A BIOCHEMICAL AND CYTOCHEMICAL STUDY OF ADENOSINE TRIPHOSPHATASE ACTIVITY IN THE PHLOEM OF *NICOTIANA TABACUM*

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ABSTRACT

A biochemical and cytochemical study has been made of the distribution of ATPase in mature and differentiating phloem cells of *Nicotiana tabacum* and of the substrate specificity and effects of fixation on enzyme activity. Homogenates of unfixed leaf midveins and midveins fixed in formaldehyde-glutaraldehyde were assayed for enzyme activity by determining the amount of P_i, liberated per milligram of protein from various substrates in a 30 min period at pH 7.2. In fresh homogenates, hydrolysis of ATP was not significantly different from that of ITP, CTP, and UTP. Hydrolysis of GTP was slightly higher than that of ATP. ATP hydrolysis by fresh homogenates was 17% more extensive than that of ADP, 76% more extensive than that of 5′-AMP, and was inhibited by fluoride and *p*-chloromercuribenzoate (PCMB). There was little or no hydrolysis of the competitive inhibitors 2′- and 3′-AMP nor with the alternate substrates *p*-nitrophenylphosphate (PNP) or β-glycerophosphate (β-GP). In homogenates of material fixed in formaldehyde-glutaraldehyde for 1¼ h, ATPase activity was 13% preserved. Hydrolysis of ATP by fixed homogenates was not significantly different from that of ADP, 5′-AMP, ITP, CTP, and GTP. Hydrolysis of UTP was lower. Fluoride and PCMB inhibited fixed ATPase activity. The results of cytochemical localization experiments using a lead phosphate precipitation technique were in agreement with the biochemical results. Similar localization patterns were obtained with the nucleoside triphosphates ATP, CTP, GTP, ITP, and UTP. Activity was also localized with ADP and 5′-AMP but not with the competitive inhibitors 2′- and 3′-AMP, nor with PNP or β-GP. Little or no reaction product was deposited in other controls incubated without substrate or with substrate plus fluoride, PCMB, or *N*-ethylmaleimide. ATPase activity was demonstrated chiefly at the plasma membrane of mature and differentiating phloem cells and was associated with the P-protein of mature sieve elements. It is suggested that the phloem transport system derives its energy from the demonstrated nucleoside triphosphatase activity.

INTRODUCTION

Since it is well established that metabolic energy is utilized in phloem translocation (see references 6, 24), determination of the precise sites at which this energy is released by enzymes such as ATPase is

crucial to an understanding of phloem function. Data presently available concerning the properties, distribution, and function of these enzymes in phloem cells are limited and equivocal. ATPase activity in phloem tissue has previously been detected by biochemical assay of phloem exudate from *Robinia* (39), although assays for ATPase activity in *Cucurbita* exudate were negative (8). ATPase activity in phloem cells has also been demonstrated by cytochemical localization at the light and electron microscope levels. In a light microscope study, Kuo (22) reported higher ATPase activity in phloem cells of *Cucurbita*, particularly in sieve elements and companion cells, than in surrounding tissues, and suggested that this intense activity might be involved in the translocation of solutes.

Recent studies of the intracellular sites of phloem ATPase in *Cucurbita* (7, 16), and in *Nicotiana* (17), have supported and extended these findings at the electron microscope level. The presence of ATPase activity was reported at the surfaces of mature and differentiating sieve elements and companion cells, in association with the dispersed P-protein in mature sieve elements, in mitochondria, dictyosomes, and plasmodesmata. The ATPase activity found in association with the P-protein first appeared during intermediate stages of sieve element differentiation and was most prominent in the mature sieve elements.

It is possible that P-proteins play a role in the movement of substances through the phloem, and the observations that these tissues contain high concentrations of ATP (2, 14, 15, 19, 21) have increased speculation that they may be contractile (17, 23, 28, 41). More data on the enzymatic and physical properties of P-protein are needed to resolve this question.

Other phosphatases have also been found in phloem tissue of several species by biochemical assay of phloem exudates (3, 8), by localizations at the light microscope level (3, 12, 22, 26), and by cytochemical studies at the electron microscope level (4, 11, 42). These studies showed the presence of acid phosphatase activity at the plasma membrane, in the endoplasmic reticulum (ER),[1] and in association with "slime strands."

Previous investigations of phloem ATPase in *Nicotiana* and in *Cucurbita* (7, 16) have described the subcellular distribution of enzyme in mature and differentiating cells; however, nothing was known of the substrate specificity or sensitivity to fixatives. The purpose of the present study was to expand our knowledge of the properties of phloem ATPase by combined biochemical and cytochemical investigations in order to evaluate the localization procedures. The patterns of localization and levels of activity have been studied with a variety of nucleoside phosphates, inhibitors, alternate and analog substrates. The results indicate that a fraction of ATPase activity in *Nicotiana* leaf midveins survived formaldehyde-glutaraldehyde fixation, and the enzyme which survived in the phloem was highly specific for 5′-nucleoside phosphates. Similar localization patterns were obtained with each nucleoside triphosphate, and activity could be inhibited by fluoride and by sulfhydryl blocking agents. Cytochemical reaction was positive with ADP and with 5′-AMP, but not with 2′- or 3′-AMP, nor with β-glycerophosphate (β-GP) or *p*-nitrophenylphosphate (PNP).

MATERIALS

Nicotiana tabacum L. cv. "Wisconsin 38" plants were grown from seed in vermiculite under greenhouse conditions, and were used when approximately 18–24 inches tall.

5′-ATP, 5′-ADP, 5′-AMP, 5′-GTP, 5′-CTP, and 5′-ITP, as the sodium salts, PNP, *N*-ethylmaleimide (NEM), and *p*-chloromercuribenzoate (PCMB) were obtained from Calbiochem, San Diego, Calif., in A grade; 2′-AMP, 3′-AMP, 5′-UTP, as the sodium salts, Trizma base, in Sigma grade, and sodium β-GP, in grade I from Sigma Chemical Co., St. Louis, Mo.; and 50% glutaraldehyde, from Polysciences, Inc., Warrington, Pa. Glutaraldehyde was purified by distillation before use, and showed a single peak at 280 nm (1).

METHODS

Preparation of Tissue Homogenates

Midveins of young *Nicotiana* leaves were excised in 0.1 M sodium cacodylate-buffered 4% formaldehyde-5% glutaraldehyde, pH 7.2. Segments 0.5–1 cm in length were fixed 1¼ h, rinsed in cacodylate buffer, and then washed 3 h in several changes of 0.1 M Tris-maleate buffer, pH 7.2. The tissue was homogenized in fresh Tris-maleate buffer with clean sand and filtered through sintered glass. Fresh material was excised into cacodylate buffer, cut into 0.5–1 cm lengths, rinsed in Tris-maleate buffer, and similarly homogenized and filtered.

[1] *Abbreviations used in this paper:* ER, endoplasmic reticulum; β-GP, β-glycerophosphate; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; PNP, *p*-nitrophenylphosphate.

