walls of the phloem tissue appeared to contain specific regions of ATPase activity. In the phloem of minor veins, a heavy deposit was often seen in the wall between a sieve element and companion cell (Fig. 14). A similar deposit was frequently observed in the wall between sieve elements and parenchyma cells of minor veins. This deposit however was usually less intense than that between sieve elements and companion cells (Fig. 14). Also, reaction product was often heavy in the intercellular spaces of the minor veins (Fig. 3). The appearance of activity at specific wall sites was seldom encountered in the phloem of the petiole.

ATPase activity was evident in the regions of cell connections. Deposit was observed within the plasmodesmata (Figs. 7, 11), as well as within the surrounding wall surface (Figs. 4, 7).

Discussion

Translocation of nutrients (carbohydrates and others) from sites of synthesis to sites of usage in growth and development, a process dependent on metabolic activity, is a major function of the phloem. Nevertheless, our knowledge of the specific locations and overall pattern of energy consumption involved in translocation has remained incomplete.

The present experiments demonstrate the presence of ATPase activity in the phloem by cytochemical localization. The results are in agreement with the earlier work of Kuo (1964) who used hand cut sections and histochemical techniques to demonstrate a phloem ATPase in *Cucurbita*, and the results of Wanner (1953) who demonstrated a phloem ATPase in *Robinia*. Presence of ATPase activity is indicative of energy consumption and thus further evidence that the phloem is a metabolically active tissue. The cytochemical observations now reported indicate that this energy consumption is localized at specific sites within the cells. In *Cucurbita* phloem, the primary sites of ATPase activity were located at the surfaces of sieve elements and associated cells, the dispersed P-protein of mature sieve elements, and in certain regions of the cell walls. ATPase was also localized in the mitochondria of all phloem cells, especially the companion cells and associate parenchyma cells and in the endoplasmic reticulum of mature sieve elements. The demonstration of ATPase activity in phloem tissue supports the idea that translocation is an energy linked process and indicates the sites of ATP consumption.

It has been suggested that loading and unloading of the sieve elements is regulated by metabolic activity at the source and sink (Kursanov, 1963 Eschrich, 1970· Crafts and Crisp, 1971) and also that the actual long-distance movement in the transport process is regulated by meta-

bolic activity (Kursanov, 1963; Willenbrink, 1968). Several investigators have shown that the sieve elements contain a high concentration of ATP (Kluge and Ziegler, 1964; Willenbrink, 1968· Gardner and Peel, 1968); however, studies of phloem exudate indicated that ATPase activity was not present in exudate of *Cucurbita maxima* (Eschrich *et al.*, 1971). In the present studies, the finding of ATPase activity distributed throughout the lumen of the mature sieve element and in association with dispersed P-protein indicates the possibility that metabolic activity in the form of ATP consumption is involved along the pathway in the translocation process. Previously, it had been thought that P-protein filaments were possibly structural in nature as their only known function was the plugging of sieve-plate pores on the release of hydrostatic pressure in the sieve tubes. Finding of ATPase activity in association with the dispersed P-protein suggests that P-protein fibrils in mature cells may have ATPase activity themselves, or may be closely associated with an ATPase. It is of interest that the onset of ATPase activity associated with the P-protein parallels P protein dispersal and may represent the onset of translocation by the cell. ATPase activity associated with P-protein strongly suggests that this material is somehow functional in the translocation process. It is possible, of course, that the P-protein plays a passive role as a structural protein matrix forming a three-dimensional network within the cell lumen on which enzymes and other compounds could be suspended.

Sieve elements have the capacity for rapid synthesis of callose from phosphorylated precursors, possibly UDP-glucose (Feingold *et al.*, 1958). This rapid synthesis, mainly at the region of the sieve plate pores, occurs on wounding of the sieve element after the rapid plugging of the pores by P-protein fibrils. Another possible function of the ATPase associated with the P-protein is that it is involved in the phosphorylation of callose precursors and rapid deposition of callose.

The exact pathway of assimilate movement from the sites of synthesis in the mesophyll cells to sites of loading in the sieve-element conduits is not known. Unless the assimilates travel entirely within the symplast through plasmodesmatal connections, they must pass several membranes which function as permeability barriers. In this event there are two further possibilities. The sugar could move across the membrane of the mesophyll cell into the wall and thence through the apoplast to the site of loading into the sieve element through a semi permeable membrane; or, the sugar could move from cell to cell traveling from the cytoplasm into the wall and across the wall into the cytoplasm of the adjacent cell until it enters the sieve elements. Wherever the sugar passes a permeability barrier the expenditure of energy via ATP is required and so is, most probably, the phosphorylation of the sugar. In this regard it is significant that the surfaces of sieve elements, companion cells and parenchyma cells

all had high ATPase activity. Kursanov (1963) stresses the importance of phosphorylated compounds in phloem transport and with Brovchenko has shown that exogenously applied ATP will cause an increase in the rate of movement of sugars from the mesophyll to the phloem (Kursanov and Brovchenko, 1961). Furthermore, this response to ATP was greater in young plants than in older ones. Shiroya (1968) also demonstrated that ATP treatment stimulated translocation in *Helianthus annus*.

The pattern of localization of ATPase activity is consistent with the view that carbohydrate could move from the mesophyll into the apoplast and be accumulated by parenchyma cells and companion cells prior to loading into the sieve elements of the minor veins. ATPase activity in the walls between sieve elements, companion cells, and parenchyma cells of the minor veins may indicate that carbohydrates are indeed moving in the apoplast. No ATPase was found at similar wall locations in the petiole material. Sugars have previously been detected in the free space of sugar-cane cell walls by Hawker (1968), as has the presence of acid invertase (Hawker and Hatch, 1965). Furthermore the presence of ATPase activity at the surfaces of mature sieve elements suggests that some degree of phloem loading may be possible in minor veins directly from the apoplast into the sieve element through its membrane. The surface ATPases may function in the phosphorylation of the sugar or in its transfer through the membrane or both. The possibility of additional phloem loading through the plasmodesmata is also suggested by the localization of ATPase activity at the plasmodesmata membrane cylinder and nearby wall regions.

Phloem also functions in the circulation of monovalent cations, and it is possible that phloem loading of these ions is quite different from the loading of sugars, and that the surface ATPase functions in an ion-transport system. It is also possible that a major site of sugar loading is via the sieve-element membrane and that transfer cells function mainly in the retrieval and transfer to the sieve elements of solutes which are supplied to the leaf in the transpiration stream. It is now important to distinguish between the loading of carbohydrates and cations in determining the major pathway of sugar into the sieve elements, as well as the role of ATPase in phloem loading.

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