Comparative Enzymology of the Adenosine Triphosphate Sulfurylases from Leaf and Swollen Hypocotyl Tissue of *Beta vulgaris*

**MULTIPLE ENZYME FORMS IN HYPOCOTYL TISSUE**

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**ABSTRACT**

ATP sulfurylase activity was partially purified from the swollen hypocotyl of beetroot (*Beta vulgaris*); activity was measured by sulfate-dependent PPI-ATP exchange. The ATP sulfurylase activity was separated from pyrophosphatase and ATPase activities which interfere with the assay of ATP sulfurylase activity. The ATP sulfurylase activity from hypocotyl tissue was invariably resolved into two approximately equal activities (hypocotyl I and II) by ion exchange chromatography and polyacrylamide gradient gel electrophoresis. Both enzymes catalyzed selenate- and sulfate-dependent PPI-ATP exchange; the affinity of hypocotyl II for these substrates was greater than for hypocotyl I. It is unlikely that the two activities arise by allelic variation or as an artifact of purification; they are most probably isoenzymes. Studies of the subcellular localization of the two hypocotyl enzymes were inconclusive.

ATP sulfurylase was also purified from leaf tissue. Ion exchange chromatography resolved the ATP sulfurylase from leaf tissue into a major activity (which accounted for 98% of the total leaf activity) and a minor activity. The major leaf and hypocotyl II ATP sulfurylases were indistinguishable as judged by the properties investigated.

In plants, fungi, and the assimilatory sulfate-reducing bacteria, sulfate is the principal form of sulfur required for growth (24). In yeast, it is well established that sulfate is activated by ATP in two separate reactions catalyzed by the enzymes ATP sulfurylase (ATP-sulfate adenyl transferase, EC 2.7.7.4) and APS kinase (ATP-adenylyl sulfate 3'-phosphotransferase, EC 2.7.1.25), respectively:

\[
\text{ATP} + \text{SO}_4^{2-} + \text{Mg}^{2+} \rightarrow \text{APS} + \text{PPi}
\]

\[
\text{APS} + \text{ATP} \rightarrow \text{PAPS} + \text{ADP}
\]

In yeast, PAPS is the activated form of sulfate; the sulfate moiety of PAPS is reduced and used to form the various compounds containing sulfur in the $-2$ valency state which are essential for the growth and function of all living cells. The capacity of an organism to synthesize APS and PAPS from inorganic sulfate, however, does not necessarily imply that the organism is able to reduce sulfate since it is well established that animals synthesize APS and PAPS yet are unable to reduce sulfate in significant quantities (24). In animals, PAPS is used to form a wide range of sulfate esters in reactions catalyzed by the sulfotransferases (24).

ATP sulfurylase activity has been demonstrated in a wide range of plants (1, 2, 14). The activated form of sulfate reduced by plants, however, is uncertain. In several studies APS kinase activity could not be demonstrated (5, 6, 14), and this has led to the suggestion that APS might be the activated form of sulfate (6, 14), as has been described for the disimilatory sulfate-reducing bacteria (22). APS kinase activity has been described in *Chlorella* (16), but Tsang *et al.* (29) and Schmidt (25) have determined that APS is the activated form of sulfate for reduction in this organism. APS kinase activity has been reported in higher plants (7, 20); the enzyme from spinach only catalyzed the synthesis of PAPS in the presence of 3'-AMP (7). Since the incorporation of sulfate into cysteine/cystine by isolated chloroplasts was almost entirely dependent on the addition of 3'-AMP, Burnell and Anderson (7) suggested that PAPS is an intermediate in sulfate reduction in spinach.

The ATP sulfurylase from spinach leaf tissue has been highly purified (6, 27); the enzyme catalyzes APS-dependent synthesis of ATP (6), sulfate- and selenate-dependent PPI-ATP exchange and the synthesis of APS when coupled with Mg$^{2+}$-dependent alkaline pyrophosphatase (27). In leaf tissue, ATP sulfurylase and other enzymes associated with sulfate activation have been described in chloroplasts (5–8).