Biochemical Assays

Aliquots of fresh or fixed homogenate were incubated 30 min at room temperature in a medium containing 2 mM $Mg(NO_3)_2$ and 2 mM substrate, in 0.1 M Tris-maleate buffer, pH 7.2. Substrates were ATP, ADP, 5′-AMP, 3′-AMP, 2′-AMP, CTP, GTP, UTP, ITP, β-GP, and PNP. Homogenate was also incubated in the above medium containing ATP plus either 0.01 M fluoride, 0.1 M NEM, 0.002 M PCMB, or 3.6 mM lead nitrate. Reactions were stopped by the addition of cold 30% trichloroacetic acid, and media were filtered before P_i estimations according to the method of Chen et al. (5). P_i (as microgram P_i per microgram protein) liberated by enzymatic hydrolysis during 30 min was determined by subtracting the phosphate contained in the original homogenate and that introduced by nonenzymatic hydrolysis of the substrate solutions. Protein was estimated by the method of Lowry et al. (27).

Cytochemical Localizations

Midveine sgments (0.5–1 cm) of young *Nicotiana* leaves were fixed 1¼ h in formaldehyde-glutaraldehyde buffered to pH 7.2 with 0.1 M sodium-cacodylate-acetate. After fixation, specimens were washed 1–2 h in cacodylate buffer and sectioned at 50–100 μm with a Sorvall TC2 tissue sectioner. Sections were washed 1–2 h further in 0.1 M Tris-maleate buffer, pH 7.2, before incubation in a modified Wachstein-Meisel medium containing 2 mM substrate, 2 mM $Mg(NO_3)_2$, and 2.4–3.6 mM lead nitrate, in Tris-maleate buffer. Substrates were ATP, ADP, 5′-AMP, GTP, UTP, ITP, and CTP.

Controls were incubated without substrate; with substrate in the presence of either fluoride (0.01 M), NEM (0.1 M), or PCMB (0.002 M); or with equimolar concentrations of alternate substrates PNP, β-GP, 2′-AMP, or 3′-AMP. Other controls were fixed in acrolein or postfixed in osmium tetroxide before incubation in the presence of substrate. Routine incubations were carried out for 2 h at room temperature, after which sections were washed 3–6 h in distilled water before additional processing.

Postincubation Processing

For light microscopy, specimens were embedded in glycol methacrylate, sectioned at 8–10 μm on glass knives, and treated with 2% ammonium sulfide. Specimens were viewed and photographed without further staining.

Sections for electron microscopy were postfixed overnight in cold 2% osmium tetroxide buffered to pH 7.1 with 0.1 M sodium-cacodylate. The material was stained in block in uranyl acetate (9), dehydrated with acetone, and embedded in Epon. Thin sections were cut on diamond knives with a Porter-Blum MT2B ultramicrotome, and either viewed unstained or after double staining with uranium and lead, and photographed with a Philips EM 300 electron microscope.

RESULTS

Enzyme Assays and Effects of Fixation

ATPase in filtered homogenate of fresh material liberated an average of 0.287 μg P_i per μg protein during a 30 min incubation period. For comparative purposes, this level was taken to be 100% activity. Percentages of relative hydrolysis of other substrates are given in Table I. Hydrolysis of ATP in fresh homogenate was 17% more extensive than the hydrolysis of ADP, and was 76% more extensive than that of 5′-AMP. The ATPase was inhibited 65% by fluoride, and 96% by PCMB. The hydrolysis of other nucleoside triphosphates was not significantly different from that of ATP, with the exception of GTP which was more extensively hydrolyzed. The alternate substrates 2′-AMP, 3′-AMP, β-GP, and PNP were hydrolyzed considerably less than ATP.

ATPase in filtered homogenate of formaldehyde-glutaraldehyde-fixed material liberated an average of 0.037 μg P_i per μg protein during a 30 min incubation period. For comparison with other substrates in fixed homogenate, this level was taken to be 100% activity. The relative extent of hydrolysis of other substrates by fixed homogenate are given in Table I. The hydrolysis of ATP was not significantly different from that of either ADP or 5′-AMP. Fixed ATPase was inhibited 68% by fluoride and 78% by PCMB. When fixed homogenate was incubated with ATP in the presence of NEM, the hydrolysis was not significantly different from that of ATP alone in the incubating medium. Hydrolysis of other nucleoside triphosphates by fixed homogenate was not significantly different from that of ATP, with the exception of UTP which was lower (by 51%).

Hydrolysis of the alternate substrates 2′-AMP, 3′-AMP, and β-GP was considerably less than that of ATP, but PNP hydrolysis by fixed homogenate was not significantly different from that of ATP. The ratio of ADP and 5′-AMP hydrolysis (relative to that of ATP) was greater in fixed than in fresh homogenate, indicating a selective loss of ATPase activity. This observation is also evident in the lower preservation of triphosphate hydrolysis after fixation than the preservation of di- or monophosphate hydrolysis. The percentages

TABLE I

Hydrolysis of Phosphate Esters by Nicotiana Leaf Midvein Homogenates

Substrate	Homogenate incubated for 30 min at 22°C, pH 7.2 (Mean values ± SEM from at least four experiments)					% of mean activity remaining after fixation
	Fresh homogenate		Fixed homogenate			
	μg P_i/μg total protein	% ATP level	μg P_i/μg total protein	P value'	% ATP level	
ATP	0.287 ± 0.014	100	0.037 ± 0.004	<0.001	100	13
ATP + fluoride	0.101 ± 0.020*	35 ± 6.5	0.012 ± 0.003‡	n.s.	32 ± 6	12
ATP + NEM	0.276 ± 0.004 n.s.	96 ± 3.0	0.044 ± 0.001 n.s.	<0.001	119 ± 7	16
ATP + PCMB	0.012 ± 0.0004*	4 ± 0.5	0.008 ± 0.002*	n.s.	22 ± 1.6	67
ADP	0.237 ± 0.008§	83 ± 4.3	0.037 ± 0.011 n.s.	<0.001	100 ± 8	16
2'-AMP	0.004 ± 0.0005*	1 ± 0.5	0.003 ± 0.001*	n.s.	8 ± 1	75
3'-AMP	0.015 ± 0.001*	5 ± 0.3	0.012 ± 0.003*	n.s.	32 ± 2.5	80
5'-AMP	0.070 ± 0.006*	24 ± 2.4	0.030 ± 0.005 n.s.	<0.01	81 ± 10	43
ITP	0.259 ± 0.015 n.s.	90 ± 6.0	0.029 ± 0.006 n.s.	<0.001	78 ± 6	11
CTP	0.236 ± 0.033 n.s.	82 ± 4.5	0.031 ± 0.008 n.s.	<0.001	84 ± 10	13
GTP	0.380 ± 0.027§	132 ± 12	0.041 ± 0.009 n.s.	<0.001	111 ± 11	11
UTP	0.221 ± 0.031 n.s.	77 ± 5.4	0.018 ± 0.005§	<0.001	49 ± 2	8
β-GP	0.011 ± 0.001*	4 ± 0.3	0.008 ± 0.000*	n.s.	22 ± 1.5	73
PNP	0.049 ± 0.001*	17 ± 0.7	0.032 ± 0.000 n.s.	<0.001	86 ± 10	65

* $P < 0.001$, with respect to value for ATP alone as substrate.

‡ $P < 0.01$, with respect to value for ATP alone as substrate.

