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Methionine Biosynthesis in *Lemna*: Inhibitor Studies

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ABSTRACT

A search was made for compounds that would inhibit methionine biosynthesis in *Lemna paucicostata* Hegelm. 6746. DL-Propargylglycine (0.15 micromolar) produced growth inhibition and morphological changes which were prevented by exogenous methionine. Also, DL-propargylglycine inhibits cystathionine γ-synthase activity. L-Aminoethoxyvinylglycine (0.05 micromolar) produced growth inhibition and morphological changes partially preventable by exogenous methionine. L-Aminoethoxyvinylglycine impairs the cleavage of cystathionine to homocysteine. Lysine and threonine, at concentrations which individually had little effect on growth or morphology of *Lemna*, together produced growth inhibition and morphological changes preventable by exogenous methionine. The resulting metabolic block prevented conversion of cysteine to cystathionine, presumably secondary to depletion of the supply of *O*-phosphohomoserine.

Inhibition of *Lemna* growth resulted when the molybdate:sulfate ratio in the medium was increased to 20:1 or more. Such inhibition was prevented by lowering this ratio to 0.3 or less. A non-steady-state experiment (molybdate:sulfate, 20:1) showed that molybdate inhibited sulfate uptake, but it provided no evidence of a further impairment in the organification of sulfate. Molybdate-induced growth inhibition of *Lemna* was prevented by cystine but not by cystathionine or methionine. Cystathionine is not converted by *Lemna* to cysteine rapidly enough to sustain growth.

As one means of learning more about the regulation of the methionine biosynthetic pathway, we have recently begun a search for inhibitors of specific steps in this pathway, using *Lemna paucicostata* as the experimental material. Each inhibitor was chosen with the hope that it might render the growth of the plants dependent upon exogenous methionine or a related compound, thereby providing some of the experimental advantages for study of regulation which have been afforded by auxotrophic mutants in other biological systems.

In this paper, we describe the conditions under which each of four compounds inhibits a specific step in methionine biosynthesis in *Lemna* and present data upon the extent of the metabolic block brought about by each inhibitor. A preliminary report upon this work has been made (3).

MATERIALS AND METHODS

Chemicals. AVG[2]; its saturated analog, L-2-amino-4-(2′-aminoethoxy)-butyric acid; L-2-amino-4-methoxy-*trans*-3-butenoic acid; and its saturated analog, L-2-amino-4-methoxy-butyric acid were gifts from A. Stempel of Hoffman-La Roche. PAG was purchased from Sigma, L-lysine from Mann, L-threonine from ICN, L-methionine from Schwarz/Mann, L-homocystine, L-cystine, and L-cystathionine from Calbiochem. L-Homoserine was purchased from Nutritional Biochemicals, and sodium molybdate was purchased from Merck. DL-[2-^{14}C]Propargylglycine, L-[U-^{14}C]cystine, L-[U-^{14}C]lysine, and L-[U-^{14}C]threonine were purchased from Amersham; $^{35}SO_4^{2-}$ and Protosol were purchased from New England Nuclear; L-[4-^{14}C]aminoethoxyvinylglycine was a gift from Hoffman-La Roche. L-[^{35}S,U-^{14}C]Methionine was prepared as described previously (10), as were tritiated compounds used as markers (8).

Nutritional Studies. Cultures of *Lemna paucicostata* Hegelm. 6746 were grown mixotrophically under the standard conditions described previously (4), except where noted. Medium 4 (4), which contains 20 μM sulfate, was used, except in some experiments with molybdate, where medium 7 (4), containing 2 ± 1 μM sulfate, was used. Usually, cultures were initiated with about 30 fronds and were grown in 600 ml medium for 7 d. Exceptions to this are noted. Growth is reported as MR (4). MR is equal to 7,224.72 divided by the doubling time in h.

Stock solutions of amino acids and inhibitors were filter-sterilized (0.2-μm pore size) and added aseptically to autoclaved medium. For those amino acids that are relatively insoluble, the amino acid was dissolved in the medium at the desired concentration. The resulting solution was filter-sterilized and aseptically transferred to sterile growth flasks.

Labeling to Isotopic Steady State. Colonies were pregrown in the supplemented medium for 2 to 2.5 doublings. Duplicate cultures, containing about 16 fronds each, were initiated in supplemented or control medium (2 L) containing $^{35}SO_4^{2-}$ (20 μM; 3,000 dpm/nmol) and allowed to undergo 4 to 4.2 doublings. Extracts of the harvested plants were used for analysis of ^{35}S-containing compounds essentially as previously described (5, 8).

