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In Vivo Metabolism of 5′-Methylthioadenosine in *Lemna*

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ABSTRACT

Evidence is presented that *Lemna* converts 5′-methylthioadenosine (MTA) to methionine. The methylthio moiety and four of the ribose carbons of the nucleoside contribute the methylthio and the four-carbon moieties of methionine. Plants grown in the presence of inhibitors which block methionine biosynthesis convert MTA to methionine at a rate sufficient to sustain normal growth (at least 4.4 nanomoles per colony per doubling with a molar yield of at least 65%). The pathway for conversion is shown to be constitutive in plants grown in standard medium and to function at a rate sufficient to dispose of MTA arising as a result of polyamine synthesis, and to explain the observed rate (1.4 nanomoles per colony per doubling) of preferential recycling of methionine sulfur (Giovanelli, Mudd, Datko 1981 Biochem Biophys Res Commun 100: 831–839). Rapid entry of methionine methyl into *S*-adenosylmethionine and phosphorylcholine was observed for plants grown in standard medium. Adenine generated during this cycle is efficiently salvaged into ADP and ATP.

Conversion of MTA to methionine completes the steps in methionine thiomethyl recycling (Giovanelli, Mudd, Datko 1981 Biochem Biophys Res Commun 100: 831–839) in which the sulfur of methionine is retained while the four-carbon moiety is not. The findings further show that the four-carbon moiety of methionine can be derived via the ribose moiety of MTA in addition to the established route from *O*-phosphohomoserine via transsulfuration. Previous observations (Giovanelli, Mudd, Datko 1980 Biochemistry of Plants pp 453–505) can now be interpreted as establishing that exogenous methionine down-regulates its own net synthesis via the transsulfuration pathway.

We have previously demonstrated a cycle in *Lemna* in which the sulfur atom of methionine is separated from its four-carbon moiety (9). The sulfur is reincorporated into methionine, whereas the four-carbon moiety is not. We proposed that such preferential recycling of methionine sulfur (methionine thiomethyl recycling) might proceed in *Lemna* via MTA[2] as outlined in Figure 1. In *Lemna*, and probably most other plants, the major portion of MTA is generated in polyamine synthesis (9). The same general reactions as shown in Figure 1 also occur in animals (2), bacteria (19), and yeast (21); in these studies, it was shown that the methylthio and four ribose carbons of MTA are converted to the methylthio and four-carbon moieties, respectively, of methionine. Demonstration of this pathway in higher plants has been limited to ripening apples (27) and tomatoes (24). These tissues are specialized in evolving large amounts of ethylene, and produce MTA mainly as a by-product of ethylene synthesis (1, 26). Here we demonstrate for the first time the *in vivo* operation of such a pathway in a higher plant (*Lemna*) which evolves little or no ethylene (9).

MATERIALS

MTA was obtained from Sigma, and was recrystallized from hot water. MTR was prepared by acid hydrolysis of MTA (18). Growth media containing MTA and MTR were filter sterilized.

Radioactive Compounds. [Methyl-^{14}C]MTA and [methyl-^{3}H] MTA were prepared by incubation of appropriately labeled AdoMet in 0.2 M ammonium acetate (pH 5.0) containing 10 mM β-mercaptoethanol for 30 min at 100°C (20). The product was chromatographed in solvent A, and the peak of radioactivity corresponding to MTA was eluted with 10 mM β-mercaptoethanol, lyophilized to dryness, and the residue dissolved in water saturated with N_2.

[Adenosyl-U-^{14}C]MTA was prepared from [U-^{14}C]adenosine (200 μmol, 250 μCi) via the intermediate 5′-chloro-5′-deoxyadenosine, as described by Shapiro and Schlenk (21). Chromatography of the product in solvent A showed a single radioactive peak (R_F 0.71) that comigrated with authentic MTA; less than 0.4% of the radioactivity could have resided in oxidized MTA (R_F 0.35), adenosine (R_F 0.47), or adenine (R_F 0.59). Neither solvent A nor a variety of other solvents tested resolved MTA from 5′-chloro-5′-deoxyadenosine. The presence of any unreacted 5′-chloro-5′-[^{14}C] deoxyadenosine in the preparation was therefore estimated from the amount of radioactivity that migrated with the same R_F as MTA after oxidation with H_2O_2. Separate experiments showed that oxidation (on paper) with 30% H_2O_2 converted MTA to a compound (presumably the sulfoxide and/or sulfone) with a greatly reduced migration (R_F 0.35) in solvent A, while the migration of 5′-chloro-5′-deoxyadenosine remained unchanged. Using this procedure, it was found that 98% of the radioactivity of [adenosyl-U-^{14}C]MTA was converted to a slower migrating compound, indicating that the preparation could have contained a maximum of 2% 5′-chloro-5′-[^{14}C]deoxyadenosine.

[Methyl-^{3}H, adenosyl-U-^{14}C]MTA was prepared by mixing [methyl-^{3}H]MTA and [adenosyl-U-^{14}C]MTA.

[Methyl-^{14}C]MTR was prepared by acid hydrolysis of [methyl-^{14}C]MTA (18) and purified by paper chromatography in solvent A. The preparation was 97% radiopure as judged by chromatography in the above solvent (R_F 0.66); the only other detected peak of radioactivity migrated with an R_F of 0.38, and was tentatively ascribed to MTR sulfoxide.

All other radioactive compounds were obtained from Amersham-Searle or New England Nuclear. [^{3}H]Methionine sulfoxide was present in preparations of [methyl-^{3}H]methionine that had been stored for extended periods, and was characterized by its comigration with authentic methionine sulfoxide during paper chromotography in solvent B, and by its reduction to methionine by incubation with β-mercaptoethanol as described under "Radioactive Methionine Sulfoxide."

