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Regulation of Adenosine 5′-Phosphosulfate Sulfotransferase Activity by H_2S and Cyst(e)ine in Primary Leaves of *Phaseolus vulgaris* L.*

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Abstract. During chloroplast development in the primary leaves of *Phaseolus vulgaris,* the extractable activity of adenosine 5′-phosphosulfate sulfotransferase increased ten-fold. When chloroplast development took place in air enriched with 3.5 µl $H_2S \cdot l^{-1}$ there was a decrease in adenosine 5′-phosphosulfate sulfotransferase activity. Cyst(e)ine in concentrations up to 1 mM (in the external medium) did not affect the increase in adenosine 5′-phosphosulfate sulfotransferase activity in intact plants. In plants with excised roots, 0.75 mM cyst(e)ine inhibited this increase. In green primary leaves, H_2S or cyst(e)ine treatment resulted in a decrease of extractable adenosine 5′-phosphosulfate sulfotransferase activity. In intact plants, this effect of cyst(e)ine was observed at a concentration of 1 mM, and in plants with excised roots, 0.25 mM had a comparable effect.

In developing plants, the extractable activities of O-acetyl-L-serine sulfhydrylase (EC 4.2.99.9) and ribulosebisphosphate carboxylase (EC 4.1.1.39.) were not affected by H_2S or cyst(e)ine.

Key words: Adenosine 5′-phosphosulfate sulfotransferase – Cysteine – *Phaseolus* – Sulfide.

Introduction

Adenosine 5′-phosphosulfate sulfotransferase, an enzyme of assimilatory sulfate reduction in algae and higher plants, transfers the sulfonyl group of APS to a carrier to form Carrier-S-SO_3^- (Schmidt et al., 1974; Schmidt, 1976). We have previously shown that H_2S, or cyst(e)ine, decreases the extractable activity of APSSTase in *Lemna minor* (Brunold and Schmidt, 1976, 1978) This finding is in contrast with those reported for *Euglena* and cell cultures of *Catharantus roseus:* In Euglena (Brunold and Schiff, 1976), addition of cysteine to the culture medium did not affect extractable APSSTase activity. In cell suspension cultures of *Catharantus roseus* (Schwenn et al., 1978, 1979), cyst(e)ine or methionine induced a six-fold increase in APSSTase activity and a complete suppression of PAPS sulfotransferase, thereby questioning the role of APS sulfotransferase in assimilatory sulfate reduction.

These conflicting results prompted us to examine whether the regulation of APS sulfotransferase by cyst(e)ine and H_2S was restricted to *Lemna minor* which, under natural conditions, grows on ponds where H_2S can be detected (Kuchar, 1954). In this paper we present evidence that in the primary leaves of *Phaseolus vulgaris*, H_2S and cyst(e)ine reduce the level of extractable APS sulfotransferase.

In order to exclude unspecific effects of H_2S and cyst(e)ine, the following control-parameters were included in the experiment: OASSase, RubPCase, chlorophyll.

Material and Methods

Plant Material

Seeds of beans (*Phaseolus vulgaris* L. var. Saxa (Radio) Stamm Vatter) were sterilized for 1 h in 1% (w/v) sodium hypochlorite, rinsed three times with sterile water and then soaked in sterile aerated water for 24 h. After a further rinsing, the seeds were transferred to a grid in a PVC cultivation tank (610·410·355 mm) containing 10 l of sterilized nutrient solution. The level of the nutrient solution was 2 mm below the seeds. The nutrient solution contained: 1.5 mM $Ca(NO_3)_2$; 1 mM KNO_3; 0.75 mM KH_2PO_4; 0.75 mM $MgSO_4$; 0.4 µM $MnCl_2$; 2 µM H_3BO_3; 0.07 µM $ZnSO_4$; 0.05 µM $CuSO_4$; 0.08 µM MoO_3; and 30 µM Fe^{3+}-EDTA and

* This is no. 8 in the series "Regulation of Sulfate Assimilation in Plants." The term "cysteine" is used when it is clear that cystine is not involved; "cyst(e)ine" is used for an undefined mixture of cysteine and cystine. The concentrations are expressed in all cases relative to cysteine

