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In Vivo Regulation of *De Novo* Methionine Biosynthesis in a Higher Plant (*Lemna*)

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ABSTRACT

Administration of methionine to growing *Lemna* had essentially no effect on accumulation of sulfate sulfur in protein cysteine, but decreased accumulation into cystathionine and its products (homocysteine, methionine, *S*-methylmethioninesulfonium salt, *S*-adenosylmethionine, and *S*-adenosylhomocysteine) to as low as 21% that of control plants, suggesting that methionine regulates its own *de novo* synthesis at cystathionine synthesis. Methionine caused only a slight reduction (to 80% that of control plants) in the accumulation of sucrose carbon into the 4-carbon moieties of cystathionine and products. This observation was puzzling since cystathionine synthesis proceeds by incorporation of equivalent amounts of sulfur (from cysteine) and 4-carbon moieties (from *O*-phosphohomoserine). The apparent inconsistency was resolved by the demonstration in *Lemna* (Giovanelli, Datko, Mudd, Thompson 1983 Plant Physiol 71: 319–326) that *de novo* synthesis of the methionine 4-carbon moiety occurs not only via the established transsulfuration route from *O*-phosphohomoserine, but also via the ribose moiety of 5′-methylthioadenosine. It is now clear that the more accurate assessment of the flux of sulfur (and 4-carbon moieties) through transsulfuration is provided by the amount of ^{35}S from $^{35}SO_4^{2-}$ that accumulates in cystathionine and its products, rather than by the corresponding measurements with ^{14}C. These studies therefore unequivocally demonstrate in higher plants that methionine does indeed feedback regulate it own *de novo* synthesis *in vivo*, and that cystathionine synthesis is a locus for this regulation.

Plants play an indispensable role in producing the ultimate source of most methionine required in the diet of humans and other nonruminant animals (15). In spite of this crucial role, the basic questions of whether methionine regulates its own *de novo*[2] synthesis *in vivo* in plants, and if so, the identity of the regulatory loci, remain unanswered (15, 23).

Two available lines of evidence bear on these questions, but fail to provide definitive answers. The first is based on isotope competition studies. Experiments with Paul's scarlet rose cells (11) showed that exogenous methionine decreased the accumulation of ^{14}C from [^{14}C]glucose into protein methionine to 20% that of control cells, suggesting that methionine regulates its own *de novo* synthesis in these cells. However, this interpretation was questioned by Davies (10) on the basis that ^{14}C 'trapped' in soluble methionine was not determined. Neither was account taken in these studies of excretion of ^{14}C-compounds into the medium (27), or the possibility that synthesis of the methyl and 4-carbon moieties of methionine might be regulated independently. Bright *et al.* (2) observed that exogenous methionine did not affect incorporation of radioactivity from [^{14}C]acetate into soluble plus protein methionine in cultured wheat embryos. These results suggest that methionine biosynthesis is insensitive to feedback control in this issue, but the authors themselves pointed out that other interpretations of the data are possible, since their measurements of combined radioactivity in the methyl and 4-carbon groups of methionine need not provide a valid assay of net synthesis of methionine through the transsulfuration pathway. For example, transmethylation reactions could result in a disproportionate labeling of the methyl group relative to that of the 4-carbon moiety. The effect of exogenous methionine on accumulation of ^{35}S from $^{35}SO_4^{2-}$ into soluble methionine was studied in *Chlorella* (29). Exogenous methionine had no effect on the accumulation in wild type cells, but increased that in ethionine-resistant cells. Interpretation of these results is equivocal, since ^{35}S in protein methionine, the major end product of *de novo* methionine biosynthesis (15), was not determined. The second line of evidence is based on experiments with plant variants resistant to the methionine analog, ethionine, or the combined presence of lysine and threonine. In normal plants, the latter condition inhibits synthesis of precursor homoserine required for methionine synthesis (23). The concentration of soluble methionine in these variants was increased many-fold (1, 19, 20, 26, 29). However, soluble methionine is normally a minor component of total cellular methionine, and, when measured, the amounts of total methionine (*i.e.* methionine in soluble plus protein forms) were only fractionally higher in the variants (1, 25, 26). This evidence, therefore, fails to establish that the variants are regulatory mutants in which methionine is being synthesized appreciably faster.

