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# AMINO-ACID METABOLISM IN FOREST SOIL—ISOLATION AND TURNOVER OF ORGANIC MATTER COVALENTLY LABELLED WITH $^{35}$S-METHIONINE

J. W. FITZGERALD
Department of Microbiology, University of Georgia, Athens, GA 30602, U.S.A.

and

M. E. WATWOOD
Department of Biology, University of New Mexico, Albuquerque, NM 87131, U.S.A.

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**Summary**—Organic matter was extracted with pyrophosphate-NaOH buffer, pH 8.0 from the 02 horizon of a hardwood forest and exposed to $^{35}$S-methionine for 18 h to allow the incorporation of the amino-acid into the extract. Compared with other amendments, supplementation of the incorporation medium with $Na_2SO_4$ yielded the highest recoveries of the labelled organic matter. This material was chemically recalcitrant, requiring treatment for 6 h at 121°C with 6 M NaOH for complete release of the incorporated methionine. A and B horizon soils from several forests were exposed to labelled material following dialysis to remove unlabelled components and a minor $^{35}$S-labelled component. All samples began to release $^{35}$S-methionine after 48 h. Further metabolism of this amino-acid included mineralization and conversion to cysteine. A portion of the sulfate-S generated from mineralization was incorporated into organic matter and was recovered only by acid and alkali extraction. $^{35}$S-labelled methionine and cysteine were also found in these latter fractions, indicating that these amino-acids had been directly incorporated into organic material during exposure.

## INTRODUCTION

Inorganic sulfate is a major anionic component of acidic precipitation and leaching of forest soils with this anion is associated with the loss of cation nutrients (Johnson, 1980). Interconversions between sulfate and organic S will influence the mobility of this anion, and thus this negative effect of increased sulfate loading (Strickland and Fitzgerald, 1984; Watwood *et al.*, 1986; Fitzgerald *et al.*, 1988). Studies using a $^{35}$S-label indicate that exogenous sulfate may be subject to leaching loss, or may be retained by adsorption or by covalent incorporation into organic matter (Strickland and Fitzgerald, 1985; Schindler *et al.*, 1986). Furthermore, organic S formed by the latter process may be reconverted to sulfate (David *et al.*, 1983; McLaren *et al.*, 1985; Strickland *et al.*, 1986).

Apart from acidic precipitation, a potentially important source of sulfate for the S-cycle is the S-containing amino-acid, methionine (Harwood and Nicholls, 1979) which enters soil primarily from leaf decomposition in the 02 component of the forest floor. Fitzgerald and Andrew (1984) found that methionine was subject to mineralization. In addition, a portion of the methionine was also incorporated into soil organic matter possibly via the formation of petpide linkages (Fitzgerald *et al.*, 1984). Incorporation of this amino-acid was shown to be inhibited by various antibiotics, by sodium azide and by autoclaving (Fitzgerald and Andrew, 1985). Many of the methods for tracing the fate of methionine are destructive, however, and have provided only indirect evidence for these conversions. Thus, despite results suggesting that the process is biologically mediated, the possibility that methionine can be incorporated covalently into organic matter is inferred from findings that a portion of the added amino-acid was released by acid and base treatment and hence under conditions which release organic matter. Direct evidence for this conversion was obtained in the current study using a pyrophosphate-NaOH buffer (pH 8) which solubilizes organic matter with minimum destruction of covalent S linkages (Fitzgerald *et al.*, 1985; Strickland *et al.*, 1986). This extractant was utilized to recover organic matter before and after incubation with $^{35}$S-methionine so that covalent rather than ionic interaction of the amino-acid with organic matter could be proven. The biological fate of this form of organic S in forest soil was also determined.

## MATERIALS AND METHODS

Field moist samples were collected in April 1987 from a 29-yr-old eastern white pine (*Pinus strobus*) plantation (watershed 1), and from two mixed mature hardwood catchments (watersheds 2 and 18), located within the Coweeta basin near Franklin, N. Carolina. Large roots were removed by hand and samples, maintained at 5°C, were assayed within 1 wk after collection. A detailed description of the research site is available (Swank and Crossley, 1988).