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[2] Abbreviations: MTA, 5′-methylthioadenosine; MTR, 5-methylthioribose; AdoMet, *S*-adenosylmethionine; PAG, DL-propargylglycine (*i.e.* DL-2-amino-4-pentynoic acid).

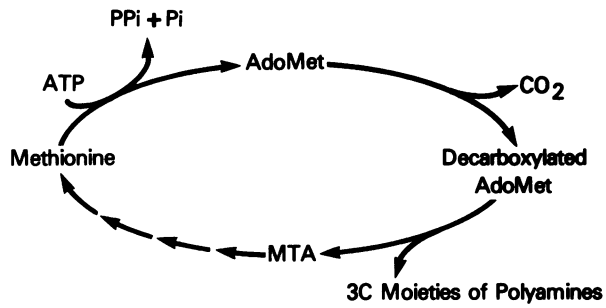

FIG. 1. Proposed sequence of reactions through which thiomethyl portion of methionine is recycled but four-carbon moiety is not.

METHODS

Chromatography. Chromatography on Dowex 50-H^+ was performed with columns 0.9 x 3 cm. The following solvents (containing 10 mM β-mercaptoethanol) were frequently used during paper chromatography: solvent A, 1-butanol:acetic acid:H_2O (12:3:5, v/v); solvent B, 1-butanol:propionic acid:H_2O (250:124:175, v/v).

Determination of Radioactivity. Radioactivity of aqueous samples and paper chromatograms was determined as previously described (10). Slurries containing TCA-insoluble material were first evaporated to dryness, dissolved in 0.2 ml of Protosol (New England Nuclear), and radioactivity determined after addition of 10 ml of Complete Counting Cocktail 3a20 (Research Products International). For determination of ^3H in the presence of Protosol, 100 μl of acetic acid was added to prevent chemiluminescence.

Growth Conditions. Cultures of *Lemna paucicostata* Hegelm. 6746 were grown mixotrophically with 20 μM inorganic sulfate under the standard conditions described previously (5).

Measurement of Uptake. Uptakes of MTA, amino acids, and MTR were studied over periods ranging from 10 min (in 10 ml of medium) to 24 h (in 300 ml of medium) by measuring accumulation of radioactive compounds in washed plants as described (4). Clearance (μl/frond · d) of MTA was determined as follows. Plants were preincubated for 2 h in medium containing either 0.4 or 250 μM MTA, then incubated for 2 h with 50 ml medium containing [methyl-^{14}C]MTA at the corresponding concentration. Radioactive medium was aspirated from the plants, which were then incubated under the same conditions for 2 h in 50 ml medium containing unlabeled MTA (0.4 or 250 μM). Less than 4% of the radioactivity associated with the plants was removed during this washing procedure. The amounts of radioactivity in these washed plants were used to calculate a mean clearance of MTA.

Products of MTA Metabolism. Plants incubated with [methyl-^3H, adenosyl-U-^{14}C]MTA were grown either under standard conditions, or pregrown for 6 d in 250 μM MTA under otherwise identical conditions. Incubations were initiated by aseptic aspiration of growth medium from the fronds and addition of growth medium supplemented with radioactive MTA. To terminate incubations, radioactive medium was aspirated and the plants washed two to six times with the (unlabeled) medium used for growth. Plants were collected on a Büchner funnel with Whatman No. 5 filter paper, and homogenized in 10% TCA containing 300 nmol each of carrier methionine and AdoMet, and 1 μmol of carrier MTA. The time required between aspiration of radioactive medium and homogenization in TCA is specified for each experiment in "Results."

Homogenates were centrifuged to yield TCA-insoluble and soluble fractions. The TCA-insoluble fraction was resuspended in 10% TCA, recentrifuged, and the supernatant solution combined with the soluble fraction. The TCA-insoluble fraction was suspended in water, and an aliquot removed for determination of radioactivity. Other aliquots were used for determination of radioactive lipid components (6).

The soluble fraction was extracted four times with ether, and chromatographed in solvent B. The amounts of radioactivity in TCA-soluble components were then determined from the chromatogram, as described below. In general, inasmuch as it was not possible to correct for any losses during elution of radioactivity from paper chromatograms and electrophoretograms, the amount of radioactivity in each compound was routinely calculated from the proportion of total radioactivity on the paper that was present in the particular compound. Separate experiments with plants incubated with [methyl-^{14}C]MTA showed that the values for radioactive methionine and its sulfoxide obtained by this method were in good agreement with corresponding values obtained after correction for any losses based on recovery of authentic tritiated compounds.

Radioactive Methionine, and Estimates of Maximal Radioactivity in MTA, Adenine, and MTR. After chromatography of each extract, a prominent radioactive peak was observed that migrated with an R_F of 0.59 to 0.74, corresponding to that of authentic methionine. Since methionine did not separate consistently from MTA, MTR, and adenine, the peak of radioactivity was eluted, oxidized with dimethylsulfoxide (14), and applied to a column of Dowex 50-H^+. Radioactivity not absorbed to Dowex 50-H^+ provided an estimate of the maximal amount of radioactive MTR; no attempt was made to characterize this radioactivity. The column was eluted with 10 ml of 3 N NH_4OH in order to recover radioactivity in methionine sulfoxide, and in any adenine and oxidized MTA. Essentially, the same results were obtained if fractionation on Dowex 50-H^+ was carried out before oxidation with dimethylsulfoxide. Chromatography of the oxidized products in solvent B showed one major peak of radioactivity that comigrated with authentic methionine sulfoxide (R_F 0.24–0.28). Radioactivity in this peak provided a measure of that originally present in methionine. The ratio of $^3H/^{14}C$ determined on an eluate of this peak was used to calculate the relative number of carbons in methionine that were derived from the methyl and adenosyl carbons of MTA. Minor peaks of radioactivity, when observed, corresponded to adenine (R_F 0.69–0.72) and oxidized MTA (R_F 0.52). Inasmuch as these minor peaks were not further characterized, radioactivity in them represents an upper estimate of radioactivity originally present in adenine and MTA.