Here we describe the effects of exogenous methionine on the labeling patterns of sulfur amino acids in *Lemna* growing on $^{35}SO_4^{2-}$ or [U-^{14}C]sucrose. The results of these studies show conclusively for the first time in a higher plant that methionine does indeed regulate its own *de novo* synthesis *in vivo*, and that a major locus for this regulation is at cystathionine synthesis. Parts of this study have been presented in preliminary form (15).

MATERIALS

Radioisotopes. $Na_2{}^{35}SO_4$ was purchased from New England Nuclear, and specific radioactivities were based on an estimated

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[2] The term is used here to describe specifically the net synthesis of the entire molecule, *i.e.* the sulfur, 4-carbon, and methyl groups. In plants, this synthesis occurs predominantly, perhaps exclusively, by the transsulfuration pathway, *i.e. via* the reactions *O*-phosphohomoserine + cysteine→cystathionine→homocysteine→methionine (15). The recently discovered synthesis of methionine from 5′-methylthioadenosine (17) results in a net synthesis of only the 4-carbon moiety of methionine, while the methyl and sulfur groups of methionine are independently recycled (16).

concentration of 20 μM SO_4^{2-} in the medium. Due to the presence of contaminating SO_4^{2-} in certain components of the medium (8), the estimated concentration of SO_4^{2-} is subject to a 10% error. Other radioisotopes were prepared as described (6, 7, 14), or were obtained commercially.

Chemicals. Rexyn 203 (16–50 mesh), a polystyrene amine resin, was obtained from Fisher Chemical. Dowex 50 (AG 50W-X4, 200–400 mesh) and Dowex 1 (AG 1-X10, 200–400 mesh) were from Bio-Rad.

METHODS

General Methods. The following solvents were used for paper chromatography: solvent A, 2-propanol:88% HCOOH:H_2O (7:1:2, v/v); solvent B, 1-butanol:propionic acid:31.2 mM aqueous 2-mercaptoethanol (250:124:175, v/v). Protein was determined as described (6). Unless stated otherwise, ion exchange chromatography was at room temperature with columns of 0.9 × 3 cm bed volume, and Dowex 50-H^+ chromatography was by the method of Giovanelli *et al.* (13).

Labeling of *Lemna* to Isotopic Steady State. Plants were grown under the standard conditions for mixotrophic growth in 20 μM sulfate (8). Plants used as inocula for methionine-supplemented cultures were pregrown in the presence of the appropriate concentration of methionine for 6 to 7 d. An inoculum of 3 to 11 colonies of control or methionine-supplemented plants was added to 4L of appropriate medium containing either $^{35}SO_4^{2-}$ or [U-^{14}C]sucrose and allowed to undergo between 3.7 and 4.5 doublings. The amounts of methionine removed from the medium were no greater than 29% for medium supplemented with 2 μM methionine, and 12% for medium supplemented with 0.6 / μM methionine.

Harvesting and Extraction of Plants. Plants were harvested and extracted by the 'simplified procedure' to yield TCA-insoluble (pellet P) and -soluble fractions (9).

Protein Amino Acids. Protein amino acids were determined with an amino acid analyzer as described (14).

Analysis of ^{35}S-Compounds. *Protein [^{35}S]Cysteine and Protein [^{35}S]Methionine.* Determinations were made as described in a previous study ('third experiment') (14).

Soluble ^{35}S-Amino Acids. For experiments 1 and 2 (Table I), the soluble fraction was processed as described (9) into nonamino acid and amino acid fractions. The latter fraction was separated, after performic acid oxidation, into acidic and neutral oxidation products. After addition of marker [^{3}H-methyl]methionine sulfone, [^{35}S]methionine (sulfone) and *S*-methyl-[^{35}S]methioninesulfonium salt in the neutral oxidation products were determined by fractionation on Dowex 50-NH_4^+, followed by paper electrophoresis or paper chromatography of the appropriate fraction (9). An upper limit for [^{35}S]homocysteine was obtained by electrophoresis of the acidic oxidation products in the presence of marker [^{3}H]homocysteic acid (14). [^{35}S]AdoMet[3] and [^{35}S] AdoHcy were determined as described (9), and [^{35}S]cystathionine was assayed by the method of Giovanelli *et al.* (14), except that reduction with hydriodic acid was omitted.