§ $P < 0.05$, with respect to value for ATP alone as substrate.

n.s. = not significant, with respect to value for ATP alone as substrate.

'P values with respect to the corresponding value for fresh homogenate.

of substrate hydrolysis which are preserved after formaldehyde-glutaraldehyde fixation are given in Table I. ATP and ADP are hydrolyzed equally by fixed homogenate although less of the ATPase activity is preserved. Hydrolysis of 5'-AMP is 19% less than that of ATP, even though it is 43% preserved.

When 3.6 mM lead nitrate was included in the assay mixture of fresh homogenate, ATPase activity was 60% inhibited. The enzymatic hydrolysis of ATP by fixed homogenate, however, was apparently increased by 6% when 3.6 mM lead nitrate was included in the incubation medium. The ratio of enzymatic to nonenzymatic hydrolysis of ATP when both rates were measured in the presence of lead was 8:1.

These observations in homogenate are interesting when compared with the more specific cytochemical localizations within the phloem cells. Although the hydrolysis of 5'-AMP is not significantly different from that of ATP in fixed homogenate, the reaction given by intact phloem cells is stronger with ATP than with 5'-AMP. Two possible explanations for these findings are (*a*) that the enzymes detected by biochemical assay are not active in similar fashion when observed cytochemically due to differential inhibition by lead ions, or (*b*) that the enzymes are not evenly distributed throughout all cells of the midvein.

The values for enzyme activity in fresh and in fixed homogenates are based on Lowry determinations of protein concentration. It has been mentioned, however, that Lowry determinations may be inaccurate with fixed materials (20). When activity in these samples is computed on the basis of microgram P_i liberated per milligram fresh weight of tissue, the ATPase activity is 9.5% preserved. This figure is in reasonable agreement with the 13% survival figure based on protein determinations. In the case of plant tissues, fresh weight of material may itself be inaccurate due to differences in anatomy and (in fixed tissues) to loss of soluble contents and changes in water retention by cells and cell walls.

Cytochemistry

Incubations were carried out under varying conditions to determine those most favorable for cytochemistry. Optimum localizations were routinely obtained in *Nicotiana* phloem tissue after 2 h of incubation at room temperature in medium containing 2 mM substrate with a 5′-phosphate, and 3.6 mM lead nitrate (as judged by intensity and nature of reaction product deposits, amounts of background lead, and morphological preservation). Preliminary experiments were conducted with incubation times varying from 15 min to 3 h at room temperature. Deposition of lead phosphate reaction product could be obtained in this range of incubation times; however, the material was judged to be overincubated after 3 h at room temperature as reaction product was very heavy, and nonspecific background lead was also present. The ATP:lead ratio was varied by using lead concentration of 2.4 to 3.6 mM with 2 mM ATP. In this range of lead concentrations there were no detectable differences in the localization of reaction product. Specimens were washed in several changes of buffer for at least 3 h before incubation or homogenization in order to remove any residual fixative.

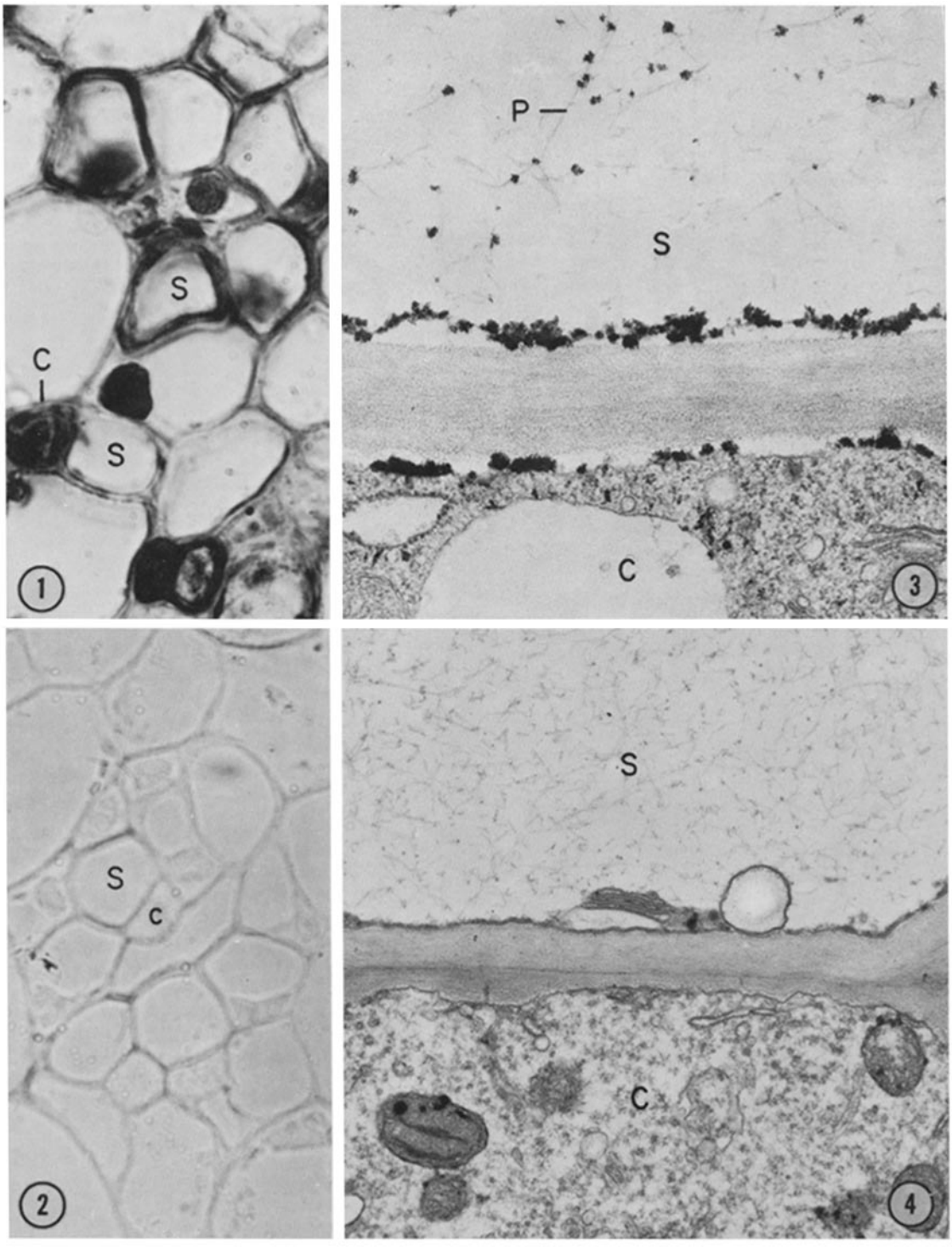
Microscopy

8–10-μm sections of specimens incubated with ATP and stained with ammonium sulfide for light microscopy showed heavy deposits of black lead sulfide (reaction product) in the phloem companion cells and outlining the sieve elements and phloem-parenchyma cells (Fig. 1). Activity was considerably stronger in the phloem, especially in the companion cells, than in the surrounding tissue. Many cells also showed nuclear staining. When tissue was incubated in identical conditions except for the absence of substrate, no lead deposits were formed in the cells (Fig. 3).

