Planta (1989) 179:228–234

Planta

Localization of enzymes of assimilatory sulfate reduction in pea roots

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Abstract. The localization of enzymes of assimilatory sulfate reduction was examined in roots of 5-d-old pea (*Pisum sativum* L.) seedlings. During an 8-h period, roots of intact plants incorporated more label from $^{35}SO_4^{2-}$ in the nutrient solution into the amino-acid and protein fractions than shoots. Excised roots and roots of intact plants assimilated comparable amounts of radioactivity from $^{35}SO_4^{2-}$ into the amino-acid and protein fractions during a 1-h period, demonstrating that roots of pea seedlings at this stage of development were not completely dependent on the shoots for reduced sulfur compounds. Indeed, these roots contained activities of ATP-sulfurylase (EC 2.7.7.4), adenosine 5′-phosphosulfate sulfotransferase, sulfite reductase (EC 1.8.7.1) and O-acetyl-L-serine sulfhydrylase (EC 4.2.99.8) at levels of 50, 30, 120 and 100%, respectively, of that in shoots. Most of the extractable activity of adenosine 5′-phosphosulfate sulfotransferase was detected in the first centimeter of the root tip. Using sucrose density gradients for organelle separation from this part of the root showed that almost 40% of the activity of ATP-sulfurylase, adenosine 5′-phosphosulfate sulfotransferase and sulfite reductase banded with the marker enzyme for proplastids, whereas only approximately 7% of O-acetyl-L-serine sulfhydrylase activity was detected in these fractions. Because their distributions on the gradients were very similar to that of nitrite reductase, a proplastid enzyme, it is concluded that ATP-sulfurylase, adenosine 5′-phosphosulfate sulfotransferase and sulfite reductase are also exclusively or almost exclusively localized in the proplastids of pea roots. O-Acetyl-L-serine sulfhydrylase is predominantly present in the cytoplasm.

Abbreviation: APSSTase = adenosine 5′-phosphosulfate sulfotransferase

Key words: ATP-sulfurylase-adenosine 5′-phosphosulfate sulfotransferase – *Pisum* (sulfate reduction) – Proplastid – Sulfite reductase

Introduction

It is generally accepted that in leaves assimilatory sulfate reduction is localized in the chloroplasts (Schiff 1983; Schmidt 1986), since all the enzymes involved in this pathway have been detected in these organelles (Schmidt 1986) and since isolated chloroplasts can form [^{35}S]cysteine from $^{35}SO_4^{2-}$ (Trebst and Schmidt 1969; Schwenn and Trebst 1976). On the other hand, cell, tissue and organ cultures from a wide variety of plant parts including roots can be grown heterotrophically on media where sulfate is the sole source of sulfur (Schiff 1983), and non-green tissues of plants such as beet discs (Ellis 1963) and roots (Pate 1965) reduce sulfate. Indeed, enzymes of sulfate reduction such as ATP-sulfurylase (EC 2.7.7.4; Ellis 1969), adenosine 5′-phosphosulfate sulfotransferase (APSSTase; Schmidt 1976; Fankhauser and Brunold 1978), sulfite reductase (EC 1.8.7.1; Mayer 1967; Tamura and Hosoi 1979) and O-acetyl-L-serine sulfhydrylase (EC 4.2.99.8; Tamura et al. 1976; Fankhauser and Brunold 1979) have been detected in roots.

New interest in assimilatory sulfate reduction in roots comes from the fact that heavy metals induce the formation of phytochelatins (Grill et al. 1985) in plant roots (Fujita and Kawanishi 1987). These compounds have the structure $(\gamma\text{-glutamylcysteine})_n$ glycine ($n = 2$ to 11). In view of their high cysteine content, it seems doubtful (Klapheck et al. 1987) that the transport of reduced sulfur from leaves to roots (Bonas et al. 1982) could cover

the need for phytochelatin synthesis. Indeed, we detected an increase in the activity of ATP-sulfurylase and APSSTase in extracts from roots of maize seedlings cultivated in the presence of cadmium and synthesising phytochelatins (Nussbaum et al. 1988).