Abbreviations: APS = adenosine 5′-phosphosulfate; APSSTase = adenosine 5′phosphosulfate sulfotransferase; BSA = bovine serum albumin; DTE = dithioerythritol; EDTA = ethylenediaminetetraacetic acid; OASSase = O-acetyl-L-serine sulfhydrylase; PAPS = adenosine 3′-phosphate 5′-phosphosulfate; POPOP = 1,4 Di 2-(5-phenyloxazolyl)-benzene; PPO = 2,5-diphenyloxazol; RubP = ribulose-bisphosphate; RubPCase = ribulosebiphosphate carboxylase

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was sterilized by autoclaving at 120° C for 1 h. The nutrient solution was continuously renewed at a flow rate of 10 l per day. The temperature in the tank was kept constant at 26 ± 0.5° C. The seedlings were kept in darkness for 3 days, then illuminated continuously at 4,000 l× (Philips TL 33 fluorescent tubes). In the light, the relative humidity was 60–80% and 100% in the dark. Tanks were aerated with air at a rate of $60\,l \cdot h^{-1}$ during the dark period and $1500\,l \cdot h^{-1}$ during the light period. Seedlings used for cysteine treatments were transferred either intact or without roots to 150 ml Erlenmeyer flasks containing nutrient solution with different cyst(e)ine concentrations. The nutrient solutions were renewed daily. Cyst(e)ine was added to the sterile nutrient solutions with sterile Milipore filters.

Preparation of Extracts

Primary leaves were homogenized in a chilled glasshomogenizer with 0.1 M Tris-HCl (pH 8.0) containing 0.1 M KCl, 2 mM $MgCl_2$, and 10 mM DTE. The quantity of homogenization buffer was increased with leaf age; 0 days in light, 2 ml per 5 leaves, 1 day in light, 2 ml per 2 leaves, 2 days in light, 2 ml per leaf, three and four days in light, approximately 10 ml per g fresh weight. The homogenate was centrifuged at 9,000 *g* for 10 min at 4° C. The supernatant was used directly for the assay of APSSTase and RubPCase. For the assay of OASSase, the supernatant was diluted with homogenization buffer to a concentration of 25 to 100 µg protein per ml.

Enzyme Assay

OASSase was measured using a modified method of Pieniazek (1973). The complete assay mixture contained, in a final volume of lml: Tris-HCl (pH 7.5), 200 µmol: Na_2S, 2.5 µmol; DTE, 10 µmol; O-acetyl-L-serine, 30 µmol; 200 µl of extract. Incubation was for 5 min at 37° C.

APSSTase was measured according to Schiff and Levinthal (1968), as modified by Brunold and Schmidt (1978). The counting fluid was toluene-Triton X-100 (2:1 v/v) with $4\,g \cdot l^{-1}$ PPO and $120\,mg \cdot l^{-1}$ POPOP.

RubPCase was measured according to Buchanan and Schürmann (1973), using the following modifcations: The complete assay mixture contained in a final volume of 100 µl in a Eppendorf-tube: Tris-HCl (pH 8.0), 20 µmol; $MgCl_2$, 5 µmol; NaEDTA, 6 nmol; reduced glutathione, 0.5 µmol; $NaH^{14}CO_3$ (0.3 µCi), 5 µmol; RubP, 70 nmol and 20 µl of the extract. The reaction was started by adding extract and RubP simultaneously. Incubation was for 10 min at 20° C. The reaction was stopped by filtering 20 µl of the assay mixture onto a Whatman GF/A glass fibre filter (∅ 24 mm) and adding simultaneously 100 µl of a acetone/formiate mixture (15:4, v:v). The counting fluid was toluene with 4 g PPO and 120 mg POPOP per litre.

Estimation of Chlorophyll

1 ml homogenate was mixed with 2 ml acetone and centrifuged for 5 min at 9,000 *g*. The pellet was washed with 2 ml acetone and centrifuged. This treatment resulted in a white pellet. The supernatants were combined and chlorophyll determined according to Strain et al. (1971).