The material prepared for cytochemical study was well preserved after the incubation procedures; in the presence of nucleoside triphosphate, enzymatic activity could be consistently localized at specific sites within the cells. When sections were incubated in medium containing ATP, an ATPase activity was found at the surfaces of sieve elements and companion cells, and in close association with the dispersed P-protein in mature sieve elements (Fig. 2). Little or no activity was found in the ER of companion cells, although ATPase was seen in sieve element reticulum. When similar tissue was incubated in the absence of ATP, no lead deposits were formed in the cells (Fig. 4). Deposition or reaction product was strongly inhibited by fluoride and by the sulfhydryl blocking agents NEM and PCMB. As shown in Figs. 5 and 6, little or no lead deposit was formed in the presence of ATP as substrate in tissues inhibited with these agents. Specimens incubated in the presence of ATP and inhibited with fluoride (Fig. 5) or with NEM (Fig. 6) were generally free of reaction product, and were strongly inhibited compared to the ATPase activity illustrated in Fig. 3. Assays of fixed homogenate indicate that ATPase activity was 68% inhibited by fluoride, and 78% inhibited by PCMB. Enzyme activity was also severely inhibited, or destroyed, by fixation in acrolein or by postfixation is osmium tetroxide before incubation. Specimens incubated in the presence of ATP after such fixations were indistinguishable from the no-substrate controls (Fig. 4).

Patterns of localization obtained with other nucleoside triphosphates were similar in both mature and differentiating cells to that obtained with ATP. For example, specimens incubated in the presence of ITP (Fig. 7) contained reaction product in the sieve element reticulum at the surfaces of sieve elements and companion cells, and closely associated with the P-protein dispersed in the sieve element lumens (Fig. 7). When sections were incubated with UTP as substrate (Fig. 8), reaction product was deposited at similar locations within the cells. Fig. 8 shows a lower magnification view of a specimen incubated with UTP, illustrating the activity present at the surfaces of the sieve element and companion cell, and associated with the P-protein. This specimen also shows considerably less deposit at the surfaces of the adjacent parenchyma cells (Fig. 8); however, this observation was noted in samples reacted with other nucleoside phosphates and was not limited to the substrate UTP.

Specimens incubated in the presence of CTP (Fig. 9) or GTP (Fig. 10) also gave characteristically similar localization patterns. Fig. 9 illustrates a sieve element and companion cell incubated with CTP, and shows the activity at the plasma membranes and the activity associated with the P-protein. A profile of ER, located in the companion cell between the nucleus and cell wall (Fig. 9), contains no reaction product, a finding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which was also evident in samples reacted with ATP (Fig. 3), 5′-AMP (Fig. 14), and with other substrates. This lack of activity in ER may be due to the formaldehyde-glutaraldehyde fixative used in specimen preparation. Widnell (40) has suggested that 5′-nucleotidase in the ER of intact tissue is probably inactivated during fixation. Positive reactions in the phloem cells which were given by ITP, CTP, GTP, and UTP could be inhibited by fluoride, by NEM, and by PCMB.

During all stages of differentiation of sieve elements, ATPase activity was found at the plasma membranes. Cells which had reached an intermediate stage of development also showed ATPase activity in association with the dispersing P-protein. Similar patterns of activity during differentiation were observed with the other 5′-nucleoside phosphates ADP, 5′-AMP, UTP, GTP, CTP, and ITP. Fig. 10 shows a sieve element at an intermediate stage of differentiation which had been incubated with substrate GTP. The developing sieve element in this section shows reaction product at the cell surface, and lighter deposits are also present within the dispersing P-protein. Cells adjacent to the differentiating sieve element also showed activity at their surfaces (Fig. 10).

Control specimens incubated in the presence of equimolar concentrations of alternate substrates β-GP (Fig. 11) or PNP (Fig. 12) showed little or no activity. The section of a specimen incubated with β-GP at pH 7.2, illustrated in Fig. 11, shows a mature sieve element and one at a late stage of differentiation. Little or no reaction product was formed in these cells, nor in their adjacent companion cells and parenchyma cells. An enzyme which does give a positive reaction with β-GP has been found in phloem tissue at pH 5.5 (see Introduction), but it was not active in these preparations at pH 7.2. A low magnification view of the phloem (Fig. 12) showing portions of a mature sieve element, two differentiating sieve elements, and associated cells illustrates the lack of reaction product typical of specimens incubated with PNP as substrate. In this case, the cytochemical results are not in agreement with the biochemical findings. Two possible explanations for this discrepancy are that the hydrolysis in homogenate is nonenzymatic, or that the enzyme is not located in the phloem.

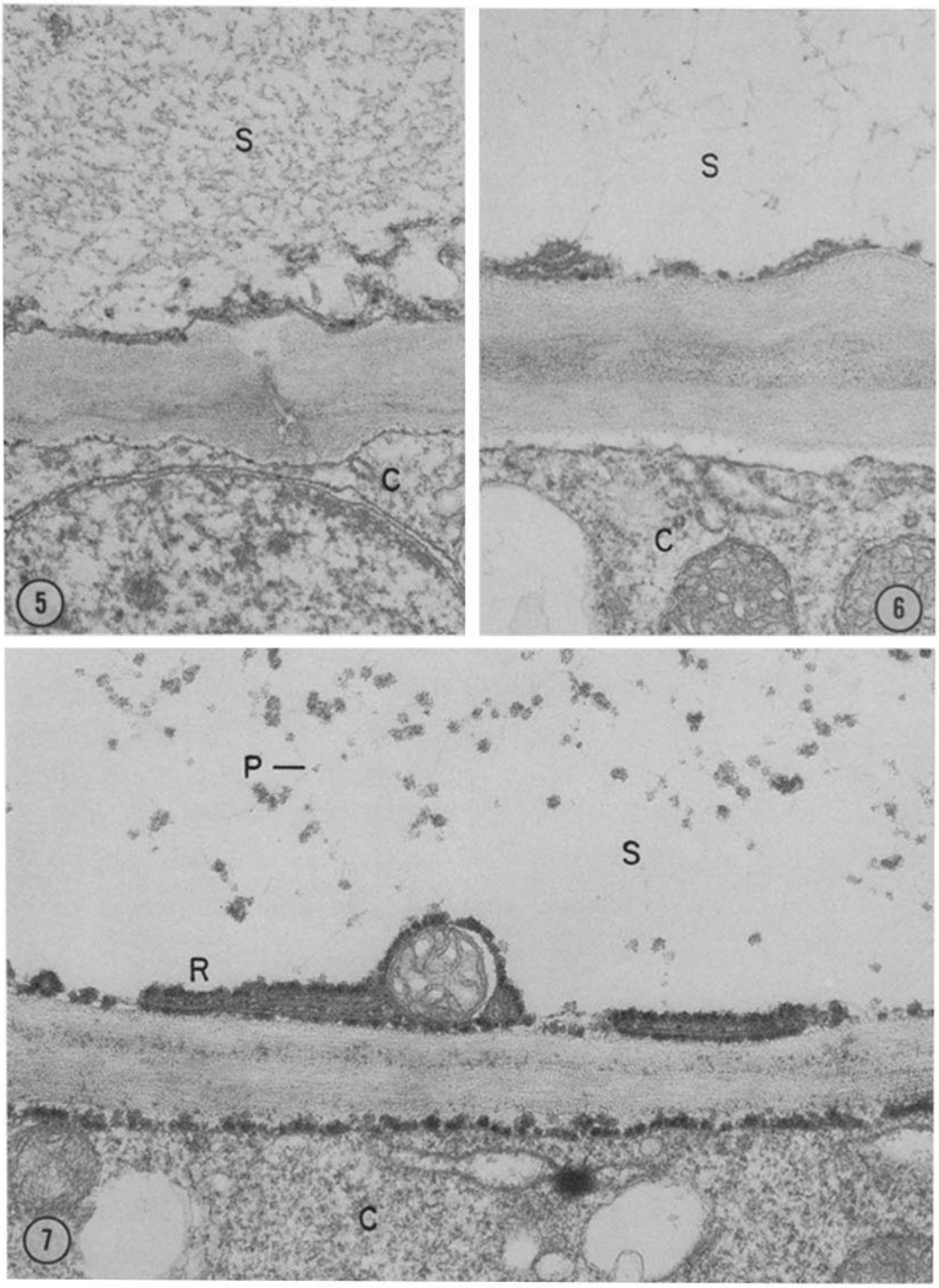
Adenosine nucleosides other than ATP also gave a positive reaction (Figs. 13 and 14); however, this was less consistent and generally less intense than the localization seen with ATP. Sections incubated in the presence of ADP (Fig. 13) contained reaction product at most phloem cell surfaces as shown in low magnification view (Fig. 13). Activity was also associated with dispersed P-protein in the lumens of mature sieve elements (Fig. 13). 5′-AMP gave a weak positive reaction at similar sites, as illustrated in Fig. 14; however, there was no deposition of reaction product in the presence of competitive inhibitors 2′- or 3′-AMP. A specimen demonstrating the lack of activity in the presence of analog substrate 2′-AMP is illustrated in Fig. 15.

