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Sulfur-containing Compounds in *Lemna perpusilla* 6746 Grown at a Range of Sulfate Concentrations

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ABSTRACT

***Lemna perpusilla* 6746, grown photoautotrophically at a series of sulfate concentrations ranging from 0.32 to 1,000 μM, was labeled to radioisotopic equilibrium with $^{35}SO_4^{2-}$. Sulfur-containing compounds were isolated and purified from the colonies. Radioactivity in each compound was a measure of the amount of that compound present in the tissue. The following compounds were identified and quantitated: inorganic sulfate, glutathione, homocyst(e)ine, cyst(e)ine, methionine, S-methylmethionine sulfonium, S-adenosylmethionine, S-adenosylhomocysteine, cystathionine, chloroform-soluble (presumed to be sulfolipid), protein cyst(e)ine, and protein methionine. γ-Glutamylcyst(e)ine, *erythro*- and *threo*-thiothreonine, and S-methylcysteine were not detected. No volatile ^{35}S compounds were formed during plant growth at 1,000 μM sulfate, nor were significant amounts of ^{35}S compounds excreted into the medium.**

The amount of each component present in colonies grown over the 3,000-fold range of medium sulfate was relatively constant except for inorganic sulfate. This increased about 30-fold from the lowest to the highest medium sulfate concentration. The total soluble sulfur amino acids increased about 1.5- to 2-fold, due primarily to an increased amount of glutathione. Protein cyst(e)ine and protein methionine were the major organic sulfur compounds in *Lemna*, and the amounts of these compounds remained virtually constant despite the variation in external sulfate concentration.

Procedures for the analysis of S-adenosylmethionine, S-methylmethionine sulfonium, and S-adenosylhomocysteine are presented.

Recent work in this laboratory has led to the development of a phytostat which permits the growth of substantial amounts of vegetatively reproducing small aquatic plants under conditions such that any specified nutrient may be maintained at a relatively very low, but constant, concentration. The use of this apparatus to grow *Lemna perpusilla*[2] at a series of low, constant concentrations of inorganic sulfate is described in the accompanying paper (3). As a result of these experiments, samples of *Lemna* were available which had been labeled to isotopic equilibrium with $^{35}SO_4^{2-}$ at concentrations of sulfate in the medium covering almost a 3,000-fold range. In the present paper we report modifications and extensions of previous analytical methods (5, 6) which permit quantitative determination of the major sulfur-containing compounds in these samples. The effects of variation in growth medium sulfate concentration upon the accumulation of these compounds will be described.

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[2] According to a recent publication of W. S. Hillman (7) from whom we obtained this strain, *Lemna perpusilla* Torr. 6747 is now designated *Lemna paucicostata* Hegelm. 6746.

MATERIALS

Biological Materials. The conditions for maintenance of stock cultures of *L. perpusilla* 6746 and for the photoautotrophic growth of experimental colonies in both batch and semicontinuous culture in the phytostat are described in the accompanying paper (3). Cultures were examined for the possible presence of contaminating microorganisms (1) at the time of harvest. No contaminants were found in the cultures used here.

Chemicals. The method of preparation of 5′-methylthioadenosine, either from S-adenosyl-L-methionine or from S-adenosyl-L-[*methyl*-^{3}H]methionine (Amersham/Searle) has been described (5). Sodium [^{35}S]thiosulfate (outer S labeled) was from New England Nuclear and S-[*methyl*-^{14}C]methylmethionine sulfonium was from ICN. The sources of other chemicals have been described (5).

METHODS

Methods for chromatography on Dowex 50-H^+, paper chromatography, and paper electrophoresis have been described (1, 5, 6). Solvents used for paper chromatography included solvent A, 2-propanol-88% formic acid-H_2O (7:1:2, v/v/v); solvent B, 1-butanol-acetic acid-H_2O (12:3:5, v/v/v); solvent C, methanol-pyridine-1.25 N HCl (37:4:8, v/v/v); solvent D, 2-propanol-88% formic acid (6:4, v/v); solvent E, isobutyric acid-0.5 N NH_4OH (5:3, v/v); and solvent F, ethanol-pyridine-15.1 N NH_4OH-H_2O (6:2:0.8:1.2, v/v/v/v). Solvents for electrophoresis included solvent G, 0.05 M pyridine-0.32 M formic acid (pH 2.9); and solvent H, 0.46 M formic acid (pH 1.9).

HARVESTING OF COLONIES

Colonies were transferred from the culture vessel to a holding solution, the chemical composition of which was identical to that used in the growth medium, where they were counted. With the exception of colonies grown at the lowest sulfate concentration, the holding solutions contained no ^{35}S. For colonies grown in 0.32 μM sulfate, the holding solution contained $^{35}SO_4^{2-}$ of the same specific radioactivity as that used in the growth medium. This procedure was adopted as a precaution against the possibility that the pool sizes of soluble sulfur compounds in colonies grown in 0.32 μM sulfate might be reduced such that significant losses of ^{35}S in these pools could occur if the colonies were subjected to a "chase" of unlabeled sulfate during counting. After the colony count, the holding solution was aspirated and the colonies rapidly washed with a nonradioactive solution chemically identical to that used in the growth medium. The solution used for washing was aspirated just prior to killing. In order to minimize displacement from steady-state conditions, the time between removal of the colonies from the growth vessel and killing of the colonies was kept as short as possible and conditions of light and temperature were maintained the same as those employed during growth.