Uptake Studies. Uptakes of amino acids and inhibitors were studied over periods ranging from 1 h to several d by use of ^{14}C-labeled compounds and following disappearance of radioactivity from the medium or accumulation of radioactivity in the washed plants. Generally, the two methods gave the same result within experimental error. To determine accumulation of radioactivity, colonies were harvested and separated into TCA-soluble and -insoluble fractions. Radioactivity in TCA-soluble fractions was determined directly after appropriate dilution and, in the TCA-insoluble fractions, after they had been dissolved in 0.4 ml Protosol. Organic sulfur was defined as TCA-insoluble sulfur plus that portion of the TCA-soluble material which was retained by Dowex 50-H$^+$.

Uptakes measured for 24 h or longer were expressed on the basis of frond × d, derived by integration of the growth curve:

$$\text{frond} \times \text{d} = \frac{f_0\left[\frac{t}{\log_2(f_t/f_0)}\right]}{0.693} \times \left[\frac{f_t}{f_0} - 1\right]$$

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[2] Abbreviations: AVG, L-aminoethoxyvinylglycine (*i.e.* L-2-amino-4-(2′-aminoethoxy)-*trans*-3-butenoic acid); PAG, DL-propargylglycine (*i.e.* DL-2-amino-4-pentynoic acid); MR, multiplication rate.

where t is the interval over which uptake is determined, expressed in d; and f_0 and f_t are frond number at time 0 and t, respectively.

Because this expression for frond × d is subject to large errors when calculated over periods when f_t is close to f_0, uptakes measured for short intervals were expressed on the basis of the geometric mean of frond number = $\sqrt{f_0 \cdot f_t}$. All uptakes measured were found to be linear with time, permitting uptake measured over short intervals to be expressed also as uptake/frond × d.

RESULTS AND DISCUSSION

Growth Effects of L-Methionine and Intermediates in its Biosynthesis. Normal plants in the standard medium grew with a mean MR of 190 (range 165–216; number of experiments, 227). Preliminary experiments were carried out to investigate the effects of L-methionine and certain of its precursors upon growth of *Lemna*. The concentrations of these compounds which could be tolerated by *Lemna* with little or no effect upon multiplication rate and morphology were as follows: L-methionine, 2 μM (produced a 10% decrease in MR accompanied by a slight decrease in frond size after several doublings); L-cystathionine, 250 μM (produced some decrease in frond size); L-cystine, 31 μM (slight decrease in frond size).

L-Methionine Uptake. At 0.67 μM L-methionine, the uptake was 0.64 nmol/frond × d; at 2.0 μM L-methionine, uptake was 1.5 nmol/frond × d. On a frond basis, uptake was linear with time between 2 and 24 h. After a 2-h period of uptake, less than 3% of the radioactivity originating in L-methionine washed out of the plants during 60 min in control medium. From these results, it can be shown that, during the interval required to form a new colony of *Lemna*, each colony growing in 0.67 μM methionine absorbs about 6 nmol methionine, slightly more than is required to provide the total protein methionine content of the newly formed colony (10).

These measured rates of uptake are rapid. At 0.67 μM and 2.0 μM methionine, each frond (volume approximately 0.6 μl) during each 24-h interval removed from the medium the amount of amino acid contained in 0.95 and 0.75 ml of medium. In designing experiments in which *Lemna* is to be grown under essentially invariant steady-state conditions in the presence of exogenous methionine, it is important to realize that, unless the volume of medium is kept rather large with respect to the number of plants, the concentration of amino acid in the medium may change appreciably during the course of an experiment. For example, if it is desired that during a 1-week experiment with 2 μM methionine the concentration of this compound in the medium decrease by no more than 10%, it will be necessary to provide at the beginning of the experiment 260 ml of medium for each frond in the inoculum. As shown below, the uptakes of those inhibitors used in the present studies which are themselves structurally α-amino acids occur at roughly comparable rates, and similar considerations must be borne in mind in designing experiments in which these compounds are used.

L-Cystine Uptake. At 31 μM L-cystine, the uptake was 1.8 nmol/frond × d, an amount sufficient to provide 32 ng-atom sulfur/colony during the interval required to form a new colony. After a 2-h period of uptake, less than 2% of the radioactivity originally in L-[U-^{14}C]cystine washed out during a 2-h incubation in medium containing 31 μM nonradioactive L-cystine.

