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Formation of organic sulfur in forest soils: a biologically mediated process[1]

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The ability of soils from hardwood, clear-cut, and pine forests to incorporate sulfur from added inorganic sulfate into salt-extractable (adsorbed) and nonsalt-extractable forms was investigated. At least 65% of the added sulfate was adsorbed while 8–27% of the sulfate added was recovered only after treatment of salt-extracted samples with acid and base (nonsalt-extractable sulfur). The incorporation of sulfur into this latter fraction was dependent upon incubation time, temperature, and depth and exhibited both spatial as well as seasonal variation in samples taken along a transect of one of the watersheds. Sulfur incorporation into the nonsalt-extractable fraction was inhibited 75–87% by sodium azide, 62–84% by erythromycin, and 41–68% by candicidin suggesting that the process is mediated by bacteria and fungi. Data on factors influencing sulfur incorporation suggest that sulfate was incorporated into organic matter as a covalent linkage and released after rupture of this linkage during acid and base treatment. The observations that ^{35}S incorporation was inhibited 93–99% by unlabelled sulfate and stimulated 21–65% by increased carbon availability are consistent with this suggestion.

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Les auteurs ont étudié l'adsorption du soufre ajouté sous forme de sulfate inorganique à des sols de forêts feuillues, de parterres de coupe et de forêts de pin. Au moins 65% du sulfate ajouté fut adsorbé, tandis que 8–27% fut récupéré après traitement des échantillons avec un acide et une base (soufre extractible sous forme autre qu'un sel). L'incorporation du soufre dans cette dernière fraction dépend du temps d'incubation, de la température et la profondeur, et présente des variations spatiales et saisonnières pour des échantillons prélevés le long d'un transect d'un des bassins. L'incorporation du soufre dans la fraction extractible sous forme autre qu'un sel est inhibée à 75–87% par l'azide de sodium, à 62–84% par l'érythromycine et à 41–68% par la candicidine, suggérant un processus biologique. Les données relatives aux facteurs qui influencent l'incorporation du soufre suggèrent que le sulfate est incorporé sous forme organique par un lien covalent et est libéré par rupture de ce lien par traitement avec un acide et une base. Les observations à l'effet que l'incorporation de ^{35}S est inhibée à 93–99% par un sulfate non marqué et stimulé à 21–65% avec l'augmentation de la disponibilité de la source de carbone sont à l'appui de cette suggestion.

[Traduit par le journal]

Introduction

During studies on the ability of forest soils to degrade 6-[^{35}S]sulfoquinovose (Strickland and Fitzgerald 1983), it was observed that salt extraction after incubation released about 65% of the radioactivity as inorganic sulfate. The remaining ^{35}S was recovered in organic matter (Schnitzer and Skinner 1968) during acid hydrolysis and subsequent base extraction. Analysis of the acid fraction by electrophoresis (Fitzgerald et al. 1982) revealed the presence of inorganic sulfate as well as a number of ^{35}S-labelled organics. Since ester-sulfate linkages hydrolyse in acid to yield inorganic sulfate (Fitzgerald 1976), the occurrence of this anion in the hydrolysate was considered by Fitzgerald et al. (1982) to result from the hydrolysis of these linkages present in soil organic matter.

The occurrence of soil ester sulfate is well documented for agricultural systems (see, for example, Freney 1961, 1967; Tabatabai and Bremner 1972; Bettany et al. 1979; Anderson et al. 1981) and recent work has confirmed the presence of these linkages in forest-soil organic matter (David et al. 1982). Although considerable effort has been made to define steady-state levels of soil organic sulfur, with a few exceptions (Freney 1979; Saggar et al. 1981; Strick et al. 1982), the flux of sulfur into this pool has not been investigated. Moreover, it has generally been assumed that soil microflora are responsible for the formation of organic sulfur. Although this assumption may be valid for agricultural systems which do not tend to accumu-

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late inorganic forms of sulfur (see, for example, Fitzgerald 1978), it warrants serious consideration in studies of forest systems which frequently exhibit high capacities for inorganic sulfate adsorption (Johnson et al. 1982). The adsorption of this anion on Fe and Al oxides (Fitzgerald and Johnson 1982) occurs without apparent involvement of microorganisms.