It is well established that nonphotosynthetic tissues contain ATP sulfurylase activity (2, 14) and incorporate sulfate into compounds containing sulfur in the $-2$ valency state (13, 18, 21). Slices of the nonphotosynthetic hypocotyl tissue of beetroot incorporate sulfate into cysteine (13); the incorporation was enhanced by serine, implying that sulfate is reduced to sulfide and incorporated into either serine or O-acetyls erine in a reaction catalyzed by serine sulphydrylase or O-acetyls erine.

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4. Abbreviations: APS: adenosine 5'-sulfotophsphate; PAPS: adenosine 3'-phosphate 5'-sulfotophosphate; DEAE-cellulose: diethylaminoethyl cellulose.
sulfhydrase, respectively. This suggests that the ATP sulfurylase activity of nonphotosynthetic tissue is associated with the reduction of sulfate. Since the ATP sulfurylase activity of leaf tissue is associated with chloroplasts, this raises the question as to whether two forms of the enzyme exist in plants. This paper describes a comparative study of the ATP sulfurylases from the leaf and swollen hypocotyl tissues of the beetroot plant (*Beta vulgaris*), a plant which has previously been demonstrated to carry out nonphotosynthetic reduction of sulfate in hypocotyl tissue (13). Leaf tissue contains one major ATP sulfurylase, but hypocotyl tissue contains two enzymes of approximately equal activity. One of the hypocotyl enzymes was indistinguishable from the leaf enzyme.

**MATERIALS AND METHODS**

**Chemicals and Enzymes.** Chemicals were obtained from the sources described previously (26, 27). In addition, bovine serum albumin was obtained from the Commonwealth Serum Laboratories, Parkville, Vic., Australia and polyacrylamide slab gels (7.2 × 7.2 × 0.3 cm) containing 5 to 20% polyacrylamide gradient were obtained from Gradient Pty. Ltd., Lane Cove, N.S.W., Australia. **59**Pi was prepared from **32**Pi by pyrolysis (17), and the specific radioactivity was adjusted to 0.25 c/mole with unlabeled Na2**32**P04.

**Mg**<sup>2+</sup>-dependent alkaline pyrophosphatase was purified from spinach as described by Shaw and Anderson (27); the activity of purified enzyme was 60 units/ml, the definition of an enzyme unit described by Shaw and Anderson (27) being used. ADP sulfurylase, ATP sulfurylase and ATPase activities were absent from purified pyrophosphatase although the purified enzyme is known to contain some 3'-nucleotidase activity (7).

**Plant Material.** Beetroot plants (*Beta vulgaris*) with well developed swollen hypocotyls were obtained directly from market gardens; only plants of good quality were used. De-ribbed upper leaves were used as the source of leaf tissue. Hypocotyls were washed in distilled water, and the outer 0.5 cm was removed by peeling. The peeled tissue was diced into cubes (approx. 8 cm<sup>3</sup>) and used as the source of hypocotyl tissue.

**Extraction and Purification of the Hypocotyl ATP Sulfurylases.** Diced hypocotyl tissue was extracted with 100 mM tris-HCl buffer, pH 8.0, containing 10 mM potassium thioglycolate (medium 1) in a Waring Blender using 0.5 ml of medium 1 per g fresh weight. The homogenized material was strained through muslin to remove cell debris. Particulate material was removed from the filtered solution by centrifugation (20,000 g for 20 min); the supernatant solution containing the ATP sulfurylase activity is referred to as crude extract. Solid (NH4)2SO4 (0.561 g/ml, equivalent to 80% saturation) was added to the crude extract. Precipitated protein was recovered by centrifugation and redissolved in medium 1 (0.2 ml/g) of original weight of tissue; this procedure effected the separation of protein from the bulk of the phenolics in the crude extract and also served to concentrate the extract. This fraction is referred to as concentrated crude extract.