The intracellular localization of the enzymes of assimilatory sulfate reduction in roots is not clear: O-actyl-L-serine sulfhydrylase was found in the proplastid fraction of spinach roots (Fankhauser and Brunold 1979), whereas sulfite reductase, detected in the particulate fraction from pea roots, was tentatively ascribed to the mitochondria (Mayer 1967). It seems interesting in this connection that in mutants of *Euglena gracilis* lacking plastids all the enzymes of assimilatory sulfate reduction were localized in the mitochondria (Brunold and Schiff 1976; Saidha et al. 1985). In this paper we present evidence that in 5-d-old pea seedlings the roots contain appreciable levels of all enzyme activities of assimilatory sulfate reduction and that the roots are not completely dependent on the shoots for the necessary amount of reduced sulfur.

Material and methods

Pisum sativum L. cv. Fruehbusch (Vatter, Berne, Switzerland) was grown aseptically either in glass jars covered with polyethylene foil in continuous light (5000 lx) provided by two fluorescent tubes (TL 40 W/33; Philips, Eindhoven, The Netherlands), at 22° C and a relative humidity in the culture room of 80±3%, or in the dark in aluminium boxes at 27° C. Perlite moistened with nutrient solution (Wyss and Brunold 1979) was used as a substrate (Perlite: nutrient solution=2:1; v/v). Seeds were sterilized for 30 min in 10% (w/v) sodium hypochlorite and rinsed five times with sterile water. The aluminium boxes were aireated under sterile conditions at a rate of 25 $l \cdot h^{-1}$. In-vivo assimilation of radioactive sulfate was examined by adding sterile carrier-free $^{35}SO_4^{2-}$ to the nutrient solution of pea seedlings arranged on stainless-steel grids resting on the vessels which contained the solution. The level of the solution (200 ml) was such that the roots were immersed while the cotyledons and the shoots were kept dry. After the appropriate time interval the roots of the seedlings were washed for 30 min with H_2O at 4° C. After the isolation of roots and shoots, extraction and separation into sulfate, amino-acid and protein fractions was performed according to Brunold and Suter (1984). Excised roots were fed with $^{35}SO_4^{2-}$ after covering the cut stem with the foremost 0.5 cm of a disposable 1-ml pipet tip filled with cotton wool. For xylem-sap analysis the cotton was eluted with 1.5 ml of 0.5 M $HClO_4$. The sap was separated into sulfate and amino-acid fractions according to Brunold and Suter (1984).

Extracts for measuring enzyme activities in roots and shoots were routinely prepared in a glass homogenizer at 0–4° C in 0.1 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris)-HCl, pH 8.0, containing 10 mM KCl, 20 mM $MgCl_2$, and 10 mM tithioerythritol. For 1 g of plant material, 2 ml of buffer were used. When the enzyme activities of whole plants including the cotyledons were determined, homogenization was performed with a Polytron (Cinematica, Luzern, Switzerland) in 10 ml buffer solution per gram of plant material. The homogenates were centrifuged at 10000·*g* for 10 min at 4° C. The supernatants were used immediately for the enzyme assays.

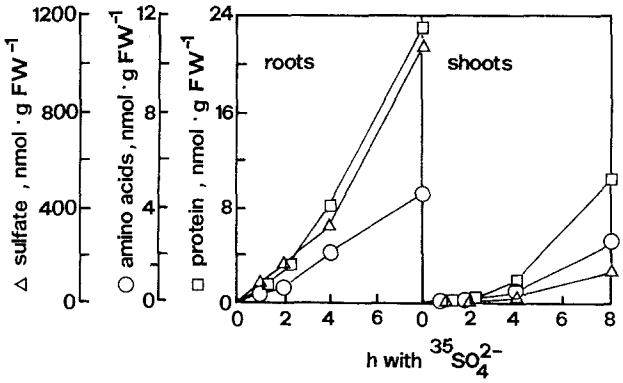

Fig. 1. Radioactive sulfur in the sulfate, amino-acid and protein fractions of roots and shoots from 5-d-old pea seedlings cultivated for various times with nutrient solution containing $^{35}SO_4^{2-}$. The shoots and roots of the five seedlings analysed had mean fresh weights of 91.0±17.2 and 95.2±6.0 mg, respectively