Radioactive Methionine Sulfoxide. The peak of radioactivity that corresponded to methionine sulfoxide (R_F 0.24–0.28) during the initial chromatography in solvent B was eluted, reduced to methionine by incubation in 0.7 M β-mercaptoethanol in 10 mM K-phosphate (pH 7.5) for 1 h at 100°C, then rechromatographed in solvent B. One dominant peak of radioactivity was observed, migrating with authentic methionine (R_F 0.62–0.66). Radioactivity in the peak provided a measure of that previously present as methionine sulfoxide. The ratio of $^3H/^{14}C$ determined on an eluate of the peak was used to calculate the relative number of carbons in methionine that were derived from the methyl and adenosyl carbons of MTA.

Radioactive AdoMet. The peak of radioactivity that comigrated with carrier AdoMet during the initial chromatography with solvent B (R_F 0.15–0.17; detected by its UV absorbance) was eluted and degraded as described under "Radioactive Compounds." The resulting MTA and homoserine/homoserine lactone were resolved by paper chromatography in solvent B. Radioactivity that comigrated (R_F 0.80) with MTA (formed from carrier AdoMet and detected by UV absorbance) was a measure of the amount of radioactivity originally present in the MTA moiety of AdoMet. Radioactivity that migrated with an R_F of 0.30, corresponding to that of authentic homoserine, was a measure of radioactivity in the four-carbon moiety of AdoMet.

Radioactive Phosphorylcholine. Estimates of radioactive phosphorylcholine were made from the amounts of radioactivity present in peaks migrating with an R_F of 0.21 to 0.29 (solvent B),

corresponding to that of authentic phosphorylcholine. Radioactivity migrating in this region was characterized as phosphorylcholine in an experiment[3] in which control plants were incubated with 5 μM [methyl-^{14}C]MTA. Radioactivity migrated toward the anode at a rate corresponding to that of authentic phosphorylcholine during electrophoresis in 1.38 M triethanolamine chloride (pH 8.0) for 30 min at 44 v/cm and 4°C. After treatment with alkaline phosphatase, radioactivity comigrated toward the cathode with authentic [^{3}H]choline under the same conditions of electrophoresis.

Radioactive Adenine Nucleotides. The large peaks of radioactivity (R_F 0.05) obtained during initial chromatography in solvent B of the soluble fractions were eluted and rechromatographed with carrier AMP, ADP, and ATP in isobutyric acid:H_2O:15 M NH_4OH:0.1 M Na_2EDTA (100:55.8:4.2:1.6,v/v). Radioactivity corresponding to marker ATP (R_F 0.36–0.38) and ADP (R_F 0.43–0.47) provides a measure of radioactivity in ATP and ADP, respectively. No radioactivity was detected in AMP (R_F 0.53–0.57).

Radioactive areas on each chromatogram were combined, eluted, and hydrolyzed in 1 N HCl for 1 h at 100°C. The hydrolysate was evaporated to dryness *in vacuo*, and applied to a column of Dowex 50-H$^+$. The column was washed with 5 ml of water, then eluted with 5 ml of 3 N NH_4OH. Chromatography of the ammonia eluate in 1-butanol:H_2O (172:28, v/v) showed that radioactivity was present only in the UV-absorbing region produced by carrier adenine. No attempt was made to characterize the radioactivity that was not adsorbed to Dowex 50-H$^+$.

Products of MTR Metabolism. Methods for determination of the products of MTR metabolism were essentially the same as those described above for metabolism of MTA. At the completion of incubation, radioactive medium was aspirated and the plants washed three times with nonradioactive medium containing 250 μM MTA and 10 μM MTR. Plants were homogenized in 10% TCA containing 200 nmol each of MTA, MTR, AdoMet, and [^{3}H] methionine (sulfoxide).

RESULTS

Effect of MTA on Growth. Plants grown in medium supplemented with 250 μM MTA had a multiplication rate (13) of 185 (SE = 5; n = 5; range, 173–198), only slightly, if at all, below that of control cultures grown concurrently (multiplication rate, 193, SE = 3; n = 7; range, 182–200). The mean frond/colony ratio was 3.62 (SE = 0.03), significantly below the control ratio of 4.04 (SE = 0.1). In one experiment, 500 μM MTA led to a 35° decrease in multiplication rate with accompanying abnormal colony types and bleached fronds. Therefore, a concentration of 250 μM MTA was chosen for most subsequent experiments.

MTA Prevention of Growth Inhibition due to Methionine Deprivation. We have previously demonstrated that exposure of *Lemna* to 150 nM PAG, or 72 μM lysine plus 8 μM threonine, leads to severe growth inhibition by preventing methionine synthesis via transsulfuration (4, 22). This inhibition is relieved by supplemental methionine (4). As shown in Figure 2, 250 μM MTA also prevented the growth inhibition produced by 150 nM PAG, by 72 μM lysine plus 8 μM threonine (Fig. 2A), or by combination of these two inhibitory regimens (Fig. 2B). MTA at a concentration of 100 μM was far less effective in preventing lysine plus threonine or PAG inhibition.

MTA (250 μM) had no significant effect on the uptake of 72 μM lysine (in the presence of 8 μM threonine) and caused a small (approximately 11%) decrease in the uptake of 8 μM threonine (in the presence of 72 μM lysine). Since even 36 μM lysine plus 4 μM threonine causes virtually complete inhibition of *Lemna* growth (4), the decrease in threonine uptake due to 250 μM MTA would not explain the observed prevention of lysine plus threonine growth inhibition. MTA (250 μM) did not affect the uptake of PAG. Thus, the observed prevention by 250 μM MTA of growth inhibition cannot be attributed to an effect on uptake of PAG. Lysine (72 μM) plus 8 μM threonine inhibited uptake of PAG by approximately 40%. Therefore, a concentration of 250 nM PAG was used in the experiments investigating the effect of MTA in the combined presence of PAG, lysine, and threonine (Fig. 2B). Under these conditions, the rate of uptake of PAG would be at least as rapid as the uptake with 150 nM PAG alone.