Estimation of Sulfate-S and Total S

Sulfur was determined according to Johnson and Nishita (1952). For sulfate, the leaves were dried for 24 h at 80° C, homogenized in a glass homogenizer, and transferred to the reduction tube. For the determination of total sulfur, 1 ml of 30% H_2O_2, p.a. grade was added to the dried plant material which then was taken to dryness at 90° C. The treatment with H_2O_2 was repeated twice. Finally, the residue was dissolved in about 4 ml of reaction mixture and placed in the reduction apparatus. A background absorbance of 0.02 was subtracted from the absorbance at 670 nm measured against water.

Proteins were determined according to Brunold and Schmidt 1978. $AP^{35}S$ was prepared according to Tsang et al. (1976). OAS was obtained from Serva, RubP from Sigma Chemical Co., $^{35}SO_4^{2-}$ from Radiochemical Center Amersham, U.K., DTE and BSA from Fluka AG, Buchs, Switzerland.

The different H_2S-concentrations in air were produced by injecting a solution of $Na_2S \cdot 9H_2O$ into a 1,000 ml flask containing 500 ml 0.35 N H_3PO_4 and bubbling air ($100\,l h^{-1}$) through the acid. The injection was performed using a Perfusor (Braun, Melsungen). The air enriched with H_2S was mixed with an air stream ($1550\,l h^{-1}$) containing no H_2S. The mixture was then introduced into the cultivation tank. H_2S-concentrations were varied by changing the concentrations of the $Na_2S \cdot 9\,H_2O$-solution or the injection rate.

H_2S-concentrations were measured using a Jonoflux (Hartmann and Braun, Frankfurt). There was no measurable difference in the H_2S-concentrations in the air before or after passage through the cultivation tank.

Radioactivity was measured with a Picker Nuclear Liquimat 220 liquid scintillation spectrometer.

Results

Changes in the extractable specific APSSTase-activity of the primary leaves of bean seedlings during light-induced chloroplast development are presented in Fig. 1. There was an initial loss in specific activity during the first day in the light, followed by a rapid increase and, after two days, a level of enzyme activity was reached which was about ten times higher than the dark level.

Figure 1 also shows the extractable specific activities of OASSase and RubPCase together with the chlorophyll content per mg fresh weight, and the sulfate-S and total S content per mg dry weight. There was an increase in RubPCase activity and chlorophyll content. After four days in the light, OASSase activity and the sulfate-S and total-S contents were not appreciably different from the initial dark values.

Figure 2 shows the results of a similar experiment where the beans were fumigated with air containing $3.5 \pm 0.5\,\mu l \cdot l^{-1}$ H_2S during illumination. The extractable APSSTase activity decreased during the first day in the light and remained low during the following 3 days. The enzyme activity after four days in the light was about 10% of the initial dark level.

Figure 3 shows the APSSTase activity in extracts from primary leaves of bean seedlings which were treated with air containing $11 \pm 1\,\mu l \cdot l^{-1}$ H_2S during the first two days in the light and then with air containing no H_2S for the following 2 days. As soon as H_2S was omitted, there was a rapid increase in APSSTase activity, so that after two days a level of