PAG. The sulfur and 4-carbon moieties of methionine are brought together with the formation of cystathionine from *O*-phosphohomoserine and cysteine in a reaction catalyzed by cystathionine γ-synthase. PAG causes growth inhibition of *Bacillus subtilis*, relieved by methionine (19), and is an active-site-directed inhibitor of an *O*-succinylhomoserine-dependent bacterial cystathionine γ-synthase (14). Therefore, we selected PAG for our studies. At concentrations of 0.10 μM and above, this compound produced rapid and very severe growth inhibition of *L. paucicostata*, accompanied by frond detachment. Inclusion of 2 μM L-methionine or 250 μM L-cystathionine completely prevented these effects (Fig. 1). Growth was logarithmic and sustained during subculture in the presence of either of these intermediates. Cystine (31 μM) also permitted continued growth in the presence of 0.15 μM PAG, although at only approximately 75% of the control rate. The latter result was unexpected, because cystine precedes the putative metabolic block at cystathionine γ-synthase.

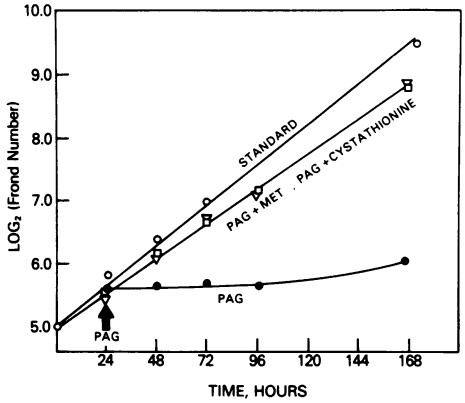

FIG. 1. Growth inhibition of *L. paucicostata* caused by 0.15 μM PAG and its prevention by 250 μM L-cystathionine (□) and by 2 μM L-methionine (▽). The growth of a typical control culture under standard conditions is also shown. The arrow indicates time of addition of PAG.

To help define the mechanism(s) by which methionine, cystathionine, or cystine reversed the growth inhibition caused by PAG, uptake studies were performed. The rates of uptake of PAG were linear with concentrations from 0.02 μM to 0.10 μM, so that, over this concentration range during each 24-h period, each frond absorbed the amount of the L-isomer of PAG contained in 2.2 ml medium (G. A. Thompson, S. H. Mudd, A. H. Datko, unpublished observations). At 0.15 μM PAG, the corresponding value decreased slightly to 2.0 ml/frond × d. At 0.15 μM PAG, inhibitions of uptake of this compound were observed in the presence of added amino acids as follows: L-methionine, 2 μM, 26% inhibition; L-cystathionine, 250 μM, no inhibition; L-cystine, 31 μM, 5% inhibition. Thus, even in the face of the 26% inhibition due to 2 μM methionine, the rate of uptake from 0.15 μM PAG is slightly greater than that at 0.10 μM PAG in the absence of methionine. Therefore, none of the compounds in question affected uptake sufficiently to explain its prevention of PAG-induced growth inhibition.

To gain additional information upon the effects of PAG, $^{35}SO_4^{2-}$-labeling experiments were performed. *Lemna* was pregrown in nonradioactive medium in the presence of 0.15 μM PAG and 2 μM L-methionine. Subcultures were then made into medium of the same chemical composition, containing $^{35}SO_4^{2-}$, and growth was continued for an additional 4 to 4.2 doublings. For comparison, radioactively labeled samples were prepared with plants grown in standard medium and in medium containing L-methionine. At the end of the experiment, the ^{35}S contents of plant protein cyst(e)ine and protein methionine, as well as glutathione, soluble cyst(e)ine, cystathionine, soluble methionine, and *S*-methylmethionine sulfonium, were determined. The results, reported in dpm per frond, are shown in Table I. To allow for possible differences in the sizes or protein contents of the fronds and to correct for any changes in the specific radioactivity of soluble cysteine, which is a common precursor of each of the amino acids listed, the ^{35}S content of each of these compounds was normalized relative to the ^{35}S content of protein cyst(e)ine in the same plant sample. The relative amounts of radioactivity which pass from cysteine into cystathionine and its products—soluble methionine,

Table I. *Radioactivity in Various Compounds after Labeling to Steady State with $^{35}SO_4^{2-}$*

Mean MRs for duplicate cultures were: no addition, 179 (SD, 6.4; SE, 4.5); L-methionine, 157 (SD, 0.7; SE, 0.5); PAG and L-methionine, 149 (SD, 7.8; SE, 5.5); AVG and L-methionine, 158 (SD, 2.1; SE, 1.5); and L-lysine, L-threonine, and L-methionine, 192 (SD, 13.4; SE, 9.5).