Effect of MTA Concentration on Uptake of MTA. Uptake of MTA was a linear function of MTA concentration over the range 0.4 to 250 μM. A clearance of 3.2 μl/frond·d was determined, *i.e.* during each day each frond took up the amount of MTA in 3.2 μl medium. Thus, at 250 μM MTA the uptake was equivalent to 6.8 nmol/colony·doubling time of 39 h.

Metabolism of the Methyl and Adenosyl Moieties of MTA, and the Number of Carbon Atoms from the Adenosyl Moiety Entering Methionine. Control plants or plants grown in 250 μM MTA were incubated with 250 μM [methyl-^{3}H, adenosyl-U-^{14}C]MTA, and the TCA-soluble fractions were examined in detail (Table I).

Metabolism of the methyl moiety of MTA is illustrated by the labeling patterns obtained for tritium (Table I, columns 1, 3, and 5). The relatively small amounts of tritium remaining in MTA indicate extensive metabolism of this compound. For plants pregrown in 250 μM MTA (Table I, columns 1 and 3), the major tritiated product was methionine. For plants grown in standard medium (Table I, column 5), again the chief product of metabolism of the methyl group of MTA was methionine. However, a significant amount of tritium had progressed to the products of methionine metabolism, AdoMet and phosphorylcholine. Chemical degradation showed that the tritium of AdoMet was confined largely to the methyl-containing MTA moiety (Table I, column 5; see also Fig. 4).

The labeling patterns for tritium in Table I further show that MTR accounted for no more than 9% of the radioactivity originating in the methyl group of MTA. Tritium compounds appearing in ether-soluble and TCA-insoluble fractions were not characterized. The 'lipid component' of the TCA-insoluble fraction was not radioactive phosphatidylcholine since it migrated at the front during TLC on silica gel with choloroform:1-propanol:propionic acid:H_2O (2:3:2:1, v/v), while authentic phosphatidylcholine migrated with an R_F of 0.28.

Separate experiments[3] using [methyl-^{14}C]MTA as substrate showed that the patterns of radioactive products obtained were essentially the same as those reported for [methyl-^{3}H]MTA.

The labeling patterns for ^{14}C originating in the adenosyl moiety of MTA were more complex. ^{14}C was distributed uniformly in the administered MTA, and the adenine and ribose moieties each have five carbon atoms. Each of these moieties therefore contains 50% of the total ^{14}C in the precursor MTA, and their fates must be considered separately. For each incubation, major amounts of ^{14}C were contained in ATP and ADP (Fig. 3; Table I, columns 2, 4, and 6). Acid hydrolyses of the combined ATP and ADP fractions showed that ^{14}C in the adenine moieties accounted for 78, 96, and 100% of the ^{14}C contents of these fractions in the respective incubations. From these results, it was calculated that ^{14}C in the adenine of these nucleotides, together with the ^{14}C present in adenine itself (Table I), accounted for at least 29 to 42% of the total ^{14}C in the plants, sufficiently close to the theoretical value of 50% as to indicate that there had been little or no net dispersion of ^{14}C from the adenine portion of MTA into non-adenine moie-

[3] Details are provided in National Auxiliary Publications Service document No 04041 containing five pages. Order from NAPS % Microfiche Publications, P.O. Box 3513, Grand Central Station, New York, NY 10163. Remit in advance in U.S. funds only $7.75 for photocopies or $4.00 for microfiche. Outside the U.S. and Canada, add postage of $4.50 for photocopies, $1.50 for microfiche.

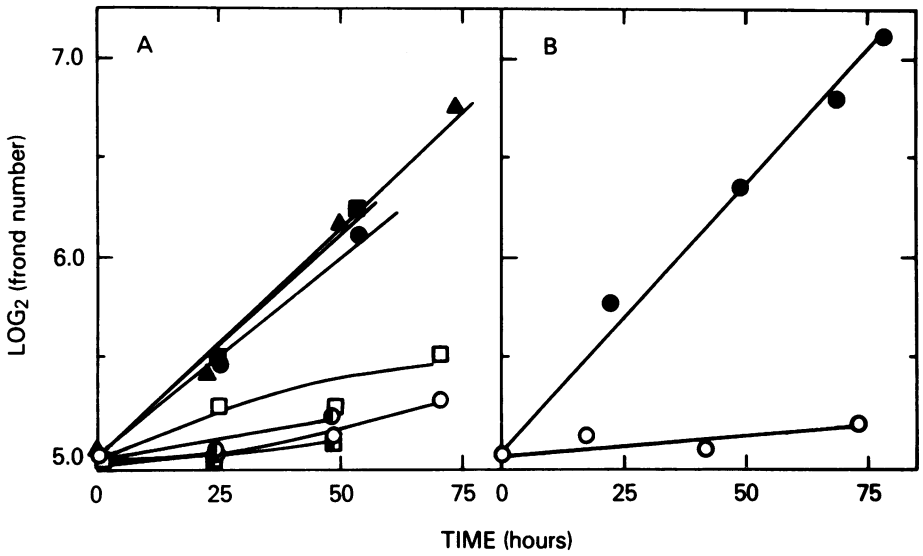

FIG. 2. Effect of MTA on growth inhibition caused by PAG, and lysine plus threonine. (A), PAG (150 nM) without (□) and with MTA (100 μM, ◧; 250 μM, ■); lysine (72 μM) plus threonine (8 μM) without (○) and with MTA (100 μM ◐; 250 μM, ●); MTA only (250 μM, ▲). Plants were pregrown in the corresponding concentrations of MTA 24 h before addition of inhibitors. B, PAG (250 nM) and lysine (72 μM) plus threonine (8 μM) without (○) and with MTA (250 μM, ●). Plants were pregrown in 250 μM MTA 6 d before addition of inhibitors. Each point on these curves has been normalized as though the initial value for $\log_2$ (frond number) was 5.0. Inoculum size and volume of growth medium were adjusted so that depletion of components did not exceed 20%.

ties.