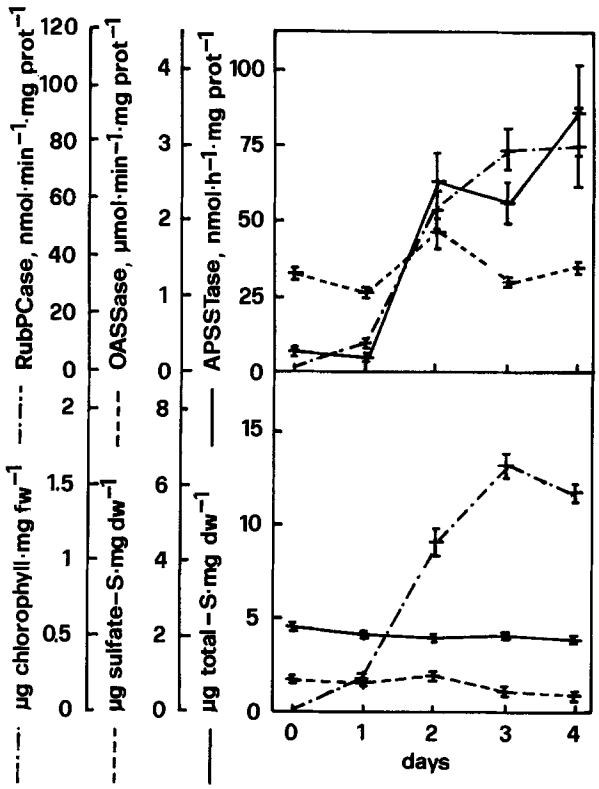

Fig. 1. Extractable activities of adenosine 5′-phosphosulfate sulfotransferase (APSSTase), O-acetyl-L-serine sulfhydrylase (OASSase) and ribulosebisphosphate carboxylase (RubPCase) and chlorophyll, sulfate-S, and total-S content of greening primary leaves of beans. The mean values of 6 measurements are presented. Vertical lines indicate ± standard error

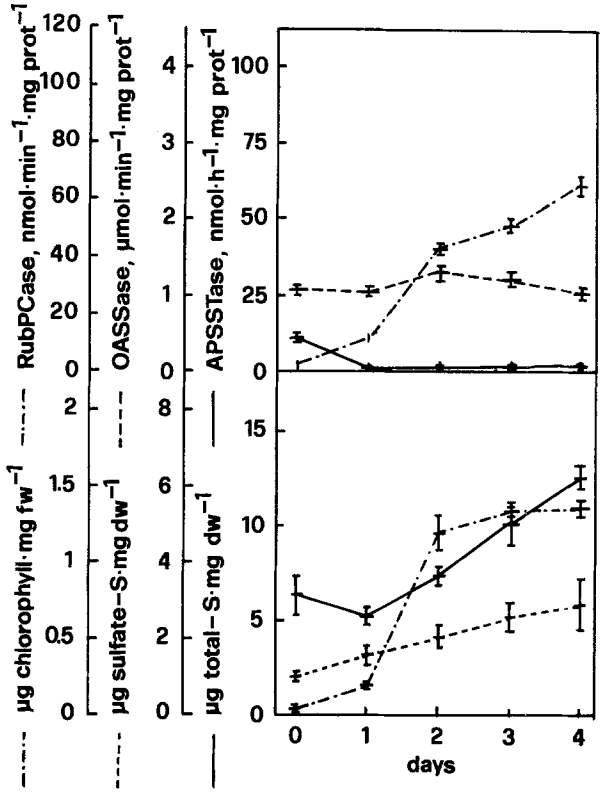

Fig. 2. Extractable activities of adenosine 5′-phosphosulfate sulfotransferase (APSSTase), O-acetyl-L-serine sulfhydrylase (OASSase) and ribulosebisphosphate carboxylase (RubPCase) and chlorophyll, sulfate-S and total-S content of greening primary leaves of beans fumigated with air containing 3,5 ± 0.5 µl $H_2S\,l^{-1}$. The mean values of 6 measurements are presented. Vertical lines indicate ± standard error

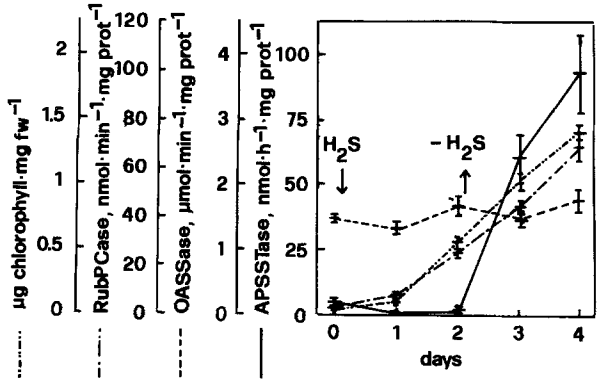

Fig. 3. Extractable activities of adenosine 5′-phosphosulfate sulfotransferase (APSSTase), O-acetyl-L-serine sulfhydrylase (OASSase) and ribulosebisphosphate carboxylase (RubPCase) and chlorophyll-content of greening primary leaves of beans fumigated with air containing 11 ± 1 µl $H_2S \cdot l^{-1}$ during the first two days in the light and with air during the next two days. The mean values of 6 measurements are presented. Vertical lines indicate ± standard error