Component	Addition to Medium, μM				
	None	L-Methionine, 2	PAG, 0.15; L-Methionine, 2	AVG, 0.05; L-Methionine, 2	L-Lysine, 36; L-Threonine, 4; L-Methionine, 2
	dpm per frond				
Soluble	4,236	3,461	2,471	3,045	3,358
Nonamino acid	3,596	2,460	2,005	2,217	2,598
Amino acid	254	778	163	627	316
Acidic oxidation products	112	228	190	213	160
Glutathione	44	98	94	95	76
Cyst(e)ine	7	20	12	13	14
Neutral oxidation products	121	573	64	380	147
Methionine	16	411	0	256	72
S-Methylmethionine sulfonium	23	59	0	40	17
Cystathionine	2	1	0	49	0
Protein	5,603	2,148	1,321	1,847	1,747
Cyst(e)ine	1,866	1,453	1,084	1,310	1,278
Methionine	3,495	215	4	127	65
Total S	9,839	5,609	3,792	4,893	5,105

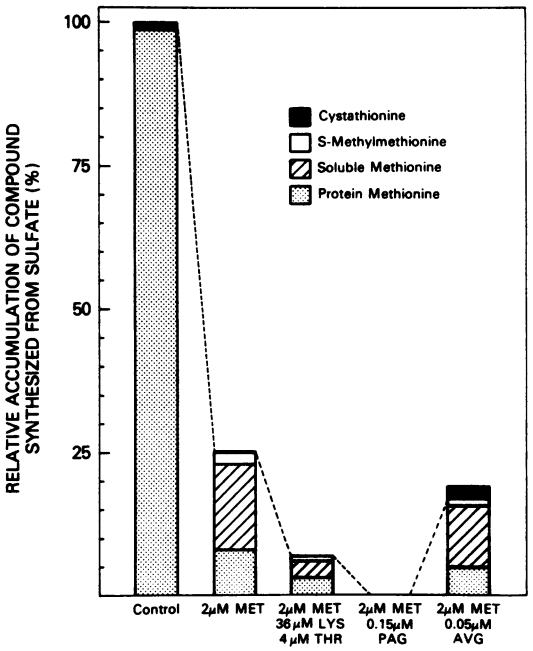

FIG. 2. Relative accumulation of cystathionine and its metabolites synthesized from $^{35}SO_4^{2-}$ by *L. paucicostata*. Radioactivity in cystathionine, *S*-methylmethionine sulfonium, and soluble and protein methionine was divided by radioactivity in protein cyst(e)ine in the same plant sample. The resulting values were expressed as percentages, taking as 100% the sum of such values in plants grown in standard medium. - - -, Total radioactivity, relative to the control, accumulated after the metabolic block.

protein methionine, and *S*-methylmethionine sulfonium—may then be validly compared. In Figure 2, the resulting values are displayed as percentages, taking 100% as equal to the sum of the values for cystathionine and its products (*i.e.* soluble methionine, protein methionine, and *S*-methylmethionine sulfonium) in plants grown in standard medium. The presence of L-methionine by itself caused an increase in the amount of ^{35}S accumulating in soluble methionine, presumably due to the trapping effect of an increased pool of soluble methionine. However, this increase was more than offset by a decrease in the ^{35}S in protein methionine. The net result was a 75% decrease in the entry of ^{35}S into cystathionine and its products, considered as a whole. This result is in agreement with previous observations made in this laboratory. We have suggested that it may well be due to control by methionine of the net rate of entry of sulfur into methionine and that the locus of the regulatory effect is at cystathionine synthesis (9). The further addition of PAG brought about a virtually complete cessation of ^{35}S entry into cystathionine and its products (Fig. 2).

The steady-state concentration of cystathionine, itself, is of special interest in this experiment, because this is the immediate product of cystathionine γ-synthase. Figure 3 depicts, for each plant sample, the area of a paper chromatogram to which the final isolate of cystathionine migrated, and it clearly shows that PAG led to a decrease in the amount of [^{35}S]cystathionine to values below those found in normal plants or in plants grown in the presence of L-methionine alone.

The combined results of the nutrition, uptake, and ^{35}S-labeling experiments thus provide for the first time evidence that PAG at low concentrations may be used to inhibit the *O*-phosphohomoserine-dependent cystathionine γ-synthase of plants. Direct support of this proposed site of action has recently been furnished by our demonstration that PAG inhibits *Lemna O*-phosphohomoserine-dependent cystathionine γ-synthase both *in vivo* and *in vitro* (21) (G. A. Thompson, S. H. Mudd, A. H. Datko, unpublished observations).