Beyond adenine-containing compounds, the largest amounts of TCA-soluble ^{14}C for each of the incubations were in methionine (and its sulfoxide) (Table I, columns 2, 4, and 6). From the relative amounts of ^{3}H and ^{14}C in methionine (and its sulfoxide), the relative contributions to methionine of carbon from the methyl moiety as compared to carbon from the adenosyl moiety of MTA could be calculated (Table II). For plants pregrown in 250 μM MTA and incubated with 250 μM [methyl-^{3}H, adenosyl-U-^{14}C] MTA for 10 min, 0.83 to 0.90 atoms of methyl carbon were present in methionine for each four carbon atoms of methionine which had originated in the adenosyl portion of MTA. For the same plants incubated for 20 min, the value was slightly lower, 0.78 to 0.84. This decrease may be attributed to operation of the cycle: methionine→AdoMet→AdoHcy→Hcy→methionine which we have previously shown functions rapidly in *Lemna*, and leads to removal of the methyl carbon of methionine relative to its sulfur and four-carbon moieties (9). In agreement, the corresponding value for control plants incubated with 250 μM [methyl-^{3}H, adenosyl-U-^{14}C]MTA was still lower, 0.51 to 0.61 (Table II), due presumably to the decreased turnover time of the pool of soluble methionine in these plants, as demonstrated by the enhanced passage of methyl-^{3}H into AdoMet and phosphorylcholine. Extrapolation of these values to zero time very strongly suggests that for each methyl carbon from MTA which enters methionine, four carbons from the adenosyl portion of this nucleoside also enter. These findings can be explained only if the carbons coming from the adenosyl moiety of MTA enter the four-carbon portion of methionine. To obtain further evidence in this regard, radiolabeled AdoMet was subjected to chemical degradation to MTA and homoserine. The results obtained (Table I, column 6; Fig. 4) confirm that the ^{14}C coming from the adenosyl of MTA resided chiefly in the four-carbon moiety of methionine. The smaller and indeterminate amount of ^{14}C in MTA formed by chemical degradation of AdoMet is tentatively attributed to ^{14}C in adenine entering AdoMet during the reaction of [adenine-^{14}C]ATP with methionine.

A further experiment was performed to investigate the possible contribution to [^{14}C]methionine formation of the 5'-chloro-5'-[^{14}C] deoxyadenosine present as a minor (maximum of 2%) contaminant of the [methyl-^{3}H, adenosyl-U-^{14}C]MTA (see "Methods"). Control plants had a clearance of 5'-chloro-5'-[^{14}C]deoxyadenosine comparable to that of radiolabeled MTA, but formed no detectable [^{14}C]methionine during a 20-min incubation with 13 times as much of the chloro compound as could have been present in the incubation (described above) of control plants with [methyl-^{3}H, adenosyl-U-^{14}C]MTA.

Uptake and Metabolism of [Methyl-^{14}C]MTR. The uptake of MTR was determined at a concentration of 2.5 μM only. The clearance was 2.5 μl/frond·d. Table III summarizes the radioactive products obtained by incubation of plants grown in 250 μM MTA with the same medium containing 2.5 μM [methyl-^{14}C]MTR. The patterns of metabolism of the methyl group of MTR were very similar to those obtained for metabolism of the methyl group of MTA by plants grown under identical conditions (Table I, columns 1 and 3). MTR was extensively metabolized, as indicated by the minor amounts of label remaining in this compound. Methionine was the major product, with little or no radioactivity progressing into AdoMet or phosphorylcholine. No label was detected in MTA.

DISCUSSION

Evidence presented here establishes for the first time that a plant tissue other than a ripening fruit can form methionine from the methylthio moiety and four of the ribose carbons of MTA. Growth inhibition of *Lemna* deprived of methionine by administration of PAG and/or lysine plus threonine, was prevented by supplementation with MTA (Fig. 2), showing that MTA can furnish the *in vivo* requirements for net accumulation of methionine, and suggesting that MTA provides both the methylthio and four-carbon moieties of methionine. Compelling evidence for methionine biosynthesis from the methylthio and four of the ribose carbons of MTA was provided by experiments with double-labeled MTA which showed that four adenosyl carbons for each methyl carbon of MTA were incorporated in high yield by *Lemna* into the four-carbon and methyl moieties, respectively, of methionine (Table II). That these four carbons derive from the ribose of MTA is indicated by the fact that the adenine portion of MTA

Table I. *Products of [Methyl-^{3}H, Adenosyl-U-^{14}C]MTA Metabolism*

The specific activity of [methyl-^{3}H, adenosyl-U-^{14}C]MTA was 13,450 dpm/nmol for ^{3}H, and 3,146 dpm/nmol for ^{14}C. For plants grown in 250 μM MTA, 528 fronds (10 min) or 254 fronds (20 min) were incubated with radioactive MTA. For plants grown in the absence of MTA, 504 fronds were incubated. Total radioactivity (dpm/frond) in the plants was 71.8 (column 1), 17.5 (column 2), 126.4 (column 3), 31.1 (column 4), 121.1 (column 5), and 31.4 (column 6). The times between aspiration of radioactive medium and homogenization in TCA were approximately 7 min for plants grown in the absence of MTA, and 7 and 5.5 min for plants grown in 250 μM MTA and incubated for 10 and 20 min, respectively. Any radioactive methionine sulfoxide detected on the initial chromatograms of the soluble fractions was presumed to arise by chemical oxidation of methionine during the isolation procedure, and was included in the combined value for 'methionine and sulfoxide.' ND = not detected; values in parentheses indicate maximal values that could have escaped detection. Values not in parentheses and preceded by the symbol '≤' are maximal estimates based on radioactivity that was tentatively characterized by its chromatographic properties (MTA, MTR, and adenine), or was resolved incompletely from radioactivity in other compounds (4-carbon and MTA moieties of AdoMet).

