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Regulation of Sulfate Assimilation in Plants

7. CYSTEINE INACTIVATION OF ADENOSINE 5'-PHOSPHOSULFATE SULFOTRANSFERASE IN *LEMNA MINOR* L.[1]

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ABSTRACT

When 0.5 mM cysteine is added to cultures of *Lemna minor* L. growing with sulfate as the sole sulfur source, there is a rapid 80% loss of extractable adenosine 5'-phosphosulfate sulfotransferase. This loss is accompanied by an inhibition of sulfate uptake; however, lack of sulfate is not responsible for the decreasing adenosine 5'-phosphosulfate sulfotransferase activity.

Cultivation with cysteine causes an increase in the cyst(e)ine pool of *L. minor*. This fact taken together with the observed inactivation of adenosine 5'-phosphosulfate sulfotransferase in crude extracts by cysteine suggests that the cysteine pool is involved in the *in vivo* regulation of the enzyme.

The activity of adenosine 5'-phosphosulfate sulfotransferase is restored within 24 hours after transfer to a culture medium without cysteine. This restoration is partially blocked by 6-methyl purine and actinomycin D and completely by cycloheximide.

Cycloheximide added to cultures of *L. minor* L. causes a loss of extractable APSTase comparable to the one obtained with cysteine. This loss may be in part due to cysteine, since cycloheximide causes a pronounced increase in the cysteine pool of *L. minor*.

Little information is available on the regulation of sulfate assimilation in higher plants, although they are major producers of reduced S compounds (29). The only detailed work was on the regulation of ATP-sulfurylase in *Lemna gibba* (7). The activity of this enzyme is higher in extracts of plants grown with L-cysteine, L-methionine, or glutathione than of control plants cultivated with sulfate as the sole S source.

Since that time, a pathway of assimilatory sulfate reduction with carrier-bound intermediates has been described for *Chlorella* (1, 26). One major difference from the previously described bacterial pathway (6, 14) is the involvement of a nucleotide sulfonyl donor, which is PAPS[2] in bacteria and APS in *Chlorella*. In this latter organism the sulfonyl group of APS is transferred to a carrier via APSTase to form carrier-S-SO_3^-. It was shown that APS is also the sulfonyl donor in higher plants (23–25) and other photosynthetic organisms (30).

In the duckweed *Lemna minor* L. H_2S causes a rapid loss of extractable APSTase activity (5). H_2S was detected in the natural biotop of *Lemna* (15) and the observed regulation may be a means for stopping sulfate reduction when sufficient S at the thiol level is present in the environment. Free H_2S does not seem to be an intermediate in the sulfate reduction pathway of plants (27).

Since we wished to study the regulatory properties of a compound which forms part of the sulfate reduction pathway we turned to cysteine.[3] In this paper we show that cultivation with cysteine reduces the level of extractable APSTase activity in *L. minor* and we present evidence that cysteine might be involved in APSTase inactivation.

MATERIALS AND METHODS

L. minor L., strain 6580 of Landolt's collection of Lemnaceae (16), was cultivated aseptically under steady-state conditions with a device described previously (8) on E-NO_3 medium (9) at 25 C and 5,000 lux. Sulfate-free medium was prepared by replacing the sulfate salts of Mg^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}, and Co^{2+} of the E-NO_3 medium by equimolar amounts of the respective chloride salts. Nutrient solutions containing cysteine were prepared immediately before use by adding a freshly prepared 100 mM cysteine solution to E-NO_3 medium. For prolonged culture periods with cysteine the nutrient solution was renewed daily.

Specific growth rates, r, were estimated from counts of the number of fronds on 2 consecutive days:

$$r = \ln \text{ frond number day}(n+1) - \ln \text{ frond number day } n$$
(ln = natural logarithm)

For the preparation of extracts *Lemna* plants were rinsed with deionized H_2O at 4 C for a min and then homogenized with 0.1 M Tris-HCl (pH 8) containing 0.1 M KCl, 20 mM $MgCl_2$, and 10 mM DTT in a mortar cooled in ice water. For 1 g of plant material 2 ml of buffer were used. The homogenate was centrifuged at 4,500g for 5 min, and the supernatant was used immediately for the assay of APSTase and OASSase.

