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# Mobilization of recently formed forest soil organic sulfur[1]

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Soils from a mixed mature hardwood forest were assayed for their capacity to mobilize sulfur which had been previously immobilized into a nonsalt-extractable (insoluble) form. These soils rapidly released soluble organic sulfur and inorganic sulfate from this fraction. It is suggested that the former component is a depolymerization product of a more complex organic sulfur matrix. The activity of preformed extracellular depolymerase and sulfohydrolase enzymes in the soil may be responsible for the depolymerization and subsequent desulfation of the organic sulfur matrix. This is supported by observations that treatment of soil samples with sodium sulfate, sodium azide, erythromycin, or candicidin failed to inhibit the capacity of A1-horizon soils to mobilize the organic sulfur fraction. The rates and final levels of sulfur mobilization increased with an increase in temperature and decreased with sample depth.

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Au moyen de bio-essais, les auteurs ont étudié la capacité de sols de forêts mixtes adultes à mobiliser le soufre préalablement immobilisé sous une forme non extractible par une solution saline. Ces sols ont libéré rapidement du soufre organique soluble et du sulfate inorganique. Ces observations suggèrent que le soufre organique est un produit de dépolymérisation de composés organiques sulfurés plus complexes. L'activité des enzymes endogènes extracellulaires, dépolymérase et sulfohydrolase, peut être responsable de la dépolymérisation et de la désulfatation subséquente de la matrice de soufre organique. Cette possibilité est supportée par les observations montrant que le traitement des échantillons de sol avec le sulfate de sodium, l'azide de sodium, l'érythromycine, ou la candicidine n'ont pas inhibé la capacité de l'horizon A1 des sols à mobiliser le soufre organique. Les taux et niveaux finals de la mobilisation du soufre ont augmenté avec l'augmentation de la température et diminué avec la profondeur de l'échantillon.

[Traduit par le journal]

## Introduction

Sulfate accumulations of up to 13 kg·ha$^{-1}$·year$^{-1}$ have been observed in various watersheds at the Coweeta Hydrologic Laboratory in the Southern Appalachian mountains (Swank and Douglass 1977). Solution sulfate concentrations diminish as throughfall passes through the forest floor and percolates through the solum (Swank and Swank 1983). As water exits the system in streamflow, input–output discrepancies indicate a net retention of at least 91% of sulfur inputs. Fitzgerald et al. (1982) found that a small portion of sulfur accumulation at Coweeta is in the form of soluble, salt-extractable organosulfur compounds such as ester sulfates, sulfonates, and other carbon bonded sulfur metabolites. The largest amount of organic sulfur (5–100 times the levels of soluble forms) was recovered in a nonsalt-extractable form. Recovery of this fraction could only be achieved by treatment with 2.0 *N* NaOH coupled with autoclaving for 12 h in 6.0 *N* HCl. In laboratory experiments, potential incorporation of sulfate into the nonsalt-extractable form was estimated to be threefold greater than observed input quantities (W. T. Swank, J. W. Fitzgerald, and J. T. Ash, unpublished data). The characteristics of alkali and acid extractability of this fraction (Flaig et al. 1975; Schnitzer and Khan 1972) and the inability to recover it using solutions commonly employed to desorb anionic soil components suggest that this sulfur pool may be a product of humification processes.

In view of the possibility that sulfur incorporated into organic matter can be mobilized (Strickland and Fitzgerald 1983), work was undertaken to ascertain the levels of sulfur mobilization which might be achieved in soils at Coweeta. Our objectives were to determine the following: (*i*) rates of mobilization and the influence of free-sulfate concentrations in regulating this process; (*ii*) involvement of soil microflora; (*iii*) the effect of temperature; and (*iv*) the capacity of soils sampled from differing depths to mobilize nonsalt-extractable sulfur. Mobilization will be used here to describe the solubilization of a previously nonsalt-extractable sulfur pool. Preliminary characterization of components released from this sulfur fraction suggests that the solubilization process involves depolymerization and desulfation. Although Johnson et al. (1982) obtained indirect evidence suggesting organic sulfur mineralization, to our knowledge this is the first report of direct evidence for organosulfur mobilization in forest soils.