The activity of ATP-sulfurylase was measured in the back-reaction by determining the ATP formed from in organic pyrophosphate and adenosine 5′-phosphosulfate (APS) using a luciferin-luciferase system (Schmutz and Brunold 1982). Activity of APSSTase was measured by the production of $^{35}SO_3^{2-}$ assayed as acid-volatile radioactivity from [^{35}S]APS in the presence of DTE (Tsang et al. 1976; Brunold and Suter 1983). Sulfite-reductase activity was routinely determined in a coupled assay system using O-acetyl-L-serine sulfhydrylase to measure the sulfide formed (von Arb and Brunold 1983). In the fractions of the sucrose density gradients, sulfite-reductase activity was estimated by measuring the methylene blue formed from H_2S according to Brunold and Schiff 1976. The activity of O-acetyl-L-serine sulfhydrylase was routinely determined according to Pieniacek et al. (1973), by measuring the cysteine formed from O-acetyl-L-serine and S^{2-}. In the fractions of the sucrose density gradients, this enzyme activity was also determined according to Becker et al. (1969).

Subcellular fractions from pea roots using sucrose density gradients were prepared according to Emes and Fowler (1979). Centrifugation was for 30 min at 19000 rpm at 4° C using a Kontron (München, FRG) TST 28.38 rotor. Fractions of 1 ml were collected. Sucrose concentrations were determined by measuring the refractive index with a Zeiss (Oberkochen, FRG) refractometer.

Proteins were estimated according to Bradford (1976), using bovine serum albumin as a standard.

[^{35}S]Adenosine 5′-phosphosulfate was prepared according to Tsang et al. 1976. APS was purchased from Sigma, $^{35}SO_4^{2-}$ from the Radiochemical Centre, Amersham (UK), the luciferin-luciferase system from Lumac, Basel, Switzerland, and all other chemicals from Fluka, Buchs, Switzerland.

Results

Figure 1 shows the time-course of the incorporation of radioactive sulfur from $^{35}SO_4^{2-}$ in the nutrient solution into the sulfate, amino-acid and protein fractions of 5-d-old pea seedlings. After

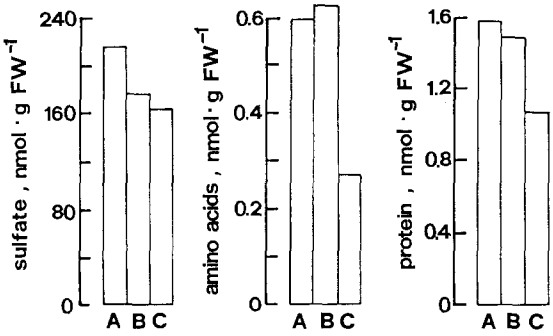

Fig. 2. Radioactive sulfur in the sulfate, amino-acid and protein fractions of roots from intact plants (*A*), of roots without shoots but with cotyledons (*B*), and of excised roots (*C*) from 5-d-old pea seedlings cultivated for 1 h with nutrient solution containing $^{35}SO_4^{2-}$. The radioactivity in the sulfate and the amino-acid fraction of the xylem sap of (*B*) was 0.09 and 2.5% and of (*C*) 0.05 and 3.75%, respectively, of the total radioactivity of the two fractions measured in the roots