APSTase was measured by the production of sulfite-^{35}S, assayed as acid-volatile radioactivity from $AP^{35}S$ in the presence of DTT (12, 22). The complete assay mixture contained in a final volume of 400 µl: $AP^{35}S$ (78–190 cpm/nmol), 75 nmol; Tris-HCl (pH 9.25), 50 µmol; DTT, 4 µmol; Na_2SO_4, 400 µmol; and 50 µl of *Lemna* extract, containing 150 to 300 µg of protein. Incubation was for 30 min at 37 C under N_2.

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[2] Abbreviations: PAPS: adenosine 3'-phosphate 5'-phosphosulfate; APS: adenosine 5'-phosphosulfate; APSTase: adenosine 5'-phosphosulfate sulfotransferase; OASSase: O-acetyl-L-serine sulfhydrylase; OAS: O-acetyl-L-serine; CH: cycloheximide.

[3] The term "cysteine" is used when it is clear that cystine is not involved. The term "cyst(e)ine" is used for an undefined mixture of cysteine and cystine.

OASSase was measured by the method of Pieniazek *et al.* (20). The complete assay mixture contained in a final volume of 1 ml: Tris-HCl (pH 7.5), 200 μmol; Na_2S, 2.5 μmol; DTT, 10 μmol; OAS hydrochloride, 2.5 μmol; and 20 μl of *Lemna* extract, containing 60 to 120 μg of protein. Incubation was for 10 min at 37 C. The reaction was stopped by adding 1 ml of ninhydrin reagent (250 mg of ninhydrin in a mixture of 4 ml of concentrated HCl and 16 ml of glacial acetic acid). The test tubes were then placed in a boiling water bath for 5 min, and then cooled in an ice bath. After addition of 2 ml of 96% ethanol, the *A* was measured at 546 nm in an Eppendorf photometer. Serine, OAS, and cystine do not give a color reaction with ninhydrin under the assay conditions.

ATP-sulfurylase was assayed according to Robbins (21). The addition of pyrophosphatase was omitted, because the *Lemna* extracts have high pyrophosphatase activity. The rates of ATP-sulfurylase were routinely measured with extracts which had been passed through Sephadex G-25 after centrifugation according to Loussaert (18). One hundred μl of eluate containing 300 to 600 μg of protein were used.

Sulfate uptake was studied by placing 20 to 30 fronds of *Lemna* into round glass dishes with a diameter of 4 cm and a height of 2.5 cm kept at 25 C and 5,000 lux. The dishes contained 5 ml E-NO_3 medium with $^{35}SO_2^{2-}$ (1,097 cpm/nmol) and the same cysteine concentration as the cultivation medium. The uptake period was 30 min. The fronds were then rinsed with H_2O at 4 C for 1 min, placed on H_2O at 4 C for 30 min, and rinsed a second time with H_2O. The frond number was determined and the plants were transferred to vials for scintillation counting. After addition of 2 ml of 30% H_2O_2 the fronds were taken to dryness at 90 C. The treatment with H_2O_2 was repeated twice. Finally the residue was dissolved in 1 ml of 0.1 N HCl and 10 ml of counting fluid were added.