The above procedures were modified for assay of ^{35}S in soluble methionine, AdoMet, and *S*-methylmethioninesulfonium salt in Table II. The soluble fraction, after addition of marker [^{3}H-methyl]AdoMet and *S*-[^{3}H-methyl]methylmethioninesulfonium salt, was extracted with ether to remove TCA, then chromatographed at 4°C on Dowex 50-NH_4^+ (9). Material that passed through the column was chromatographed on Dowex 50-H^+ to yield an amino acid fraction in which [^{35}S]methionine was determined as the sulfone (14). The NH_4OH eluate from Dowex 50-NH_4^+ chromatography was lyophilized to dryness, and the residue immediately dissolved in 10 mM HCOOH. The solution was then chromatographed with solvent A, and the areas corresponding to AdoMet (R_F, 0.1) and *S*-methylmethioninesulfonium salt (R_F, 0.28–0.44) were eluted to yield eluates 1 and 2, respectively. Each eluate was then boiled at pH 5.0 (9). This procedure converts [^{35}S]AdoMet to 5′-methyl-[^{35}S]thioadenosine (and homoserine, via the lactone). *S*-Ribosyl-[^{35}S]methionine, which co-migrates with *S*-methylmethioninesulfonium salt in solvent A, is converted to 5′-methyl-[^{35}S]thioribose. The procedure has no effect of *S*-methylmethioninesulfonium salt. Each of the boiled eluates was rechromatographed in solvent A containing 10 mM 2-mercaptoethanol. Boiled eluate 1 yielded a peak of radioactivity corresponding to 5′-methylthioadenosine, which was eluted and rechromatographed in solvent B. The ratio of ^{35}S/^{3}H in 5′-methylthioadenosine eluted from this chromatogram was used to calculate [^{35}S]AdoMet. For boiled eluate 2, essentially all radioactivity migrated in the area corresponding to *S*-methylmethioninesulfonium salt, and no 5′-methyl-[^{35}S] thioribose (R_F approximately 0.72) was detected. The area corresponding to *S*-methylmethioninesulfonium salt was eluted and further purified by paper chromatography, first in solvent B, then in 1-butanol:pyridine:H_2O (1:1:1, v/v). The ratio of ^{35}S/^{3}H in the product obtained from the latter chromatography was used to calculate values for *S*-methyl-[^{35}S]methioninesulfonium salt.

Uncharacterized ^{35}S-Compounds. Volatile ^{35}S-compounds formed during growth were determined after being trapped in mercuric acetate (9). ^{35}S in uncharacterized amino acids from plants was given by that in neutral oxidation products other than methionine sulfone and *S*-methylmethioninesulfonium salt. Organic ^{35}S in the nonamino acid fraction from plants was determined from the amount of ^{35}S in this fraction that was not precipitated as $BaSO_4$ (9). Organic ^{35}S in the medium was determined from the amount of ^{35}S that was not precipitated as $Ba^{35}SO_4$, and was further fractionated by Dowex 50-H^+ chromatography into nonamino acid and amino acid fractions.

Analysis of ^{14}C-Compounds. Analysis of ^{14}C-compounds is technically more difficult than of ^{35}S-compounds, because it is necessary to purify each ^{14}C-compound from the large excess of other cellular ^{14}C-compounds resulting from labeling to isotopic equilibrium with [U-^{14}C]sucrose. After their purification, methionine, AdoMet, and *S*-methylmethioninesulfonium salt were degraded to determine ^{14}C localized in 4-carbon moieties.

Protein [^{14}C]Methionine and [^{14}C]Threonine. The TCA-insoluble fraction was hydrolyzed with 2 ml of 3 N mercaptoethanesulfonic acid (Pierce) (24). [2-^{3}H]Methionine and [G-^{3}H]threonine were added to an aliquot of the protein hydrolysate equivalent to a maximum of 500 fronds (3–3.5 mg protein). The solution was diluted with 40 ml water and applied to a column of Rexyn 203 (free base) with a bed volume of 13 ml/meq of mercaptoethanesulfonic acid. The column was washed with two bed volumes of 10 mM 2-mercaptoethanol. Mercaptoethanesulfonic acid was retained by the column, while amino acids were recovered in the effluent and wash fractions. These combined fractions were then chromatographed successively on Dowex 1 (equilibrated with pyridine-formate, pH 5.9) and Dowex 50-NH_4^+ to remove acidic and basic amino acids, respectively. After reduction with 2-mercaptoethanol (17), the resultant neutral amino acid fraction was chromatographed in solvent B to separate [^{14}C]methionine and [^{14}C]threonine. Each of these ^{14}C-amino acids (localized by ^{3}H marker) was eluted separately, and further purified as described below.

