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SHORT COMMUNICATION

BACTERIAL PRODUCTION OF ORGANIC SULPHUR IN A FOREST LITTER EXTRACT

T. C. STRICKLAND
Department of Forest Science, Oregon State University, Corvallis, OR 97331, U.S.A.

and

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

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Incorporation of sulphate-S into organic matter in soil and litter of deciduous forests in North Carolina has been postulated as a mechanism contributing to sulphur accumulation in these ecosystems (Fitzgerald *et al.*, 1982; Strickland and Fitzgerald, 1984; Swank *et al.*, 1984). Field studies (Strickland *et al.*, 1986) have demonstrated that organic sulphur is accumulated as ester sulphate-S, sulphonate-S, and amino-acid-S and indirect evidence (Fitzgerald *et al.*, 1983) suggests that the process is microbially mediated. The formation and release of organic-S by bacteria is well documented (Fitzgerald, 1976). We used the organic matter extraction–incubation method of Strickland and Fitzgerald (1985) to examine the possible role of exoenzymes in the extracellular sulphation of soil organic matter. This method extracts organic matter without rupturing organic-S linkages and after dialysis and amendment with organic-S precursors, yields a partially defined medium which can be used to monitor rates of organic-S formation. Since organic-S biosynthetic pathways utilize adenosine 5′-phosphosulphate (APS) and 3′-phosphoadenosine-5′-phosphosulphate (PAPS) as "active" intermediates during synthesis (Benson, 1963; De Meio, 1975; Dodgson and Rose, 1975; Flavin, 1975; Greenberg, 1975; Harwood, 1980), these nucleotides were prepared with a ^{35}S label for use as a tracer. The aim of our study was to show the direct involvement of soil micro-organisms in organic-S formation.

Litter from the O2 horizon was collected in August of 1982 from watershed 18, a mixed mature hardwood forest located at the Coweeta Hydrologic Laboratory in North Carolina. This site is part of a permanent sampling transect established in 1982 to monitor sulphur transformations on this watershed (Fitzgerald *et al.*, 1983; Swank *et al.*, 1984). Litter samples were sieved (< 1 cm) and stored in sealed polyethylene bags at 10°C. Organic matter was extracted from the litter at pH 8.0 by the method of Strickland *et al.* (1986). The resulting extract was supplemented with NaCl, Na_2SO_4, NH_4Cl (each 0.5 g l^{-1}), $MgCl_2$ (0.15 g l^{-1}), agar (20 g l^{-1}) and autoclaved. Before plates were poured, stock solutions of glucose and ATP were sterilized by filtration (0.2 μm) and added to the sterilized extract to final concentrations of 20 and 2 mM, respectively. Bacteria were isolated by streaking the unsterilized extract onto this agar. One bacterial isolate was identified on the basis of colony morphology, picked off and maintained on slants of the same medium. The bacterium is a Gram-negative rod 1–2 μm long × 0.5 μm in width. The isolate was cultured in a minimal salts medium as above supplemented with the organic extract (5% v/v) which had been sterilized by autoclaving. Cells were harvested by centrifugation and pellets were resuspended in and washed 3 times with a minimal salts solution (50 ml) containing (g l^{-1}) KH_2PO_4 (1.5), K_2HPO_4 (3.5); and NaCl, NH_4Cl and $MgCl_2$ at the concentrations used above. To prepare inocula, the washed cells were resuspended in sterile 5 mM $Na_4P_2O_7$-NaOH, pH 8.0.

Three media (10 ml in 125 ml Erlenmeyer flasks) were inoculated after addition of 2.0×10^3 counts min^{-1} of either $^{35}SO_4^{2-}$, $AP^{35}S$ or $PAP^{35}S$. The media used were: (1) extract medium, unsterilized organic matter extract supplemented with NaCl, $MgCl_2$, NH_4Cl, glucose and ATP at the concentrations used above; (2) extract medium as in (1) lacking the glucose and ATP; and (3) a minimal salts medium containing the salts, glucose and ATP as in (1), with KH_2PO_4 and K_2HPO_4 (1.5 and 3.5 g l^{-1} respectively). The final pH of each medium was adjusted to 8.0 with dilute NaOH. Cultures were incubated at 30°C with shaking, and periodically, aliquots (80 μl) were collected and immediately subjected to electrophoresis.