EXTRACTION OF TISSUES AND ASSAY OF ^{35}S COMPOUNDS

Initially analyses were performed on samples of colonies grown in 0.32, 4.5, and 1,000 μM sulfate by a procedure which requires a series of solvent extractions in order to measure chloroform-soluble compounds. When values for chloroform-soluble compounds were not required, a simplified extraction method was used. The simplified procedure also incorporated new methods for analysis of S-methylmethionine sulfonium, S-adenosylmethionine, and S-adenosylhomocysteine that were developed during the course of our studies. Colonies assayed by the simplified procedure were those grown in 14.3 μM sulfate and a second sample grown in 1,000 μM sulfate. The amounts of ^{35}S-amino-acids determined by the two procedures were in good agreement, as demonstrated by the separate assays of colonies grown in 1,000 μM sulfate (see Table II). All tissue extractions were performed at 0 to 4 C unless stated otherwise.

INITIAL PROCEDURE FOR EXTRACTION OF TISSUE AND ASSAY OF ^{35}S COMPOUNDS

Extraction of Tissue. Plants were killed by addition of 1 ml of boiling 80% ethanol-0.01 M acetic acid/colony. To the suspension of colonies in ethanol-acetic acid was added 20 μmol of H_2SO_4 and 100 nmol each of the sulfur amino acid carriers previously described (5). The liquid was decanted from the colonies, which were transferred to a graduated tissue grinder and homogenized with 10 ml of 80% ethanol-0.01 M acetic acid. The homogenate was centrifuged to yield a supernatant fluid, which was combined with the decanted solution described above. The pellet resulting from the centrifugation was homogenized, first with 10 ml of 80% ethanol-0.01 M acetic acid, then with 10 ml of methanol-chloroform-water (60.25:15, v/v/v). The supernatant solution obtained from each homogenization was also combined with the decanted solution described above, and the combined soluble extract was reduced in volume to approximately 0.5 ml. This solution was then diluted with water to a final volume of 10 ml, and clarified by centrifugation. The supernatant fraction (supernatant 1) was decanted, and the small pellet combined with the main pellet fraction described above. The combined pellet fraction was then incubated with DTT (5). The pellet obtained after DTT treatment and precipitation with 5% trichloroacetic acid is pellet P. The soluble fraction was obtained by combination of the supernatant solutions obtained after DTT incubation with supernatant 1, and extraction with ether to remove trichloroacetic acid.

An aliquot of the soluble fraction was titrated with 1 M NH_4OH to a pH of 5, 0.1 ml of 1 M ammonium acetate (pH 5.0) was added, and the mixture incubated in a boiling water bath for 10 min. This procedure converts the relatively unstable compound S-adenosylmethionine to 5'-methylthioadenosine (12), and was adopted as a precaution against the uncontrolled degradation of S-adenosylmethionine to products that might possibly interfere with the assay of other ^{35}S compounds. The heated aliquot was fractionated into the chloroform-soluble fraction and water-soluble fraction by addition of methanol and chloroform so that the final ratio of methanol to chloroform to water was 1:4:5. After shaking and separation into two phases, the methanol-water phase was removed and the chloroform phase and interfacial emulsion was washed with 1.5 volumes of water. The water wash was combined with the methanol-water fraction to yield the water-soluble fraction.

Analysis of Water-soluble Compounds. In brief, the soluble fraction was separated into "non-amino-acid" and "amino acid" fractions. The latter was further separated, after performate oxidation, into "acidic oxidation products" (containing the sulfonic acids derived from glutathione, homocyst(e)ine, cyst(e)ine, γ-glutamylcyst(e)ine, *erythro*-thiothreonine, and *threo*-thiothreonine) and "neutral oxidation products" (containing S-methylmethionine sulfonium and the sulfones of methionine and S-methylcysteine) (5). Any 5'-methylthioadenosine formed from S-adenosylmethionine would also be present in the neutral oxidation products, but this fraction was not used for estimation of S-adenosylmethionine since it was not established that 5'-methylthioadenosine could be recovered quantitatively after performic acid oxidation and subsequent purification steps. Although cystathionine could also be assayed in the neutral oxidation products, a more satisfactory procedure was to assay this compound in its unoxidized form (5). S-Adenosylhomocysteine was assayed along with cystathionine in an unoxidized aliquot of the water-soluble fraction. Recoveries in these procedures were monitored by the use of internal standards of ^3H-labeled authentic compounds (5).

Specific details of the determination of ^{35}S in the various compounds are as follows. Radioactivity in inorganic sulfate was determined in the non-amino-acid fraction essentially as previously described (5). The $^{35}SO_4^{2-}$ eluted from the electrophoretogram was shown to be free of [^{35}S]thiosulfate by chromatography for 48 hr with solvent E, in which sulfate moved with an R_F of approximately 1.4 relative to that of thiosulfate.

The acidic oxidation products were assayed using minor modifications of the described procedure (5). γ-Glutamylcysteic acid co-purified with glutathione sulfonic acid in the acetic acid electrophoresis step but the two compounds were well resolved when the glutathione sulfonic acid fraction (along with carrier γ-glutamylcysteic acid) was chromatographed in solvent D for 63 hr (mobility of γ-glutamylcysteic = 1.24 to 1.31 relative to that of glutathione sulfonic acid).

Erythro- and *threo*-thiothreonine sulfonic acids were purified by sequential acetic acid electrophoresis (5) and chromatography with solvent D, in which *erythro*-thiothreonine sulfonic acid moved with an $R_{\text{cysteic acid}}$ of 1.45 and *threo*-thiothreonine sulfonic acid moved with an $R_{\text{homocysteic acid}}$ of 1.45.