[^{3}H]Methionine was purified via the sulfoxide and carboxymethylsulfonium salt derivates (J. Giovanelli, S. H. Mudd, in preparation). ^{14}C in the 4-carbon moiety of methionine was determined by degradation of methioninecarboxymethylsulfonium salt (J. Giovanelli, S. H. Mudd, in preparation).

[3] Abbreviations: AdoMet, *S*-adenosylmethionine; AdoHcy, *S*-adenosylhomocysteine.

[^{14}C]Threonine was determined by periodate oxidation (18). It was necessary to remove other ^{14}C amino acids in the eluate which are also sensitive to periodate. Serine is known to be periodate sensitive (22). Cysteine, cystine, tryptophan, and tyrosine may possibly also exhibit some periodate sensitivity, since these amino acids have been reported to react with periodate in undefined ways (3). Chromatography in solvent B resolved threonine (R_F, 0.35) from cystine (R_F, 0.02), tryptophan (R_F, 0.61), and tyrosine (R_F, 0.50), but possibly not completely from serine (R_F, 0.20) and cysteine (R_F, 0.43). The eluate from the chromatogram developed with solvent B was therefore chromatographed with ethanol: H_2O:14.5 M NH_4OH (90:5:5, v/v) for 49 h, resulting in complete resolution of threonine (migrating 21 cm) from serine (migrating 10 cm, and localized with marker [^{3}H]serine) and cysteine (remaining at origin). ^{14}C corresponding to [^{3}H] threonine was eluted from this chromatogram, and chromatographed on Dowex 50-H^+ to decrease the amounts of any compounds that might interfere with periodate oxidation. After the periodate oxidation, less than 10% of ^{14}C was retained by Dowex 50-H^+. Protein [^{14}C]threonine was calculated from the ratio of periodate-sensitive ^{14}C/^{3}H.

Soluble ^{14}C-Amino Acids. After addition of *S*-adenosyl-[2-^{3}H] methionine and *S*-methyl-[2-^{3}H]methioninesulfonium salt, the TCA-soluble fraction was chromatographed on Dowex 50-NH_4^+. AdoMet and *S*-methylmethioninesulfonium salt in the NH_4OH eluate were separated by paper chromatography. Details of these procedures are given in the assay of ^{35}S-compounds. AdoMet was degraded to homoserine (and 5′-methylthioadenosine) as described for [^{35}S]AdoMet, and *S*-methylmethioninesulfonium salt was degraded to homoserine by refluxing at pH 8.3 (6). After each of the degradations, [^{14}C]homoserine was purified by successive paper chromatography with solvents A and B, and was detected by [^{3}H]homoserine formed from the parent sulfonium compound. The ratio of ^{14}C/^{3}H in homoserine was used to calculate ^{14}C in the 4-carbon moiety of AdoMet or *S*-methylmethioninesulfonium salt.

[2-^{3}H]Methionine, [^{3}H]cystathionine, and [^{3}H]AdoHcy were added to the fraction not retained by Dowex 50-NH_4^+, and methionine was separated from cystathionine and AdoHcy by Dowex 50-H^+ chromatography (14). [^{14}C]Methionine was further purified, and ^{14}C in its 4-carbon moiety determined as described for protein [^{14}C]methionine. The fraction containing cystathionine and AdoHcy was reduced to convert any sulfoxide derivatives to their thioether forms (17) and chromatographed with solvent B. The negligible amounts of ^{14}C that co-migrated with [^{3}H] cystathionine and [^{3}H]AdoHcy did not warrant localization of ^{14}C in the 4-carbon moieties of these compounds. Consequently, upper limits of ^{14}C in the 4-carbon moieties of cystathionine and AdoHcy were provided by the total amounts of ^{14}C that co-migrated with corresponding ^{3}H marker.