For the determination of the free amino acids *Lemna* plants with a fresh wt of 300 to 500 mg were washed following the procedure described for the sulfate uptake experiments. The washed fronds were put into 50 ml of boiling 80% methanol in a 150-ml Erylenmeyer flask. The methanol was kept boiling for 10 min, replacing the evaporating methanol continuously. The extract was decanted and the fronds were extracted a second time for 10 min in 50 ml of boiling 20% methanol. The extracts were combined and reduced to 1 to 2 ml at 11 mm Hg and 50 C. The extract was transferred to a graduated centrifuge tube and made up to 5 ml with H_2O. Then 6 ml of chloroform-methanol (2:1, v/v) were added. After centrifuging for 5 min at 4,500*g*, the water-methanol phase was collected and reduced to 1 to 2 ml at 11 mm Hg and 50 C. This extract was transferred to a Dowex 50-w (X8 200–400 mesh, H^+ form) column (5 × 30 mm). After washing the column three times with 2 ml of H_2O (pH 5), the basic compounds were eluted with 6 ml of 10% NH_3. This eluate was taken to dryness at 11 mm Hg and 50 C and dissolved in 3 ml of lithium-citrate buffer (0.15 N Li^+) (pH 2.2 ± 0.1) containing 1% thiodiglycol (v/v) and 0.1% phenol (w/v). An aliquot of 1 ml was introduced into a Spinco amino acid analyzer and free amino acids were determined according to Benson (2) with a DC-6A column. The flow rate for the five buffers (pH 2.79, 2.9, 3.00, 3.42, and 3.22) was 70 ml · hr^{-1} and 35 ml · hr^{-1} for the ninhydrin reagent (19). The amounts of free amino acids were determined with a computing integrator (Autolab, Spectra Physics, Mountain View, Calif.).

Cysteine, CH, 6-methyl purine, and actinomycin D were dissolved in E-NO_3 medium and added to the culture medium through Millipore filters (HA 0.45 μm).

Radioactivity was measured with a Picker Nuclear Liquimat 220 liquid scintillation spectrometer. The counting fluid was toluene-Triton X-100 (2:1, v/v) with 4 g/l PPO and 120 mg/l POPOP.

For the measurement of acid-volatile sulfide in the plant material, 500 μg of plants were homogenized in liquid N_2 in a mortar. The resulting powder was acidified with 2.5 ml of 1 M H_3PO_4. N_2 was bubbled through the suspension for 45 min at room temperature. The gas stream was passed through 850 μl of zinc acetate solution (13), and sulfide determined according to Johnson and Nishita (13), using a molar extinction coefficient of 35 × 10^3 for the methylene blue formed.

Proteins were determined as described previously (25); but turbidity of the trichloroacetic acid precipitate was measured at 750 nm; BSA was used as a standard.

^{35}S-labeled sulfate was purchased from Büchler (Amersham) for the preparation of $AP^{35}S$ and from Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland, for the sulfate uptake experiments.

$AP^{35}S$ was prepared according to Hodson and Schiff (11) as modified by Schmidt (24).

OAS hydrochloride, DTT, CH, and BSA were obtained from Sigma; 6-methyl purine and cysteine were purchased from Fluka AG., Buchs, Switzerland.

RESULTS

The results in Figure 1 show the activity of extractable APSTase of *L. minor* at various times after the addition of cysteine to the culture medium. After the rapid loss of APSTase activity a new steady-state level of the enzyme activity is established. This new level is about 20% of the control plants cultivated with SO_4^{-2}. The growth rate is not affected significantly by the cysteine concentration used, indicating that we are looking at a physiological regulation and not at a harmful effect of a high cysteine concentration.

Evidence that we are dealing with a physiological response is further established by the results in Table I, which show the extractable activities of ATP-sulfurylase, APSTase, and OASSase

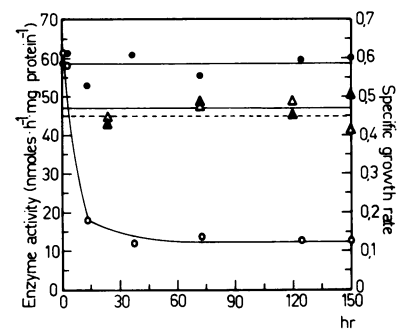

FIG. 1. Specific growth rate (△- - -△) and extractable activity of APS sulfotransferase (○——○) of *L. minor* at different times after addition of 5 · 10^{-4} M cysteine to culture medium. Control cultures had sulfate as the sole S source ([▲——▲]: specific growth rate; [●——●]: APS sulfotransferase). Before the addition of cysteine the plants had been cultivated under steady-state conditions for 7 days with 8.8 · 10^{-4} M sulfate as the S source.