The neutral oxidation products were chromatographed in solvent A for 15 hr. [^{35}S]Methionine sulfone was further purified as described (5). Both S-methylmethionine sulfonium and S-methylcysteine sulfone, which would be contained in the neutral oxidation products, migrate with an $R_{\text{methionine sulfone}}$ of about 0.5 in this solvent. For each sample, this area was eluted and the eluates subjected to electrophoresis in solvent G. Two peaks of radioactivity which moved toward the cathode were obtained. (*a*) The faster migrating material which had moved 23 to 25 cm (*i.e.* the area in which S-methylmethionine sulfonium would be expected) was eluted and chromatographed with solvent B. In most instances, the ^{35}S in the S-methylmethionine sulfonium peak after this procedure was taken as a measure of this compound. For one sample the radiopurity of this material was further confirmed by electrophoresis in solvents G and H, and chromatography with solvents B, E, F and *t*-butanol-88% formic acid-H_2O (7:1.5:1.5, v/v/v) and ethanol-*t*-butanol-11.3 N NH_4OH-H_2O (75:25:1:18.8, v/v/v/v). (*b*) The more slowly migrating material which had moved 4 to 5 cm (*i.e.* the area in which S-methylcysteine sulfone was expected) was eluted and shown by sequential chromatography with solvent B, electrophoresis in solvent H and, if needed, chromatography with solvent F in each instance to be free of detectable ^{35}S-methylcysteine sulfone.

[^{35}S]Cystathionine and S-adenosyl[^{35}S]homocysteine were assayed in the water-soluble fraction. Aliquots of this fraction were treated with DTT and purified by Dowex 50-H^+ chromatography (5). S-Adenosylhomocysteine carrier (100 nmol) was added to the samples prior to their electrophoresis in solvent H for 1.7 hr. [^{35}S]Cystathionine was then measured as described (5) except that the eluates obtained after chromatography in solvent A (containing, in this case, 10 mM mercaptoethanol) were subjected to additional chromatography in acetone-0.5% (w/v) urea (3:2, v/v) containing 10 mM 2-mercaptoethanol. S-Adenosylhomocysteine, visualized in UV light, was located about 33 cm toward the cathode (*i.e.* about 5 cm ahead of cystathionine). The area of the electrophoretogram corresponding to the added S-adenosylho-

mocysteine was eluted and a known amount of S[*G*-^{3}H]adenosyl-L-homocysteine was added to the eluates. The eluates were subjected to chromatography in solvent A. Using the tritiated marker as a guide, the appropriate areas were eluted and the eluates were subjected to electrophoresis in solvent G. Using the tritiated marker as a guide, the appropriate areas were eluted and chromatographed in solvent E. The ratios of $^{35}S/^3H$, determined on the eluates after the last chromatographic step, were used to calculate the S-adenosyl[^{35}S]homocysteine contents of the original samples.

Determination of Protein [^{35}S]Cyst(e)ine and Protein [^{35}S]Methionine and Chemical Assay of Various Protein Amino Acids. Pellet P was oxidized with performic acid, acid-hydrolyzed, and the hydrolysate fractionated on Dowex 50-H$^+$ (5). Amino acids were recovered in the fraction retained by the column. By procedures previously described (5) [^{35}S]cysteic acid and [^{35}S]methionine sulfone were further purified.

α-Amino nitrogen was determined in the fraction of the protein hydrolysate that was retained by Dowex 50-H$^+$. The method of Smith *et al.* (21) was used, except that to remove NH_3 from the alkaline sample, it was placed in a boiling water bath and sparged with N_2 for 30 min. Chemical determinations of protein amino acids were performed with an amino acid analyzer (5).

SIMPLIFIED PROCEDURE FOR EXTRACTION OF TISSUE AND ASSAY OF ^{35}S COMPOUNDS

Extraction of Tissue. Colonies were frozen in liquid N_2 and ground to a powder. The powder was extracted successively with cold 5% trichloroacetic acid containing 20 μmol of H_2SO_4 and 100 nmol of each of the sulfur amino acid carriers (5) and cold 5% trichloroacetic acid. The pellet was resuspended in 4 ml of water. The suspension was extracted with ether to remove trichloroacetic acid, and incubated with DTT (5). The steps described in the initial procedure were then followed to yield pellet P and the soluble fraction.

Analysis of Water-soluble ^{35}S Compounds. For analysis of radioactivity in all compounds except S-adenosylmethionine, an aliquot of the soluble fraction was boiled as described for the initial procedure. The details described for the initial procedure were then followed for assay of radioactivity in all water-soluble compounds except S-adenosylmethionine and S-methylmethionine sulfonium. For analysis of S-adenosylmethionine, an aliquot of the soluble fraction was mixed with carrier S-adenosylmethionine (100 nmol) and S-adenosyl-L-[*methyl*-^{3}H]methionine, and electrophoresed for 1.25 hr in solvent G (Fig. 1A). Most of the ^{35}S migrated either toward the anode (inorganic sulfate) or no further than 15 cm toward the cathode (not shown in Fig. 1A). S-Adenosylmethionine migrated 27 to 29 cm toward the cathode. S-Methylmethionine sulfonium migrated 29 to 32 cm toward the cathode, resolving poorly from S-adenosylmethionine. Those paper strips containing the S-adenosyl[^{3}H]methionine marker were eluted with 0.01 M ammonium acetate (pH 5.5), and the eluate incubated in a boiling water bath as described for the initial procedure. Reelectrophoresis of the heated eluate (Fig. 1B) under the same conditions as described above yielded a major ^{35}S peak that migrated 7 cm toward the cathode and corresponded with ^{3}H marker. Concomitantly, most of the radioactivity disappeared from the 25- to 30-cm area. The peak at 7 cm was eluted with 24 mM formic acid and chromatographed with solvent A (Fig. 1C). Two peaks of radioactivity were obtained with R_F values of 0.31 and 0.56. Each of the two peaks corresponded with UV absorbing material derived from carrier S-adenosylmethionine added to the soluble fraction, and for any one sample had identical $^{35}S/^3H$ ratios. The compound at R_F 0.56 co-chromatographed with authentic 5'-methylthioadenosine (visualized by absorption of UV light). The compound at R_F 0.31 was formed in variable amounts, and probably consists predominantly of an oxidized product of

