Soil Biol. Biochem. Vol. 18, No. 5, pp. 463–468, 1986
Printed in Great Britain

0038-0717/86 $3.00 + 0.00
Pergamon Journals Ltd

IN SITU MOBILIZATION OF ^{35}S-LABELLED ORGANIC SULPHUR IN LITTER AND SOIL FROM A HARDWOOD FOREST

T. C. STRICKLAND* and J. W. FITZGERALD
Department of Microbiology, University of Georgia, Athens, GA 30602, U.S.A.

and

W. T. SWANK
Southeastern Forest Experiment Station, Coweeta Hydrologic Laboratory, Otto, NC 28763, U.S.A.

(Accepted 10 *March* 1986)

Summary—^{35}S-labelled inorganic sulphate (SO_4^{2-}) was incorporated into the organic matter of an extract from 02 horizon litter to yield an organic-S preparation of high specific radioactivity (459 Ci mmol^{-1}; 1380 T Bq mmol^{-1}). In the labelled organic-S preparation, 13, 47 and 40% of the total S was present as ester sulphate, amino acid-S and sulphonate-S, respectively. The *in situ* mobilization of this organic-^{35}S preparation was monitored in surface horizons of a hardwood forest. Of the ^{35}S added as organic-S, 7 and 10% was converted to SO_4^{2-} in the 02 and A1-Ap horizons, respectively after 2 days of incubation. After 7 days, up to 28% of the added S had been mineralized to SO_4^{2-}, and 76 and 40% was metabolized to other forms of organic-S in each respective horizon indicating rapid transformation rates among organic-S pools *in situ*.

INTRODUCTION

The accumulation of inorganic sulphate (SO_4^{2-}) as insoluble organic-S has been observed in many ecosystems (Freney *et al.*, 1971; Saggar *et al.*, 1981; Strick *et al.*, 1982; Swank *et al.*, 1983). Once organic-S is formed, it may be mobilized to yield soluble S available for biological uptake or leaching loss (Bettany *et al.*, 1980; Johnson *et al*, 1982). As originally defined (Strickland *et al.*, 1984), the term mobilization refers to the solubilization of organic-S due to depolymerization of a larger matrix to yield smaller components which may or may not undergo S-mineralization.

Monitoring specific transformation dynamics within this organic-S pool has been hampered by deficiencies in methodology. ^{35}S was used by Freney *et al.* (1975) to label organic-S fractions in soil before planting with sorghum. Under these conditions, transfer of ^{35}S from soil organic matter to the plants was observed. However, as with the experiments of Strickland *et al.* (1984), such procedures require the removal of SO_4^{2-} from samples before organic-S cycling determinations, thereby creating an artificial S deficiency. These procedures can also only provide an indirect estimate of organic-S mobilization, since there is no way to non-destructively determine the amount of organic-S available for mobilization. Strickland and Fitzgerald (1984) attempted to circumvent this problem by adding ^{35}S-labelled organic matter free of SO_4^{2-} to samples which contained *in situ* amounts of unlabelled SO_4^{2-}. The utility of this method was nevertheless hampered by the low specific radioactivity of the organic-S which was available, necessitating the addition of large quantities of organic-^{35}S to samples.

In this paper, a new method for preparing ^{35}S-labelled organic-S is given which provides a substrate with high specific radioactivity. Field exposure of this preparation in surface horizons of a hardwood forest was carried out and S transformations within the organic-^{35}S pool were monitored for 2 and 7 days in order to obtain direct evidence for organic-S mobilization.

MATERIALS AND METHODS

Field exposures were carried out during July, 1984 in a mixed, mature hardwood forest located at the Coweeta Hydrologic Laboratory, near Franklin, NC. A study site adjacent to the stream (plot 9 of a permanent transect, Swank *et al.*, 1984) was chosen to examine mobilization of organic-^{35}S prepared from 02 layer material collected from the same site. The soil at this site is a sandy loam Ashe, one of the Typic Dystrochrepts.

Preparation of organic-^{35}S from 02 layer organic matter

This procedure is summarized in Fig. 1. Litter from the 02 horizon (about 30 g wet wt) was sieved (< 1 cm) to remove large roots and then shaken for 18 h at 30°C in a 0.1 M $Na_4P_2O_7$-NaOH buffer which had been adjusted to pH 8.0 with NaH_2PO_4 crystals (litter : buffer = 1 : 5). This type of extraction was shown by Fitzgerald *et al.* (1985) to recover organic-S with minimum rupture of organic-S linkages. The

*Present address: Department of Forest Science, Oregon State University, Corvallis, OR 97331, U.S.A.