RESULTS AND DISCUSSION

De novo synthesis of the sulfur moiety of methionine in *Lemna* growing on inorganic sulfate as a sole source of sulfur may be summarized by the reactions (15):

$$SO_4^{2-} \rightarrow \rightarrow \text{(soluble) cysteine} \rightarrow \text{cystathionine} \rightarrow \text{homocysteine} \rightarrow \text{methionine}$$

(O-phosphohomoserine)

Inorganic sulfate is first assimilated into soluble cysteine, the portal for all organic reduced sulfur compounds. Cysteine then enters the transsulfuration pathway via cystathionine to yield homocysteine and methionine. If methionine regulates its own *de novo* synthesis, *Lemna* growing in the presence of exogenous methionine should exhibit decreased accumulation of sulfate sulfur in compounds distal to the site of regulation. The amount of feedback regulation of *de novo* methionine biosynthesis was therefore assessed from the relative accumulations in control and methionine-supplemented plants of ^{35}S from $^{35}SO_4^{2-}$ into protein cysteine, a major product of soluble cysteine metabolism (15), and into cyastathionine and its products. Plants were grown under steady state conditions on $^{35}SO_4^{2-}$, either in the absence or presence of supplementary methionine, essentially to isotopic equilibrium. The ^{35}S in various compounds, therefore, provides valid measurements of the amounts of sulfur derived from sulfate that accumulates in these compounds. For initial studies, a concentration of 2 μM methionine was selected. This condition was expected to provide maximal opportunity for detection of any feedback regulation, because 2 μM supplemental methionine: (a) approximates the maximum that can be tolerated without causing more than 10 to 20% decrease in growth rate (4, 5, 16); (b) provides for uptake of an amount of methionine exceeding the total (soluble + protein) accumulating in control plants (4); (c) increases the pool sizes of soluble methionine, *S*-methylmethioninesulfonium salt and AdoMet by approximately 300-, 40-, and 10-fold, respectively (5).

Supplementary 2 μM methionine caused a small (27%) reduction in accumulation of ^{35}S from $^{35}SO_4^{2-}$ into protein cysteine, from 20,700 dpm/frond in control plants to 15,200 dpm/frond in methionine-supplemented plants. This decrease was accompanied by identical decreases (see legend to Table I) in protein cysteine[4] and other protein amino acids, and a comparable decrease in total protein, and was therefore ascribed primarily to a decreased protein content of these methionine-supplemented plants. These results show that, on a protein basis, supplementary methionine had essentially no effect on accumulation of sulfate sulfur into protein cysteine.

The effects of supplementation with 2 μM methionine on the accumulation of ^{35}S into cystathionine and its products are illustrated in Table I (compare experiments 1 and 2). To correct for differences in protein content of plants, the amount of ^{35}S accumulated in each compound was normalized to the corresponding amount of ^{35}S in protein cysteine in the same plant sample. These values, therefore, provide a valid comparison of the relative amounts of sulfate sulfur that accumulate in cystathionine and its products in control and methionine-supplemented plants. To facilitate comparisons, in Table I the normalized values have been expressed as percentages, taking as 100% the sum of such values for cystathionine and its products in control plants. Thus, the extent to which the total percentage in the methionine-supplemented plants falls below 100% is a measure of the decrease brought about by methionine in the accumulation of sulfate sulfur into the methionine branch.

The overall effect of methionine supplementation was to decrease the total ^{35}S accumulated in cystathionine and its products to between 21 and 22% of that in control plants. The 35-fold increase in soluble [^{35}S]methionine in methionine-supplemented plants was ascribed to the trapping effect of the expanded pool of this amino acid. However, this increase, and the much smaller increase in radioactivity accumulating in *S*-methylmethioninesulfonium salt, were more than offset by the decline of ^{35}S in protein methionine. Both control and methionine-supplemented plants accumulated relatively small amounts of ^{35}S in uncharacterized compounds in the volatiles, in the plants, and in the media. Even on the unlikely assumption that all the ^{35}S in uncharacterized compounds was derived from cystathionine,

[4] The specific activities of protein cysteine isolated from control (23,600 dpm/nmol) and 2 μM methionine-supplemented (23,000 dpm/nmol) plants were therefore essentially the same, and in good agreement with the respective values of 25,000 and 25,400 dpm/nmol calculated for 3.7 (control) and 4.0 (methionine-supplemented) doublings in $^{35}SO_4^{2-}$. These findings corroborate other evidence (4, 5, 12) that methionine sulfur is not transferred to cysteine at a measurable rate.