DISCUSSION

Enzyme activity found in fresh and in fixed homogenates represents the overall activity present in midveins rather than specific activity of the phloem cells. However, localization experiments do permit the resolution of activities at specific sites within the phloem cells. In all cases, the presence or absence of activity toward the nucleoside phosphates corresponded to the biochemical results. ATP was extensively hydrolyzed in fixed tissue, especially

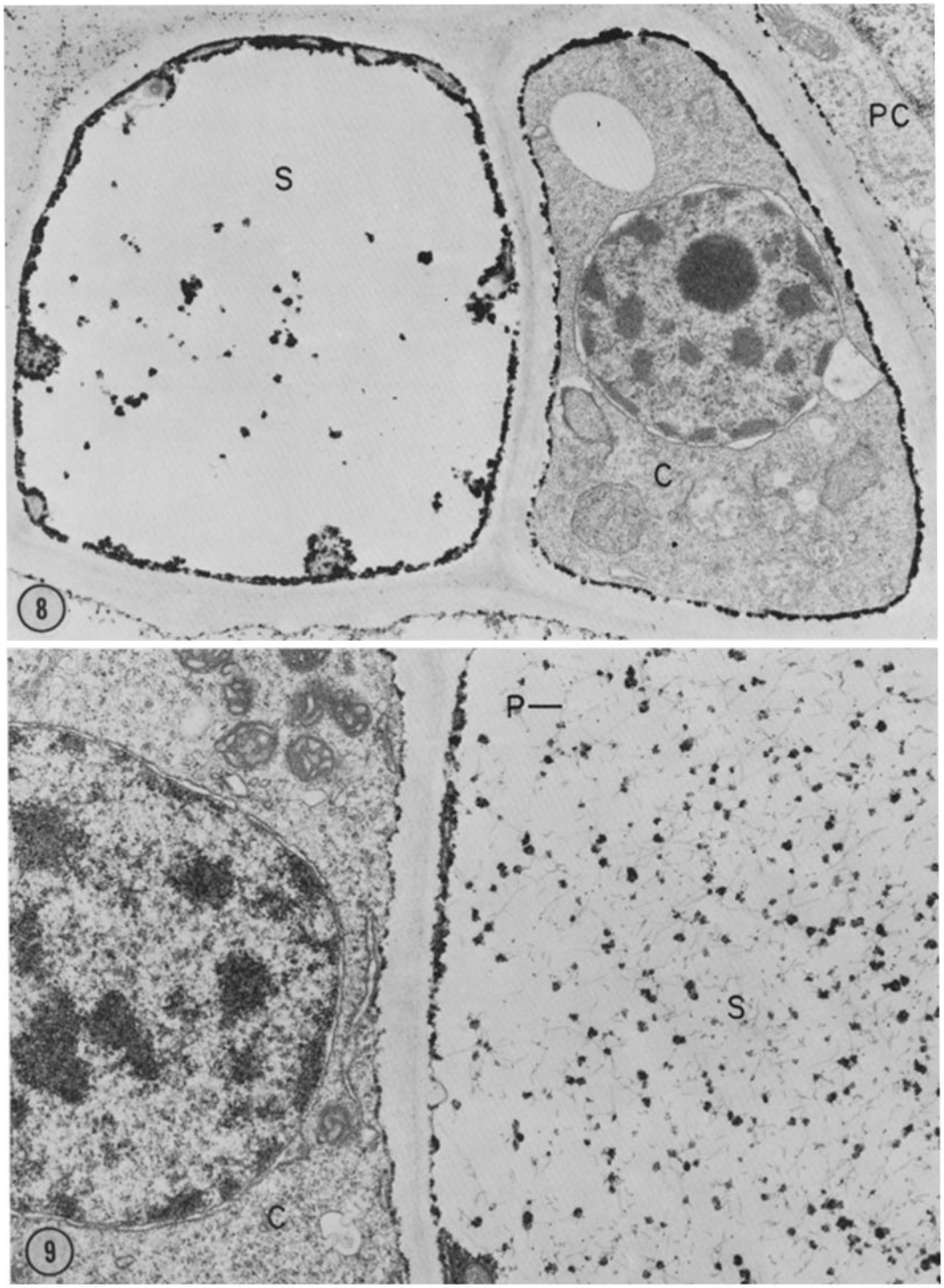
FIGURES 1 and 2 Light micrographs of *Nicotiana* phloem in transection. Specimens stained with ammonium sulfide after 2 h of incubation. Fig. 1 shows the presence of ATPase activity at the surfaces of the sieve elements (*S*) and companion cells (*C*). Reaction product is heavier in the phloem than in surrounding parenchyma cells. Many cells also exhibit nuclear staining. Fig. 2, incubated as a no-substrate control, shows the general lack of reaction product in specimens incubated without ATP. Fig. 1, × 800; Fig. 2, × 800.

FIGURES 3 and 4 Electron micrographs of sieve elements (*S*) and companion cells (*C*) from *Nicotiana* phloem. Cells in Fig. 3 were incubated with ATP, and demonstrate an ATPase activity at the cell surfaces, and in association with the dispersed P-protein (*P*) in the sieve element lumen. Fig. 4, incubated under identical conditions except without ATP, contains little or no reaction product. Fig. 3, × 40,100; Fig. 4, × 20,900.