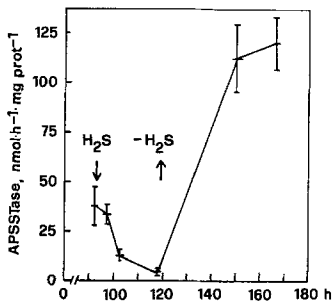

Fig. 4. Extractable activity of adenosine 5′-phosphosulfate sulfotransferase (APSSTase) of green primary leaves of beans. Fumigation with air containing 12 ± 2 µl $H_2S\,l^{-1}$ started, when the beans had developed in light for four days. H_2S was omitted after 26 h. The mean values of 6 measurements are presented. Vertical lines indicate ± standard error

enzyme activity was reached comparable to that of the control experiment. OASSase activity, RubPCase activity, chlorophyll content, sulfate-S and total-S were comparable to the values given in Fig. 1.

At this point it seemed interesting to know whether treatment with H_2S could induce a decrease in APSSTase in the leaves of seedlings cultivated in the light for a certain period of time without H_2S. Figure 4 shows the rapid decrease in APSSTase activity when fumigation with air containing 12 ± 2 µl

Table 1. Effect of different cyst(e)ine concentrations on the extractable activity of adenosine 5′-phosphosulfate sulfotransferase, O-acetyl-L-serine sulfhydrylase and ribulosebisphosphate carboxylase and the chlorophyll content of greening primary leaves of beans. After one day in the light the seedlings were transferred either intact or minus their roots to the different nutrient solutions containing cyst(e)ine. The listed parameters were measured two days after the transfer. Mean values of two independent experiments are presented

Cysteine-concentration (M)	APSSTase ($nmol \cdot h^{-1} \cdot mg\ prot.^{-1}$)		OASSase ($\mu mol \cdot min^{-1} \cdot mg\ prot.^{-1}$)		RupPCase ($nmol \cdot min^{-1} \cdot mg\ prot.^{-1}$)		Chlorophyll ($\mu g \cdot mg\ fw^{-1}$)	
	with roots	without roots	with roots	without roots	with roots	without roots	with roots	without roots
0	72.3	25.3	1.16	1.18	57.8	40.2	1.20	1.36
$5 \cdot 10^{-5}$	79.5	28.4	1.00	1.10	57.8	48.3	1.18	1.22
$7.5 \cdot 10^{-5}$	48.8	42.3	1.13	1.19	79.4	59.6	0.70	1.10
10^{-4}	85.7	23.6	0.87	1.53	59.2	70.5	1.10	1.29
$2.5 \cdot 10^{-4}$	40.5	21.0	0.97	0.62	63.7	60.9	1.18	1.26
$5 \cdot 10^{-4}$	56.1	28.7	1.08	1.30	68.5	79.0	1.21	1.20
$7.5 \cdot 10^{-4}$	48.9	5.4	1.10	1.14	77.0	54.5	1.26	1.14
10^{-3}	61.9	6.6	1.05	1.10	75.5	52.8	1.34	1.13

Table 2. Effect of different cyst(e)ine concentrations on the extractable activity of adenosine 5′-phosphosulfate sulfotransferase, O-acetyl-L-serine sulfhydrylase and ribulosebisphosphate carboxylase and the chlorophyll content of primary leaves of beans. After three days in light the seedlings were transferred either intact or minus their roots to the different nutrient solutions containing cyst(e)ine. The listed parameters were measured one day after the transfer