Table I. Activity of Adenosine 5'-Phosphosulfate (APS) Sulfotransferase, ATP-Sulfurylase and O-Acetyl-L-serine Sulfhydrylase in Extracts of Lemna minor Cultivated with Sulfate, Cysteine, or Cysteine and Sulfate as Sulfur Source.

Growth Condition	APS-Sulfotransferase	ATP-Sulfurylase	O-Acetyl-L-serine Sulfhydrylase
	nmol · hr^{-1} · mg $protein^{-1}$		
8.8·10^{-4} M sulfate	55.5 ± 7.86	878 ± 86.4	4340 ± 285.6
5·10^{-4} M cysteine for 7 days	8.6 ± 0.84	1441 ± 133.0	4186 ± 186.0
5·10^{-4} M cysteine and 8.8·10^{-4} M sulfate for 7 days	12.6 ± 0.73	1174 ± 79.1	4510 ± 410.3

Each value is presented as the mean of 6 assays ± standard error.

of *L. minor* cultivated with sulfate, cysteine, or sulfate plus cysteine as sulfur sources. With cysteine alone, the activity of APSTase was about 30% lower than with sulfate and cysteine, and about 85% lower than with sulfate. Compared to plants with sulfate as S source, the level of ATP-sulfurylase was 30% higher with cysteine and sulfate, and 60% higher with cysteine alone. The level of OASSase was not affected significantly by cysteine.

A mixture of an extract from *Lemna* grown with sulfate and of an extract from *Lemna* cultivated with cysteine has an APSTase activity which is essentially equal to the sum of the activities of the individual extracts. We conclude therefore that the low level of APSTase activity found in extracts of *L. minor* cultivated with cysteine is not due to an inhibitor present in extracts from these organisms.

In view of our previous work which showed the cultivation with H_2S affected the APSTase level in *L. minor* (5), we determined whether plants grown with cysteine have increased levels of sulfide formed from cysteine. They contained 13.8 ± 1.36 nmol of acid-volatile sulfide/g fresh wt (five determinations), which is not significantly more than 11.4 ± 1.05 nmol/g fresh wt (five determinations) of plants grown with sulfate as S source. It seems unlikely therefore that H_2S formed from cysteine is involved in the observed decrease of extractable APSTase.

An inhibition of sulfate uptake by cysteine has been described for tobacco cells (10). We therefore tested to determine if the observed loss of APSTase activity was due to a lack of sulfate.

Figure 2 shows that in *L. minor* 0.5 mM cysteine rapidly inhibits sulfate uptake. The remaining rate of sulfate uptake after 7 hr was 50% of the initial rate. In order to stop sulfate uptake completely, we transferred *L. minor* to a nutrient solution without a sulfur source and determined the activity of extractable APSTase.

Figure 3 shows that under these conditions the level of APSTase was not decreased. It seems unlikely therefore that a lack of sulfate is responsible for the decreasing APSTase activity when cysteine is added to the culture medium.

We therefore evaluated whether the cysteine pool could regulate the APSTase activity in *Lemna*.

Table II shows the changes in the pools of the free amino acids in *L. minor* at different times after the addition of 0.5 mM cysteine to E-NO_3 medium. The cysteine estimated by the method used represents the total of the cysteine and the cystine initially present in the extracts. The large increase in cyst(e)ine suggested to us that cyst(e)ine may actually be involved in the regulation of APSTase activity *in vivo*. We therefore turned to *in vitro* experiments.

Table III shows that crude extracts of *L. minor* lost about 40% of their initial APSTase activity when stored in 2 mM cysteine at 25 C for 2 hr; the loss was 90% at 10 mM cysteine. Table III furthermore shows that addition of up to 10 mM cysteine to the assay mixtures did not inhibit the enzyme (0 hr values).