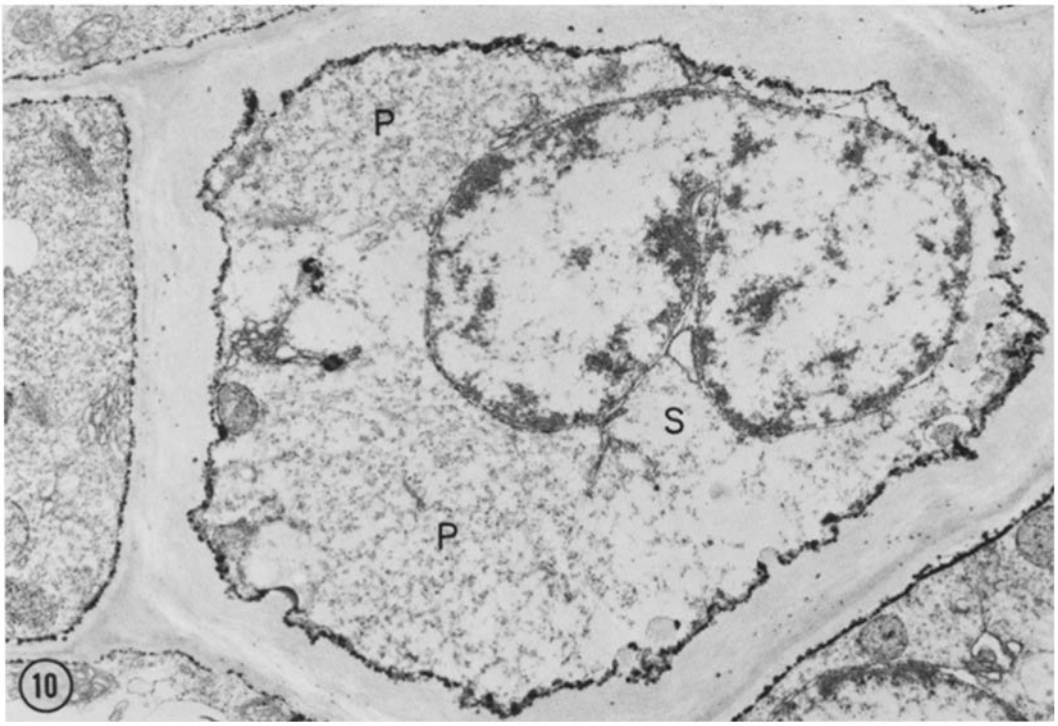


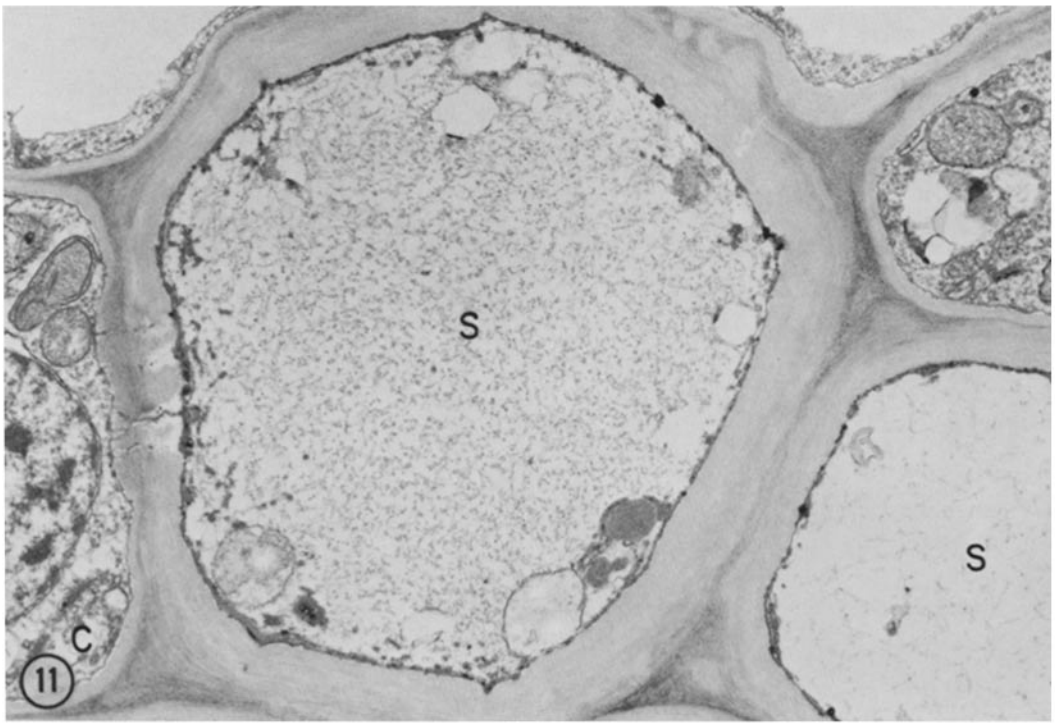
FIGURES 5 and 6 *Nicotiana* phloem incubated with ATP in the presence of enzyme inhibitors NEM or fluoride. Fig. 5 illustrates a sieve element (*S*) and companion cell (*C*) with little or no reaction product after inhibition with sulfhydryl blocking agent NEM in the presence of ATP. Fig. 6 shows similar cells which had been inhibited with fluoride in the presence of ATP. Both specimens strongly inhibited compared to the ATPase activity illustrated in Fig. 2. Fig. 5, × 28,000; Fig. 6, × 27,100.

FIGURE 7 A sieve element (*S*) and companion cell (*C*) of *Nicotiana* which had been incubated with ITP as substrate. Reaction product is observed at the cell surfaces, in the sieve element reticulum (*R*), and in association with the dispersed P-protein (*P*) in the sieve element lumen. × 34,500.