A possible explanation for the capacity of cystine to protect against PAG-induced growth inhibition is that 31 μM cystine in the medium may bring about an increased concentration of tissue cysteine. Such a build-up of one of the cosubstrates for cystathionine γ-synthase might promote sufficient flux through this step to

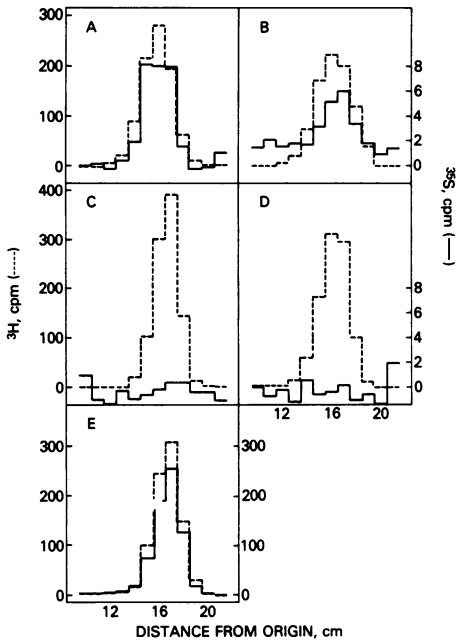

FIG. 3. Chromatography of [^{35}S]cystathionine. [^{35}S]Cystathionine (——) was purified, along with added authentic marker [^{3}H]cystathionine (- - -), from aqueous extracts of $^{35}SO_4^{2-}$-grown *Lemna*, as described in "Materials and Methods." Representations of chromatograms of the final isolates shown here were from *Lemna* grown in standard medium (A) or with the following additions to the standard medium: 2 μM L-methionine (B); 36 μM L-lysine, 4 μM L-threonine, and 2 μM L-methionine (C); 0.15 μM PAG and 2 μM L-methionine (D); 0.05 μM AVG and 2 μM L-methionine (E). Each chromatogram was loaded with cystathionine derived from 174 to 210 *Lemna* fronds.

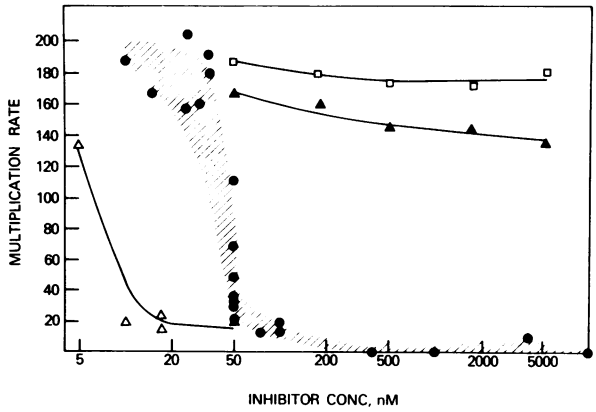

FIG. 4. Growth response of *L. paucicostata* to increasing concentrations of AVG (●); its saturated analog, L-2-amino-4-(2'-aminoethoxy)-butyric acid (□); L-2-amino-4-methoxy-*trans*-3-butenoic acid (△); and its saturated analog, L-2-amino-4-methoxy-butyric acid (▲).

permit the observed rate of growth, if even a small portion of this enzyme activity remains uninhibited, as direct assays show is indeed the case (G. A. Thompson, S. H. Mudd, A. H. Datko, unpublished observations).

AVG. A second enzyme essential for methionine biosynthesis is β-cystathionase, which cleaves cystathionine to the immediate methionine precursor, homocysteine. Previous studies from this laboratory showed that rhizobitoxine (2-amino-4-(2'-amino-3'-hydroxypropoxy)-*trans*-3-butenoic acid) brings about an active-site-directed, irreversible inhibition of β-cystathionase partially purified from spinach (11). Although rhizobitoxine is not, at present, readily available, a structural analog, AVG, has been isolated (16) and shown to act as does rhizobitoxine in inhibiting plant ethylene production (15). In hopes that plant β-cystathionase would also be inhibited by AVG, we included this compound in the present studies.

AVG (0.05 μM) produced a progressive inhibition of the growth of *L. paucicostata*. In some experiments, growth inhibition was virtually complete. Inclusion of L-methionine in the medium almost completely prevented these effects and permitted continued growth. A dose-response curve for the effect of AVG upon *Lemna* growth is presented in Figure 4. Little effect was detected at a concentration of 0.025 μM. Inhibition was almost complete at 0.075 μM. Figure 4 also presents the results of studies of several structural analogs of AVG. L-2-Amino-4-methoxy-*trans*-3-butenoic acid was more potent than AVG, producing almost complete growth inhibition at a concentration of 0.01 μM, whereas the saturated analog of either of these compounds was virtually without effect at concentrations up to 5 μM.