1 and 2 h with $^{35}SO_4^{2-}$, appreciable radioactivity was detected in the fractions prepared from the roots, whereas only very low radioactivity was measured in those from the shoots, indicating that these roots were reducing sulfate and were not completely dependent on the shoots for providing the necessary reduced sulfur compounds. This was substantiated by the results presented in Fig. 2, which shows the amount of radioactivity in the sulfate, amino-acid and protein fractions of roots from intact plants, of roots without shoots but with cotyledons, and of excised roots, all kept on nutrient solution with $^{35}SO_4^{2-}$ for 1 h. Consistent with the conclusion from Fig. 1, both preparations of roots without shoots incorporated ^{35}S from radioactive sulfate into the protein and amino-acid fractions at rates comparable to those of roots from intact plants. This indicated that these roots should contain all the enzymes of assimilatory sulfate reduction. Indeed, the total activities of sulfite reductase and O-acetyl-L-serine sulfhydrylase in roots were 120 and 100%, respectively, of the levels in shoots of 5-d-old pea seedlings (Fig. 3). The ATP-sulfurylase and APSSTase activities in the roots were 50% and 30%, respectively, of those in the shoots. Appreciable levels of all four enzyme activities were measured in the cotyledons. In all three organs, total APSSTase activity was extremely low, whereas total O-actyl-L-serine sulfhydrylase activity was very high compared with the other two enzymes of sulfate reduction. During day 6 all four enzyme activities increased rapidly in the shoot. An especially marked increase was measured for ATP-sulfurylase and APSSTase, the levels of which were only 20% and 10%, respectively, in roots compared with shoots of 6-d-old seedlings.

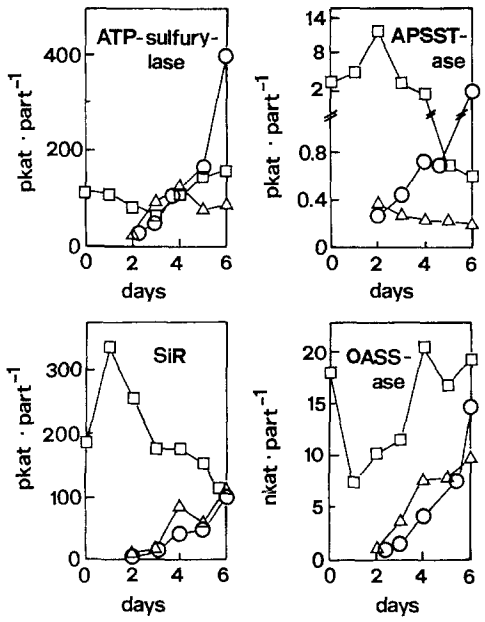

Fig. 3. Total extractable activity of ATP-sulfurylase, APSSTase, sulfite reductase (*SiR*) and O-acetyl-L-serine sulfhydrylase (*OASSase*) in cotyledons (□), roots (△) and shoots (○) from pea seedlings cultivated in continuous light for various times

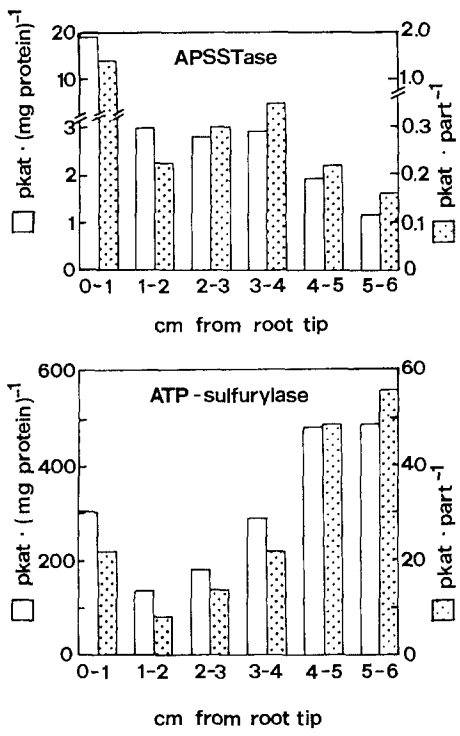

Fig. 4. Total and specific extractable activities of APSSTase and ATP-sulfurylase in 1-cm sections of roots from 5-d-old pea seedlings

When the activities of ATP-sulfurylase and APSSTase were compared in 1-cm-long segments along the root axis of 5-d-old pea seedlings (Fig. 4), a high level was detected in the first seg-