In the present work, evidence is presented which suggests that sulfur recovered from soil after acid and base treatment represents a reasonable index of the organic sulfur generated by microbial activity during exposure of forest ecosystems to exogenous inorganic sulfate. A brief account of some aspects of this and related work has already been given (Fitzgerald and Johnson 1982).

Materials and methods

Site description

The capacity to incorporate sulfur into the acid- and base-extractable fraction of soils from a number of forest ecosystems was examined. These catchments, representing five experimental watersheds and a pitch pine stand, are located near Franklin, NC. The watersheds (WS) are in the Coweeta basin at the Coweeta Hydrologic Laboratory and consist of two undisturbed, mature mixed-hardwood forests (WS 2 and 18), a 27-year-old white pine (*Pinus strobus* L.) plantation (WS 17), and two clear-cut forests (WS 7 and 48). The clear-cut forests differed in the age of hardwood regrowth following cutting (1-year-old regrowth, WS 48; 3-year-old regrowth, WS 7 as of sampling date). The white pine forest, which is essentially a monoculture, is on a catchment that was clear-cut in 1940 and, after repeated cutting of hardwood sprouts in succeeding years, was subsequently planted to pine. The pitch pine (*Pinus rigida* Mill.) stand is located on a 45-year-old undisturbed site about 10 km from the Coweeta basin. This stand, consisting of a pine overstory and mixed-hardwood understory, is typical of early forest succession on more xeric sites in the region. A variety of soils were represented by the study sites. Soils sampled on watersheds 17 and 18 were in the sandy loam Ashe series, and watersheds 2 and 48 were in the sandy loam Chandler series; both members of the Typic Dystrochrepts. Soils on watershed 7 were in the fine-loamy Tusquitee series, a Humic Hapludult, and the pitch pine was located on the sandy loam Evard series, a Typic Hapludult. Additional descriptions for all study sites are available (Johnson and Swank 1973; Swank and Douglass 1977; Swank and Swank 1983).

Sampling and laboratory incubations

Unless otherwise indicated in the text, one sample (approximately 15 g wet weight, 0–5 cm in depth) was taken at random from the A-horizon of each site during September 1981. These were maintained in sealed bags at 15°C and root material was removed by hand prior to analysis within a week following collection. Samples (1.0 g wet weight, not seived) were incubated for 48 h with 7–8 nmol $Na_2{}^{35}SO_4$ (approximately 3.3×10^{10} Bq · mmol^{-1}) at temperatures indicated in the text. After incubation, the sample was washed three times with water and these washes were pooled to yield a combined fraction designated as soil water. The sample was then sequentially extracted with 1 *M* Na_2SO_4, 1*M* NaH_2PO_4, and 1*M* LiCl and washed with water once again. This wash was added to the combined salt extract and the sequential extraction was repeated twice. After hydrolysis in 6 *N* HCl at 121°C for 12 h, the residue was washed with water once, held in contact with 2 *N* NaOH for 12 h at room temperature, and finally washed again with water. Water washings were combined with the hydrolysate and base extract, respectively. Volumes of 0.4 mL were utilized for all extractants and water washes. In some cases, acid and base fractions were combined to yield a fraction designated in the text as nonsalt-extractable sulfur. In experiments designed to examine the influence of temperature and various soil amendments on the incorporation of ^{35}S into this combined fraction, samples were immediately extracted with salt without prior collection of soil water. Under these conditions, the incorporation of the label is terminated by isotope dilution as soon as contact is made with the first extractant (Na_2SO_4). In all cases, assays on a given sample were made in triplicate (variation ≤ 0.05% from the average) and average values are reported.

TABLE 1. Recovery of ^{35}S from soil samples taken from various forest ecosystems

Fraction	% recovery from: WS 2	WS 7	WS 17	WS 48
Soil water	2.9	12.2	3.5	9.9
Salt extract	73.2	65.4	77.2	73.6
Acid hydrolysate	11.5	10.2	8.4	5.9
Base extract	3.4	2.1	2.4	2.1
All fractions	91.0	89.9	91.5	91.5

NOTE: A-horizon samples incubated at 28°C.