Table I. *Effects of 2 μM Supplementary Methionine on Labeling Patterns of Sulfur and 4-Carbon Moieties of Cystathionine and Products*

Control (experiment 1) and 2 μM methionine-supplemented (experiment 2) plants were grown on $^{35}SO_4^{2-}$ of specific activity 27,000 dpm/nmol. Respective doubling times of 55.1 and 51.2 h, number of doublings of 3.7 and 4.0, frond/colony ratios of 3.0 and 2.7, and protein/frond of 7.7 and 6.3 μg were obtained in experiments 1 and 2. Control plants (experiment 1) contained the following amounts of protein amino acids (nmol/frond): cysteine (0.7), methionine (1.8), aspartate (8.8), threonine (5.1), serine (5.7), glutamate (8.7), proline (4.8), glycine (9.2), alanine (8.7), and valine (6.1). Supplementation with methionine (experiment 2) decreased the corresponding amount of each protein amino acid by a mean of 27% (SE, 0.6). Control (experiment 3) and methionine-supplemented (experiment 4) plants were grown on [U-^{14}C]sucrose with respective specific activities of 46.9 and 183 dpm/nmol. Respective doubling times of 36.5 and 45.3 h, number of doublings of 4.5 and 4.5, and frond/colony ratios of 3.5 and 2.9 were obtained in experiments 3 and 4. ND, not determined.

Component	Addition to Medium			
	$^{35}SO_4^{2-}$		[U-^{14}C]Sucrose	
	None (Exp. 1)	Methionine (Exp. 2)	None (Exp. 3)	Methionine (Exp. 4)
	radioactivity (% of control[a])			
Cystathionine and products				
Protein methionine	97.0	7.3[b]	100.0[c]	24.9
Soluble methionine	0.3	10.5[b]	<1.2[d,e]	43.2
AdoMet	1.1	1.1	ND (<1)[f]	3.2
S-Methylmethioninesulfonium salt	1.5	2.1	ND (<1)[f]	8.8
Cystathionine	<0.1	<0.1	<3.1[d]	<1.1[d]
Homocysteine	<0.4	<0.5		
AdoHcy	<0.01	<0.02	<1.3[d]	<2.2[d]
Total	99.9[g]–100.4	21.0–21.6	100.0–107.6	80.1–83.4
Uncharacterized compounds				
Volatile sulfur	0.01	0.03		
Organic sulfur in plants				
Amino acids	10.1	9.6		
Nonamino acids	4.2	4.3		
Organic sulfur in medium				
Amino acids	0.5	0.2		
Nonamino acids	0.7	1.1		
Total	15.51	15.23		
Total	115.4–115.9	36.2–36.8		

[a] Values for ^{35}S accumulations were calculated as follows: (a) Radioactivity in each compound was expressed on the basis of radioactivity in protein cysteine in the same sample. (b) Each value so determined was then expressed as a percentage of the sum of values in protein methionine, soluble methionine, AdoMet, and *S*-methylmethioninesulfonium salt in corresponding control plants; the negligible upper limits of ^{35}S accumulating in cystathionine, homocysteine, and AdoHcy were not included in the summation. Values for ^{14}C accumulation were calculated as follows: (a) ^{14}C in the 4-carbon moiety of each compound was expressed on the basis of protein [^{14}C]threonine in the same sample. (b) Each value so determined was then expressed as a percentage of that for protein methionine in controls plants. ^{14}C in cystathionine and its soluble products in control plants was not included in this calculation because even the small amounts reported were gross overestimates (see Footnote 'd').

[b] Protein methionine and soluble methionine had the same specific activity (1500 dpm/nmol), indicating complete mixing of methionine taken up from the medium with that synthesized by the plant.

[c] Ratio of ^{14}C in 4-carbon/methyl = 3.0. The reason for this nonuniform distribution of ^{14}C is not clear, and is discussed in a separate report (J. Giovanelli, S. H. Mudd, in preparation).

[d] Upper limit based on total ^{14}C in partially purified compound. Since the total ^{14}C was negligible, ^{14}C localized in the 4-carbon moiety was not determined.

[e] Purified only as far as the sulfoxide derivative; upper limit is based on total ^{14}C in this derivative.