Concentrated crude extract was fractionated by the addition of 0.176 g of solid (NH4)2SO4/ml of extract (equivalent to 30% saturation). The supernatant solution was recovered, and a further 0.127 g/ml of solid (NH4)2SO4 was added (50% saturation); precipitated protein was recovered by centrifugation and dissolved in 0.04 ml of 20 mM tris-HCl buffer, pH 8, containing 5 mM potassium thioglycolate (medium 2) per g original weight of tissue. The (NH4)2SO4 fraction was extensively dialyzed against medium 2 to remove residual (NH4)2SO4. The dialyzed fraction was subjected to gel filtration on a Sephadex G-200 column (60 × 2.5 cm) equilibrated with 20 mM tris-HCl, pH 8 (medium 3). Fractions containing ATP sulfurylase activity were applied to a DEAE-cellulose column (10 × 2 cm) equilibrated with medium 3, and the column was developed with a linear gradient of KCl (0–240 mM). Two peaks of ATP sulfurylase activity were eluted from the column (Fig. 1). The ATP sulfurylase activity eluted by 110 mM KCl is designated hypocotyl I enzyme, and the activity eluted by 150 mM KCl is referred to as hypocotyl II enzyme. In some instances isolated hypocotyl I and II enzymes were treated on a second DEAE-cellulose column as described by Shaw and Anderson (27); this treatment effected a 4-fold concentration of activity. All operations were conducted at 2 to 4°C.

![Fig. 1](https://www.plantphysiol.org/)

**Fig. 1.** Ion exchange chromatography of the hypocotyl and leaf ATP sulfurylases on a DEAE-cellulose column. The enzymes were subjected to chromatography following (NH4)2SO4 fractionation and gel filtration on Sephadex G-200 as described in the text. The KCl gradient was applied in a volume of 1 liter. **A:** Sulfate-dependent PPI-ATP exchange; **B:** protein. The bold line depicts the concentration of KCl.
per g fresh weight of leaf tissue, and the solutions were combined. The combined fractions were dialyzed to remove (NH₄)₂SO₄ and subjected to gel filtration and ion exchange chromatography as described for the purification of the hypocotyl enzymes, except that the linear gradient of KCl used to develop the DEAE-cellulose column was 0 to 280 mM. Most of the ATP sulfurylase activity (98%) was eluted from the DEAE-cellulose column by approximately 150 mM KCl; a small amount of activity was eluted by approximately 80 to 110 mM KCl (Fig. 1). All operations were conducted at 2 to 4°C.

**Assay of Enzyme Activities.** ATP sulfurylase activity was measured by sulfate-dependent PPI-ATP exchange (26, 27). Assays were conducted at 35°C for 15 min and contained 2 μmoles of Na₂K₂ATP; 2 μmoles of Na₂P₂O₇ (0.5 μc); 10 μmoles of MgCl₂; 10 μmoles of NaF; 40 μmoles of K₂SO₄; 100 μmoles of tris-HCl buffer, pH 8.0; and enzyme in a final volume of 1 ml. Fluoride was added to inhibit pyrophosphatase activity (26) but was omitted from assays of enzyme preparations which were known to be uncontaminated with pyrophosphatase activity. Concentrated crude extracts mixed contained 120 μmoles of KCl in lieu of K₂SO₄ to maintain the ionic strength. Reactions were terminated with 2 ml of trichloroacetic acid (7.5%, w/v). ³²P-ATP was separated from ³²P-PPI by the method of Davie et al. (12) and is expressed as sulfate-dependent PPI-ATP exchange in μmoles/min (ATP sulfurylase units); specific activity is expressed as units/mg of protein.

**Polyacrylamide Gradient Gel Electrophoresis of the ATP Sulfurylases.** The method was essentially that of Skyring et al. (28); ATP sulfurylase activity on the gels was detected by the molybdate-dependent formation of Pi (31). Electrophoresis was performed in tris-glycine buffer (30 mM with respect to glycine), pH 8.2, at 4°C and 150 v; the gels were prerun for 25 min prior to applying the sample. ATP sulfurylase samples, adjusted to 5% (v/v) sucrose and containing not less than 0.15 unit of activity, were applied to the gels in a volume not exceeding 35 μl. The samples were subjected to electrophoresis for either 3 or 15 hr; the amperage generally decreased from 15 to 8 mA during electrophoresis.