### *Preparation and supplementation of organic extracts*

The organic extract preparation and $^{35}$S-methionine incorporation procedure is summarized in Fig. 1.

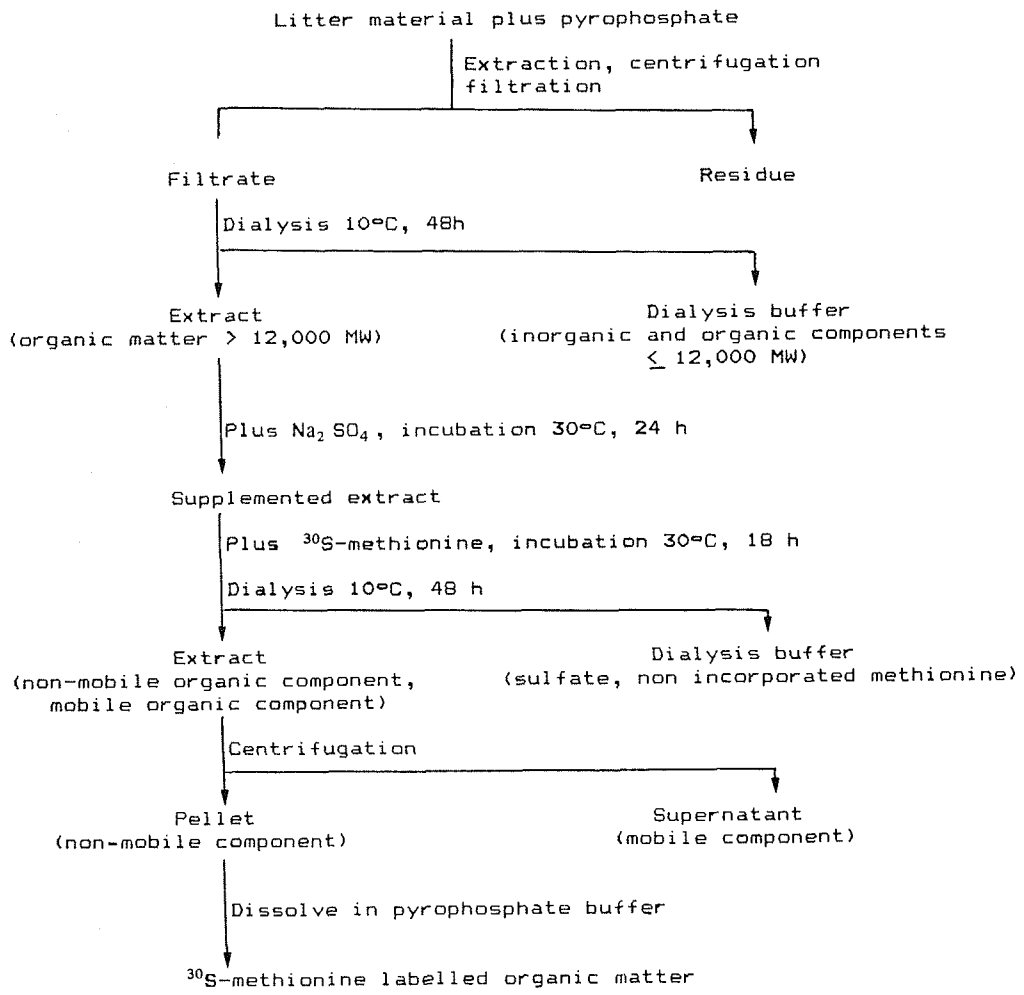


Fig. 1. Procedure for the preparation of $^{35}S$-methionine-labelled organic matter.