We used DTT to determine whether the effect observed with cysteine could be based on the reduction of —SH groups on the enzyme resulting in inactivation. Table IV shows that the activity of APSTase was lost rapidly in crude extracts prepared and stored

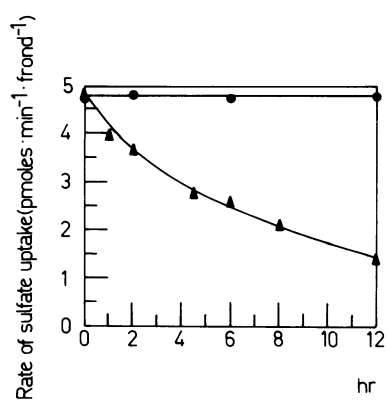

FIG. 2. Rates of sulfate uptake of *L. minor* on $8.8 \cdot 10^{-4}$ M sulfate (●——●) and after addition of $5 \cdot 10^{-4}$ M cysteine to the sulfate medium (▲——▲). At the intervals shown *Lemna* plants (20–30 fronds) were transferred to a medium containing $^{35}SO_4^{2-}$ (1,097 cpm/nmol) for 30 min, then rinsed and analyzed for radioactivity.

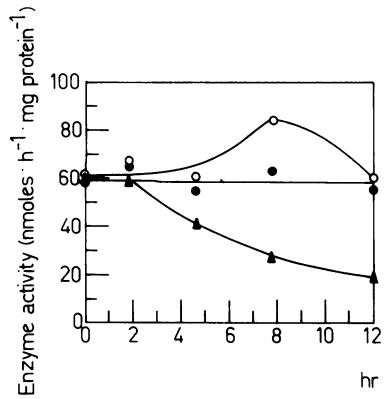

FIG. 3. Activity of APS sulfotransferase in extracts from *L. minor* at different times after transfer to sulfur-free medium (○——○) or after the addition of $5 \cdot 10^{-4}$ M cysteine (▲——▲). Control cultures (●——●) had sulfate as the sole S source. Before the transfer or the addition, the plants had been grown under steady-state conditions for 7 days with $8.8 \cdot 10^{-4}$ M sulfate as the S source.

Table II. Free Amino Acids in Extracts of Lemna minor at Different Times After the Addition of $5 \cdot 10^{-4}$M Cysteine to the Culture Medium.

Amino Acid	Hr with Cysteine in Culture Medium 0	3	6	11	24
	nmol · mg dry weight^{-1}				
P-Ser	2.9	3.9	4.9	4.5	3.4
Asp	14.9	14.7	16.1	12.6	16.9
Thr	2.8	3.5	3.2	3.1	3.1
Ser	12.8	12.9	13.8	13.4	13.0
Asn	39.8	65.0	70.4	85.0	72.3
Gln	23.4	41.5	45.5	48.1	37.8
Pro	0.3	0.7	1.1	0.9	0.8
Glu	23.1	32.2	30.3	25.3	30.1
Citrulline	1.5	4.3	7.0	6.4	5.6
Gly	4.2	8.4	7.9	6.5	5.3
Ala	15.5	20.2	23.8	22.2	15.3
Val	4.5	6.2	6.8	6.4	5.5
Cystine	0.6	4.3	4.6	6.7	5.5
Cystathionine	1.1	0.9	1.0	1.4	1.1
Met	0.5	0.6	0.3	0.7	0.2
Ile	1.2	0.6	0.3	0.8	0.7
Leu	1.2	0.9	0.9	1.1	0.9
Tyr	0.1	0.2	0.2	0.3	0.3
Phe	1.3	1.9	2.0	1.0	1.1
γ-Aminobutyric Acid	8.8	6.9	5.8	4.8	3.2
Ornithine	0.7	0.8	0.6	0	0
Lys	1.0	0.7	0.8	1.0	0.6
His	1.7	2.2	1.9	1.6	1.8
Arg	0	0	0	0.1	0.4

The cystine estimated represents the total of the cysteine and the cystine initially present in the extracts. Before the addition the plants had been cultivated under steady-state conditions for 7 days with $8.8 \cdot 10^{-4}$M sulfate as the sole sulfur source.

Table III. Loss of Activity of Adenosine 5'-Phosphosulfate (APS) Sulfotransferase in Extracts of Lemna minor at 25 C with Cysteine.