Component	250 μM [Methyl-^{3}H, Adenosyl-U-^{14}C]MTA in Incubation Medium					
	250 μM MTA in growth medium				0 μM MTA in growth medium	
	10-min incubation		20-min incubation		10-min incubation	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
	% of total radioactivity					
	(1)	(2)	(3)	(4)	(5)	(6)
TCA-soluble fraction	96.3	95.8	94.7	92.9	77.3	89.6
Methionine (and sulfoxide)	54.0	21.5	40.7	15.0	21.7	8.7
AdoMet						
4-carbon moiety	ND (<0.04)	≤0.7	ND (<0.02)	≤1.2	ND (<0.1)	3.4
MTA moiety	1.2	≤1.8	1.7	≤2.5	4.5	≤2.3
Phosphorylcholine	ND	ND	ND	ND	8.1	ND (<1.8)
MTA	≤3.0	≤2.7	≤4.1	≤3.0	≤4.2	≤2.5
MTR	≤5.6	≤2.4	≤9.3	≤2.8	≤6.6	≤2.9
ATP	ND	20.3	ND	18.9	ND	23.1
ADP	ND	17.2	ND	16.3	ND	19.2
Adenine	ND	≤4.6	ND	≤2.7	ND	≤3.9
Ether soluble	5.7	1.9	4.9	2.2	2.1	1.1
TCA-insoluble fraction	3.7	4.2	5.3	7.1	22.7	10.4
Lipid components	ND (<0.15)	0.4	0.2	0.4	2.4	0.5

was converted concurrently almost entirely to adenine, ADP, and ATP (Table I; Fig. 3). The efficient conversion of [methyl-^{14}C] MTR (which lacks the adenine moiety) to methionine (Table III) also supports this conclusion.

Similar conversions of MTA or MTR to methionine have been reported recently in animal tissues (2), yeast (21), bacteria (19), and with ripening apples (27) and tomatoes (24). Although many details of these overall conversions remain to be clarified, in rat liver it is clear that the first step is a phosphorylytic cleavage of MTA to methylthioribose-1-phosphate (3), catalyzed by MTA phosphorylase (see 25 for review). In contrast, with apples (27) and tomatoes (24) it is suggested that MTR, formed from MTA in the presence of MTA nucleosidase (11, 26), is an early intermediate. The data reported here with *Lemna* are consistent with those reported for apples and tomatoes in that radioactivity from MTR was incorporated into methionine *in vivo* (Table III). Recently, we have defined conditions under which cell-free extracts of *Lemna* convert MTA, but not MTR, to methionine (J Giovanelli, SH Mudd, unpublished), suggesting that in *Lemna* MTR may be a side product of MTA metabolism rather than a direct intermediate upon the pathway to methionine, and may require a step such as phosphorylation to enter this pathway. Guranowski and Paszewski (12) have mentioned briefly the presence of MTR kinase activity in plants.

An alternative scheme for formation of methionine from MTA was proposed at one time for plant tissues (1), and has recently been shown to operate in *Aspergillus* (12). In this scheme, the methylthio group of MTA is transferred (possible as methyl mercaptan) to a four-carbon unit to form methionine. *O*-Phosphohomoserine, the physiologically important four-carbon donor in methionine biosynthesis in plants (8), would be a likely participant in the latter condensation, inasmuch as a reaction of this type has been demonstrated in crude plant tissue extracts (7). In *Lemna*, this reaction would almost certainly be catalyzed by cystathionine γ-synthase which is not absolutely specific for cysteine as its sulfhydryl substrate (23). However, two findings exclude this pathway as being responsible for the observed conversion by *Lemna* of MTA to methionine. (a) MTA can be converted to methionine by plants growing in the combined presence of 72 μM lysine, 8 μM threonine, and 250 nM PAG (Fig. 2), conditions which both prevent the formation of *O*-phosphohomoserine and severely inhibit cystathionine γ-synthase (4, 22). (b) Four nonmethyl carbons from MTA are incorporated into methionine (Table II).

The present studies also provide some indication of the *in vivo* rates of synthesis of methionine from MTA. When cystathionine γ-synthase-mediated methionine biosynthesis was prevented by lysine, threonine, and PAG, and 250 μM MTA was provided in the medium, sufficient methionine was formed to maintain a virtually normal growth rate (Fig. 2). Because *Lemna* accumulates a little more than 4.4 nmol methionine/colony·doubling (9), the rate of synthesis of methionine from MTA under the conditions