To extract organic matter, a bulk sample (30 g wet weight, not seived) of the 02 horizon from watershed 2 was shaken at 30°C for 18 h in a 0.1 M sodium pyrophosphate-NaOH buffer adjusted to pH 8.0 with $NaH_2PO_4$ crystals. The sample to buffer ratio was 1:5. This extraction procedure was shown (Fitzgerald *et al.*, 1985) to recover organic S with minimum rupture of covalent linkages. The slurry was centrifuged and the supernatant filtered (2.0 $\mu$m) to remove particulate debris. The filtrate was dialyzed (4.8 $\mu$m pore size) at 10°C against 5 mM pyrophosphate-NaOH, pH 8.0 (buffer to extract ratio, 20:1). The buffer was replaced 10 times during 48 h. The dialyzed extract was divided into four 20 ml aliquots and three aliquots received various supplements as follows. Glucose and adenosine 5′-triphosphate (ATP, sterilized by filtration, 0.22 $\mu$m pore size) were added to two of the extracts to final concentrations of 20 and 50 mM, respectively. One of these extracts received $Na_2SO_4$ (final concentration 0.1 mM) and a third, containing no glucose or ATP, also received $Na_2SO_4$ to the same final concentration. These supplemented extracts as well as an unsupplemented control were shaken at 30°C for 24 h before the addition of methionine.

*Methionine incorporation*

After this initial incubation to increase microbial activity, the extracts were exposed to $^{35}S$-methionine for various times at 30°C to determine conditions requisite for maximum incorporation of the amino-acid into organic matter. An aqueous solution containing 0.3 nmol $^{35}S$-labelled methionine (51.0 TBq $mmol^{-1}$, Amersham) was added to each extract. Immediately following this addition and for every 3 h thereafter, subsamples (20 $\mu$l) of each mixture were subjected to electrophoresis on Whatman No. 1 paper in 0.1 M $KH_2PO_4$-$K_2HPO_4$ buffer, pH 8.0 for 2 h at 200 V. Radioactive components, separated by electrophoresis (Fitzgerald and Andrew, 1984), were located on dried paper strips with a radio-electrophoretogram scanner. The presence of unincorporated methionine on electrophoretograms was confirmed by coelectrophoresis with an authentic standard. Irrespective of the amendment, quantitation of components by triangulation indicated that amounts of the unincorporated amino-acid remained constant after 18 h. Following exposure for 18 h the remainder of each extract was dialyzed (4.8 $\mu$m pore size) at 10°C against 5 mM pyrophos-

phate-NaOH buffer, pH 8.0 in order to remove sulfate, unincorporated methionine and any other component of molecular weight $\leqslant$12,000 dalton. The buffer to extract ratio was 20:1 and the buffer was replaced 10 times during 48 h. Electrophoresis and scanning of electrophoretograms confirmed that each dialyzate contained a major organic $^{35}S$ component which remained at the origin during electrophoresis. A mobile organic component, containing less than 10% of the $^{35}S$, was also present in each extract. This component remained in the supernatant after centrifugation for 1 h (23628 *g*, SS-34 rotor Sorvall RC2-B). A significantly higher incorporation of the label was observed with the sulfate only supplemented extract and accordingly, the pellet derived from the centrifugation of this extract was dissolved in 5 mM pyrophosphate-NaOH buffer, pH 8 and used in subsequent work.

*Chemical recalcitrance*

To verify that the $^{35}S$-organic component had been formed exclusively by the direct incorporation of methionine, and to determine the chemical stability of the material, aliquots were treated with 3 and 6 M HCl or NaOH at 121°C for up to 24 h. Changes in radioactive composition were determined by electrophoresis and scanning of resulting electrophoretograms.

*Metabolism of $^{35}S$-organic component*

Field moist subsamples (1 g wet weight, not sieved) of A horizon soils from watersheds 1, 2 and 18, as well as the 02 and B horizons from watershed 2 were held at 20°C for up to 28 days with the $^{35}S$-organic preparation (800 $\mu$l, about $3.7 \times 10^6$ dis $min^{-1}$). Following exposure, samples were washed three times with water to remove soluble $^{35}S$. A salt extraction sequence was then utilized to remove $^{35}S$ which had been retained by the samples non-covalently (e.g. adsorbed $^{35}S$). This sequence consisted of three successive washes each with separate solutions of 1 M $Na_2SO_4$, $NaH_2PO_4$, LiCl and 5 mM pyrophosphate-NaOH buffer, pH 8.0. Remaining $^{35}S$, present as organic ester sulfate, was recovered as inorganic sulfate by hydrolysis with 6 M HCl at 121°C for 12 h followed by contact with 2 M NaOH for 12 h at room temperature. Details of the extraction procedure have been described by Fitzgerald *et al.* (1982). Total radioactivity of water, combined salt and acid-base fractions was determined by scintillation counting and $^{35}S$-labelled components present in these fractions were separated by electrophoresis, located by scanning and quantified by triangulation. Total recoveries of added $^{35}S$ were $\geqslant$95% using this extraction sequence.