## Materials and methods

Unless indicated otherwise, all determinations were made on soil samples (0–5 cm) collected in September 1980 from the A1 horizon of watershed 2 at the Coweeta Hydrologic Laboratory near Franklin, N.C. Soil samples from differing depths were taken in September 1981 using a Fenn auger of 5 cm diameter. Depths sampled were as

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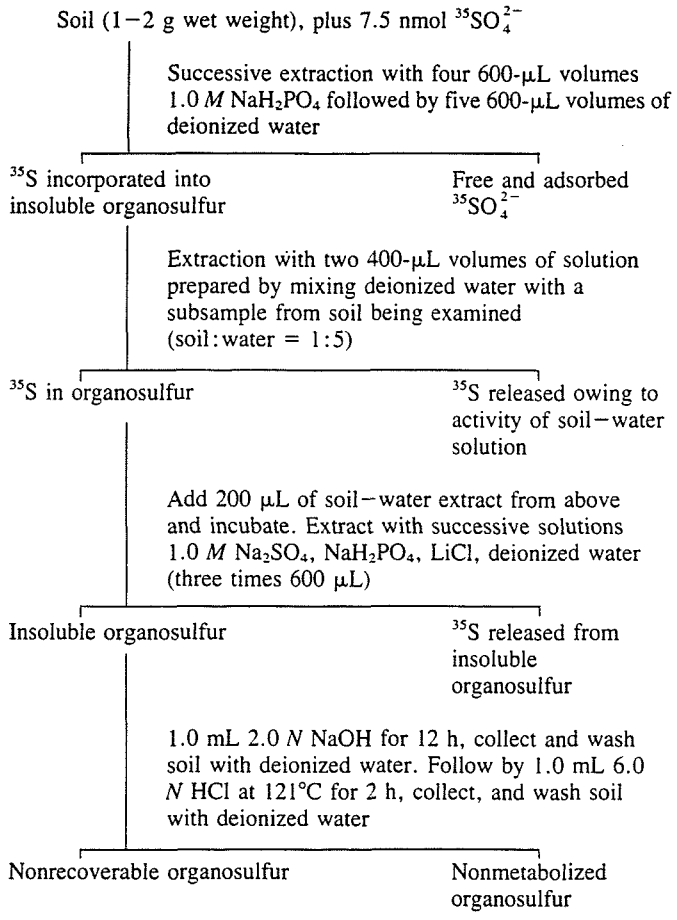


FIG. 1. Procedure for determination of $^{35}S$ mobilization from nonsalt-extractable fraction.

follows: 0−5 cm (A1 horizon), 10−25 cm (B1 horizon), 30−40 cm (B2 horizon), 60−80 cm and 70−100 cm (C horizon). The vegetation in this catchment consists of mixed mature hardwoods. Typic Dystrochrept is the predominant soil type in the watershed. More detailed descriptions of watersheds in the Coweeta Basin may be found in Johnson and Swank (1973) and Swank and Douglass (1977). $^{35}S$-labelled sodium sulfate (1087.3 mCi $mmol^{-1}$; $4.02 \times 10^{10}$ Bq $mmol^{-1}$) was obtained from New England Nuclear.