Cysteine concentration (M)	APSSTase ($nmol \cdot h^{-1} \cdot mg\ prot.^{-1}$)		OASSase $\mu mol \cdot min^{-1} \cdot mg\ prot.^{-1}$)		RubPCase ($nmol \cdot min^{-1} \cdot mg\ prot.^{-1}$)		Chlorophyll ($\mu g \cdot mg\ fw^{-1}$)	
	with roots[a]	without roots[b]	with roots[b]	without roots[b]	with roots[b]	without roots[b]	with roots[b]	without roots[b]
0	77.0 ± 27.8	25.3	1.21	1.65	65.4	76.4	1.44	1.31
$5 \cdot 10^{-5}$	64.7 ± 11.2	38.1	1.38	1.70	74.3	92.2	1.38	1.34
$7.5 \cdot 10^{-5}$	98.6 ± 10.2	44.0	1.46	1.38	79.6	93.7	1.40	1.65
10^{-4}	71.7 ± 11.3	18.3	1.51	1.54	64.3	89.9	1.13	1.46
$2.5 \cdot 10^{-4}$	83.6 ± 12.8	6.0	1.44	1.68	79.5	112.5	1.29	1.18
$5 \cdot 10^{-4}$	50.4 ± 14.7	2.1	1.27	1.58	65.9	122.5	1.71	1.42
$7.5 \cdot 10^{-4}$	39.4 ± 17.1	0.74	1.03	1.25	67.8	95.9	1.31	1.46
10^{-3}	9.9 ± 2.3	0.86	1.27	1.51	86.5	127.5	1.37	1.50

[a] Mean values of 6 measurements ± standard error
[b] Mean values of 2 measurements

$H_2S\ l^{-1}$ began after 4 days in the light. When H_2S was omitted, APSSTase activity increased rapidly.

Table 1 shows the effect of different concentrations of cyst(e)ine on the extractable activities of APSSTase, OASSase, RubPCase, and the chlorophyll content of the primary leaves of bean seedlings. After 24 h in the light, when the seedlings had very low APSSTase activity (Fig. 1), they were transferred either intact, or after removing their roots to a culture medium containing cyst(e)ine. The enzyme activities and chlorophyll were determined 48 h after this transfer. At cyst(e)ine concentrations up to 1 mM, extractable APSSTase activity reached a level comparable to the level in the controls (minus cysteine). When the seedlings were treated with higher cyst(e)ine concentrations, the roots deteriorated and the APSSTase activity decreased to lower levels (data not shown), while OASSase, RubPCase, and chlorophyll were not affected. In the extracts of seedlings with excised roots, there was a complete inhibition of the increase in APSSTase activity at cyst(e)ine concentrations of 0.75 mM.

When the same type of experiment was performed with bean seedlings which were kept in the light for 72 h and correspondingly had appreciable APSSTase activity (Fig. 1), cyst(e)ine-treatment for 24 h caused a rapid decrease in activity (Table 2): in intact seedlings the cyst(e)ine concentration necessary for a significant effect on extractable APSSTase activity was 1 mM; in seedlings with excised roots, a decrease in APSSTase activity can be observed at 0.25 mM cyst(e)ine. OASSase, RubPCase and chlorophyll content were not affected much by the cyst(e)ine concentrations used.

When extracts from seedlings with low APSSTase activity, resulting from cyst(e)ine (5.0 mM) or H_2S

($16 \pm 1.2\ \mu l \cdot l^{-1}$) treatment, were mixed with extracts from seedlings with high APSSTase activity, the APSSTase activities in the extracts were found to be additive, indicating that no activator was present in the extract with the high activity and that no inhibitor was present in the extract with the low activity. This result was further substantiated with an experiment in which leaves of plants treated with cyst(e)ine or H_2S were homogenized together with leaves of untreated plants and intermediary activities were measured (results not shown).