## RESULTS AND DISCUSSION

Exposure of organic matter to $^{35}S$-methionine resulted in the formation of a $^{35}S$-organic component which remained at the origin during electrophoresis. The organic extract which had initially been supplemented with sulfate alone formed the largest amount of this component after 18 h (Table 1). Supplementation of organic matter extracts before exposure to $^{35}S$-methionine was carried out in order to stimulate the incorporation of methionine. Evidence for microbial involvement in methionine incorporation was provided by Fitzgerald *et al.* (1984) and Fitzgerald and Andrew (1985) who found the process to be time- and temperature-dependent and substantially diminished by a variety of antibiotics. Stimulation of sulfate incorporation into organic matter, another microbially mediated process, was enhanced by glucose and ATP supplements (Fitzgerald *et al.*, 1983; Strickland and Fitzgerald, 1985). In this study, sulfate was added to some of the extracts before the addition of methionine in order to prevent mineralization of the amino-acid which might occur in response to low amounts of sulfate in the dialyzed extract. This anion causes repression of the synthesis of enzymes (sulfohydrolases) which are involved in the mineralization of organic S (Fitzgerald, 1976; Fitzgerald and Strickland, 1987).

Table 1. Influence of exogenous S, C and energy on the incorporation of $^{35}S$-methionine into organic matter[a]

| Supplement | Organic S formed (% $^{35}S$ recovered after 18 h)[b] |
|---|---|
| None | 31.7 |
| Sulfate (0.1 M) | 58.4 |
| ATP (50 mM) + glucose (20 mM) | 24.5 |
| ATP + glucose + sulfate | 24.1 |

[a]Dialyzed extracts containing each supplement were held at 30°C for 24 h before addition of $^{35}S$-methionine. Amended extracts were then exposed to the amino acid for 18 h. See Fig. 1 for steps in recovery of labelled organic matter. Only the component with zero electrophoretic mobility was quantified.
[b]Period for maximum uptake of amino-acid.

$^{35}S$-labelled inorganic sulfate and traces of an electrophoretically-mobile radioactive species were also generated and these components were removed by dialysis and centrifugation, respectively. Retention of most of the label during dialysis confirmed that incorporation of $^{35}S$ via covalent linkage formation, rather than physicochemical adsorption, had occurred because adsorbed $^{35}S$ would be released and would pass through the dialysis tubing. Following dialysis and centrifugation, all of the $^{35}S$ in the organic extract which had received the sulfate only supplement was present as the organic component of interest. Treatment of this component with strong acid or alkali (Table 2) resulted in the release of $^{35}S$-methionine as the sole degradation product. This result provided proof that methionine had been incorporated directly into organic material. This was considered important in view of observations that methionine-S can be mineralized and that sulfate can be incorporated into organic matter in forest floor

Table 2. Release of methionine from organic S following treatment with acid or alkali[a]

| Treatment conditions | $^{35}S$-Methionine released (% of total radioactivity) |
|---|---|
| 3 M HCl for 1 h | 65.7 |
| for 24 h | 80.2 |
| 6 M HCl for 6 h | 83.5 |
| 3 M NaOH for 1 h | 82.7 |
| for 24 h | 96.4 |
| 6 M NaOH for 6 h | 100.0 |

[a]$^{35}S$-labelled organic matter produced according to Fig. 1 was treated at 121°C. Methionine was separated from other reaction components by electrophoresis and quantified by triangulation.