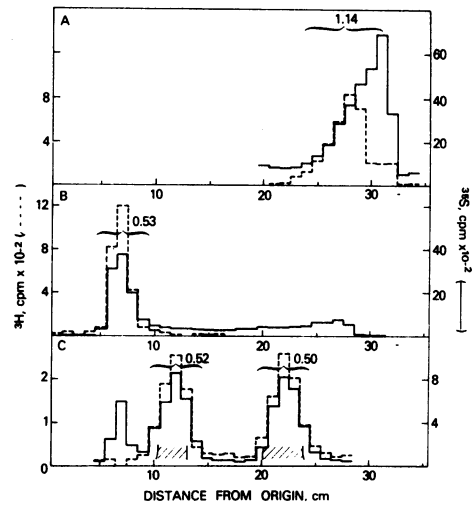

FIG. 1. Purification of S-adenosyl[^{35}S]methionine. S-Adenosyl[^{3}H]methionine and carrier S-adenosylmethionine were added to an aliquot of the soluble fraction from *Lemna* grown in 14.3 μM $^{35}SO_4^{2-}$, and the aliquot was electrophoresed in solvent G for 1.25 hr (Fig. 1A). Strips indicated by the brace were combined and eluted. The eluate was subjected to the boiling procedure described under "Methods" and then reelectrophoresed in solvent G for 1.25 hr (Fig. 1B). Strips indicated by the brace were combined and eluted, and the eluate was chromatographed in solvent A (Fig. 1C). (——): ^{35}S; (---): ^{3}H. Ratio of ^{35}S(dpm) to ^{3}H(dpm) for the eluate from each peak is indicated above each brace. Shaded areas of Fig. 1C illustrate UV absorbing material derived from carrier S-adenosylmethionine. UV absorbing material on the electrophoretograms (A and B) could not be visualized because of the presence of residual pyridine.

5'-methylthioadenosine.[3] Calculation of the amount of S-adenosyl[^{35}S]methionine was based on the amount of ^{3}H added as S-adenosylmethionine and the ratio of $^{35}S/^3H$ in the peak corresponding to 5'-methylthioadenosine after chromatography with solvent A.

For assay of S-methylmethionine sulfonium, an aliquot of the neutral oxidation products, obtained as described for the original procedure, was evaporated to dryness, dissolved in 1 ml of 0.01 M ammonium acetate (pH 5.5) and applied to a column of Dowex 50 which had been equilibrated with the same buffer. At a pH of 5.5, S-methylmethionine sulfonium has one net positive charge, and is retained by the column. All other compounds in the neutral oxidation products are not retained and are washed through the column with 4 ml of the buffer to yield fraction F. [^{35}S]Methionine sulfone is present in this fraction, and may be assayed as described in the original procedure. The column was eluted with 5 ml of 3 N NH_4OH to yield fraction E, containing S-methylmethionine sulfonium. Fraction E was electrophoresed in solvent G for 1 hr. Of the total ^{35}S in this fraction, 88% or more migrated in one peak 24 cm toward the cathode. The ^{35}S compound co-migrated with authentic S-methylmethionine sulfonium both during the above electrophoresis, and during chromatography in solvent A (mobility of S-methylmethionine sulfonium = 0.67 relative to that of methionine sulfone). The amount of [^{35}S]methylmethionine sul-

[3] Evidence that the compound of R_F 0.31 is predominantly an oxidized product (probably the sulfoxide) of 5'-methylthioadenosine is as follows. (*a*) The amount of radioactivity in the compound at R_F 0.31 relative to that in 5'-methylthioadenosine was reduced to some extent when 5'-methylthioadenosine was eluted from the electrophoretogram with 10 mM 2-mercaptoethanol or when additional carrier 5'-methylthioadenosine was added to the eluate. In subsequent experiments, 10 mM 2-mercaptoethanol has also been incorporated into the solvents used for electrophoresis and chromatography of 5'-methylthioadenosine. (*b*) At least 70% of the radioactivity in the compound at R_F 0.31 was converted to 5'-methylthioadenosine by incubation with 1 M HI (2). This procedure reduces sulfoxides (20) but not sulfones (2, 19).

fonium was calculated as: (radioactivity in fraction E relative to that in the neutral oxidation products) × (proportion of radioactivity in fraction E that co-migrated with S-methylmethionine sulfonium during the electrophoresis).