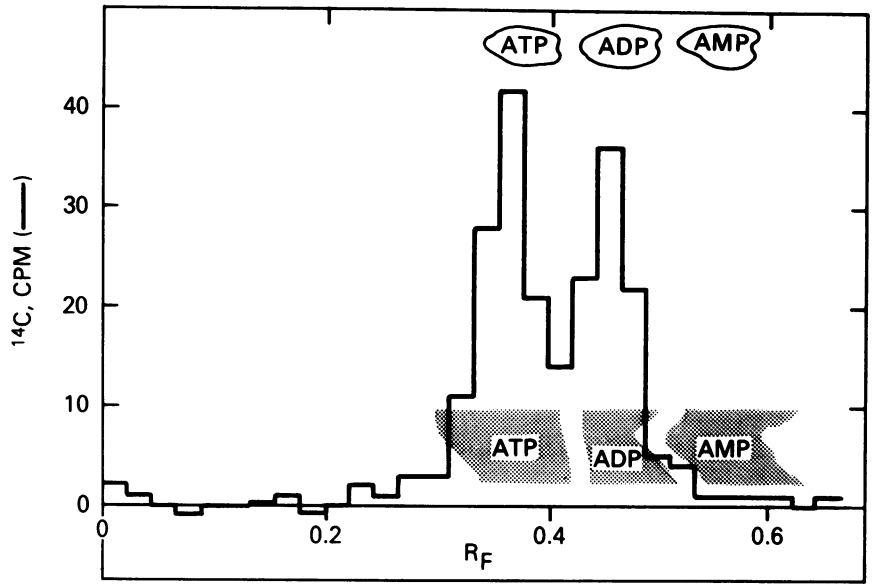

FIG. 3. Representative identification of [^{14}C]ATP and [^{14}C]ADP synthesized from [methyl-^{3}H, adenosyl-U-^{14}C]MTA. TCA-soluble fractions were chromatographed in solvent B, and the large peaks of radioactivity (^{14}C, but no ^{3}H) at approximately R_F 0.05 were eluted, mixed with carrier ATP, ADP, and AMP, and chromatographed as described in "Methods." The chromatogram shown was obtained with the extract from *Lemna* grown in 250 μM MTA and incubated with 250 μM [methyl-^{3}H, adenosyl-U-^{14}C]MTA for 10 min; similar results were obtained with extracts from plants incubated under the other conditions listed in Table I. The UV-absorbing areas produced by internal nucleotide markers are shown as shaded areas, while those of separate markers are shown above.

Table II. *Number of Methyl Carbons for Each Four Adenosyl Carbons of MTA Incorporated into Methionine*

Values were calculated from the expression: [^{3}H/^{14}C in methionine (sulfoxide)] × 0.4 ÷ (^{3}H/^{14}C in MTA). Values for ^{3}H/^{14}C in methionine and its sulfoxide were determined on eluates of chromatograms as described in "Methods."

MTA	Incubation Time	No. of Methyl Carbons per 4 Adenosyl Carbons	
		Methionine	Methionine sulfoxide
μM[a]	*min*		
250	10	0.90	0.83
250	20	0.84	0.78
0	10	0.61	0.51

[a] The concentration of MTA present in the medium used to grow plants for incubation with [methyl-^{3}H, adenosyl-U-^{14}C]MTA.

described must be no less than this value. Consistent with this conclusion is the finding that the uptake of 250 μM MTA (6.8 nmol/colony·doubling) is more than adequate to support this net rate of methionine accumulation. In contrast, at 100 μM MTA, which does not prevent growth inhibition (Fig. 2), the uptake of 2.7 nmol MTA/colony·doubling would not be adequate to support the normal net rate of methionine accumulation. For plants growing in MTA alone, efficient conversion of MTA to methionine was demonstrated with radiolabeled MTA (Table I). At both 10 and 20 min, virtually all of the MTA taken up had been metabolized. The per cent yield of radiolabeled methionine did not increase between 10 and 20 min incubation, *i.e.* no lag was observed. These findings suggest that the rate of formation of radiolabeled methionine was limited by the rate of uptake of MTA, and that the observed rates of formation of methionine from MTA were minimal ones. For plants grown in 250 μM MTA, the pool of soluble methionine was probably expanded, as shown by the following facts. (a) The plants exhibited similar morphological changes as when they were grown in 2 μM methionine, a concentration which increases soluble methionine at least 200-fold (8). (b) Radioactivity from MTA was 'trapped' in soluble methionine in such plants to a greater extent than in plants grown without MTA (Table I). (c) Cystathionine γ-synthase activity was down-regulated (GA Thompson, SH Mudd, AH Datko, unpublished), as is the case in the presence of increased methionine (23). Together, these results suggest an efficient conversion of MTA to methionine at a rate of at least 4.4 nmol/colony·doubling, and with a molar yield of at least (4.4/6.8) x 100 = 65%.

That the pathway for conversion of MTA to methionine is constitutive is demonstrated by the ability of plants grown in control medium to convert radiolabeled MTA to methionine at at least the rate specified above (Table I). In ripening apples and tomatoes, ethylene synthesis is a major source of MTA (1, 26). In *Lemna*, which evolves little or no ethylene, MTA probably arises chiefly from methionine (via AdoMet and decarboxylated AdoMet) as a product of polyamine synthesis (9). The methionine→AdoMet→decarboxylated AdoMet→MTA→→ methionine cycle would conserve the sulfur of methionine while introducing 'new' (*i.e* ribose) carbons into methionine. The rate of such sulfur recycling in *Lemna* has been estimated as 1.4 nmol/colony·doubling (9). Thus, the capacity for MTA to methionine conversion demonstrated here is more than sufficient to dispose of MTA and to account for the observed rate of methionine sulfur recycling (9).

Phosphorylcholine is widely distributed in plant saps (15), and is an intermediate in the synthesis of phosphatidylcholine (16). The present demonstration of rapid flux of methyl groups from methionine into AdoMet and phosphorylcholine (Table I, column 5) is thus consistent with our previous findings that transmethylation is a dominant pathway for methionine metabolism in *Lemna*, and that synthesis of the methyl groups of phosphatidylcholine accounts for a large proportion of this transmethylation (9).