*$^{35}S$ incorporation into the nonsalt-extractable fraction*

Glass centrifuge tubes having two open ends and fitted with coarse scintered glass filters (25−50 μm retention) were dried at 50°C and weighed. Soil samples (1.0 g wet weight) were added to each tube and treated as in Fig. 1. To each soil sample was added 7.55 nmol (approximately 8.2 μCi) (1 Ci = 37 GBq) $^{35}S$-labelled sulfate dissolved in deionized water. Water (200 μL) was then added to wash in the $^{35}S$ and ensure even distribution of the label. The tubes were placed in 50-mL capacity polypropylene tubes, capped, and incubated for 24 h at temperatures designated in the text. Following incubation, 600-μL volumes of the following extractants were passed successively through each sample via centrifugation for 8 min at 850 relative centrifugal force (2750 rpm in a 4.25-in. rotor) (1 in. = 25.4 mm). The extraction sequence was four volumes 1.0 M $NaH_2PO_4$ (2.4 mmol $PO_4^{3-}$ total; P:$^{35}S$ ratio approximately $3 \times 10^5 : 1$), then five volumes water followed by two volumes of a supernatant obtained by mixing water with a subsample of the test soil (soil:water = 1:5). The addition of the latter supernatant serves to replace any microflora and soluble soil constituents which may have been lost owing to extraction. To facilitate analysis of the combined extract, volumes were reduced relative to those utilized in more conventional extraction procedures. However, phosphate will replace even specifically adsorbed sulfate on a 1:1 molar basis (Johnson and Cole 1980). Previous work by Fitzgerald et al. (1982) demonstrated that following incubation of soils with $^{35}S$-labelled sulfate and successive extraction with 1.0 M solutions of $Na_2SO_4$, $NaH_2PO_4$, and LiCl followed by water, 8−12% of the added $^{35}S$ was consistently immobilized into a nonsalt-extractable fraction in the soil. Therefore, recoveries obtained in the current work of ≥80% of the added $^{35}S$ in the combined extracts ensure complete removal of nonmetabolized inorganic sulfate. The percentage of $^{35}S$ recovered in the combined extracts was determined using a Beckman LS 9000 liquid scintillation counting system with energy windows set at 0−655 meV. The nonsalt-extractable $^{35}S$ remaining was characterized after partial purification (T. C. Strickland, unpublished data). The C:N:S ratio and ester-sulfate content of this fraction together with data showing that its formation was temperature dependent and inhibited by antimicrobial agents (Fitzgerald et al. 1983) collectively suggest that the remaining $^{35}S$ is in organic combination. The metabolism of this fraction was investigated as follows.

*Mobilization of nonsalt-extractable fraction*

Following sulfur incorporation, 200 μL of the soil−water supernatant was added to each sample and the samples were incubated for a second period. Following incubation, samples were extracted successively with three 600-μL volumes of the following: 1.0 M $Na_2SO_4$, $NaH_2PO_4$, LiCl followed by two 600-μL volumes of water. Any $^{35}S$ extracted in this procedure will be from the previously nonsalt-extractable fraction. This extraction sequence is sufficient for complete recovery of added sulfate from soils (Fitzgerald et al. 1982) and forest floor layers (Fitzgerald et al. 1983). Attempts to increase yields by increasing the number of extractions, for example, have routinely failed. The soil residue was then treated with 1 mL of 2.0 N NaOH for 12 h at room temperature and extracted by centrifugation. The residue was extracted further with 400 μL of 2.0 N NaOH followed by two 400-μL volumes of water. The residue was finally hydrolyzed for 2 h at 121°C in 6 N HCl (1.0 mL) and washed with water. The salt and alkali−acid extracts were pooled separately, and the amount of sulfur mobilized from the nonsalt-extractable fraction was determined as a percentage of the sulfur which was incorporated into this fraction. The amount of sulfur incorporated was determined as the sum of the amount which was mobilized after the second incubation period and the amount which was extracted with alkali−acid. For comparative purposes, the average oven-dry mass of all samples was determined by drying at 50°C for 24 h, and all values are standardized to a 0.8187-g mass.

*Experimental variables examined*

*Effect of temperature*

Samples were incubated at 30, 20, and 5°C during the incorporation and mobilization stages. Fluctuations in $^{35}S$ released from the nonsalt-extractable fraction were monitored using 10 replicates, each sampled at varying time intervals up to 48 h.

*Antimicrobial agents*

Volumes (200 μL) of sodium azide (0.128 g/200 μL), erythromycin (0.5 mg/200 μL), or candicidin (10.66 mg/200 μL) were added to samples prior to the addition of soil−water and prior to the beginning of mobilization experiments.

*Soil depth*

Soil samples from depths indicated earlier were incubated at 30°C for 48 h during the mobilization stage.