Cysteine	APS - Sulfotransferase Activity 0 hr	1 hr	2 hr
	nmol · hr^{-1} · mg protein^{-1}		
mM			
0	61.5	40.6	37.5
2	63.7	36.4	23.1
10	59.0	5.5	6.5

Cysteine concentrations indicated were added to the crude extracts and to the assay mixtures.

with high concentrations of DTT. At 10 mM DTT, which was routinely used, the activity is conserved best over 2 hr after extraction. The omission of DTT or low concentrations result in low initial APSTase activities and rapid inactivation.

Asparagine, glutamine, citrulline, and proline, which also increase in *L. minor* when cysteine is added to the culture medium (Table II), have no effect if included in the culture medium up to 1.5 mM (data not shown).

When *L. minor* was transferred from E-NO_3 medium with cysteine plus sulfate as S sources to a nutrient solution with sulfate alone, an increase in APSTase activity was detected (Fig. 4). After 24 hr the APSTase level typical of *Lemna* grown with sulfate was reached.

Table IV. Activity of Adenosine 5'-Phosphosulfate (APS) Sulfotransferase in Extracts of Lemna minor Prepared and Stored with Different DTT Concentrations.

DTT Concentration	APS - Sulfotransferase Activity 0 hr [a]	2 hr [b]	% of initial activity
mM	nmol·hr^{-1}·mg protein^{-1}		
0	15.0	3.3	22.0
5	37.5	21.1	56.3
10	64.3	48.3	75.1
20	53.0	34.6	65.3
30	61.3	24.5	40.0
50	54.8	13.2	24.1

a activity measured immediately after extraction
b activity measured 2 hr after extraction and storing at 25 C.

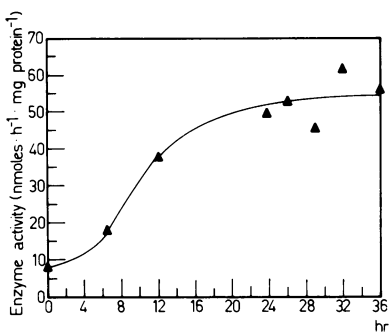

FIG. 4. Activity of APS sulfotransferase in extracts from *L. minor* at different times after the transfer from culture medium with sulfate plus cysteine to one lacking cysteine. Before the transfer, the plants had been cultivated under steady-state conditions for 11 days with $5 \cdot 10^{-4}$ M cysteine and $8.8 \cdot 10^{-4}$ M sulfate as S sources.

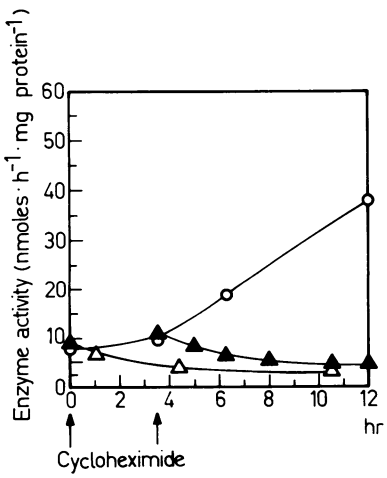

FIG. 5. Activity of APS sulfotransferase in extracts from *L. minor* at different times after transfer to cysteine-free medium. CH (4 μg/ml) was added at the time of transfer (△——△) or 3.5 hr later (▲——▲). Control cultures had no CH added (○——○). The plants were pretreated for 7 days as in Figure 4.

Figure 5 shows that this increase was inhibited by CH, when added immediately after the omission of cysteine or 3.5 hr after the transfer.

Table V shows that the addition of CH to the culture medium caused a pronounced increase in the cyst(e)ine pool of *L. minor*. Therefore, besides its effect on protein synthesis, CH could act on the level of APSTase activity via cysteine.

Figure 6 shows the decrease of extractable APSTase activity after the addition of cysteine, CH, or a combination of cysteine and CH to plants growing with sulfate. These results are consistent with the idea of a CH effect via cysteine. When cysteine alone was added, there was a lag period of about 2 hr, then a rapid loss of activity with a half-life of about 5 hr. The kinetics of the decrease of activity is comparable with that produced by CH or by a combination of cysteine and CH, although there was no detectable lag period when CH was involved.