TABLE 2. Recovery of ^{35}S from the 01 and 02 forest-floor layers and the underlying A-horizon soil

Fraction	% recovery from: 01	02	A	A[a]
Soil water	37.6	49.1	14.7	13.0
Salt extract	6.1	14.7	65.1	62.1
Acid hydrolysate	41.4	22.8	12.7	12.2
Base extract	8.1	3.6	2.4	3.3
All fractions	93.1	90.3	94.8	90.6

NOTE: Samples collected from WS 18 September 1982 and incubated at 20°C. For these particular samples, the 01, 02, and the A horizon occurred at depths of 3, 2, and 5 cm, respectively.

[a]Washed six times with water and subjected to three successive salt-extraction sequences.

Electrophoresis and determination of radioactivity

To determine the amount of ^{35}S present as sulfate, fractions from A-horizon soils were analysed by electrophoresis on Whatman No. 1 paper for 2 h at 250 V in barium and sodium acetate – acetic acid buffers, pH 4.5. In the former buffer, authentic sulfate remains at the origin because it precipitates as $BaSO_4$, whereas in the sodium acetate buffer, sulfate migrates about 14 cm from the origin. Electrophoresis in each

TABLE 3. Influence of sample depth and incubation temperature on recoveries of ^{35}S from soil samples taken from a pitch pine stand

Fraction	Sample depth[a] (cm)	% recovery after incubation at:[b] 5°C	28°C
Soil water	5	9.0	4.9
	25	7.4	4.2
Salt extract	5	70.8	73.2
	25	74.8	73.9
Nonsalt-extractable S	5	9.5	17.4
	25	12.1	7.7
All fractions	5	89.3	95.5
	25	89.0	91.1

[a]The 5 and 25 cm depths correspond with the A_1, and the mid-B_{22}, horizons, respectively. Samples were taken from a single pit selected at random within this stand.

[b]Incubation was for 48 h; the time at which maximum levels of nonsalt-extractable sulfur was formed judging from time courses run with each sample at each temperature.

buffer enables the identity of a given radioactive component to be confirmed as inorganic sulfate. Radioactive components were located on dried strips by scanning at slow speed using a Packard radiochromatogram scanner (Fitzgerald and Dodgson 1971). The total radioactivity of fractions from all samples was determined in a Beckman LS9000 scintillation counter using the ^{14}C full-spectrum energy window.

Results

Sulfur from exogenous inorganic sulfate was incorporated into the acid and base fractions of soils from both undisturbed and managed hardwood forests as well as in soils from the two pine stands (Tables 1–3). These nonsalt-extractable forms ranged from 8 to 17% of added ^{35}S and thus it appears that the ability of soil to mediate this conversion is not restricted by forest type, at least within the Coweeta region. As anticipated from the work of Johnson et al. (1980), the A-horizon soils in this area can adsorb substantial quantities of sulfate and between 65–71% of the added ^{35}S was recovered after salt extraction. Total recoveries of between 89 and 95% of added ^{35}S were obtained with all samples analyzed (Tables 1–3). The inability to completely recover ^{35}S was noted previously (Fitzgerald et al. 1982) and likely reflects experimental error. Inorganic sulfate was the only radioactive component detected when the salt extracts were examined by electrophoresis. The ability to adsorb sulfate does not, however, appear to be related to the capacity to incorporate sulfur into either the acid or base fraction. Thus, results of studies utilizing the 01 and 02 horizons (see Table 2 for an example) showed that these forest-floor layers adsorbed little added sulfate and yet incorporated substantially more sulfur into the acid and base fraction