Uptake of AVG was essentially linear with time (from 2 to 24 h) and with concentration (in the range of 0.015 to 0.050 μM), so that, during each 24-h period, each frond absorbed the amount contained in 0.6 ml medium. Uptake of 0.05 μM AVG was decreased by 24% by 2 μM L-methionine. Considered together with the very steep concentration-dependence of growth inhibition by AVG, the latter result raised the question as to whether methionine prevents growth inhibition solely by retarding the uptake of AVG or partially by this effect and partially by providing a metabolite essential under these conditions. To help decide this question, an experiment was performed in which the effect of 0.05 μM AVG was compared to that of 0.065 μM AVG plus 2 μM methionine. Under these conditions, the uptakes of AVG should be the same. Approximately 30 fronds were inoculated into 2.5 L of medium containing either 0.05 μM AVG or 0.065 μM AVG plus 2 μM methionine. After 5 d, subcultures were performed, and growth was continued for 4 additional d. On a frond basis, the MR in the presence of 0.05 μM AVG was 101 during the first passage and 68 during the second. In 0.065 μM AVG plus 2 μM methionine, the corresponding values were 114 and 189. Analyses of protein content permitted the calculation that, during the second passage, the protein MR values were no more than 98 in the absence, and at least 180 in the presence, of exogenous methionine. These results indicate that methionine restored a normal MR by providing a metabolite essential under the specified conditions.

When *Lemna* plants were labeled to isotopic steady-state with $^{35}SO_4^{2-}$ by growth in the presence of 0.05 μM AVG and 2 μM L-methionine, there was a striking increase in the amount of ^{35}S in cystathionine (Figs. 2 and 3; note the change in the scale of the ordinate for [^{35}S]cystathionine in Fig. 3, panel E). Based on ^{35}S in protein cysteine, [^{35}S]cystathionine had risen about 35-fold in these plants. Clearly, this finding supports the postulated location of an AVG-induced metabolic block at β-cystathionase.

Figure 2 also shows that, in spite of the block at β-cystathionase, the accumulation of ^{35}S in products resulting from the cleavage of cystathionine was not markedly decreased in the presence of both AVG and methionine below the amount accumulating in the presence of methionine only. This result suggests that only an incomplete inhibition of β-cystathionase was achieved at the concentration of AVG used and that the remaining active β-cystathionase, in company with the demonstrably elevated cystathionine concentration, was sufficient to permit a relatively undiminished entry of ^{35}S into the products of cystathionine cleavage. A similar result was obtained by Giovanelli *et al.* (12), studying the effects of rhizobitoxine in corn seedlings. A 60% inhibition of β-cystathionase activity produced a striking accumulation of cystathionine, but only small decreases in the flux of ^{35}S from sulfate into methionine were detected after 3 to 6 h.

Lysine Plus Threonine. To inhibit an earlier step in methionine biosynthesis, the combination of lysine plus threonine was studied.

These compounds are known to act synergistically to inhibit growth of a number of plants or plant tissues. The inhibition is often relieved by methionine or homoserine. Bryan (2) recently reviewed this area and concluded that the results of several studies are consistent with an experimentally induced methionine limitation brought about by interference with the conversion of aspartate to homoserine.

L-Lysine (36 μM), in combination with 4 μM L-threonine, produced a marked inhibition of the growth of *L. paucicostata*, which progressed in severity for the first day or two. In a series of 12 experiments using these concentrations of lysine and threonine, the MR values (calculated over intervals of 6 to 7 d after addition of lysine plus threonine) ranged from 6 to 44. Neither 36 μM L-lysine nor 4 μM L-threonine, added singly, produced severe growth inhibition or morphological changes. L-Lysine (72 μM) plus 8 μM L-threonine also produced severe growth inhibition (Fig. 5), accompanied by a progressive loss of the normal green coloration of the plants and by abnormal detachment of fronds, so that many colonies soon came to contain only one or two fronds. These effects were completely prevented by the simultaneous inclusion of 2 μM L-methionine in the growth medium (Fig. 5). Growth then became logarithmic with time and could be sustained at the same rate and with the same protein content per frond during successive subcultures.

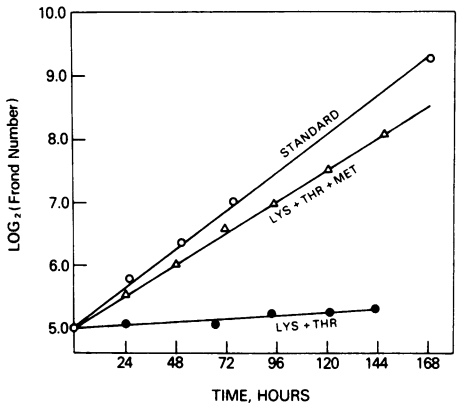

FIG. 5. Growth inhibition of *L. paucicostata* caused by 72 μM L-lysine plus 8 μM L-threonine and its prevention by 2 μM L-methionine. The growth of a typical control culture under standard conditions is also shown.