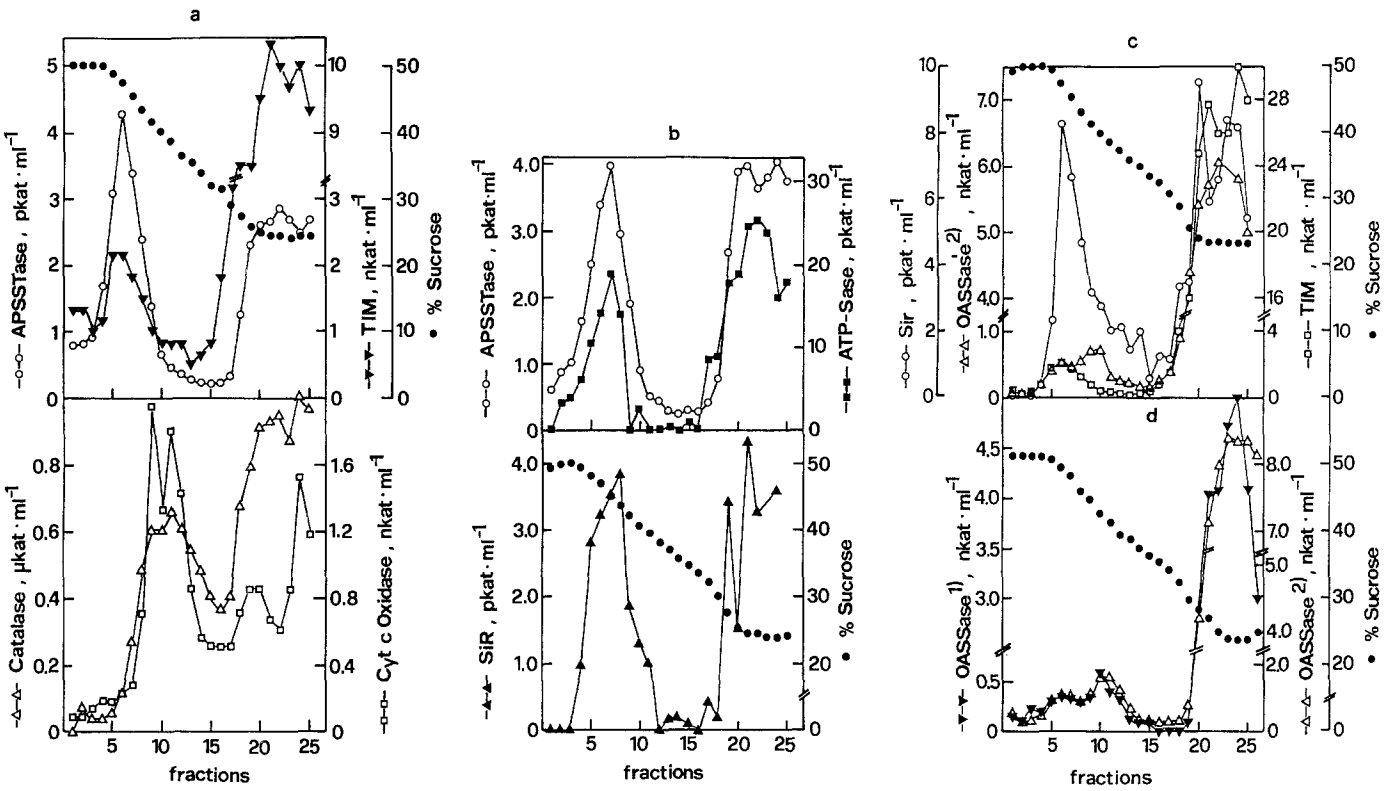

Fig. 5a–d. Distribution of the activity of enzymes of assimilatory sulfate reduction and of the marker enzymes for proplastids (triosephosphate isomerase, *TIM*), mitochondria (cytochrome-c oxidase, *Cyt c oxidase*) and microbodies (*catalase*) from roots of 5-d-old pea seedlings on sucrose density gradients. Four independent experiments are presented (**a–d**), and the following enzymes of assimilatory sulfate reduction were measured in the individual gradients: APSSTase, **a**, **b**; ATP-sulfurylase, *ATP-Sase*, **b**; sulfite reductase, *SiR*, **b**, **c**; O-acetyl-L-serine sulfhydrylase, *OASSase*, **c**, **d**, estimated according to Becker et al. 1969 (*OASSase*$^{1)}$) or to Pieniacek et al. 1973 (*OASSase*$^{2)}$)