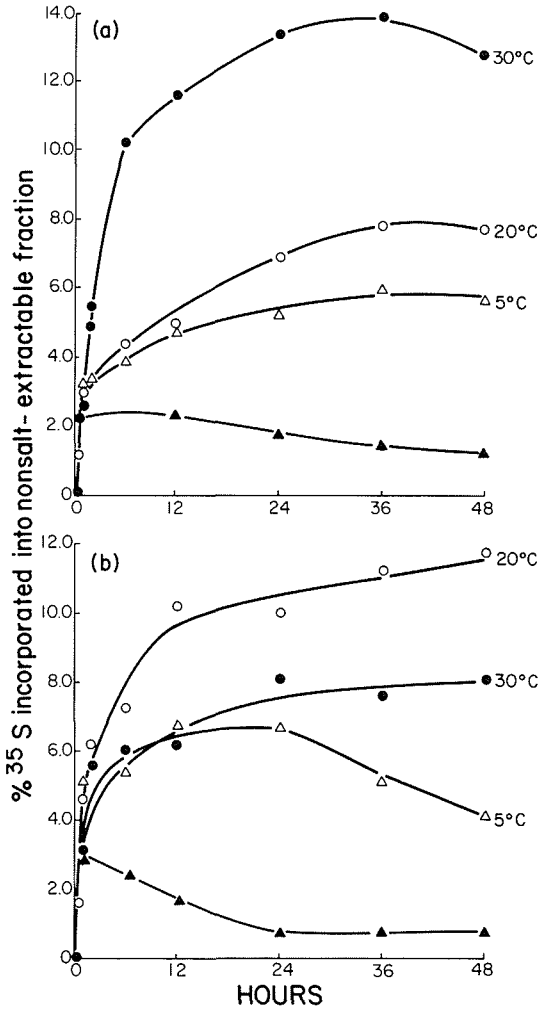

FIG. 1. Influence of incubation time, temperature, and sodium azide on the incorporation of ^{35}S into the nonsalt-extractable fraction of A-horizon soil from WS 2 (*a*) and WS 17 (*b*). △, 5°C; ○, 20°C; ●, 30°C; ▲, incubation at 30°C with 2 mmol sodium azide.

compared with the underlying A-horizon soils. Moreover, increased salt extraction of the latter samples failed to release sulfate in excess of that recovered by the standard salt-extraction procedure (Table 2).

The distribution of ^{35}S in the acid and base extracts was routinely assessed after electrophoresis of these samples. In all cases, inorganic sulfate accounted for at least 90% of the radioactivity of the acid fraction and comprised all of the ^{35}S found in the base extract (data not shown). Based upon the data of Table 2 and similar findings obtained with other hardwood sites, it is unlikely that sulfate recovered in the acid and base (nonsalt-extractable) fraction represents sulfate that failed to be extracted with salt. As suggested previously

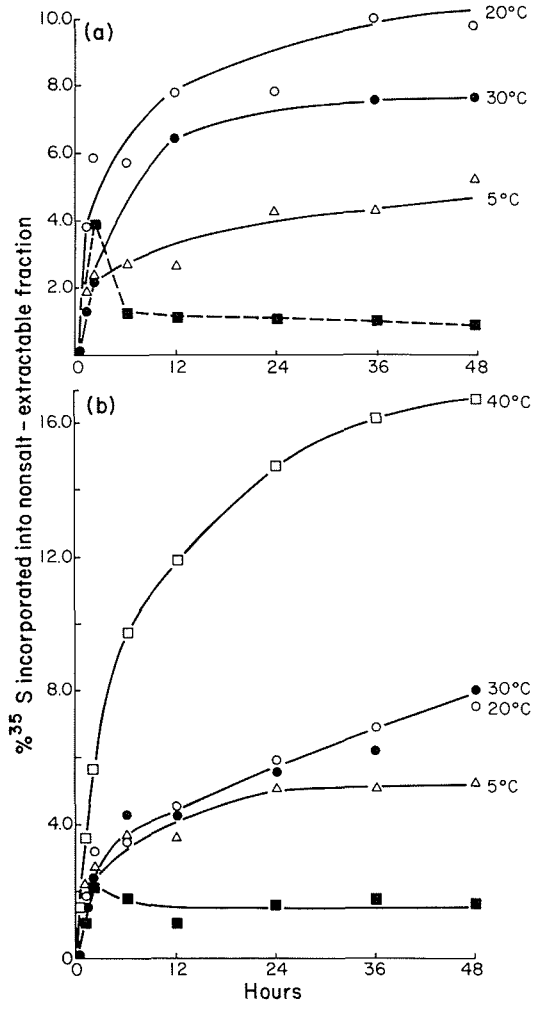

FIG. 2. Influence of incubation time, temperature, and sodium azide on the incorporation of ^{35}S into the nonsalt-extractable fraction of A-horizon soil from WS 7 (*a*) and WS 48 (*b*). △, 5°C; ○, 20°C; ●, 30°C; □, 40°C; ■, incubation at 30°C with 2 mmol sodium azide.