*Inorganic sulfate*

To determine if inorganic sulfate availability regulates mobilization of sulfur from the nonsalt-extractable fraction, sulfate was added to samples at various concentrations (up to 46 nmol·$g^{-1}$) prior to the mobilization stage but after incorporation. Sulfate solutions were used to prepare soil water mixtures instead of deionized water during the reconstitution stage and prior to the second incubation. Incubation was for 48 h at 30°C.

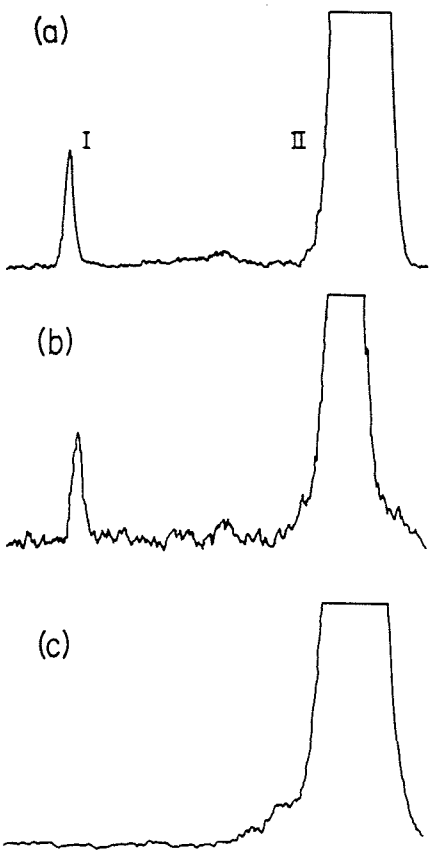


FIG. 2. Electrophoretogram scans of extracts collected during the determination of the capacity of soils to mobilize sulfur from the nonsalt-extractable fraction. (*a*) Extract collected after addition of soil–water mixture to reconstitute soil microflora and (or) other soil constituents. (*b*) Salt extracts collected after incubation. (*c*) Phosphate extract used to remove nonmetabolized sulfate. Fractions were electrophoresed in 0.1 *M* sodium acetate – acetic acid buffer, pH 4.5 for 2 h at 250 V. Component I, covalently linked sulfur; component II, inorganic sulfate.

*Electrophoresis*

Extracts (80 μL) were applied to Whatman No. 1 paper strips and subjected to electrophoresis for 2 h at 250 V in either 0.1 *M* sodium acetate – acetic acid or 0.1 *M* barium acetate – acetic acid buffers at pH 4.5. The strips were then dried at 35°C and $^{35}S$-labelled components were located by radiochromatogram scanning in a Packard model 7220/21 radiochromatogram scanner. Inorganic sulfate was identified by its rapid movement in sodium acetate – acetic acid (approximately 14 cm) and by precipitation at the origin as barium sulfate in the barium acetate – acetic acid buffer. The presence of any component migrating at a slower rate than inorganic sulfate in the sodium acetate – acetic acid buffer indicates that the component contains sulfur which is covalently linked to some larger moiety.

## Results and discussion

To better understand the sulfur mobilization process, it was necessary to determine the nature of the components released during incubation. A radiochromatogram scan of the filtrate obtained by centrifugation after reconstitution with soil water is shown in Fig. 2*a*. Two $^{35}S$-labelled components were detected. The first component remained at the origin after electrophoresis at pH 4.5. Because this component exhibited no electrophoretic mobility at this pH and was previously not extractable with phosphate (Fig. 2*c*), it is likely a form of covalently linked sulfur which was released from the nonsalt-extractable soil fraction. If this component had contained sulfur in an ionic linkage, it would have either been removed during phosphate extraction, or separated as inorganic sulfate during electrophoresis. Since the organosulfur component was not extracted with phosphate, it is not likely to be a soluble-adsorbed metabolite. Its release in response to soluble soil–water constituents (Fig. 2*a*) and during turnover incubation (Fig. 2*b*) suggests that this component is a depolymerization product of a larger insoluble organic matrix. The second component released during the process was identified as inorganic sulfate by its precipitation in barium acetate – acetic acid buffer and also by coelectrophoresis in the sodium acetate buffer with a sulfate standard (data not shown). Since extraction with phosphate removes unmetabolized inorganic sulfate (Fitzgerald et al. 1982; Chao et al. 1962), sulfate released during the mobilization stage was released from organic matter.