Table II. *L-Lysine or L-Threonine Uptake and Effects of Intermediates in the Methionine Pathway*

Uptake of L-[^{14}C]lysine (200 dpm/nmol) was measured in the presence of L-threonine, and uptake of L-[^{14}C]threonine (200 dpm/nmol) was measured in the presence of L-lysine. Uptakes were measured during 2 h in the presence of 90 to 97 fronds. The values shown are means of duplicate experiments derived from measurement of radioactivity accumulated in the plants.

Experiment	Addition			Uptake	
	L-Lysine	L-Threonine	Other	L-Lysine	L-Threonine
	μM			*pmol/frond × 2 h*	
1	36	4		298	294
	36	4	L-Methionine, 2	263	190
	72	8		367	456
	72	8	L-Methionine, 2	347	296
2	54	6		388	455
	54	6	L-Cystathionine, 250	293	391

Studies of the rates of uptake of lysine and threonine (Table II) showed that, although methionine inhibited the uptake of both of these amino acids, in the presence of 2 μM methionine the rates of uptake of lysine (at 72 μM) and threonine (at 8 μM) were at least as high as were the uptakes of these compounds at 36 μM and 4 μM in the absence of methionine. We concluded that, at these higher concentrations of lysine and threonine, methionine was functioning as an essential metabolite and was not preventing growth inhibition merely by decreasing the uptake of lysine and/or threonine.

Cystathionine (250 μM) was also able to prevent the growth-inhibitory effects of lysine plus threonine, added at concentrations up to 54 μM and 6 μM. Inhibition by cystathionine of the uptakes of lysine and threonine at these concentrations (Table II, experiment 2) was not sufficient to account for the cystathionine prevention of growth inhibition. This thioether was again providing a metabolite essential under these conditions.

An experiment in which plants were labeled with $^{35}SO_4^{2-}$ to isotopic steady-state in the presence of 36 μM lysine, 4 μM threonine, and 2 μM methionine demonstrated that the presence of lysine plus threonine brought about a decrease in the entry of ^{35}S into cystathionine and its products to 6% of the value for control plants and to 25% of the value for plants grown in methionine alone (Fig. 2), as well as a decrease in the [^{35}S]cystathionine content (Fig. 3). These results support the localization of the block at a step prior to cystathionine formation and complement and extend previous reports that, in relatively short-term labeling experiments with other plant systems, the accumulation of radioactivity from either $^{35}SO_4^{2-}$ (1) or [^{14}C]aspartate (6, 13) into methionine is diminished.

Molybdate. This anion, a structural analog of sulfate, was used in an attempt to interfere with the reduced sulfur branch of the methionine pathway. Molybdate has been shown to inhibit sulfate uptake by several tissues (17, 22–24). Furthermore, experiments using cell extracts or purified enzymes have shown that molybdate inhibits sulfate incorporation into adenosine 5′-phosphosulfate in the reaction catalyzed by ATP-sulfurylase (18, 25) and, thereby, has the potential to interfere with reduction and organification of sulfur.

The standard medium used in the present studies contains 20 μM sulfate and 6 μM molybdate. To examine the effect of molybdate on *Lemna* growth, the medium was modified by lowering the sulfate concentration to 2 ± 1 μM and increasing the molybdate concentration. At 391 μM molybdate, growth of *Lemna* was severely inhibited (Table III). The inhibition was accompanied by abnormal frond detachment, as indicated by a decrease in the ratio of frond/colony. Inclusion of 3 mM sulfate completely prevented these effects.

To gain insight into the mechanism of the molybdate effect, experiments utilizing $^{35}SO_4^{2-}$ were performed. Preliminary experiments showed that uptake of $^{35}SO_4^{2-}$ by control cultures was virtually linear for at least 120 min and that a readily measurable proportion of the $^{35}SO_4^{2-}$ taken up became organic within 60 min. Little, if any, of the ^{35}S taken up washed back out into the medium over a 60-min period, far longer than was required to rinse and harvest colonies. Matched cultures of *Lemna* were pregrown in medium containing 391 μM molybdate for varying periods of time up to 24 h. Carrier-free $^{35}SO_4^{2-}$ was added to the growth medium (containing 20 μM SO_4^{2-}) for each culture 60 min before it was to be harvested. A control culture grown in the standard medium containing 6 μM molybdate also received a 60-min period of labeling with $^{35}SO_4^{2-}$. Total ^{35}S taken up and the proportion of such radioactivity which had become organic were then determined. The rate of $^{35}SO_4^{2-}$ uptake was inhibited 82% during 1-h exposure to 391 μM molybdate. The inhibition increased to about 90% by 12 h and remained unchanged for the next 12 h. Of the total ^{35}S taken up by the control culture, 21% became organic