Determination of Total Organic [^{35}S]Sulfur in Culture Medium. An aliquot of the 14.3 μM medium in which *Lemna* were grown was examined for the presence of organic ^{35}S. All radioactivity in the medium that was not precipitated as $BaSO_4$ was assumed to be present as organic sulfur. To an aliquot of 40 ml of culture medium was added 0.7 mmol of carrier Na_2SO_4 and 0.1 ml of 12 N HCl. The solution was titrated with $BaCl_2$ and the resulting precipitate of $BaSO_4$ removed by filtration (13). The filtrate contained 0.9% of the original radioactivity. Additional carrier Na_2SO_4 (0.7 mmol) was then added to the filtrate, and the precipitation of $BaSO_4$ repeated. The second filtrate contained 0.5% of the original radioactivity. The relative constancy of the amounts of radioactivity recovered in the two filtrates indicates that precipitation of $^{35}SO_4^{2-}$ was essentially quantitative. The quantitative precipitation of $^{35}SO_4^{2-}$ as $BaSO_4$ was confirmed by a parallel experiment in which the procedure described above was repeated on a sample of the medium to which a 50-fold excess of radioactivity had been added in the form of $H_2{}^{35}SO_4$.

Determination of ^{35}S-Amino-Acids in Medium. Aliquots (100 ml) of the 14.3 μM and 1,000 μM (sample B) medium in which the *Lemna* were grown were examined for the presence of ^{35}S-amino-acids. To each aliquot was added 100 nmol each of the sulfur amino acid carriers (5) and 100 nmol K_2SO_4. The samples were centrifuged and the supernatant fluid was subjected to boiling as described for the initial procedure. The samples were diluted to 180 ml and passed through Dowex 50-H^+ columns (1.5 × 5.6 cm). After a wash with 25 ml of water, each column was eluted with 30 ml of 3 N NH_4OH to yield an amino acid fraction. To insure complete removal of inorganic $^{35}SO_4^{2-}$ each fraction was taken to dryness, dissolved in water with 1,000 nmol of carrier H_2SO_4, and chromatographed again on Dowex 50-H^+ columns, (0.9 × 2.9 cm). After a wash with 7 ml of water, each column was eluted with 5 ml of 3 N NH_4OH to yield a medium amino acid fraction. The amount of radioactivity in this fraction was used to calculate the amount of ^{35}S-amino-acids excreted into the medium.

DETERMINATION OF VOLATILE ^{35}S COMPOUNDS

A batch culture, similar to the 1,000 μM sulfate cultures described in Table I, was used to determine the amount of volatile ^{35}S compounds formed by *Lemna* grown under the conditions used here. Colonies were grown for 9 days in medium containing 1,000 μM $^{35}SO_4^{2-}$ (3,250 dpm/nmol). The culture was bubbled with 1% CO_2 in air, and the effluent gas was passed through 50 ml of 5% (w/v) mercuric acetate. At the time of harvest radioactivity was measured in aliquots of the mercuric acetate. After the colonies were harvested the medium was brought to pH 1.9 with formic acid to permit the volatilization of any residual H_2S and mercaptans, and it was bubbled for a further 2 to 3 hr. The effluent gas was passed through 30 ml of fresh 5% (w/v) mercuric acetate, and radioactivity was determined in aliquots of the mercuric acetate.

RESULTS

Lemna was cultured in media which varied in sulfate content from 0.32 to 1,000 μM. Table I summarizes the data concerning growth conditions and exposure to radioactive sulfate. Each sample analyzed had been grown at the specified concentration of $^{35}SO_4^{2-}$ for a time sufficient to permit at least three doublings, so that in each case isotopic equilibrium would have been approached closely.

Protein Content. In an effort to ascertain a reliable basis for expression of the results, the acid hydrolysate of the protein fraction of each sample was subjected to automated amino acid analysis. From these analyses, the values for eight protein amino acids were summed. These amino acids (Table I) were selected because each is recovered in good yield after performate oxidation and acid hydrolysis, and each yields a well defined and reproducible peak during routine analysis on the amino acid analyzer. For comparison, α-amino N determinations were carried out on the acid hydrolysates of the protein fractions from several samples. Values obtained by the two methods (Table I) had a relatively constant ratio (sum of eight amino acids/α-amino N = 0.49 ± 0.04 [SD]; range: 0.46–0.54), indicating that either of these parameters provided a reliable measure of the relative original protein content of the hydrolyzed samples.

When the protein contents of the various samples were expressed on a colony basis, there was a somewhat more than 2-fold variation in the resulting values (Table I). The two samples derived within a brief period from the same culture (4.5 μM sulfate after 3.4 or 5.8 doublings) agreed closely in protein per colony. On the other hand, the two samples grown at 1,000 μM sulfate (samples A and B) differed approximately 2-fold. Although the extraction methods used for the latter samples differed (see legend of Table I), control experiments indicated that these differing methods produced similar yields of insoluble protein when applied to paired subsamples of a single batch of *Lemna*. Samples A and B were produced in different experiments carried out 7 months apart, and it is concluded that undefined variables may have affected the growth conditions and led to these changes in protein per colony. In any case, protein content would appear to be preferable to colony number as a base upon which to compare the metabolic activities or chemical compositions of different samples, and the values for S-containing metabolites appearing in Table II have been reported on this basis.