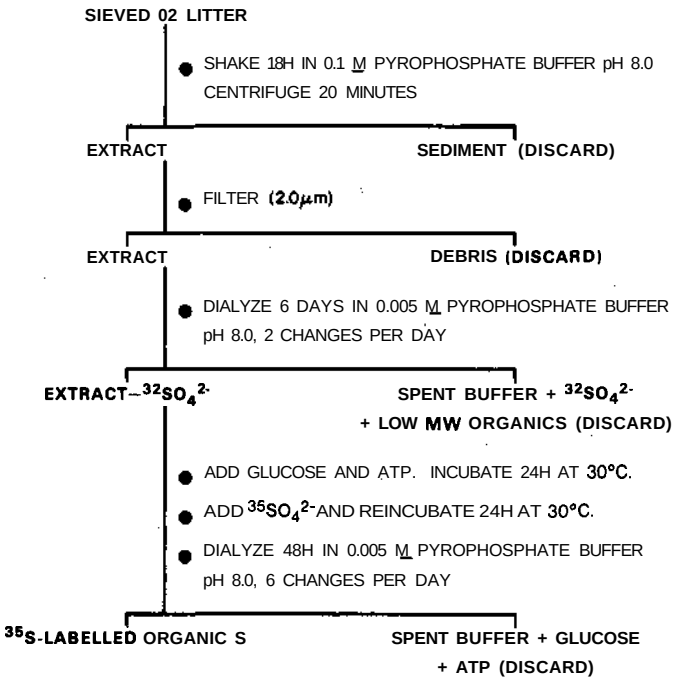

Fig. 1. Procedure for the preparation of organic-^{35}S.

mixture was centrifuged, and the supernatant was filtered (2.0 μm) to remove floating debris. The clear filtrate (extract) was dialyzed for 6 days at 10°C against 5 mM $Na_4P_2O_7$-NaOH buffer, pH 8.0. The buffer: extract ratio was 20:1 and the buffer was changed twice daily. This dialysis completely removed all unlabelled SO_4^{2-} from the extract while retaining organic components ⩾ 12,000 daltons in molecular weight.

Stock solutions of glucose and adenosine 5′-triphosphate (ATP) were sterilized by millipore filtration (0.22 μm) and added to the extract to final concentrations of 20 and 50 mM, respectively. This supplemented extract was maintained at 30°C with shaking for 24 h to stimulate microbial activity. Sterile ^{35}S labelled SO_4^{2-} (approx. 3 x 10^{10} Bq) was then added, and the extract was held again at 30°C for 24 h with shaking. To remove residual glucose and ATP following incubation, the extract was dialyzed as above except that the buffer was changed 12 times during 48 h. After examination by electrophoresis to ensure complete incorporation of ^{35}S, the preparation was frozen until needed.

Field exposure and sample analysis

Two samples (25 x 25 x 25 cm) of the uppermost horizons of the study site were excavated and placed into containers of the same dimensions which were perforated to ensure adequate drainage. One side of each container was hinged to allow insertion of the sample with minimal disturbance, and care was taken to maintain the integrity and orientation of the 02 litter layer and soil. Sampling in this manner included the Al (0–10 cm) and the Ap (10–25 cm) components of the solum. The samples in these containers were then placed in larger individual containers (catch basins) which had been sealed to prevent water loss. In order to ensure proper drainage, the interior sample containers were placed on supports to yield a 5 cm elevation from the bottom of the catch basin. Approximately 1.5 x 10^{10} Bq of ^{35}S labelled organic-S was added to each sample. Additions of this preparation (100 ml final volume) were made in 10 ml increments dropwise as evenly as possible over the surface of each sample. Each addition of the label was followed by the addition of an equal volume of deionized water. The sample containers were then left open at the study site for 2 or 7 days. There was 2.3 cm of rainfall during the initial 2 day exposure and 7.8 cm accumulated between days 2 and 7. Following field exposure, the 02 and A horizons in each sample were separated and roots were removed by hand. After sieving (< 1 cm) and thorough mixing, eight sub-samples of each horizon were extracted sequentially by the method of Fitzgerald *et al.* (1983). This procedure yields complete recoveries of water-soluble S, salt-extractable S, and acid-alkali extractable S (insoluble organic-S). Determination of ^{35}S in each fraction was by liquid scintillation counting and combined yields from the three fractions was taken as 100%.