TABLE 1. Effect of sulfate on mobilization of $^{35}S$ nonsalt-extractable organosulfur[a]

| Sulfate added (nmol) | % $^{35}S$ mobilized (g·dry weight$^{-1}$)[b] |
|---|---|
| None | 86.7 |
| 4.0 | 76.3 |
| 8.0 | 70.0 |
| 23.0 | 86.0 |
| 46.0 | 81.0 |

[a]Samples collected from top 5 cm of A1 horizon in September 1980.
[b]Error ± 5.0% using three replicates.

Treatment of soil samples with unlabeled $Na_2SO_4$ at levels ranging from 4–46 nmol sulfate·g$^{-1}$ consistently failed to inhibit mobilization of sulfur from the nonsalt-extractable fraction (Table 1). Since sulfohydrolase synthesis by microbial populations in pure culture has been shown to be repressed by sulfate (Fitzgerald 1976), significant inhibition of mobilization might be expected. However, unlike incorporation of sulfur into organic matter (Fitzgerald et al. 1983), mobilization of organic sulfur does not seem to be directly linked to microbial metabolism. Treatment of samples with sodium azide (a metabolic poison inhibiting oxidative phosphorylation), erythromycin (an antibacterial antibiotic), or candicidin (an antifungal antibiotic) failed to inhibit the mobilization of organic sulfur in the samples examined. In these experiments it was not possible to test the effect of autoclaving since the organic sulfur present in the sample could undergo hydrolysis resulting in the release of inorganic sulfate as an artifact (see Fitzgerald 1976).

While the mobilization of organic sulfur does not appear to be a result of active microbial metabolism, it does appear to be mediated by enzymes, depolymerases and sulfohydrolases, existing extracellularly in the soil. The varying amounts of sulfur mobilized at different temperatures suggest differing temperature activity optima for the enzymes involved in mobilization. During the initial 3 h of incubation at 30 and 20°C (Figs. 3 and 4), up to 22% of the incorporated $^{35}S$ was released per gram of dry soil per hour. Levels of mobilization reached a maximum after approximately 18 h at these two temperatures. When samples were incubated at 5°C (Fig. 4) the rate of mobilization during the initial 3-h period was only one fifth that achieved at 20 or 30°C (3.67% released per gram of dry soil per hour). After 18 h at 30 and 20°C, the levels of extractable sulfur fluctuated (Figs. 3 and 4), most notably at 30°C, while there

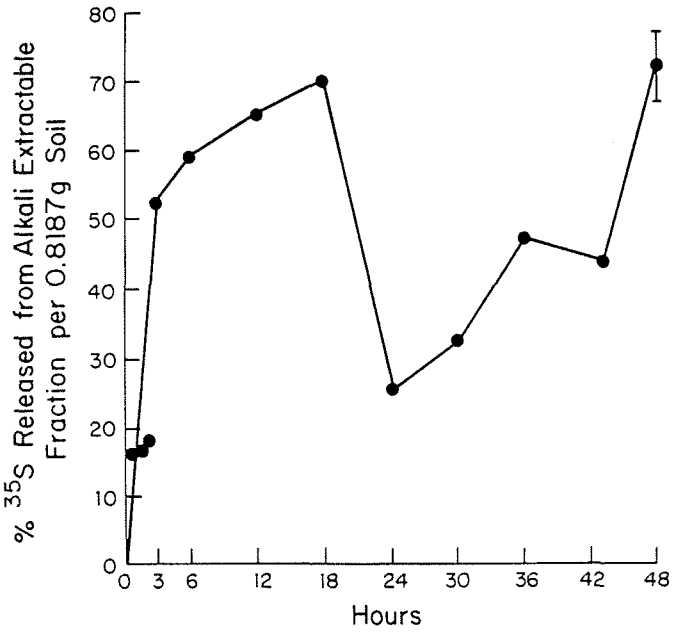


FIG. 3. Mobilization of $^{35}$S from the nonsalt-extractable fraction of A-horizon soil during incubation at 30°C. Error bar represents maximum error at any time interval.