Following electrophoresis, the gels were sliced in two, parallel to their face. One half was incubated at 35°C for 30 min in a complete mixture (with molybdate) containing 5 mM Na₂K₂ATP; 20 mM MgCl₂; 10 mM Na₂MoO₄; 100 mM tris-HCl buffer, pH 8.0; and purified spinach pyrophosphatase (final concentration 6 units/ml) in a total volume of 20 ml. The gels were washed with distilled water, and Pi produced by the action of ATP sulfurylase and ATPase if present) was detected by the method of Allen (3); gels were placed in 20 ml of HClO₄ (6%, w/v), and 4 ml of ammidol reagent and 2 ml of 6.6% (w/v) ammonium molybdate were added in that order. After 10 min the treated gels were transferred to distilled water and photographed. The other half of the gel was treated identically except that molybdate was omitted from the incubation mixture. The formation of any Pi in gels incubated without molybdate was assumed to be due to ATPase activity.

**Determination of Protein.** Protein in crude extracts and (NH₄)₂SO₄ fractions was determined by the method of Elman (15) using bovine serum albumin as standard. Purer protein from Sephadex G-200 and DEAE-cellulose columns was measured by the method of Warburg and Christian (30).

**RESULTS**

**Optimal Conditions for the Extraction and Assay of ATP Sulfurylase from Hypocotyl tissue.** The ATP sulfurylase activity of concentrated crude extracts prepared without thioglycolate was extremely low. The activity recovered in concentrated crude extracts was increased when thioglycolate was included in the extracting medium (Fig. 2). Higher concentrations of thioglycolate were inhibitory.

In preliminary studies with concentrated crude extract, KCl was omitted from the control incubations. Under these conditions, PPI-ATP exchange was frequently greater in the controls than in incubations containing sulfate. The endogenous exchange was not decreased by dialysis. Addition of KCl (equivalent in ionic strength to the substrate concentration of K₂SO₄) to control incubations decreased endogenous PPI-ATP exchange (Fig. 3). Accordingly, 120 mM KCl was included in all subsequent assays of crude extracts and (NH₄)₂SO₄ fractions.

**Purification of the ATP Sulfurylases from Beetroot Hypocotyl and Leaf Tissue.** The estimation of ATP sulfurylase by sulfate-dependent PPI-ATP exchange is subject to interference by pyrophosphatase and ATPase activities (26, 27). Concentrated crude extracts of beetroot hypocotyl tissue contain pyrophosphatase and ATPase activities (Table 1). Fractionation with (NH₄)₂SO₄ removed much inactive protein but failed to effect a satisfactory separation of ATP sulfurylase from the two interfering enzymes. Fractionation with (NH₄)₂SO₄ also failed to decrease the very high endogenous exchange activity in control incubations. Gel filtration of the 30 to 50% (NH₄)₂SO₄ fraction on Sephadex G-200 effected separation of the pyrophosphatase and ATP sulfurylase activities and decreased the endogenous exchange to a negligible level. A single peak of ATP sulfurylase activity was eluted from Sephadex G-200 columns. ATPase activity was largely removed by ion exchange chromatography on DEAE-cellulose. The small endogenous PPI-ATP exchange of ATP sulfurylase fractions purified by gel filtration was not decreased by KCl.

Ion exchange chromatography of the fractions subjected to gel filtration resolved the ATP sulfurylase activity into