Contents of S-containing Compounds. The concentrations of a variety of S-containing compounds in each *Lemna* sample are reported in Table II. Protein methionine, protein cyst(e)ine, and inorganic sulfate were the major sulfur-containing compounds, together accounting for 83 to 86% of total tissue sulfur. Soluble amino acids composed only 5.6 to 8.7% of total tissue sulfur. Among the amino acids, glutathione was the dominant component, with smaller amounts of cyst(e)ine, homocyst(e)ine, methionine, S-adenosylmethionine, and S-methylmethionine sulfonium being readily detectable. Cystathionine was barely detected in most samples. None was detected in the sample derived from the plants grown at 0.32 μM sulfate, presumably due to the lower sensitivity in this experiment in which relatively low specific radioactivity $^{35}SO_4^{2-}$ was used to label the plants. S-Adenosylhomocysteine also was barely detected in those samples in which it was sought.

γ-Glutamylcyst(e)ine was not detected in any of the present *Lemna* samples but was detected in samples of *Lemna* exposed to very high specific radioactivity $^{35}SO_4^{2-}$ for very short time periods (unpublished results). Thus, the γ-glutamyl cycle (9) may be operative in *Lemna* but the pool size of γ-glutamylcyst(e)ine appears to be too small to have been detected in the present experiments. In contrast, *threo-* and *erythro*-thiothreonine were neither detected when *Lemna* was grown to isotopic equilibrium (Table II) nor when *Lemna* was exposed to $^{35}SO_4^{2-}$ for very short time periods (unpublished results). Formation of these compounds from H_2S, reported to occur in peas (17, 18), may be negligible during growth in the absence of exogenous H_2S.

Neither adenosine-5′-phosphosulfate nor adenosine-3′-phosphate-5′-phosphosulfate would have been detected in these experiments as the phosphosulfate bond is unstable to the acid used in the extraction procedure (15).

Together, the determinations reported permit identificaion and quantitation of most of the tissue sulfur in the form of known compounds. In the three samples for which complete data are available, known compounds account for 87 to 90% of total tissue sulfur. Among the soluble subfractions, sulfate accounts for 46 to

Table I. *Culture of Lemna in Various Concentrations of Radioactive Sulfate*

Sulfate concn	Culture type	Specific radioactivity of sulfate	Doubling time	Number of doublings in $^{35}SO_4^{2-}$ medium at specified concn	Number of colonies analyzed	Protein content	
						8 selected amino acids[1]	α-amino N
μM		*dpm/nmole*	*hr*			*nmoles/colony*	
0.32	phytostat[2]	2470	53	3 at 0.37 μM or less	200	122[4]	263
4.5	phytostat[3]	20660	52	3.4 at 4.8 μM or less	57	207[4]	449
			54	5.8 "	218	232[4]	485
14.3	batch	29970	49	4	157	131[5]	—[6]
1000	batch, Sample A	33700	57	3.8	267	201[4]	372
1000	batch, Sample B	9200	49	4	157	98[5]	—

[1]Sum of values for glutamate, methionine (sulfone), glycine, alanine, valine, isoleucine, leucine, and phenylalanine.
[2]Experiment 3 described in Table I, accompanying paper (3).
[3]Experiment 1 described in Table I and Fig. 2, accompanying paper (3). The smaller colony sample was removed from the phytostat at day 11 and the larger at the termination of the experiment at day 16.
[4]Protein pellet prepared by precipitation with 80% ethanol-0.01 M acetic acid (initial method).
[5]Protein pellet prepared by precipitation with 5% trichloroacetic acid (simplified method).
[6]Not measured.

98% of the non-amino-acid fraction, the per cent rising as tissue sulfate rises due to increases in medium sulfate. Glutathione, homocyst(e)ine, and cyst(e)ine together account for 62 to 82% of the acidic oxidation products. In the neutral oxidation products fractions the identified compounds listed account for less than half of the total sulfur. A variety of unidentified compounds, which are minor insofar as their contribution to total tissue sulfur is considered, are probably present in this fraction.

Effects of Increasing Medium Sulfate Concentration. As the sulfate concentration was increased in the growth medium by approximately 3,000-fold, the *Lemna* responded by accumulating 2.3- to 3.8-fold more total tissue sulfur (Table II). This increase was almost entirely due to the accumulation of more inorganic sulfate (Fig. 2). Total protein sulfur amino acid concentrations were virtually unaffected. Among the soluble amino acids, glutathione and S-methylmethionine sulfonium may have undergone small rises in concentrations whereas homocyst(e)ine, cyst(e)ine, methionine, and S-adenosylhomocysteine concentrations were little changed. The net result was that the total tissue sulfur amino acid content was unaffected, within the error of our present measurements, by the very substantial rise in medium sulfate. Since the sulfur amino acids, together with inorganic sulfate, account for by far the predominant portion of total tissue sulfur, these observations suggest that the rate of formation of reduced organic sulfur is closely regulated so as to be unaffected by wide variations in medium sulfate concentration.

Lack of Excretion of Organic S-containing Compounds into Medium. To evaluate further the possibility that increases in external sulfate concentrations had negligible effects upon total reduced organic sulfur formation, excretions of organic sulfur into the growth media were examined. Nonsulfate sulfur excreted into the medium by plants growing in 14.3 μM sulfate amounted to about 1.0 nmol/μmol of eight protein amino acids, or only 1.5% of the total tissue sulfur amino acid content. In additional experiments, aliquots of spent media (14.3 and 1,000 μM, sample B) were fractionated into non-amino-acid and amino acid fractions. The amounts of radioactivity found in the media amino acid fractions were equivalent to 0.6 and 1.0 nmol of sulfur/μmol of eight protein amino acids for the 14.3 and 1,000 μM sulfate samples, respectively. These values are 0.9 and 1.5% of the sulfur amino acids accumulated by the plants. Together, these results indicate that excretion of organic sulfur-containing compounds into the medium by *Lemna* growing under our conditions is negligible compared to the amounts of organic sulfur-containing compounds accumulating within the plants.