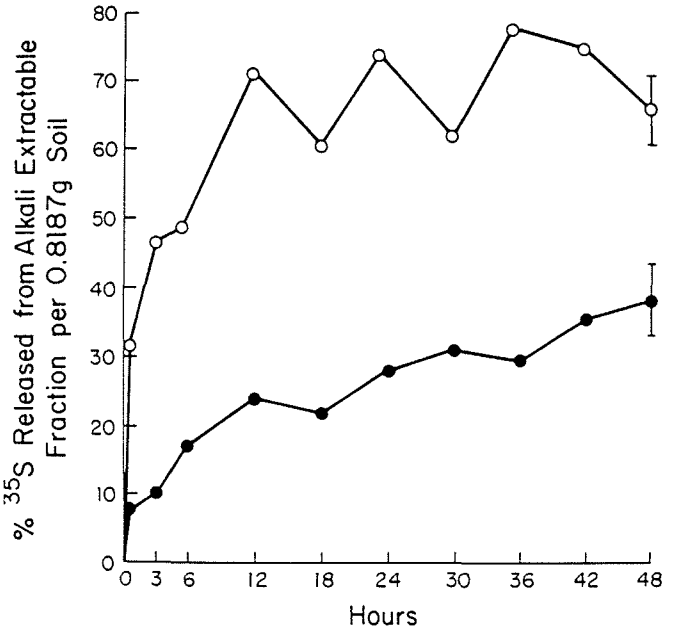


FIG. 4. Influence of incubation temperature on the release of sulfur from the nonsalt-extractable fraction of an A-horizon soil at 5°C (●) and 20°C (○). Error bars are representative of maximum error exhibited at any time interval.

was very little fluctuation at 5°C (Fig. 4). Since the rates of activity for the enzymes involved in mobilization would remain constant at a given temperature, the variation in extractable-sulfur levels probably reflects variation in microbial metabolism. The incorporation of sulfur into the nonsalt-extractable fraction is dependent on microbial metabolism in forest soils (Fitzgerald et al. 1983; Fitzgerald and Johnson 1982) and the observed fluctuation in sulfur release may be due to microbial reincorporation of the released sulfur into an insoluble form.

The levels of enzymes mediating organic sulfur mobilization diminished with increasing depth in the soil profile. After incubation at 30°C, samples from the A1, B1, and B2 horizons exhibited a capacity to mobilize 67.7, 61.2, and 25.7%, respectively, of the sulfur present in the nonsalt-extractable fraction. Although the capacity for mobilization decreased with depth, the amount of sulfur incorporated in samples taken at various depths did not vary appreciably (standard error of mean = 0.061). Collectively, this suggests that enzymes involved in mobilization are synthesized and released in the upper 25 cm of the soil profile. This was not an unexpected observation because generally the majority of microbial biomass is found in the upper soil horizons. These horizons also contain various forms of macrofauna and the majority of plant root biomass. The production of sulfohydrolases by gastropods has been documented (Dodgson and Powell 1959) and these animals may be a major source of the enzyme in soils. Moreover, plant roots are known to hydrolyze the sulfate ester of choline (Nissen 1968). Animal waste may also contribute to enzyme activity in soils since animals have been shown to excrete sulfohydrolases in urine (Breslow and Sloan 1972; Stevens et al. 1975).

Data reported here supports the suggestion of Houghton and Rose (1976) that organic sulfur components may be sequentially attacked by depolymerase–sulfohydrolase enzyme complexes to release sulfur in a form available for biological uptake. The response of this process to temperature variation and lack of response to microbial poisons or inorganic sulfate would be expected if the enzymes responsible for mobilization were present *in situ* and not dependent on *de novo* microbial synthesis for activity. When an ecosystem is impacted, as with conversion to agricultural land use and subsequent removal of nutrients, this preformed enzyme complex may serve as an important means of providing inorganic sulfate for biological uptake.

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