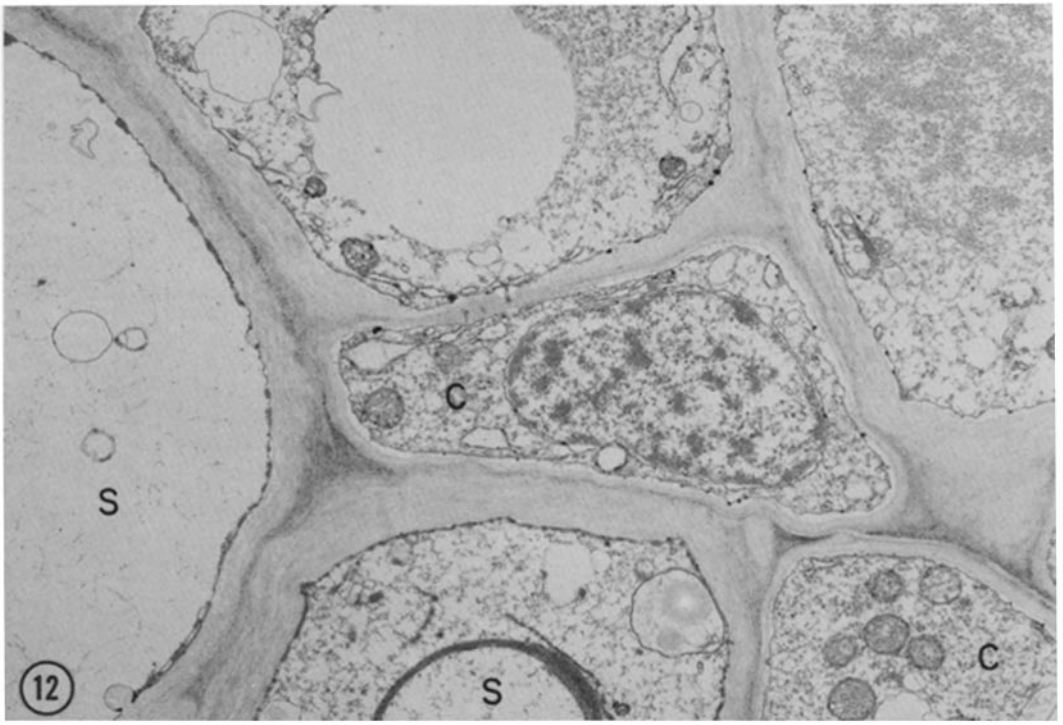


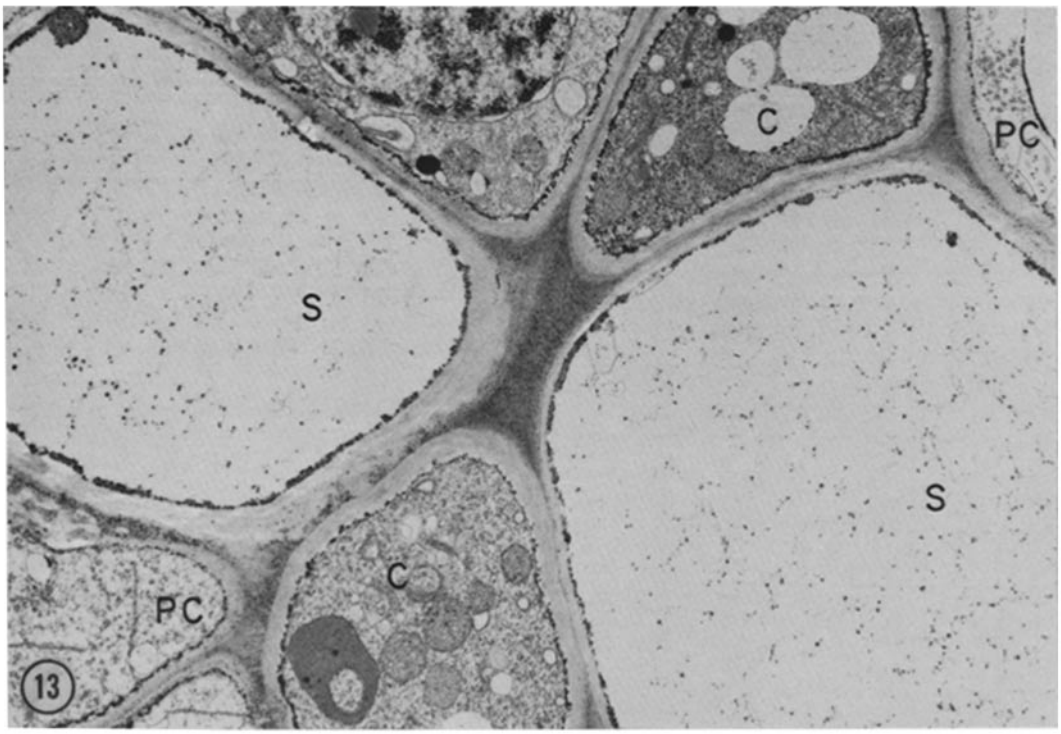
FIGURES 8 and 9 *Nicotiana* phloem cells which had been incubated in the presence of nucleoside triphosphates CTP and UTP. Fig. 8 shows a sieve element (*S*), companion cell (*C*), and portions of two parenchyma cells (*PC*) which were incubated with UTP. Reaction product can be seen in the sieve element lumen, and strongly deposited at the sieve element and companion cell surfaces. Lighter deposits are seen at the parenchyma cell surfaces, and in mitochondria of the companion cells. Fig. 9 shows portions of a sieve element (*S*) and companion cell (*C*) incubated with CTP as substrate. The presence of activity is indicated at the cell surfaces and in association with the dispersed P-protein (*P*). Fig. 8, × 15,700; Fig. 9, × 24,000.