Lack of Formation of Volatile S-containing Compounds. Wilson *et al.* (24, 25) reported that following sulfate administration a variety of plant tissues may emit volatile sulfur compounds (of which at least 50% is H_2S). Such emission was light-dependent. Conditions which brought about sudden rises in sulfate influxes enhanced the rates of emission which, maximally, were comparable to the maximal activities of several enzymes of sulfate assimilation in the plants. To determine whether a significant portion of the sulfur taken up by *Lemna* growing under our conditions was emitted in the form of volatile compounds, *Lemna* was grown photoautotrophically on 1,000 μM $^{35}SO_4^{2-}$ medium. The effluent gas from the culture was passed through mercuric acetate to trap volatile ^{35}S-containing compounds. At the end of the experiment it was found that radioactivity in the mercuric acetate was so low that its presence was equivocal. The maximum amount of ^{35}S deposited in the trapping agent was less than 0.22% of the ^{35}S incorporated by the plants into protein during the 9 days the culture grew in radioactive medium. Thus, under conditions which might be expected to promote the emission of volatile sulfur compounds (*i.e.* relatively high light intensity and high medium sulfate concentration) *Lemna* growing under our standard conditions emitted negligible amounts of volatile sulfur, as compared to the amounts assimilated and accumulated as organic sulfur compounds.

DISCUSSION

During the present work, our methods for extraction and analysis of the sulfur-containing constituents of plant tissues have been modified and extended. Killing of the plants by treatment with liquid N_2 rather than with boiling 80% ethanol-0.01 M acetic acid was introduced to avoid breakdown of S-adenosylmethionine, which is extremely heat-labile except under strongly acidic conditions (16). Extraction of the resulting frozen slurry with 5% trichloroacetic acid and removal of sulfhydryl compounds from protein-binding by treatment with DTT (5) result in an aqueous extract which is suitable for the analysis of all of the sulfur amino

Table II. *Sulfur-Containing Compounds in Lemna perpusilla 6746 Grown at a Range of Sulfate Concentrations*

A description of the growth of the *Lemna* samples is given in Materials and Table I. Radioactivity in each component present in the tissues grown in 0.32 μM, 4.5 μM and 1000 μM (sample A) sulfate was measured by the initial procedure described in Methods; that in tissues grown in 14.3 μM and 1000 μM (sample B) sulfate by the simplified procedure given in Methods, except that SO_4^{2-} in the sample grown at 14.3 μM sulfate was determined by the barium precipitation method. The amount of each component (in nmoles) was calculated by dividing the radioactivity in the component by the specific radioactivity of inorganic sulfate in the appropriate medium (see Table I). The values for each compound in the two samples grown at 4.5 μM sulfate (see Table I, samples at 3.4 and 5.8 doublings) agreed closely, and the means of these values are reported here. For the soluble components, no correction has been made for losses occurring during the initial steps (i.e. separation of the total soluble extract into "chloroform-soluble" and "aqueous soluble" fractions; of the total aqueous soluble fraction into "non-amino acid" and "amino acid" fractions; and of the "amino acid" fraction into "acidic oxidation products" and "neutral oxidation products"). In general these losses were small, as may be judged by comparison of the total amounts of sulfur in the original fractions with the sums of the amounts of sulfur in the recovered component subfractions.

Component	Sulfate in medium (μM)				
	0.32	4.5	14.3	1000 A	1000 B
	nmole/μmole of 8 protein amino acids				
Soluble	15	31	64	108	202
Chloroform-soluble	0.2	0.2	—[1]	0.02	—
Non-amino acid	7.1	22	42	92	166
SO_4^{2-}	3.3	19	41	86	159
Amino acid	5.8	5.7	10	8.6	11
Acidic oxidation products	2.9	3.6	8.6	5.0	9.2
Glutathione	1.9	2.8	—	2.8	—
Homocyst(e)ine	0.07	0.03	—	0.05	—
Cyst(e)ine	0.22	0.17	—	0.27	—
γ-Glutamylcyst(e)ine	n.d.(<0.04)[2]	n.d.(<0.12)	—	n.d.(<0.14)	—
threo-Thiothreonine	—	—	—	n.d.(<0.01)	—
erythro-Thiothreonine	—	—	—	n.d.(<0.01)	—
Neutral oxidation products	1.2	1.5	2.6	2.9	2.6
Methionine	0.35	0.24	0.28	0.20	0.21
S-Methylmethionine sulfonium	0.12	0.21	0.55	0.40	0.44
S-Methylcysteine	n.d.(<0.01)	n.d.(<0.01)	—	n.d.(<0.01)	—
Cystathionine	n.d.	0.02	—	0.01	—
S-Adenosylhomocysteine	0.01	0.01	—	0.01	—
S-Adenosylmethionine	—	—	0.38	—	0.30
Protein					
Cyst(e)ine	21	21	23	21	22
Methionine	31	32	35	26	33
Total sulfur	67	84	122	155	257
Total sulfur amino acid	58	59	68	56	66

[1] Not measured.
[2] Not detected. The values in parentheses indicate the maximum amount of the compound which could have been present and gone undetected.

acids described in this paper. We have not investigated whether the extract obtained by liquid N_2 and trichloroacetic acid treatment would be suitable for determination of chloroform-soluble material. If such a determination is desired, it may be that a parallel sample should be prepared by use of the more laborious procedure involving boiling 80% ethanol followed by chloroform-methanol-water extraction.