![Fig. 2. Effect of concentration of potassium thioglycolate in the extracting medium upon the ATP sulfurylase activity extracted from hypocotyl tissue. Cubes of randomized hypocotyl tissue (20 g) were extracted with 20 ml of 100 mM tris-HCl buffer, pH 7.5, containing 20 mM MgCl₂ and the concentrations of thioglycolate specified. The crude supernatant solutions were recovered and concentrated crude extracts were prepared as described in the text except that the (NH₄)₂SO₄ precipitates were resuspended and dialyzed against the appropriate medium used to extract the tissue. After dialysis, activity was measured by sulfate-dependent PPI-ATP exchange. Concentrated crude extracts did not catalyze thioglycolate-dependent PPI-ATP exchange.](image-url)
two approximately equal activities (Fig. 1). Crude extracts of hypocotyl tissue, depigmented by passage through a Sephadex G-50 column equilibrated with medium 1, were also resolved into two approximately equal ATP sulfurylase activities when directly subjected to ion exchange chromatography on a DEAE-cellulose column. ATP sulfurylase purified by fractionation with acetic acid was also resolved into two approximately equal activities when subjected to chromatography on a DEAE-cellulose column. In all cases one peak of activity (hypocotyl I) was eluted by 110 mM KCl, and the other peak (hypocotyl II) was eluted by 150 mM KCl.

Leaf tissue contained much higher ATP sulfurylase activity than hypocotyl tissue; typical activities were 60 units/g fresh weight and 1.3 units/g fresh weight for leaf and hypocotyl tissue, respectively. Crude extracts of leaf tissue contained an active pyrophosphatase and some ATPase activity. The purification procedure for the beetroot leaf ATP sulfurylase, which was essentially the same as the procedure used to effect a 1000-fold purification of the spinach leaf enzyme (27), removed the pyrophosphatase activity but effected only a 6-fold increase in specific activity of the beetroot ATP sulfurylase (Table II). In all experiments essentially one peak of activity was eluted from the DEAE-cellulose column (Fig. 1); the conditions for the elution of the leaf enzyme were identical to the conditions required to elute the hypocotyl II enzyme. A small amount of ATP sulfurylase activity was eluted prior to the major leaf enzyme during ion exchange chromatography but this activity did not exceed 2% of the major activity. A similar elution pattern was obtained when the (NH₄)₂SO₄ fraction for purifying the leaf enzyme was replaced with a single step precipitation of crude extract by (NH₄)₂SO₄. In both cases, the major leaf enzyme was eluted by 150 mM KCl.

Some Properties of the Hypocotyl and Leaf ATP Sulfurylases. The rates of sulfate-dependent PPi-ATP exchange catalyzed by dialyzed concentrated crude extracts of hypocotyl tissue in the presence (●) and absence (▲) of 40 mM K₂SO₄. The ionic strength refers to the components KCl and K₂SO₄ only. Incubations with and without K₂SO₄ were adjusted to the ionic strength specified with KCl.

![Graph showing effect of ionic strength on the PPi-ATP exchange catalyzed by dialyzed concentrated crude extracts of hypocotyl tissue](image)

Table I. Typical Purification of the ATP Sulfurylases from Hypocotyl Tissue of Beetroot and Their Separation from Pyrophosphatase and ATPase Activities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein mg</th>
<th>Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP sulfurylase</td>
<td>Pyrophosphatase</td>
</tr>
<tr>
<td>Dialyzed crude extract</td>
<td>770</td>
<td>0.75</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction (30-50% saturation)</td>
<td>204</td>
<td>1.19</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>79</td>
<td>2.31</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>Peak 1 (hypocotyl I)</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>Peak II (hypocotyl II)</td>
<td>0.41</td>
</tr>
</tbody>
</table>

Table II. Typical Purification of the ATP Sulfurylase from Leaf Tissue of Beetroot and Its Separation from Pyrophosphatase and ATPase Activities

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein mg</th>
<th>Enzyme Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATP sulfurylase</td>
<td>Pyrophosphatase</td>
</tr>
<tr>
<td>Dialyzed crude extract</td>
<td>1180</td>
<td>10.1</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction (combined 3rd and 4th fractions)</td>
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<td>8.5</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>253</td>
<td>22.0</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>3.09</td>
<td>61.2</td>
</tr>
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concentrations of polyacrylamide than the hypocotyl I enzyme (Fig. 6); the resolution of hypocotyl I and II activities at 15 hr was less than at 3 hr, suggesting that although the two enzymes differed significantly in charge at pH 8.2, the differences in molecular weight were small as judged by polyacrylamide gradient gel electrophoresis.