ment from the root tip for both enzymes activities. The activity of ATP-sulfurylase decreased in the following segment, but then increased and reached the highest level at the base of the roots. By contrast, APSSTase activity was much lower in the second and the following segments and was at a minimum in the last segment. Experiments with mixtures of extracts from segments with high and low enzyme activities indicated that activating or deactivating factors were not responsible for the differences in activity along the root axis.

The intracellular localization of the enzymes of assimilatory sulfate reduction is evident from Fig. 5. Results from four independent experiments are presented (Fig. 5a–d). Figure 5a shows that APSSTase activity had its peak in the sucrose gradient in the fractions where triosephosphate-isomerase activity banded, an established marker enzyme for intact proplastids (Miflin 1974). The activity of APPSTase was also detected on top of the gradient, but it did not reach the levels measured in the gradient. Catalase and cytochrome-c-oxidase activities, markers for microbodies and mitochondria, respectively, banded together in the gradient, indicating that these organelles were not separated from each other. It is evident, however, that microbodies and mitochondria were separated from the proplastids. Several repetitions of this experiment showed that APSSTase activity banded very reliably with triosephosphate isomerase and could therefore also be used as a marker enzyme for proplastids. Figure 5b shows that both ATP-sulfurylase and sulfite-reductase activities were distributed very similarly on the gradients to APSSTase activity, indicating an almost identical intracellular localization. Repetitions of this type of experiment made it clear that sulfite reductase was also a suitable enzyme for detecting the intact proplastid fractions in the sucrose gradients. This is shown in Fig. 5c, where the peak activities of both sulfite reductase and triosephosphate isomerase in the gradient were localized in the same fractions. O-Acetyl-L-serine sulfhydrylase clearly had two peaks of activity in the gradient, one banding with the intact proplastids, the other one at a sucrose concentration where microbodies and mito-

Table 1. Enzyme activity recovered as percent of the total activity from pea root homogenates added to sucrose density gradients and as percent of the recovered activities detected in the fractions containing intact proplastids

	Enzyme activity in proplastids (%)	Enzyme activity recovered (%)
Triosephosphate isomerase	5.4	98
ATP-sulfurylase	37.5	101
Adenosine 5′-phosphosulfate sulfotransferase	39.0	61
Sulfite reductase	36.0	60
O-Acetyl-L-serine sulfhydrylase	6.9	81
Cytochrome-c oxidase	8.7	72
Catalase	6.8	63

chondria were detected, using the appropriate marker enzymes (Fig. 5a). This result was corroborated with the findings from the gradient presented in Fig. 5d, where O-acetyl-L-serine sulfurylase activity was measured using two different methods. Again, two peaks of activity were detected in the gradient, and most of the activity was on top of the gradients.

In Table 1 the percentage of the various enzyme activities detected in fractions containing intact proplastids, is presented. The very similar proportions of ATP-sulfurylase, APSSTase and sulfite-reductase activities determined in this fraction again indicates a similar intracellular localization. The last enzyme of assimilatory sulfate reduction, O-acetyl-L-serine sulfhydrylase, was found at a much lower percentage in the band containing intact proplastids, indicating a different intracellular localization.