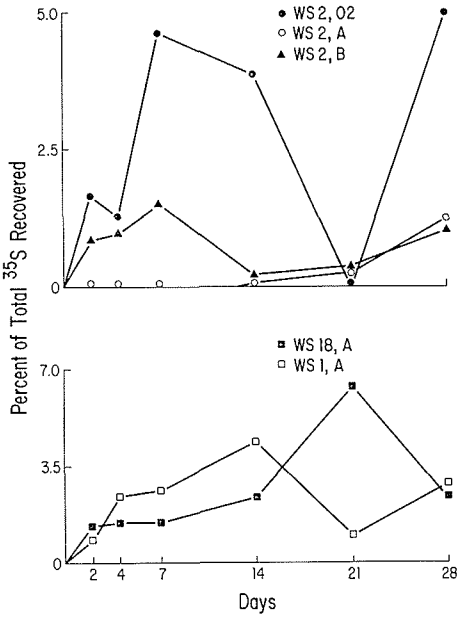


Fig. 2. Release by soil samples from watersheds 1, 2 and 18 of $^{35}$S-methionine from organic matter that had been labelled with this amino-acid.

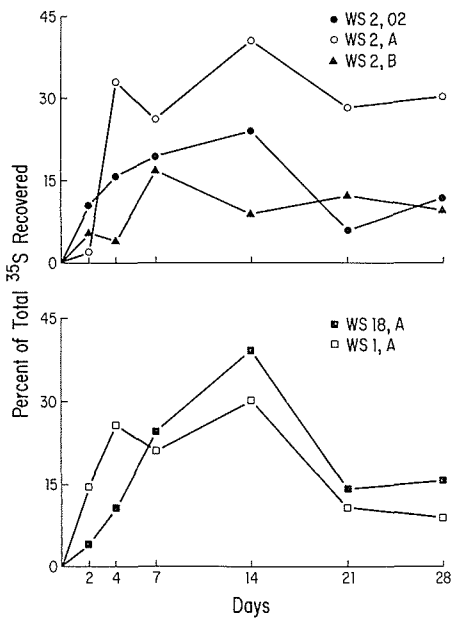


Fig. 4. Generation of $^{35}$S-sulfate during exposure to $^{35}$S-methionine labelled organic matter of samples from surface horizons of watersheds 1, 2 and 18.

and surface soil from these watersheds (Fitzgerald and Andrew, 1984; Strickland and Fitzgerald, 1985).

The homogeneity of this preparation with respect to $^{35}$S content established it as an appropriate starting material to determine if the incorporated methionine could be released biologically. Complete chemical release of methionine was achieved only at 121°C by treatment with 6 M NaOH for 6 h (Table 2) indicating that the organic $^{35}$S component was extremely recalcitrant. The biological fate of organic matter labelled with $^{35}$S-methionine is thus especially relevant in view of this extreme chemical stability. Metabolism of the labelled organic matter initially involved the release of methionine (Fig. 2). With the exception of the A horizon of watershed 2, methionine release began after 48 h, and with few exceptions, fluctuated substantially throughout the entire 28 day interval. A portion of the methionine was oxidized either after release or after acid-base extraction because oxidation products of this amino-acid (Fitzgerald and Andrew, 1985) were also recovered in trace amounts. In addition $^{35}$S-labelled cysteine (Fig. 3) as well as cystine and cysteic acid were also generated. These metabolites were identified by coelectrophoresis with authentic standards and the latter two were found only in trace quantities.

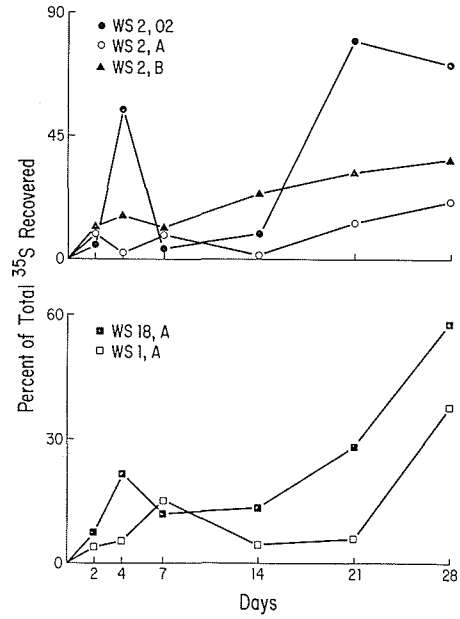


Fig. 3. Generation of $^{35}$S-cysteine during exposure to $^{35}$S-methionine labelled organic matter of samples from surface horizons of watersheds 1, 2 and 18.