Analysis by electrophoresis

The organic-^{35}S starting material (10 μl) and fractions derived from sample extraction after field exposure (80 μl) were analyzed by electrophoresis. In the case of the starting material, it was essential to ensure that the preparation was free of unincorporated $^{35}SO_4^{2-}$. Sample fractions were analyzed to determine (i) SO_4^{2-} release indicative of mineralization and (ii) forms of S not present in the starting material indicative of organic-^{35}S metabolism. Electrophoresis was for 2 h at 200 V on Whatman 3 MM paper in 0.1 M KH_2PO_4–K_2HPO_4 buffer, pH 8.0.

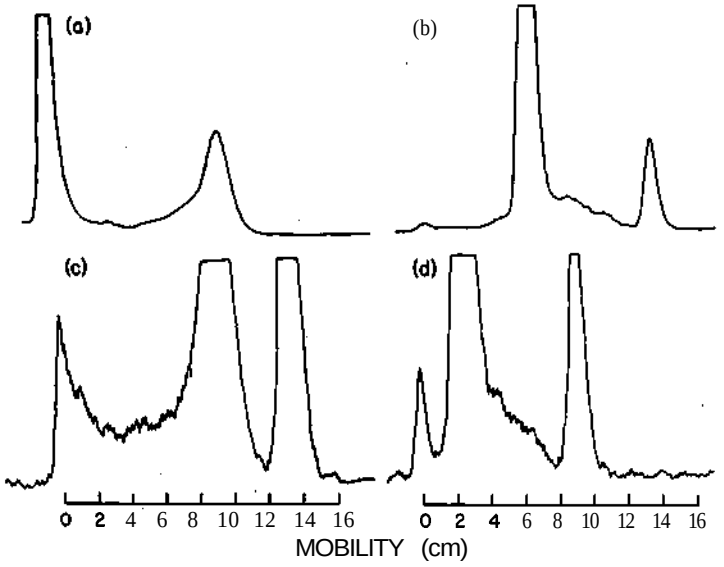

Fig. 2. Representative scans showing the electrophoretic composition of the starting material and changes associated with field exposure. (a), organic-^{35}S starting material; (b), acid hydrolysate of (a); (c), water extract of O_2 horizon after 2 days exposure; (d), acid extract of O_2 horizon. The component which migrated about 13 cm from the origin is inorganic SO_4^{2-}; other components were not identified.

^{35}S-labelled components were located on dried strips by scanning. Components represented by peaks on the chart paper (see Fig. 2) were quantified by triangulation.

Characterization of organic S pools

The S status of samples was estimated for each horizon before and after field exposure. Total S was determined by the alkaline oxidation method of Tabatabai and Bremner (1970), total hydriodic acid reducible-S (HI reducible-S) by the method of Johnson and Nishita (1952) as modified by Landers *et al.* (1983), and total non-hydriodic acid reducible-S (non-HI reducible-S) was calculated as the difference between total-S and HI reducible-S. Hydriodic acid reduction converts S in SO_4^{2-} and ester sulphate to sulphide which can be trapped and determined colorimetrically. Forms of S not reduced by hydriodic acid include amino acid and sulphonate-S. Raney nickel reducible-S (amino acid-S) was determined by the method of Freney *et al.* (1970), and sulphonate-S was estimated as the difference between non-HI reducible-S and Raney nickel reducible-S.

To determine phosphate extractable forms of S, sub-samples of each horizon (4 g wet wt) were mixed with 0.5 M Na_2HPO_4 (w/v ratio =1:5), shaken for 30 min, and centrifuged to remove particulates. All samples were treated 3 times successively in this manner and supernatant fluids were combined and filtered (2.0 μm retention). After determination of the HI reducible S present in each filtrate, sub-samples were filtered again successively through 0.45 and 0.22 μm membranes and the SO_4^{2-} content of each was determined by anion chromatography. Phosphate-extractable ester sulphate is the difference between the amounts of HI reducible-S and SO_4^{2-} present in each extract. The ester sulphate content remaining after this phosphate extraction is the difference between total HI reducible-S and HI reducible-S present in the phosphate extract.