(Fitzgerald et al. 1982), the sulfate recovered in this fraction could originate from the hydrolysis of ester linkages and (or) the oxidation of sulfonate linkages of organic matter. If this assumption is correct, then the incorporation of sulfur into the nonsalt-extractable fraction involves the formation of covalent sulfur linkages and incorporation should be affected by parameters influencing enzyme-mediated rather than physiochemical reactions such as sulfate adsorption. Table 3 shows that the incorporation of sulfur into the nonsalt-extractable fraction decreased substantially with sample depth when soils from the pitch pine stand were incubated at 28°C whereas salt-extractable sulfur remained constant. However, when incubated at 5°C, subsamples from the same batch incorporated more sulfur into the above fraction when taken at a depth of 25 cm. This result is to be expected if the process is biologically mediated since soil temperature at a depth of 25 cm is obviously simulated more closely at 5 than 28°C whereas the reverse is true for a depth of 5 cm. Thus, unlike salt-extractable sulfur, the incorporation of sulfur into the nonsalt-extractable fraction of soils from the pitch pine stand was temperature and depth dependent (Table 3).

TABLE 4. Influence of spatial and seasonal variability on ^{35}S incorporation into nonsalt-extractable sulfur

	Mean % incorporation		Standard error of mean	
Plot No.	June	August	June	August
1	9.8	10.6	1.1	0.3
5	12.3	26.8	1.0	0.2

NOTE: Three A-horizon samples from WS 18 were taken at each sampling date during 1982 by random azimuth and distance within two 0.01-ha circular plots. Distance between plots was 132 m. Samples were incubated at 20°C.

Temperature dependence was also observed for A-horizon soils taken from the Coweeta watersheds. The time courses (Figs. 1 and 2) established for these soils also show that the extent of incorporation of sulfur was time dependent; an unlikely result for physiochemical reactions involving anion adsorption. Moreover, work with soils from WS 18 indicates that this process exhibits spatial as well as seasonal variation in samples taken along a transect of this watershed. An example of the results obtained with two of the sampling stations is shown in Table 4. It can be seen that significant differences in activity between these stations occurred during August. In addition a 2.2-fold increase in the ability to incorporate sulfur was observed with the August samples relative to those taken in June for a midslope position (plot 5) but activity characteristic of a ridge position along the transect (plot 1) remained constant (Table 4). Taken together, these results suggest that the process is biologically mediated and this was confirmed when the influence of a number of effectors was tested.

The addition of glucose stimulated the incorporation of sulfur into the nonsalt-extractable fraction in most cases whereas urea inhibited the process (Table 5). Although the latter result cannot be explained, stimulation by an exogenous energy source was not unexpected since sulfate ester formation requires the expenditure of 2 mol of ATP for each mole of sulfate esterified (Dodgson et al. 1982). The incorporation of sulfur into the nonsalt-extractable fraction was also inhibited by sodium azide and by a number of antibiotics, excepting tetracycline (Table 5). Tetracycline is known to stimulate microbially mediated biosyn-

TABLE 5. Formation of nonsalt-extractable sulfur in amended and nonamended soils taken from watersheds of the Coweeta basin

Additions	Amount added (mmol × 10^{-2})	% ^{35}S incorporation into nonsalt-extractable fraction			
		WS 2	WS 7	WS 17	WS 48
None	—	12.2	7.5	8.1	8.6
Glucose	60	14.7(+21)	12.4(+65)	8.1	12.2(+42)
Urea	120	9.6(−21)	2.9(−61)	3.7(−54)	1.8(−79)
Glucose, urea	60, 120	2.1(−83)	0.9(−88)	5.3(−35)	0.9(−90)
Sodium sulfate	80	0.3(−98)	0.5(−93)	0.1(−99)	0.4(−95)
Sodium azide	200	1.6(−87)	1.2(−84)	1.2(−85)	2.2(−75)
Erythromycin	9	4.6(−62)	1.2(−84)	3.0(−63)	2.4(−72)
Candicidin	9	7.2(−41)	2.4(−68)	4.6(−43)	4.2(−51)
Tetracycline	9	19.3(+58)	13.7(+82)	11.3(+40)	20.5(+138)
Chloramphenicol	9	7.7(−37)	11.7(+56)	7.0(−14)	10.2(+19)