After 48 h, sulfate was also released from the labelled organic matter (Fig. 4). Because of the homogeneity of the preparation with respect to radioactivity, the occurrence of this anion was probably due to the mineralization of methionine after release of the amino-acid from the organic matter. Sulfate release from the 02 horizon of watershed 2 and A horizons of the other watersheds was highest after 2 wk, whereas for the B horizon the highest amounts of sulfate were found after 1 wk. Subsequent to maximal release, sulfate concentrations remained fairly constant for up to 4 wk for the A and B horizons of watershed 2, although release of this anion thereafter decreased substantially in the 02 horizon and A horizons of watersheds 1 and 18. Table 3 shows the distribution of sulfate among the various fractions obtained from samples after 2 wk. A substantial amount of the anion was recovered by salt extraction of A horizon samples. This fraction represents sulfate derived from the mineralization of released methionine which then became adsorbed within the soil matrix. Labelled sulfate was also

Table 3. Distribution of $^{35}$S-sulfate following exposure of soil to $^{35}$S-methionine labelled organic matter[a]

| Watershed horizon | Water soluble | Salt extractable | Acid extractable | Alkali extractable |
|---|---|---|---|---|
| WS18, A | 15.9[b] | 10.2 | 12.8 | 0.0 |
| WS1, A | 15.6 | 14.8 | 0.0 | 24.0 |
| WS2, 02 | 6.6 | 0.9 | 16.5 | 18.5 |
| WS2, A | 20.5 | 11.4 | 8.9 | 0.0 |
| WS2, B | 9.3 | 0.0 | 0.0 | 0.0 |

[a]2-wk exposure to soil at 20°C of labelled material produced according to Fig. 1. Sulfate was separated from other components in each fraction by electrophoresis.
[b]Values are expressed as nmol S $g^{-1}$ dry wt of sample.

recovered as water soluble for each of the soils examined and, for the B horizon, this was the only fraction found to contain sulfate. For the 02 and A horizons, the majority of the sulfate was extracted only by strong acid and, in some instances, by alkali as well. This fraction probably represents sulfate generated from the hydrolysis of sulfate ester linkages (Fitzgerald, 1976).

The occurrence of sulfate may have resulted from the mineralization of metabolites other than methionine. Thus, a portion of the sulfate generated could have resulted from the oxidation of methionine-derived cysteine. Cysteine may be formed from methionine indirectly via assimilatory reduction of mineralization-derived sulfate. Although bacteria lack the enzymes necessary to convert methionine to cysteine (Davis *et al.*, 1973), some fungi are able to carry out this conversion (Margolis and Block, 1958). Therefore, especially in the 02 horizon, which contained visible fungal hyphae, cysteine may have been formed directly from released methionine. The observations that more cysteine than methionine was found for all horizons and that the 02 horizon released more cysteine than any other horizon (over 80% after 3 wk, Fig. 4) suggest a methionine to cysteine conversion. A portion of the cysteine-S thus generated may have been oxidized to sulfate (Freney, 1960). With respect to the A horizon of watershed 2, relatively-low amounts of methionine or cysteine were recovered; however, these samples released the most sulfate. In this case sulfate may have been generated by oxidation of both methionine and methionine-derived cysteine. Direct fungal conversion of methionine to cysteine is likely in this soil because hyphae were also seen in these samples. Additional evidence for the oxidation of cysteine to yield sulfate was provided by the recovery of cystine and cysteic acid which are intermediates in the proposed cysteine oxidation pathway (Freney, 1960).

In summary, results of this work provide direct support for previous observations (Freney *et al.*, 1972) that most of the methionine in soil is present in organic matter as opposed to soil solution. After release from decaying plant matter, this amino-acid is either utilized as a source of sulfate immediately or after incorporation into and subsequent release from organic matter. Both processes of mineralization and incorporation are microbially-mediated (Fitzgerald and Andrew, 1985) and the latter may serve as a storage mechanism for S in forest soil.

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