Table 1. Sulphur status ($\mu g\ g^{-1}$) of the 02 and A horizons before and after *in situ* exposure with ^{35}S-labelled organic-S[a]

Sulphur pool	Before exposure 02	Before exposure A_1–Ap	2-day exposure 02	2-day exposure A_1–Ap	7-day exposure 02	7-day exposure A_1–Ap
Total S	908	218	1212	192	1232	181
Total HI reducible-S	275	57	330	110	346	137
Non-HI reducible-S	638	132	882	83	886	44
Amino acid-S	124	12	185	26	296	58
Sulphonate-S	451	115	697	57	590	0
Phosphate extractable						
HI reducible-S	64	38	77	32	58	25
Sulphate	21	28	19	9	30	22
Ester sulphate	44	10	58	23	29	3
Non-phosphate extractable						
Ester sulphate	211	48	253	78	288	112

[a]Means of triplicate determinations are reported.

Table 2. Changes in sulphur composition at various stages during preparation of ^{35}S-labelled organic-S from 02 layer material

Stage of preparation	Total S ($\mu g\ g^{-1}$)	Forms of S (% of total S available at each stage) HI-reducible-S	Amino acid-S	Sulphonate-S
Starting material	1090	32[a]	9	59
Undialyzed extract	315	53[a]	20	27
Dialyzed extract	152	41[b]	22	38
^{35}S addition and dialysis	173	38[b]	21	42

[a]Inorganic sulphate and ester sulphate.
[b]Ester sulphate only.

RESULTS AND DISCUSSION

Characterization of samples before and after field exposure

Carbon bonded-S (non-HI reducible-S) was the dominant form of S in the 02 horizon and sulphonate-S (as opposed to amino acid-S) was the major constituent of this pool (Table 1). Ester sulphate accounted for between 52 and 63% of the total S in the A horizons, while S in this linkage comprised only 25% of the S in the 02 horizon. A small proportion of S was in soluble forms as either SO_4^{2-} or ester sulphate, but the amounts of these were never greater than 7 or 17% in the 02 and A horizons, respectively (see phosphate extractable-S, Table 1). Analysis of samples collected before the addition of organic-S indicated an average total S content of 908 and 218 $\mu g\ S\ g^{-1}$ in the 02 and A horizons, respectively. When these values are compared to total S values of the samples after field exposure it can be seen that the addition of the organic-S extract increased the S content by 34.6% in the 02 horizon while having no effect on the S content of the A horizons. The amounts of various forms of S present in samples before exposure are also listed in Table 1.

Characterization of organic-S used in field exposures

Table 2 summarizes the changes in composition which occurred during preparation of ^{35}S-labelled organic-S. The extraction of organic matter from 02 horizon litter and subsequent treatments enriched the HI reducible-S and amino acid-S content while decreasing the amounts of sulphonate-S compared to the starting material. The total S of the final preparation was comprised of 38% ester sulphate, 21% amino acid-S and 42% sulphonate-S (Table 2). These determinations include both labelled and unlabelled forms of S. In terms of ^{35}S-labelled components only, 47, 40 and 13% of the added ^{35}S was incorporated into amino acid, sulphonate and ester sulphate linkages, respectively. Further examination of the final preparation by electrophoresis revealed the presence of two components. One component remained at the origin whereas the other migrated about 9 cm from the origin (Fig. 2a). When these components were eluted separately from the paper strips and the S content of each analyzed, it was found that about 50% of the total amino acid-S (labelled and unlabelled S), 27% of the total ester sulphate and 95% of the total sulphonate-S present in the preparation remained at origin during electrophoresis. The

Table 3. *In situ* fate of ^{35}S labelled organic-S in forest litter and soil

Form of organic-^{35}S extracted	Amount (% of total extractable ^{35}S) after: 2-day exposure 02	2-day exposure A_1–A_p	7-day exposure 02	7-day exposure A_1–A_p
Water soluble				
Total extracted	9.6 ± 0.4[a]	3.8 ± 0.3	4.4 ± 0.2	4.1 ±0.2
Non-metabolized	5.8	1.5	2.6	1.6
Metabolized[b]	0	0	0	0
Mineralized[c]	3.7	2.3	1.8	2.5
Adsorbed				
Total extracted	19.2 ± 1.0	24.7 ± 1.4	11.7 ± 0.7	33.7 ± 0.8
Non-metabolized	16.8	17.1	3.3	8.4
Metabolized	0	0	8.3	0
Mineralized	2.4	7.6	0	25.3
Acid extractable				
Total extracted	58.5 ± 0.9	56.3 ± 1.5	73.6 ± 0.8	47.9 ± 0.9
Non-metabolized	57.6	36.8	6.1	7.8
Metabolized	0	19.5	67.5	40.2
Mineralized	1.0	12.6[d]	12.6[d]	12.6[d]
Alkali extractable				
Non-metabolized	12.8 ±0.3	12.4[e]	10.3 ± 0.5	14.3 ± 0.6
Metabolized	0	2.9	0	0

[a]Mean ± SE, n = 8.
[b]Includes organic-S transformation either by polymerization, depolymerization, desulphation or SO_4^{2-} incorporation.
[c]Conversion of S to inorganic sulphate.
[d]Decrease in ester sulphate (i.e. no sulphate released during acid hydrolysis).
Total amount extracted with alkali = 15.3 ± 1.4, « = 8.

remainder of the S in these linkage groups was present in the mobile component (Fig. 2a).