The effect of inhibitors of RNA synthesis on the restoration of APSTase activity after transfer to cysteine-free medium is shown in Figure 7. With both substances (6-methyl purine and actinomycin D) applied, there was an initial rise in activity, followed by a decline after 9 hr.

Table V. Free Amino Acids in Extracts of Lemna minor at Different Times After the Addition of Cycloheximide to the Culture Medium.

Amino Acid	Hr with Cycloheximide in Culture Medium 0	1	3	6
	nmol · mg dry weight^{-1}			
P-Ser	1.77	1.43	1.49	1.98
Asp	13.28	13.25	12.92	13.53
Thr	2.81	3.92	6.15	8.24
Ser	12.01	13.03	17.58	20.00
Asn	65.38	72.04	94.96	114.34
Gln	28.19	35.15	62.87	97.80
Glu	25.25	28.84	34.50	36.28
Gly	5.22	4.20	5.42	7.44
Ala	17.90	25.02	30.24	34.69
α-Aminoadipic Acid	3.95	4.44	5.60	6.68
Val	6.37	7.65	6.87	6.28
Cystine	1.46	2.65	5.19	8.06
Met	0.37	0.23	0.29	0.42
Ile	0.44	0.87	1.67	3.56
Leu	0.34	0.74	1.79	3.11
Tyr	0.33	0.61	0.91	1.43
Phe	1.06	1.54	2.13	2.98
γ-Aminobutyric Acid	15.73	17.56	13.61	16.62
Lys	0.00	0.84	2.68	4.48
His	0.99	0.63	0.67	0.93
Arg	0.00	0.54	1.56	3.03

Before the addition the plants had been cultivated under steady-state conditions for 2 days with $8.8 \cdot 10^{-4}$M sulfate as the sole sulfur source.

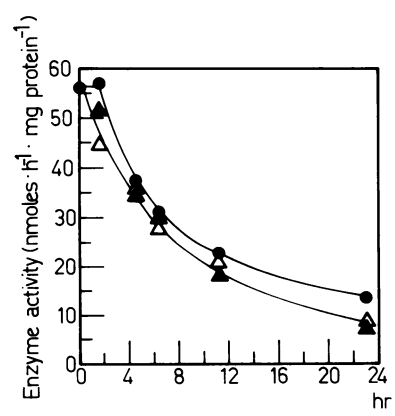

FIG. 6. Activity of APS sulfotransferase in extracts from *L. minor* at different times after addition of $5 \cdot 10^{-4}$ M cysteine (●——●), 4 μg/ml cycloheximide (△——△), or $5 \cdot 10^{-4}$ M cysteine and 4 μg/ml cycloheximide (▲——▲). Before the additions, the plants had been cultivated under steady-state conditions for 7 days with $8.8 \cdot 10^{-4}$ M sulfate as the sole S source.

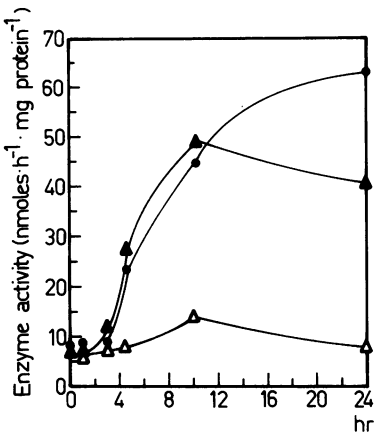

FIG. 7. Activity of APS sulfotransferase in extracts from *L. minor* after transfer to cysteine-less culture medium and simultaneous addition of 6-methyl purine (150 μg/ml) (△——△) or actinomycin D (15 μg/ml) (▲——▲). Control cultures had no addition after the transfer (●——●). Before the transfer the plants were pretreated for 7 days as in Figure 4.