Table III. *Molybdate Effects and Their Prevention by Sulfate and Intermediates of the Methionine Biosynthetic Pathway*

Concentration	No. of Experiments	MR[a]	Fronds / Colony[a]	Protein Content
		range		*μg/frond*
Molybdate, 6 μM; Sulfate, 20 μM	227	165–216	3.02–5.15	6.2–8.1
Molybdate, 52 μM; Sulfate, 20 μM	2[b]	138–159	3.89–6.70	—[e]
	8[c]	113–142	5.03–11.87	—
Molybdate, 391 μM; Sulfate, 20 μM	2	27–28	1.74–1.76	—
Molybdate, 391 μM; Sulfate, 2 μM	3	20–66	1.89–2.18	—
+ Sulfate, 3,000 μM	4	176–189	2.77–4.54	—
+ L-Cystine, 31 μM	2	175–178	3.44–3.94	9.5
	1[d]	182	3.87	9.5
+ L-Cystathionine, 250 μM	1	108	2.67	6.7
	1[d]	48	1.84	4.3
+ L-Homocystine, 48 μM	1	39	1.65	—
+ L-Methionine, 2 μM	2	47–55	1.85–1.98	7.2
	1[d]	74	2.70	
+ L-Methionine, 4 μM	1	107	2.13	7.1
	1[d]	69	1.85	—

[a] All values, except where noted, are for the initial culture of 6 to 7 d in the specified medium.
[b] Data include values for one experiment taken from Ref. 4.
[c] Values are for subculture in specified medium for as long as 42 d by inclusion of data reported in Ref 4.
[d] Values are for subculture in specified medium for an additional 6 to 7 d.
[e] Not measured.

during the 60-min period of labeling. This value, expressed as a fraction of the sulfate uptake, changed little in cultures exposed to molybdate for up to 24 h.

Together, these results define conditions under which molybdate may be used to deprive *Lemna* of adequate sulfate. Under the specified conditions, there is no indication that, in addition to inhibiting sulfate uptake, molybdate may be preventing organification of this ion, as might have been suggested by the *in vitro* inhibition of ATP-sulfurylase by molybdate (18, 25). However, the latter conclusion is not unequivocal, because in the labeling experiments a steady state was not maintained, and the size of the tissue pool of inorganic sulfate was probably falling rapidly. Further, it has not been established whether, under the specified conditions, molybdate enters the plants.

Molybdate-inhibited *Lemna* provides a system in which the importance of "reverse" transsulfuration (*i.e.* transfer of sulfur in the direction homocysteine → cysteine) may be assessed experimentally in intact plants. Thus, the growth inhibition and morphological changes due to 391 μM molybdate could be totally prevented by inclusion of 31 μM L-cystine in the growth medium (Table III). Colonies grown under these conditions also contained a normal amount of protein. L-Cystathionine (250 μM), L-homocystine (48 μM), and L-methionine (2 μM) were not effective in preventing the growth inhibition and abnormal frond detachment caused by molybdate. In those instances in which some growth appeared to occur during the first week of culture—for example, with cystathionine—growth failed to continue, and protein content decreased during subsequent subculture. Uptake of methionine was not affected by 391 μM molybdate. The failure of methionine to prevent growth inhibition by molybdate at concentrations at which methionine did relieve the growth inhibition due to either lysine plus threonine or PAG indicates that, in *Lemna*, the transfer of sulfur from methionine to cysteine does not occur at a rate sufficiently high to meet the plants' cysteine requirement for growth. Further, the inability of cystathionine to sustain growth strongly suggests that one reason for this failure is the absence of γ-cystathionase activity (which would cleave cystathionine to cysteine). This result is in agreement with our previous failure to demonstrate activity of γ-cystathionase in extracts of higher plants (7); it is also in agreement with the conclusion (based on these enzyme studies) that, in plants, transsulfuration in the direction homocysteine → cysteine is not likely to be a quantitatively important metabolic pathway (9).

In summary, in the present work, we introduce the use of PAG as an inhibitor of methionine biosynthesis in higher plants, demonstrate that AVG may inhibit at β-cystathionase as well as at its well-established site of action in ethylene production, and extend to *Lemna paucicostata* the use of lysine plus threonine to synergistically prevent the conversion of aspartate to homoserine. These inhibitors hold promise as useful tools for many sorts of experiments. As one example, we have utilized lysine plus threonine, as well as AVG, to reveal that the *O*-phosphohomoserine-dependent cystathionine γ-synthase of *Lemna* increases in activity during methionine deprivation (20).

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