Discussion

In contrast to the findings in bacteria, there are only very few examples of decreases in enzyme levels by end products of metabolic pathways in plants. In the field of assimilatory sulfate reduction, it was concluded from work with ATP-sulfurylase (Reuveny and Filner, 1977) that this enzyme is repressed when a sulfur supply sufficient to support optimal growth is present in the form of sulfate, L-cysteine, or L-methionine. In the present investigation, cysteine and H_2S were examined for any regulatory effects on APSSTase in the presence of sulfate in the nutrient solution. Cysteine is the end product of assimilatory sulfate reduction (Thompson, 1967). Free H_2S, however, does not seem to be a normal intermediate of the pathway but is produced by plants in appreciable quantities under special conditions (Wilson et al., 1978). Furthermore, free H_2S can be used for cysteine synthesis by intact chloroplasts (Hock Ng and Anderson, 1978).

The advantages of using H_2S as a regulator are that rapid non-biological oxidation reactions can be excluded and that the compound has direct access to the green cells of the plant. Two different types of experiments were performed: In the first type, H_2S or cysteine was applied to greening bean seedlings which had no appreciable APSSTase activity. In the second type, bean seedlings, which in a light-dependent process had acquired substantial APSSTase activity, were treated with the two compounds.

In the greening experiments, both H_2S and cysteine completely inhibited the light-induced increase in APSSTase. The concentrations applied did not significantly affect the control parameters, indicating that the observed inhibition is specific and not based on the toxicity of the two compounds. This observation is important in view of the fact that toxic effects of amino acids applied to plants have been reported (Feller and Erisman, 1976), and that the toxicity of H_2S to plants is well documented (Garber, 1967, Thompson and Kats, 1978). The specificity of the effect of H_2S was further demonstrated by the fact that there was a rapid increase in APSSTase when the treatment with H_2S was stopped.

The results of the second type of experiment, showing a decrease of APSSTase in green leaves of beans produced by either H_2S or cyst(e)ine, are consistent with the results obtained with *Lemna minor* (Brunold and Schmidt, 1976; 1978). We do not know to what extent synthesis or inactivation are involved in the regulation of APSSTase. It is clear, however, that some inactivation of APSSTase is involved because the decrease cannot occur at the observed rate by inhibition of APSSTase synthesis and dilution of existing APSSTase in the growing leaves alone.

An interesting point revealed in the cyst(e)ine experiment is the buffering function of the roots which makes it necessary to apply appreciably higher concentrations of cyst(e)ine to intact seedlings as compared to seedlings with excised roots, if a similar effect on APSSTase is to be expected. This buffering function of the roots has to be kept in mind when results obtained with plants like *Lemna* are to be compared with results from more typical, higher plants. A more direct access of the regulator cyst(e)ine to the green cells was created by excising the roots. Under this condition similar concentrations of cyst(e)ine affected APSSTase in *Phaseolus vulgaris* as in *Lemna minor*. Because of its direct access to the green cells, H_2S affects APSSTase of intact seedlings at comparably low concentrations. This shows that the regulation of APSSTase by H_2S and cyst(e)ine is not restricted to the relatively simple *Lemna minor*. Furthermore, the observed regulation of APSSTase by an end product of assimilatory sulfate reduction indicates that APSSTase may play a role in this process in *Phaseolus vulgaris*. The present results obtained with cyst(e)ine are in contrast to those reported for *Euglena* (Brunold and Schiff, 1976) and to the view of Schwenn (Schween et al., 1978; 1979). With *Euglena*, it has been shown (Brunold and Schiff, 1976) that APSSTase is not predominantly localized in the chloroplasts; Fankhauser and Brunold, 1978 have shown, however, that in spinach leaves APSSTase is localized predominantly or even exclusively in the chloroplasts. The results of the present paper, showing a light-induced increase in APSSTase, suggest a similar localization of the enzyme in the primary leaves of beans. It is tempting to speculate that this different localization of APSSTase in plants and *Euglena* may be of consequence in the regulatory phenomenona. The same reason may be responsible for the conflicting view of Schwenn et al. (1978, 1979). Furthermore, these authors used a different assay system for APSSTase, involving the formation of $AP^{35}S$ from $^{35}SO_4^{2-}$ by ATP-sulfurylase present in the crude

extracts, whereas, we added the substrate APS to our assay system.

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