Margolis and Kenrick (19) have related the concentration of polyacrylamide at which a protein ceases to migrate in a polyacrylamide gradient gel to the molecular weight of the protein. This relation, together with the results of 15-hr electrophoresis experiments, indicated that the molecular weights of the leaf and hypocotyl II enzymes were slightly less than the molecular weight of hypocotyl I; the molecular weights of the three enzymes were approximately 200,000 to 230,000.

Concentrated crude extracts and 30 to 50% (NH₄)₂SO₄, fractions of hypocotyl tissue were subjected to electrophoresis for 3 hr. Two molybdate-dependent bands were resolved. The fast migrating band stained slightly more heavily in the presence of molybdate than the slower moving band, but the fast moving band stained lightly in control incubations without molybdate, suggesting that the fast moving band was not separated from ATPase activity. The two ATP sulfurylases in unpurified fractions migrated at approximately the same rate as hypocotyl I and II ATP sulfurylases.

**Studies of the ATP Sulfurylase Activity of Mitochondria from Hypocotyl Tissue.** Experiments designed to determine the subcellular localization of the two hypocotyl ATP sulfurylases were inconclusive. A mitochondria-enriched fraction was prepared by the method of Raison and Lyons (23); 8% of the total ATP sulfurylase activity was associated with the particulate fraction. This fraction was homogenized with medium 3 in a Potter-Elvejhem homogenizer; only 48% of the ATP sulfurylase activity associated with the particulate fraction was recovered in the soluble fraction. The soluble fraction contained both hypocotyl I and II activities when examined by ion exchange chromatography on a DEAE-cellulose column, but the relative activities of the two enzymes varied between experiments.

**DISCUSSION**

Studies of the hypocotyl ATP sulfurylases in crude extracts were complicated by the very low activity of the tissue, inactivation of the enzymes during extraction, pyrophosphatase and ATPase activities, and very high endogenous PPI-ATP exchange. Inactivation of the enzyme was minimized by including 10 mM thioglycolate in the extracting medium (Fig. 2). Pyrophosphatase was inhibited by 10 mM NaF. Since endogenous PPI-ATP exchange was inhibited by ionic strength (Fig. 3), thereby causing an apparent K₂SO₄-dependent inhibition of endogenous exchange in some experiments, KCl of equivalent ionic strength to the substrate level of K₂SO₄ was always added to control incubations when monitoring for ATP sulfurylase activity. The effect of ATPase on sulfate-dependent PPI-ATP exchange was minimized by using dilute extracts of enzyme; under these conditions, hydrolysis of ATP by ATPase was insufficient to cause a significant decrease in sulfate-dependent PPI-ATP exchange.

Purification only effected a 15- to 25-fold increase in specific activity of the hypocotyl ATP sulfurylases. The more important aspect of purification, however, was the separation of ATP sulfurylase from enzymes which interfered with the assay of ATP sulfurylase activity. Purified ATP sulfurylase was uncontaminated with pyrophosphatase and ATPase activities, and the endogenous PPI-ATP exchange of purified enzyme was negligible (Table I). Similar remarks also apply to the leaf enzyme (Table II).
Generally speaking, the properties of the three beetroot ATP sulfurylases were similar to each other and to the enzymes described in spinach leaf (27) and the leaf tissue of four species of *Astragalus* (W. H. Shaw and J. W. Anderson, unpublished data). All enzymes were inactive in the absence of Mg²⁺, have pH optima from 7.5 to 9.0, and catalyze both sulfate- and selenate-dependent exchange. The affinity of all the enzymes for selenate was greater than for sulfate and the $V$ (selenate)/$V$ (sulfate) ratios fall within the range 0.23 to 0.29 regardless of the origin of the enzyme.