[f] No ^{14}C was detected in 4-carbon moiety. Maximal amount that could have escaped detection shown in parentheses. The sensitivity of detection of ^{14}C in this experiment was less than that of ^{35}S in experiment 1 because of the low specific activity of [^{14}C]sucrose relative to that of $^{35}SO_4^{2-}$.

[g] The expected value of 100.0 was not obtained because individual values were rounded off to first decimal place.

supplementation with 2 μM methionine must have reduced accumulation of radioactivity into cystathionine and its products to approximately 32% (36.8 × 100/115.9) of that in control plants.

In summary, these combined ^{35}S-labeling patterns showed that methionine supplementation greatly reduced accumulation of sulfate sulfur into cystathionine and products without appreciably affecting that into protein cysteine. These results suggest that methionine regulates its own *de novo* synthesis, and that the locus of this regulation is the conversion of cysteine to cystathionine, *i.e.* at the step catalyzed by cystathione γ-synthase. Since equivalent amounts of sulfur (from sulfate) and 4-carbon moieties (from *O*-phosphohomoserine) enter the transsulfuration pathway during cystathionine synthesis, we sought to confirm this suggestion by demonstrating a comparable inhibition by supplementary methionine of the *de novo* synthesis of the 4-carbon moiety of cystathionine and products.

Table I (experiments 3 and 4) shows the results obtained when plants were grown to isotopic equilibrium with [U-^{14}C]sucrose, the dominant carbon source of *Lemna* growing under the standard conditions used (8), either in the absence or presence of supplementary 2 μM methionine. By analogy with the basis for expression of ^{35}S labeling patterns, accumulation of ^{14}C was corrected for possible differences in protein content and in specific activity of the 4-carbon precursor of cystathionine, *i.e.* *O*-phosphohomoserine. Since in plants, *O*-phosphohomoserine is a common precursor for cystathioine and threonine synthesis (15), amounts of ^{14}C in the various compounds were normalized to the corresponding amount of protein [^{14}C]threonine in the same plant sample. Equal amounts of ^{14}C accumulated in protein threonine in control (109 dpm/frond) and methionine-supplemented (113 dpm/frond) plants, after adjustment was made for the 3.9-fold higher specific activity of [^{14}C]sucrose fed to the latter plants. Each of these normalized values was then expressed as a percentage of the sum of such values for cystathionine and its products in control plants. Surprisingly, methionine supplementation caused only a small decrease (to approximately 80% of control plants) in ^{14}C accumulation, compared to the major reduction in ^{35}S accumulation.

These combined results, therefore, establish a divergence in the effect of supplementary methionine on *de novo* accumulation of sulfur and 4-carbon moieties into cystathionine and products. The nature of the divergence suggested that 4-carbon units were entering cystathionine and/or its products via one or more pathways in addition to that catalyzed by cystathionine γ-synthase. This suggestion has now been confirmed by the demonstration that in *Lemna* the 4-carbon moiety of methionine is derived not only from *O*-phosphohomoserine via transsulfuration, but also from the ribose carbons of ATP via AdoMet and 5′-methylthioadenosine[5] (17).

Together, these findings made it clear that the more accurate assessment of the flux of sulfur (and 4-carbon moieties) into the transsulfuration pathway is provided by the amount of $^{35}SO_4^{2-}$ that accumulates in cystathionine and its products, rather than by the corresponding measurements with ^{14}C. The ^{35}S labeling patterns were, therefore, further explored using cultural conditions modified in two ways (Table II). First, the specific activity of $^{35}SO_4^{2-}$ was reduced, since separate experiments with control plants (data not shown) indicated that the high specific activity of $^{35}SO_4^{2-}$ in experiments 1 and 2 of Table I had probably caused the slow growth rates (doubling times greater than 50 h) of these plants. Doubling times of 38.6 and 36.0 h for control and methionine-supplemented plants, respectively, were observed when the specific activity of $^{35}SO_4^{2-}$ was reduced to 5300 dpm/nmol (legend to Table II. These doubling times are within the normal range of 34.7 to 43.0 h for control plants (8). Second, the concentration of methionine was reduced to 0.67 μM. This concentration has a minimal effect on growth (5, 16; legend to Table II), but still provides for uptake of methionine in an amount exceeding the total (soluble + protein) accumulating in control plants (4), and increases the pool sizes of soluble methionine, *S*-methylmethioninesulfonium salt and AdoMet, albeit to a lesser extent than does 2 μM methionine (5).