Discussion

In general, roots contain low levels of the enzymes of assimilatory sulfate reduction and have a weak ability to reduce sulfate (Pate 1965; Mayer 1967; Schmidt 1976; Clarkson et al. 1983). According to Pate (1965), two to four out of every 1000 atoms of ^{35}S leaving the roots of peas cultivated in the presence of $^{35}SO_4^{2-}$ are bound to organic compounds, and only 5% of the total activity of APSSTase was detected in the roots of 35-d-old sunflower plants (Schmidt 1976). On a fresh-weight basis the sulfite-reductase activity was ten-fold higher in the shoots than in the roots of 10-d-old pea plants (Mayer 1967). The present study shows that the situation is different for 5-d-old pea seedlings, since comparable activities of both sulfite reductase and O-acetyl-L-serine sulfhydrylase were detected in roots and shoots, and since ATP-sulfurylase and APSSTase activities were measured in roots at 50 and 30%, respectively, of the levels in shoots. Our results also show that these enzymes cannot only be measured in vitro, but are also active in vivo in catalysing the assimilation of sulfate-S into the amino-acid and protein fractions of the roots.

Pea plants have been studied in detail with respect to the localization of assimilatory nitrate reduction (Miflin 1974; Emes and Fowler 1979; Wallace 1986). Even in 23-d-old plants, total nitrate-reductase activity is present at comparable levels in roots and shoots (Wallace 1986). In the roots, nitrate reductase was localized in the cytoplasm, whereas 33%–35% of nitrite-reductase activity banded with the marker enzyme for intact proplastids (Miflin 1974; Emes and Fowler 1979). Using a very similar method, comparable percentages of the activities of ATP-sulfurylase, APSSTase and sulfite reductase were localized in this organelle fraction in the present study, indicating that in cells of pea roots the localization of these enzymes of assimilatory sulfate reduction is similar to that of nitrite reductase. Since most, if not all of this enzyme activity seems to be in the proplastids of pea (Miflin 1974; Emes and Fowler 1979), it seems reasonable to assume that ATP-sulfurylase, APSSTase and sulfite reductase are also localized predominantly or even exclusively in this organelle. The portion of enzyme activities on top of the gradients would then result from proplastids broken during the preparation of the gradients.

We could detect approximately 7% of O-acetyl-L-serine sulfhydrylase activity in the proplastid fractions, which corresponds well to the percentage of glutamine-synthetase activity from pea roots determined in these organelles (Miflin 1974; Emes and Fowler 1979). A second peak of activity was detected in the sucrose gradient for glutamine synthetase (Emes and Fowler 1979) as for O-acetyl-L-serine sulfhydrylase in this study, but most activity of both enzymes was measured in the supernatant. This indicates that they are predominantly not bound to an organelle fraction, but are mostly soluble cytoplasmic enzymes. If we assume from the results with the first three enzymes of sulfate assimilation that our isolation procedure resulted in about 40% of intact proplastids, one can calculate that approx. 15%–20% of O-acetyl-L-serine sulfhydrylase is localized in this organelle. This is consistent with findings from spinach leaves, where 20% of O-acetyl-L-serine sulfhydrylase was detected in the chloroplasts (Fankhauser et al. 1976). It is interesting in this respect that positive evidence

for the dual location in proplastids and the cytoplasm of triosephosphate isomerase has been found by Anderson and Advani (1970). A low percentage of the enzyme, comparable to that found in this study, has been reported in the proplastid fractions from pea roots in previous work (Miflin 1974; Emes and Fowles 1979). This indicates that triosephosphate isomerase is also predominantly localized in the cytoplasm as is O-acetyl-L-serine sulfhydrylase. It can be concluded, therefore, that even though triosephosphate isomerase is routinely used for localizing proplastids on gradients, it is not the ideal marker enzyme. On the basis of the results presented in this study it could well be replaced by APSSTase which can be measured very conveniently and in minimal time.

In the leaves the electron donor for both nitrite and sulfite reductase is ferredoxin (Krueger and Siegel 1982). For maize roots a ferredoxin-like electron carrier is active with nitrite reductase (Suzuki et al. 1985). It is tempting to speculate that a similar ferredoxin-like root electron carrier serves as an electron donor for sulfite and nitrite reductase of pea roots. This idea is consistent with the finding of a ferredoxin-sulfite reductase in barley roots (Tamura and Hosoi 1979) since ferredoxin can replace the ferredoxin-like root electron carrier (Suzuki et al. 1985).

This research was supported by Grant from the Swiss National Science Foundation.

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Received 2 January; accepted 26 April 1989