There are, however, several quantitative properties of the beetroot hypocotyl I enzyme which distinguish it from the other two beetroot enzymes. The most obvious of these differences was a difference in charge which permitted separation of the hypocotyl enzymes by ion exchange chromatography on DEAE-cellulose columns at pH 8.0 (Fig. 1) and by electrophoresis at pH 8.2 (Figs. 5 and 6). The hypocotyl I enzyme also had a lower affinity for sulfate and selenate and Table III) and a slightly higher pH optimum than the leaf and hypocotyl II enzymes (Fig. 4). The differences in molecular weight of the three enzymes was small; the enzymes were retarded but not separated by gel filtration on Sephadex G-200 columns but were resolved by electrophoresis for 15 hr on a polyacrylamide gel gradient. The leaf and hypocotyl II enzymes, on the other hand, were indistinguishable by electrophoresis at 3 or 15 hr, ion exchange chromatography, or their kinetic properties.

Hypocotyl tissue invariably contained hypocotyl I and hypocotyl II enzymes in approximately equal proportions; this rules out the possibility of allelic variation. Several lines of evidence suggest that it is unlikely that the two hypocotyl enzymes arise as an artifact. Firstly, the two activities were resolved in approximately equal amounts irrespective of whether the extracts were purified and irrespective of the method of purification. Secondly, the differences in molecular weight of the two hypocotyl enzymes is barely significant, but the molecular weight of both enzymes is in excess of 200,000. Thirdly, hypocotyl II enzyme was indistinguishable from the leaf enzyme. While we cannot exclude the possibility that leaf tissue contains an additional ATP sulfurylase (a small amount of ATP sulfurylase was eluted prior to the major leaf ATP sulfurylase on DEAE-cellulose columns), this activity was never greater than 2% of the major leaf enzyme. We conclude that the multiple forms of ATP sulfurylase in hypocotyl tissue are most probably isoenzymes.

In leaf tissue, sulfate reduction and the enzymes of the sulfate reduction pathway (including ATP sulfurylase) are associated with chloroplasts (6, 7, 24). Presumably the ATP sulfurylase of beetroot leaf tissue is a chloroplastic enzyme. We are unable to comment on the subcellular localization of the two hypocotyl enzymes, but the similarity of the leaf and hypocotyl II enzymes warrants a thorough study of the plastids of the nonphotosynthetic hypocotyl tissue for ATP sulfurylase activity. The studies on the association of the hypocotyl enzy mes with mitochondria were inconclusive, especially since the differential centrifugation technique used to prepare the mitochondria does not adequately resolve the large number of subcellular particles of size and density similar to mitochondria which occur in nonphotosynthetic tissue. It is impossible to attribute a physiological role to the two enzymes until their subcellular localization has been clearly established; in view of the problems in extracting subcellular organelles purified ATP sulfurylases from beetroot. Electrophoresis was conducted for 15 hr and ATP sulfurylase activity was detected as described in Fig. 5. At 15 hr the enzymes ceased to migrate any further into the gradient. L: leaf enzyme; HI: hypocotyl I enzyme; HII: hypocotyl II enzyme.

**Fig. 5.** Polyacrylamide gradient gel electrophoresis of the purified ATP sulfurylases from beetroot. Electrophoresis was conducted for 3 hr. The gel was incubated with molybdate as described in the text to detect molybdate-dependent formation of Pi. Pi was not detected on control gels of the purified enzymes incubated without molybdate. L: leaf enzyme; HI: hypocotyl I enzyme; HII: hypocotyl II enzyme.

**Fig. 6.** Polyacrylamide gradient gel electrophoresis of the
from beetroot hypocotyl, it would seem prudent to choose another nonphotosynthetic tissue to pursue this problem.

Nitrite, like sulfate, is reduced by both photosynthetic and nonphotosynthetic tissues. Dalling et al. (10) reported that photosynthetic tissue contains a single nitrite reductase which is associated with chloroplasts. Nonphotosynthetic tissue, on the other hand, contains two nitrite reductase activities; one of these enzymes was indistinguishable from the leaf enzyme (9). Both of the nonphotosynthetic activities were associated with plastids (11). While the subcellular distribution of two ATP sulfurylases of beetroot hypocotyl in uncertain, there is a striking similarity in the distribution of isoenzymes of ATP sulfurylase and nitrite reductase in photosynthetic and nonphotosynthetic tissue.

**LITERATURE CITED**