Table II. *Effects of 0.67 μM Supplementary Methionine on Sulfur-Labeling Patterns*

Control and 0.67 μM methionine-supplemented plants were each grown in triplicate with $^{35}SO_4^{2-}$ of specific activity 5300 dpm/nmol. Respective mean doubling times of 38.6 h (0.7) and 36.0 (0.7), number of doublings of 4.1 (0.07) and 4.0 (0.05), and frond/colony ratios of 3.8 (0.03) and 3.6 (0.09) were obtained. Values in parentheses are SE. ^{35}S in compounds was expressed as described in Table I.

Compound	Addition to Medium	
	None	Methionine
	radioactivity (% of control)	
Cystathionine and products		
Protein methionine	97.4 (0.09)	14.3 (0.5)
Soluble methionine	0.5 (0.00)	3.9 (0.6)
AdoMet	0.95 (0.04)	0.8 (0.1)
S-Methylmethioninesulfonium salt	1.2 (0.05)	2.4 (0.4)
Total	100.0	21.4 (0.8)

The ^{35}S-labeling patterns obtained for plants supplemented with 0.67 μM methionine were very similar to those reported for 2 μM methionine. Control plants contained 3040 dpm/frond (SE, 18) in protein cysteine compared to 2730 dpm/frond (SE, 140) for methionine-supplemented plants, showing that supplementation with 0.67 μM methionine had little or no effect on accumulation of sulfate sulfur into protein cysteine. For determination of the effect of 0.67 μM methionine on ^{35}S-labeling patterns of cystathionine and products (Table II), only those compounds shown (Table I) to contribute significantly were analyzed. Accumulation of ^{35}S into cystathionine and products was decreased by 0.67 μM methionine to 21% that of control plants. Assay of triplicate cultures showed that this value is quite reproducible (SE, 0.8 percentage units). There was a less pronounced increase in soluble [^{35}S]methionine and decrease in protein [^{35}S]methionine in plants supplemented with the lower concentration of methionine, consistent with the smaller pool size of soluble methionine in these plants.

Our studies on $^{35}SO_4^{2-}$ incorporation may now be interpreted as providing definitive evidence, for the first time in a higher plant, that methionine does indeed feedback regulate its own *de novo* synthesis *in vivo*, and that cystathionine synthesis is a locus of this regulation. Recent studies have provided some insights into the molecular mechanism for this regulation. The elevated concentrations of methionine, AdoMet and *S*-methylmethionine sulfonium salt in methionine-supplemented plants are consistent with one or more of these compounds being effectors in regulation of the cystathionine γ-synthase step, but do not exclude the participation of other effectors. No evidence for feedback inhibition of *Lemna* cystathionine γ-synthase activity was obtained with a variety of potential feedback inhibitors, including those listed above (31). Specific activities of cystathionine γ-synthase in *Lemna* grown with 0.67 or 2 μM methionine were reduced to 15% that of control plants (31). Down-regulation of this enzyme

[5] Accumulation of the 4-carbon moiety of methionine via 5′-methylthioadenosine appeared not to be regulated by methionine (J. Giovanelli, S. H. Mudd, A. H. Datko, in preparation), as expected from the divergent labeling patterns of ^{35}S and ^{14}C.

should contribute to the decreased flux into cystathionine reported here. However, studies with propargylglycine, a mechanism-based irreversible inhibitor of this enzyme, showed that down-regulation of cystathionine γ-synthase, by itself, is not adequate to account for this decreased flux (30). Additional factors must be involved. Some of the factors currently being examined include the potent stimulation of threonine synthase by AdoMet (18, 21), and the synergistic effect of AdoMet and lysine on the feedback inhibition of aspartokinase (28). The recent isolation of plant variants with elevated levels of soluble methionine associated with increased activity (19) or feedback desensitization (1, 20) of the lysine plus AdoMet-sensitive aspartokinase suggests that regulation at this step may normally be important *in vivo* in adjusting the amounts of *O*-phosphohomoserine synthesized from aspartate to changes in the amounts of *O*-phosphohomoserine utilized for transsulfuration.

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