In situ *transformations of organic-S*

Differences in the electrophoretic patterns of the starting material (Fig. 2a) and of soil or litter extracts after field exposure (Fig. 2c,d) enabled the determination of *in situ* transformations of organic-S (summarized in Table 3). In both horizons, much of the added ^{35}S became insoluble after 2 days and was thus not extractable with 1.0 M salt solutions. The amount of salt-extractable ^{35}S became further diminished between days 2 and 7 in the 02 horizon while, this pool increased in the A horizons. Thus, although the organic-S was soluble in pyrophosphate buffer, its addition to field samples resulted in >70% of it being located in the acid-alkali extractable fraction, thereby indicating the insolubility of the organic-S *in situ*. In the A horizons, there was a decrease in acid-alkali extractable S between days 2 and 7 which resulted in an increase in soluble and adsorbed S pools. The increase in these latter fractions was manifested as an increase in SO_4^{2-}, while the amounts of soluble and adsorbed organic-S diminished in the A horizons. Conversely, in the 02 horizon the amounts of soluble and adsorbed organic-S, as well as SO_4^{2-}, decreased during this period (Table 3). These results suggest that in the 02 horizon, organic-S accumulating and polymerizing processes dominate, resulting in the production of an insoluble organic-S matrix.

While high rates of mobilization occurred early during field exposures in the A horizons, decreased amounts of soluble and adsorbed organic-S observed after 7 days suggest that these rates became diminished. When the supply of soluble organic-S becomes exhausted, the rates of S-mineralization will be governed by the rate of depolymerization (solubilization) of the insoluble organic-S matrix *in situ*. Net changes in organic-S metabolism (Table 4) indicate that S-mineralization was about 2-fold greater after 7-days in both horizons. This indicates that conversion of organic-S to SO_4^{2-} is a continuous process *in situ* even when there is an excess of SO_4^{2-} from precipitation. After the 7-day exposure, about 3.5 $\mu g\ l^{-1}$ of SO_4^{2-}-S had entered the study site in throughfall. Table 4 also shows that total organic-S transformation (metabolism and mineralization) ranged from about 90% of added S in the 02 horizon to 80% in the A horizons after 7 days. This suggests that although there is a net retention of S in these horizons (Swank and Douglass, 1977), a large capacity for organic-S metabolism does exist.

The assessment of organic-S cycling *in situ* has been hampered by limitations to detection and by incomplete characterization of the organic-S pool (Strickland *et al.*, 1984; Fitzgerald *et al.*, 1985). However, the method of preparation utilized in our study provided organic-S with sufficiently high specific radioactivity to overcome limitations to detection when assaying large (kg) quantities of soil. In developing the procedure, advantage was taken of the observation that this carbon- and energy-dependent process is microbially mediated (Fitzgerald *et al.*, 1983). Moreover, the use of an organic matter extract as opposed to 02 layer material *per se* (Strickland and Fitzgerald, 1985), ensured that adsorption of added label did not take place thus enriching the radioactivity of the final preparation. Characterization of this preparation with respect to linkage types and electrophoretic patterns made it possible to monitor the rapid exchange of S between different pools. For example, organic-S mineralization was detected not only by the presence of SO_4^{2-} but also by a net increase in ester sulphate content (carbon bonded-S must be mineralized to supply SO_4^{2-} for esterification). Moreover, a net decrease in ester sulphate content is also indicative of mineralization. Further insight into the cycling of organic-S can be obtained from the appearance in extracts of electrophoretically-separable components which were not present in the starting material. Such components may be produced from the mineralization and reincorporation of S, or they may be depolymerization or polymerization products of components already present in the starting material. We propose that this approach has resulted in a more comprehensive view of organic-S mobilization.

Table 4. Cycling of organic-S in forest floor and soil during 2 and 7 day field exposures

Horizon	Amount (%) of added organic-S: Metabolized[a] 2-day	7-day	Mineralized 2-day	7-day
02	0[b]	75.8	7.1	14.4
A_1–Ap	22.4	40.2	22.5	40.4

[a]See footnote [b], Table 3 for explanation.
[b]Unable to detect change in starting material.