The present work further demonstrates for the first time in plants that adenine formed during conversion of MTA to methionine is efficiently salvaged for adenine nucleotide synthesis (Table I; Fig. 3). This salvage resembles that reported in animals (see 25 for review) and yeast (21), and may well proceed by the

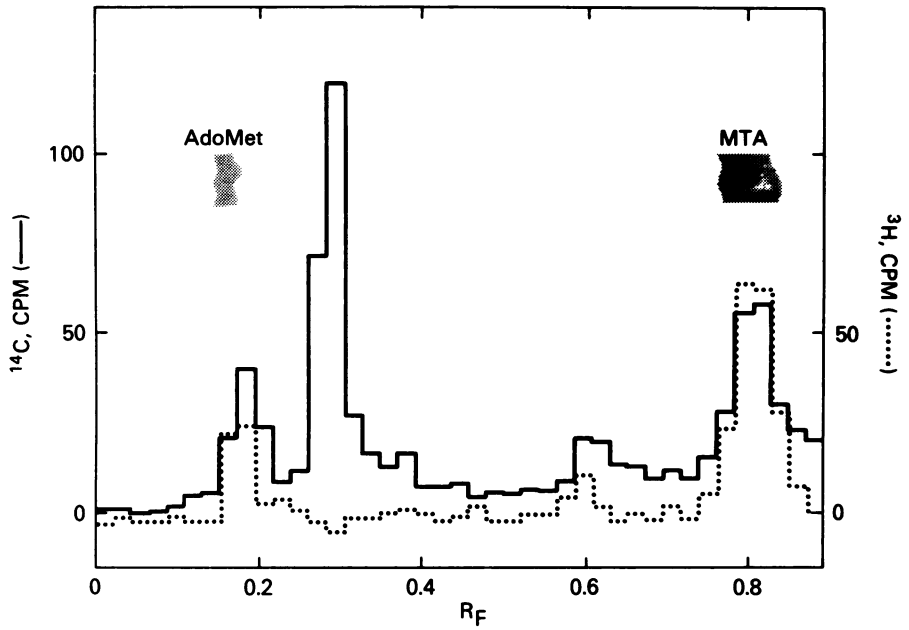

FIG. 4. Chromatography of products of chemical degradation of radioactive AdoMet formed from [methyl-^{3}H, adenosyl-U-^{14}C]MTA. Radioactive AdoMet was isolated from plants incubated with [methyl-^{3}H, adenosyl-U-^{14}C]MTA as described for columns 5 and 6 of Table I, chemically degraded to homoserine and MTA, and chromatographed in solvent B. The UV absorbance of AdoMet, added after the degradation, and of MTA formed by degradation of AdoMet, are shown by the shaded areas. External markers indicated that the ^{14}C peak migrating with an R_F of approximately 0.3 was homoserine. Homoserine lactone migrated with an R_F of approximately 0.4.

Table III. *Products of [Methyl-^{14}C]MTR Metabolism*

Plants were grown in medium containing 250 μM MTA, then incubated with medium containing both 250 μM MTA and 2.5 μM [methyl-^{14}C]MTR of specific activity 1.15×10^5 dpm/nmol. For the 10-min incubation (283 fronds), uptake was 5.51 dpm/frond; for the 20-min incubation (246 fronds), uptake was 9.87 dpm/frond. The time between aspiration of radioactive medium and homogenization was 4.7 min for the 10-min incubation, and 5 min for the 20-min incubation. See Table I for further details.

Component	Incubation Time	
	10 min	20 min
	% of total radioactivity	
TCA-soluble fraction	97.8	97.2
Methionine (and sulfoxide)	51.1	46.4
AdoMet	ND (<1)	ND (<2)
Phosphorylcholine	ND (<0.3)	ND (<2)
MTA	ND (<2)	ND (< 1)
MTR	≤22.5	≤16.6
Ether soluble	15.2	19.3
TCA-insoluble fraction	2.2	2.8

purine salvage reactions described for plants by Ross (17)[4].

It is now clear that the four-carbon moiety of methionine can be generated in two separate pathways. One is from *O*-phosphohomoserine via transsulfuration (cystathionine synthesis), and the other is from the ribose carbons of MTA via methionine thiomethyl recycling. Demonstration of the latter pathway in *Lemna* helps explain our seemingly puzzling observation (8) that supplementation of cultures of *Lemna* with methionine greatly reduced assimilation of ^{35}S from [^{35}S]sulfate into cystathionine and its products (methionine, AdoMet, and *S*-methylmethionine), without causing a commensurate decrease in the assimilation of ^{14}C from [^{14}C]sucrose. In these experiments, the more accurate assessment of the flux of sulfur (and four-carbon moiety) through transsulfuration is provided by the amount of ^{35}S from [^{35}S]sulfate, rather than the amount of ^{14}C from [^{14}C]sucrose, that accumulates in cystathionine and its products. This is so because the amount of ^{35}S accumulated is determined only by the flux into cystathionine (transsulfuration), while the amount of ^{14}C accumulated is determined by the combined fluxes of transsulfuration and methionine thiomethyl recycling. Our previous results (8) may therefore now be interpreted as showing that exogenous methionine does indeed down-regulate the *in vivo* flux through transsulfuration, *i.e.* methionine regulates its own net synthesis through this pathway. As expected from the divergent effects described above of exogenous methionine on the accumulation of ^{35}S and ^{14}C, preliminary results (J Giovanelli, SH Mudd, AH Datko, unpublished) indicate that methionine thiomethyl recycling is not appreciably regulated *in vivo* by exogenous methionine. These and other aspects of the *in vivo* fluxes through the transsulfuration pathway will be examined in a separate publication.

Acknowledgments——We are indebted to Dr. Fritz Schlenk for advice on the organic synthesis of radiolabeled MTA, and to the late Dr. Morris Lieberman for providing details of his work before publication.

[4] In the absence of data on the relative sizes of the metabolic pools of adenine and its nucleotides, and on the fluxes between these compounds, the ^{14}C labeling patterns reported for adenine and its nucleotides do not allow conclusions to be drawn on the sequence of reactions in adenine salvage. Thus, accumulation of ^{14}C in ADP and ATP in the absence of detectable labeling of AMP (Fig. 3) is not inconsistent with the established role of AMP in adenine salvage.

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