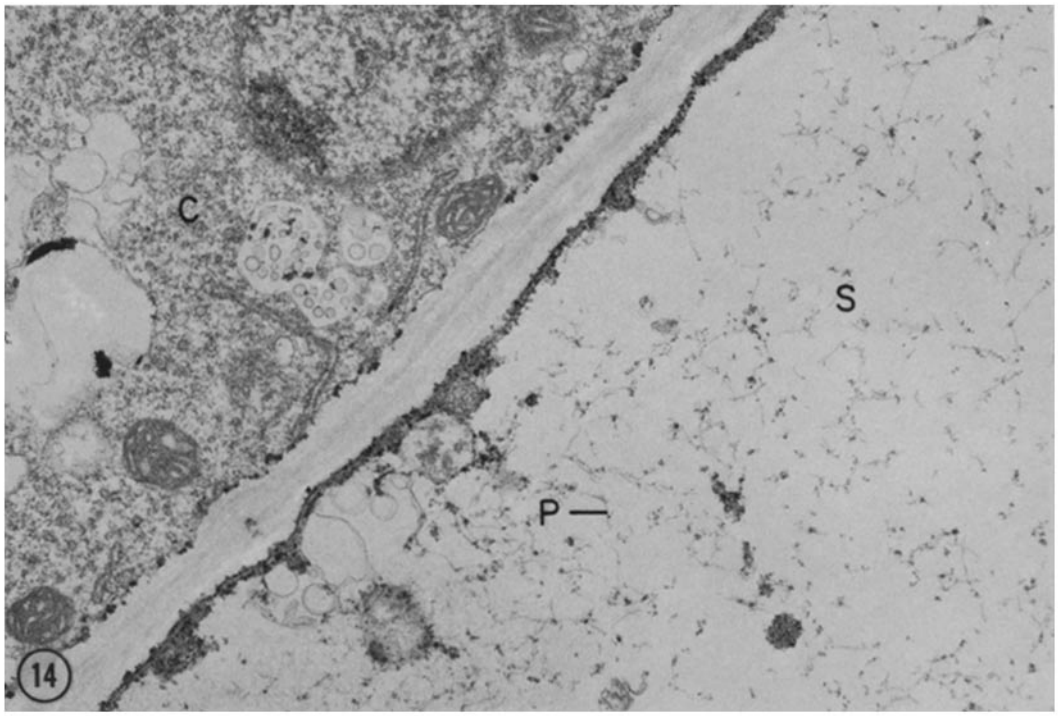


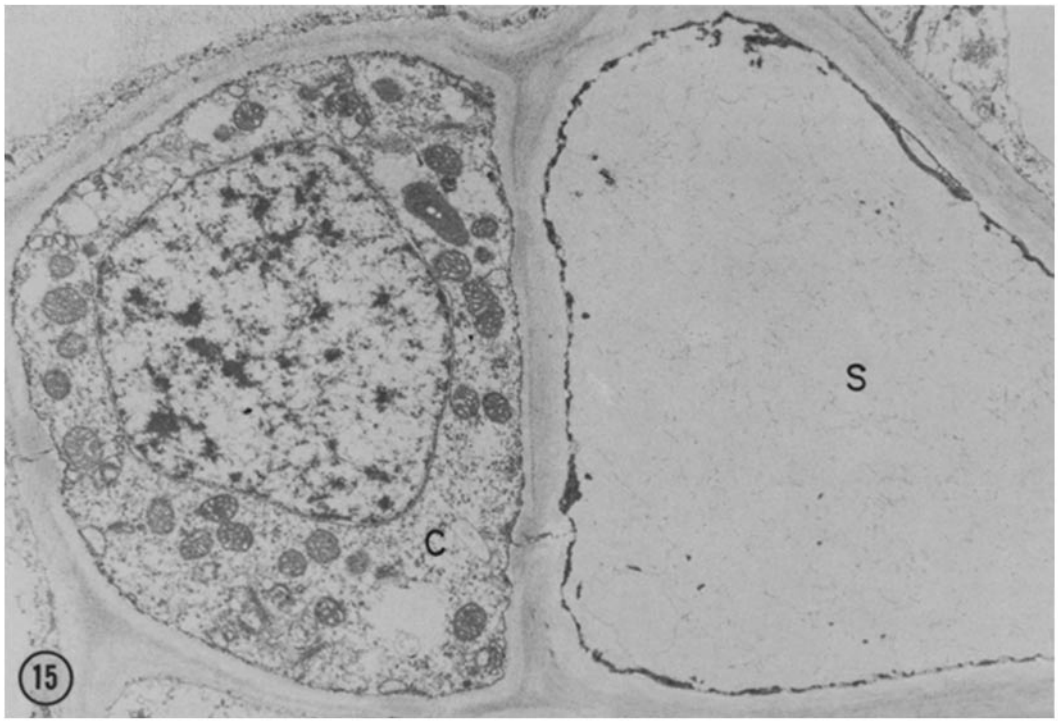
FIGURES 10 and 11 Differentiating sieve elements (*S*) and adjacent cells of *Nicotiana* which had been incubated with GTP (Fig. 10) or with β-GP (Fig. 11). With substrate GTP, reaction product was formed at the cell surfaces and within the dispersing P-protein (*P*). This activity could not be detected in the presence of β-GP. Fig. 11 also shows a portion of a mature sieve element (*S*) which formed little or no reaction product after incubation with β-GP at neutral pH. *C*, companion cells. Fig. 10, × 15,200; Fig. 11, × 12,300.





FIGURES 12 and 13 Low magnification views of *Nicotiana* phloem in transection, showing portions of several sieve elements (*S*), companion cells (*C*), and parenchyma cells (*PC*). Fig. 12: Specimens incubated with PNP formed little or no reaction product. Fig. 13: When the substrate ADP was present in the incubating medium, activity could be detected at all cell surfaces and in association with the dispersed P-protein. Also, the nuclear staining noted in the light micrographs (Fig. 1) can be seen in this specimen. Fig. 12, × 11,500; Fig. 13, × 10,300.





FIGURES 14 and 15 Sieve elements (*S*) and companion cells (*C*) from *Nicotiana* phloem which had been incubated in the presence of 5′-AMP (Fig. 14) or analog substrate and competitive inhibitor 2′-AMP (Fig. 15). Fig. 14 shows the weakly positive reaction which was found after incubation with 5′-AMP. There are light deposits at the plasma membranes and throughout the dispersed P-protein (*P*). Fig. 15 shows the lack of activity in specimens incubated with 2′-AMP. Fig. 14, × 22,400; Fig. 15, × 9,400.

in sieve elements and companion cells, despite its apparently greater sensitivity to fixation than ADP or 5′-AMP. Nucleoside phosphatase activity could not be detected after acrolein or osmium tetroxide fixation. Fixation in formaldehyde-glutaraldehyde for 1¼ h preserved 13% of the ATPase activity, which appeared to be sufficient for cytochemical localizations. The activity lost during fixation may be due to inactivation at random sites, or to a selective loss from some sites, or both. It has been suggested by Widnell (40) that 5′-nucleotidase in ER of intact tissue is inactivated by fixation. The present failure to demonstrate nucleoside phosphatase activity in this organelle supports this suggestion; however, activity was demonstrated in the sieve element reticulum which differentiates from the rough ER.

Correspondence of present biochemical and cytochemical findings indicates that these procedures allow direct subcellular localizations of nucleoside phosphatase activity in phloem cells. In these experiments, ATPase activity was demonstrated at the plasma membrane of mature and differentiating cells and was associated with the P-protein of mature sieve elements. The lead phosphate precipitation techniques for ATPase localization in fixed tissue may produce numerous artifacts, as several previous papers have shown (13, 30, 31, 33, 34). The cytochemical techniques were judged to be reliable for *Nicotiana* phloem because (*a*) consistent localizations could be repeatedly and routinely obtained with nucleoside phosphates, as expected from the biochemical results; (*b*) no lead deposits were seen in specimens incubated in media containing lead but without substrate; (*c*) when material was incubated in the presence of substrate plus any of several inhibitors, little or no reaction product was deposited, as expected from the biochemical results; (*d*) incubation with lead in the presence of alternate substrates formed little or no lead deposit in the tissue; (*e*) a positive reaction which was given by 5′-AMP could not be demonstrated with the competitive inhibitors 2′-AMP and 3′-AMP, as expected from the biochemical results. These judgments are based on the assumptions that P_i is captured *in situ* at the site of enzymatic hydrolysis, and that the precipitated lead phosphate does not migrate in the cells, assumptions which can be cautiously made as the result of many careful studies of animal tissues (18, 29, 32, 37, 38, 40).

The chief significance of the present results is the finding of nucleoside triphosphatase activity in phloem cells (which is relatively strong compared with that of surrounding cells, and which is specific for nucleoside phosphates as substrate). In its specificity the phloem ATPase was similar to other ATPases (25) in that it would also hydrolyze CTP, GTP, and ITP, and to a lesser extent UTP, each having the phosphates in 5′ position. The hydrolysis of ATP in the presence of NEM and the inhibition by PCMB indicate that the enzyme possesses two classes of sulfhydryl groups similar to those in myosin (25). Failure of the phloem enzyme to hydrolyze β-GP or PNP indicates that it does not have general (nonspecific) phosphatase activity. The enzyme did react with ADP, and to some extent with 5′-AMP. However, it was specific in the 5′-phosphate requirement and was inactive toward 2′- and 3′-AMP. Substrates which gave a positive reaction in the phloem each have comparatively high free energy of hydrolysis, whereas the nonreactive substrates have lower free energy of hydrolysis. These observations suggest that the release of and consumption of energy in phloem cells is high, and that it is specific for "high-energy" phosphates. Possibly energy released by nucleoside triphosphatase activity in the phloem could be used to drive the phloem transport system by one or more of the mechanisms that have been proposed (6, 10, 17, 28, 35, 36).

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