Acknowledgements—This research was supported by the National Science Foundation and through a cooperative agreement with the United States Department of Agriculture (Forest Service). We thank J. T. Ash and D. D. Hale for expert technical assistance, M. E. Watwood and W. T. Scott Swank for sampling assistance, and B. F. Strickland for proofreading.

REFERENCES

Bettany J. R., Saggar S, and Stewart J. W. B. (1980) Comparison of the amounts and forms of sulfur in soil organic matter fractions after 65 years of cultivation. *Soil Science Society of America Journal 44*, 70–75.

Fitzgerald J. W., Ash J. T., Strickland T. C. and Swank W. T. (1983) Formation of organic sulfur in forest soils: a biologically mediated process. *Canadian Journal of Forest Research* 13, 1077-1082.

Fitzgerald J. W., Strickland T. C. and Ash J. T. (1985) Isolation and partial characterization of recently formed forest floor and soil organic sulfur. *Biogeochemistry* 1, 155–167.

Freney J. R., Melville G. E. and Williams C. H. (1970) The determination of carbon bonded sulfur in soil. *Soil Science* 109, 310–318.

Freney J. R., Melville G. E. and Williams C. H. (1971) Organic sulphur fractions labelled by the addition of ^{35}S-sulphate to soil. *Soil Biology & Biochemistry* 3, 133-141.

Freney J. R., Melville G. E. and Williams C. H. (1975) Soil organic matter fractions as sources of plant-available sulphur. *Soil Biology & Biochemistry* 7, 217–221.

Johnson D. W., Henderson G. S., Huff D. D., Lindberg S. E., Richter D. D., Shriner D. S., Todd D. E. and Turner J. (1982) Cycling of organic and inorganic sulphur in a chestnut oak forest. *Oecologia* 54, 141-148.

Johnson C. M. and Nishita H. (1952) Microestimation of sulfur in plant materials, soil, and irrigation waters. *Analytical Chemistry* **24**, 736–742.

Landers D. H., David M. D. and Mitchell M. J. (1983) Analysis of organic and inorganic sulfur constituents in sediments, soils and water. *International Journal of Environmental and Analytical Chemistry* **14**, 245-256.

Saggar S., Bettany J. R. and Stewart J. W. B. (1981) Sulfur transformations in relation to carbon and nitrogen in incubated soils. *Soil Biology & Biochemistry* **13**, 499–511.

Strick J. E., Schindler S. C., David M. B., Mitchell, M. J. and Nakas J. P. (1982) Importance of organic sulfur constituents and microbial activity to sulfur transformations in an Adirondack forest soil. *Northeastern Environmental Science* 1, 161-169.

Strickland T. C. and Fitzgerald J. W. (1984) Formation and mineralization of organic sulfur in forest soils. *Biogeochemistry 1*, 79–95.

Strickland T. C., Fitzgerald J. W. and Swank W. T. (1984) Mobilization of recently formed forest soil organic sulfur. *Canadian Journal of Forest Research* **14**, 63–67.

Strickland T. C. and Fitzgerald J. W. (1985) Incorporation of sulphate-sulphur into organic matter extracts of litter and soil: involvement of ATP sulphurylase. *Soil Biology & Biochemistry* **17**, 779–784.

Swank W. T. and Douglass J. E. (1977) Nutrient budgets for undisturbed and manipulated hardwood forest ecosystems in the mountains of North Carolina. In *Watershed Research in Eastern North America*, Vol. 1 (D. L. Correll, Ed.), pp. 343–363. Smithsonian, Washington, DC.

Swank W. T., Fitzgerald J. W. and Ash J. T. (1984) Microbial transformation of sulfate in forest soils. *Science* **223**, 182–184.

Swank W. T., Fitzgerald J. W. and Strickland T. C. (1985) Transformation of sulfur in forest floor and soil of a forest ecosystem. In *Hydrological and Hydrochemical Approaches to the Acidification of Ecological Systems*. Nordic Hydrological Program—Report No. 10 (I. Johansson, Ed.), pp. 137–145. Published by The Coordinating Committee for Hydrology in Norden, Uppsala, Sweden.

Tabatabai M. A. and Bremner J. M. (1970) An alkaline oxidation method for determination of total sulfur in soils. *Soil Science Society of America Proceedings* **34**, 62–65.