Methods for quantitative determination of three sulfur-containing compounds not analyzed by our previous procedures (5) were introduced during the present work. (*a*) The analysis of S-adenosylmethionine involves preliminary separation of this compound by electrophoresis at pH 2.9, followed by quantitative conversion to 5′-methylthioadenosine and reelectrophoresis at pH 2.0 to obtain a radiochemically pure derivative. The tendency of 5′-methylthioadenosine to oxidize could be alleviated to some extent by avoidance of oxidizing conditions.[2] If need be, the oxidation product could be reconverted to 5′-methylthioadenosine by treatment with HI.[2] (*b*) Two methods are described for the analysis of S-methylmethionine sulfonium. Although the two procedures yield results which are in close agreement (*cf.* samples A and B, 1,000 μM sulfate, Table II), the simplified procedure involving separation of S-methylmethionine sulfonium by chromatography on Dowex 50 at pH 5.5 will probably be found preferable for most future applications. (*c*) Analysis of S-adenosylhomocysteine was

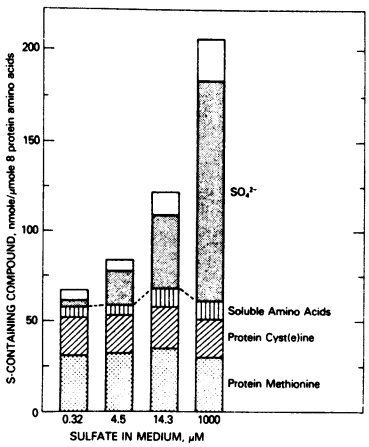

FIG. 2. Tissue contents of sulfur-containing compounds in *L. perpusilla* grown at several constant sulfate concentrations. Values are taken from Table II. Values for the 1,000 μM sample are the means of values from samples A and B. Dashed line connects the values for total sulfur amino acids. Total sulfur contents are indicated by the total heights of the bars. Differences between these values and those accounted for by the sums of protein methionine, protein cyst(e)ine, soluble amino acids, and SO_4^{2-} are due chiefly to losses during the early fractionation procedures.

best performed on an unoxidized aliquot of the water-soluble fraction to avoid degradation during performic oxidation. To obtain radiochemically pure preparations of S-adenosylhomocysteine it was necessary to purify this compound sequentially by Dowex 50-H^+ chromatography, electrophoresis at pH 1.9, and paper chromatography with two solvents.

Taken together, the methods described here permit identification and quantitation of the sulfur-containing compounds which constituted approximately 90% of the total tissue sulfur in the present samples (Fig. 2). Although a number of sulfur-containing compounds remain to be identified among the neutral oxidation products of the amino acid fraction, the present methods permit, also, the very sensitive quantitation of all of the compounds (cysteine, cystathionine, homocysteine, methionine, S-adenosylmethionine, S-adenosylhomocysteine, γ-glutamylcysteine, and glutathione) which are known to lie on the major pathways for flux from sulfide to the quantitatively major reduced sulfur-containing end products.

Our results with *Lemna* are compatible with the generalizations reached in studies of other plants (reviewed in 10 and 23). Under conditions of moderate or limited sulfate supply, most of the tissue sulfur is present as organic sulfur, and most of the organic sulfur is present in protein rather than soluble organic sulfur. When sulfate in the environment is increased, then inorganic sulfate often accumulates much more markedly than organic sulfate so that inorganic sulfate may come to represent a major portion of the total tissue sulfur.[4] The present study with *Lemna* extends previous studies by providing more comprehensive data on the individual sulfur-containing components and demonstrates a striking constancy in the rate of production of reduced organic sulfur in the face of an almost 3,000-fold variation in the medium sulfate. It is shown, also, that this constancy is not due to the secretion of excess organic sulfur-containing compounds into the medium, or to the volatilization of such compounds. These results, along with other available evidence, suggest at least two mechanisms which may play a role in the maintenance of the relatively constant rate of production of reduced organic sulfur. First, the data in this paper, and in the accompanying one (3), make it clear that total sulfate uptake by the plants was maintained relatively constant as sulfate concentration was changed in the medium. A 3,000-fold decrease in medium sulfate concentration led only to a 3- to 4-fold decrease in total tissue sulfur. This result is not unexpected in view of the frequently reported tendency of sulfate uptake by a variety of intact plants, plant parts, and cultured plant cells to approach a maximum as medium sulfate concentration is raised (11, 22). Second, as is also shown here, as less sulfate was taken up, a greater proportion of that available to the tissues was converted to organic sulfur. Reuveny and Filner (14) have recently shown that the ATP sulfurylase activity of cultured tobacco cells is markedly derepressed when the cells are provided an adequate source of nitrogen and limited in sulfate (and other sulfur sources). Should the ATP sulfurylase of *L. perpusilla* prove to be regulated in a similar manner, it maybe that as cellular uptake of sulfate decreases, an increase in ATP sulfurylase compensates, and serves to channel a greater portion of the available sulfate into the pathway leading to sulfate reduction and organic sulfur formation.

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[4] There are exceptions to these generalizations. For example, white mustard plants are reported to form nonprotein organic sulfur compounds in amounts in excess of protein sulfur (8); in brown mustard both protein and inorganic sulfur decreased in parallel as sulfate became limiting (4); and clovers showed little capacity to accumulate inorganic sulfate even when provided with high amounts of sulfate (10).

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