DISCUSSION

It was found in *Euglena* that although cells grow well on cysteine, the level of APSTase is comparable to that found in cells grown on sulfate (4). This is in contrast to the results obtained in the present study with *L. minor*. In this organism the addition of cysteine to the culture medium causes a rapid loss of extractable APSTase activity. This rapid loss of enzyme activity could easily be explained by cessation of enzyme synthesis and an *in vivo* turnover of the enzyme (17). After the rapid loss of activity, a new steady-state level is reached, where the activity of APSTase is about 20% of the level of plants cultivated with sulfate. In this new steady-state, synthesis of APSTase is necessary. Otherwise a constant loss of activity would be observed, due to turnover of the enzyme and dilution by growth, which would halve the activity every doubling time of the plants, *i.e.* about every 37 hr. We do not know at the present time if this rate of synthesis is the same as in *Lemna* grown on sulfate and/or if the low level of APSTase is only due to an increased inactivation of the enzyme.

The results of the present paper suggest that sulfate and sulfide are not involved in the regulation of APSTase; the experiments suggest, however, that cysteine is a likely regulator. In bacteria the enzymes of assimilatory sulfate reduction are repressed by cysteine (14, 28). In *L. minor*, with both H_2S (3) and cysteine as S sources, the cyst(e)ine pool is increased and the inactivation kinetic data with cyst(e)ine are similar to those found with H_2S, suggesting that the same mechanism of inactivation is involved. Further evidence for the involvement of cyst(e)ine in regulation is the *in vitro* inactivation of APSTase by cysteine.

The cystine content of *L. minor* grown on cysteine was 4.3 to 6.7 nmol/mg dry wt. If we assume that this cystine is present in the plants as cysteine confined to the cytoplasm and that the cytoplasm contains 10% of the water content of the plants, the measured content could produce a concentration of 7.78 to 12.12 mM. This concentration very efficiently inactivates APSTase *in vitro*. If a uniform distribution of cysteine in the water content of the plants is assumed, the produced concentration of cysteine is 0.78 to 1.21 mM, which is still comparable to the 2 mM concentration which inactivates APSTase *in vitro*.

The effect of high concentrations of DTT on APSTase activity suggests that the reduction of —SH groups could be involved in the inactivation of the enzyme. The restoration of the APSTase activity shows a lag of about 3 hr and takes about 24 hr. This restoration may be based on activation of inactive enzyme or on synthesis of new enzyme molecules. Inhibitors of translation have been used in similar situations to distinguish between these two possibilities. We have shown (5) that chloramphenicol has no effect on the restoration, indicating that protein synthesis on 70S ribosomes is not involved. CH, however, completely blocks the restoration and, if added to plants growing with sulfate, causes a rapid decrease of the APSTase level. In our system, CH may have two effects: (*a*) CH inhibits protein synthesis on 80S ribosomes. This effect alone can cause a decrease in the specific activity of APSTase if there is turnover of the enzyme. (*b*) CH causes an increase in the cysteine pool, probably because assimilation of sulfate is going on and cysteine is no longer consumed for protein synthesis. The accumulated cysteine may then be involved in the inactivation of APSTase. The relative contribution of the two effects to the observed loss of APSTase activity is not known, the time course of the loss of activity produced by cysteine and CH is very similar however, suggesting that in both cases inactivation mediated by cysteine is the predominant effect.

With the inhibitors of transcription, there is an initial rise in extractable APSTase activity after omission of cysteine from the culture medium, indicating that mRNA for APSTase synthesis is present during growth of *L. minor* with cysteine. This is consistent with the idea that synthesis of APSTase is necessary for maintaining the observed low, but essentially constant level of APSTase, when cysteine is added to the culture medium. It remains to be shown whether the rate of APSTase synthesis is increased during the restoration or if only the rate of APSTase inactivation is decreased.

The two other enzymes tested, ATP-sulfurylase and OASSase, display different responses to cysteine: OASSase shows no change, ATP-sulfurylase, however, increased appreciably in activity. A similar effect by cystine was observed with *L. gibba* (7), using an assay system in which the APS formed was taken as a measure of enzyme activity.

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