NOTE: A-horizon samples incubated at 28°C; % change in activity compared with the control is given in parentheses.

thetic processes (Fitzgerald and Franklin 1983) and this antibiotic also stimulated the incorporation of sulfur into nonsalt-extractable forms in soils from all watersheds examined. Because candicidin inhibited incorporation (Table 5), the process is likely mediated by both fungi and bacteria because this antibiotic is specific for fungi (Lampen 1969), whereas the others are specific for bacteria. It should be noted further (Table 5) that sodium azide failed to totally abolish activity in soils from any of the watersheds examined. Time courses of incorporation run in the presence of this effector (Figs. 1 and 2) show that similar initial levels of incorporation were obtained in the presence and absence of azide. Identical results were obtained with the antibiotics (Fitzgerald and Johnson 1982) and collectively these data suggest that incorporation of sulfur into the nonsalt-extractable fraction is mediated initially by preformed enzymes in the soil which are not inhibited by these effectors. The occurrence of extracellular hydrolases of plant or microbial origin is well documented (Speir and Ross 1978), and it is possible that enzymes responsible for sulfate incorporation (sulfotransferases) may also be released into the soil, free of the plant or microbial cells in which they were synthesized. Since azide inhibited incorporation after about 1- to 3-h exposure (Figs. 1 and 2), synthesis of the sulfotransferase by microorganisms is necessary to sustain the process after initial incorporation of the anion.

Measurements of atmospheric sulfate input and hydrologic export for a number of watersheds show large accumulations of inorganic sulfur at Coweeta (Swank and Douglass 1977). Although Johnson et al. (1980) suggested that sulfate adsorption (salt-extractable sulfate) may represent an explanation for this observation, it is clear from the current and previous work (Fitzgerald et al. 1982) that sulfur which is incorporated into nonsalt-extractable forms could also be responsible in part for the sulfur-accumulating properties of these watersheds.

To recover additional sulfur after salt extraction, samples in the current work were treated with acid under conditions which hydrolyse sulfate-ester (C—O—SO_3) linkages (Fitzgerald 1976). Thus, sulfate recovered in the hydrolysate likely represents that which was originally esterified to organic matter. The observation that unlabelled sulfate completely inhibited the incorporation of ^{35}S into the acid fraction (Table 5) is consistent with this interpretation. The apparent inhibition can be explained in terms of isotope dilution in which the incorporation of labelled sulfate is replaced by the incorporation of unlabelled sulfate. Since the reaction is followed only by determinations for radioactivity an apparent inhibition was observed. The sulfate obtained by subsequent base extraction could represent that released from sulfonate linkages present in organic matter. In addition to ester sulfate, soil organic matter is known to also contain carbon-bonded sulfur in the form of sulfonates and sulfur-containing amino acids (Freney et al. 1970; Freney et al. 1972; David et al. 1982). The sulfonate linkage (C—SO_3) is stable in acid (Fitzgerald 1976) but undergoes oxidation to sulfate at alkaline pH (Dodgson et al. 1982) whereas the sulfur-containing amino acids do not liberate sulfate at either low or high pH (T. L. Andrew, unpublished). Thus acid- and base-extractable sulfate could represent an index of the sulfur originally incorporated into organic matter as ester- and sulfonate-linked sulfur, respectively.

In conclusion, our results demonstrate that sulfate incorporated into organic forms is microbially mediated for soils in the Coweeta basin. It remains to be determined if this process represents an important consideration relative to the sulfur cycle of other forest ecosystems. In view of the ability of these systems to vary

in terms of sulfate input and sulfur accumulation, this possibility warrants further investigation.

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