$AP^{35}S$ was prepared by incubating 370 MBq of $Na_2^{35}SO_4$ with 100 units of inorganic pyrophosphatase (EC 3.6.1.1, Sigma) and 10 units of ATP sulphurylase (EC 2.7.7.4, Sigma) in Tris-HCl buffer at 30°C for 2 h as described by Strickland and Fitzgerald (1985). $PAP^{35}S$ was synthesized by the method of Renosto and Segel (1977). Briefly, a PAPS-generating enzyme system was partially purified from baker's yeast (Fraction III of Renosto and Segel). The $PAP^{35}S$ formed after incubation of this preparation with $Na_2^{35}SO_4$ (370 mBq), ATP and Mg^{2+} was separated from residual $^{35}SO_4^{2-}$ by paper electrophoresis and eluted from electrophoretograms using cold pH 8.0 pyrophosphate buffer (5 mM $Na_4P_2O_7$-NaOH).

Samples were applied to Whatman No. 1 chromatography paper and electrophoresed in 0.1 M KH_2PO_4-K_2HPO_4 buffer, pH 8.0, for 2 h at 200 V. The paper strips were dried at 40°C, and ^{35}S-labelled components were located by radiochromatogram scanning. These components are designated according to their mobility relative to that of sulphate (R_{SO4}, see values in Fig. 1).

Component quantification by area triangulation of peaks on electrophoretogram scans and identification of organic-S have been described by Strickland *et al.* (1984) and Strickland and Fitzgerald (1985).

When cells were grown in an extract medium amended with $AP^{35}S$ or $PAP^{35}S$, but not with glucose plus ATP, electrophoresis indicated that $^{35}SO_4^{2-}$ was the only form of

et al., 1982; Sawhney and Nicholas, 1976). Incorporation of ^{35}S from $PAP^{35}S$ into the origin component never exceeded 26% of the total ^{35}S when cells were grown in either medium.

Unlike $PAP^{35}S$, incubation with authentic $AP^{35}S$ resulted in substantial incorporation of ^{35}S into the origin component by cells grown in both media. Thus, about 45% of the available ^{35}S from $AP^{35}S$, was incorporated into this component after 48 h (Fig. 4a and b). However, the rate of incorporation was much lower than that observed using $^{35}SO_4^{2-}$ as the donor (Fig. 2). A ^{35}S-labelled metabolite with an $R_{SO4} = 0.75$ was also generated from $AP^{35}S$. This electrophoretic component increased initially in response to $AP^{35}S$ with bacteria grown in both media and as with $PAP^{35}S$, $^{35}SO_4^{2-}$ was also generated. However, the ambient levels of this anion were lower (7–17% of total ^{35}S) than those arising from $PAP^{35}S$ (10–35% of total ^{35}S).

Rapid incorporation of ^{35}S-sulphate into organic matter has been documented using extracts of soil and litter from hardwood forests of the Coweeta basin (Strickland and Fitzgerald, 1985). The process is dependent upon ATP concentrations and indirect evidence obtained from the use of antibiotics (Fitzgerald *et al.*, 1983; Strickland and Fitzgerald, 1984) indicated bacterial involvement. Direct evidence for this is provided in the current work, since incubation of a Gram-negative soil isolate with $^{35}SO_4^{2-}$ produced two electrophoretically-separable species of organic-S which exhibited the same mobilities as those found by Strickland and Fitzgerald (1984, 1985).

Compared to the rate of organic-S formation from sulphate, the very low rates of formation observed when the ^{35}S-labelled organic-S precursors $AP^{35}S$ or $PAP^{35}S$ were used as the S source indicate no extracellular sulphotransferase activity in the media and that the "active sulphate" components must first be transported into the cell or mineralized to sulphate before S uptake and organic-S synthesis. This was supported by the appearance of inorganic sulphate in the medium from both nucleotide sulphates. Further, although the identification of electrophoretically-mobile components ($R_{SO4} = 0.64$, 0.75 and 1.00) as being separate from starting S donors was impossible (cf. Fig. 1), the formation of a ^{35}S-labelled origin component in the minimal salts medium indicates *de novo* production of organic-S as part of the microbial biomass.

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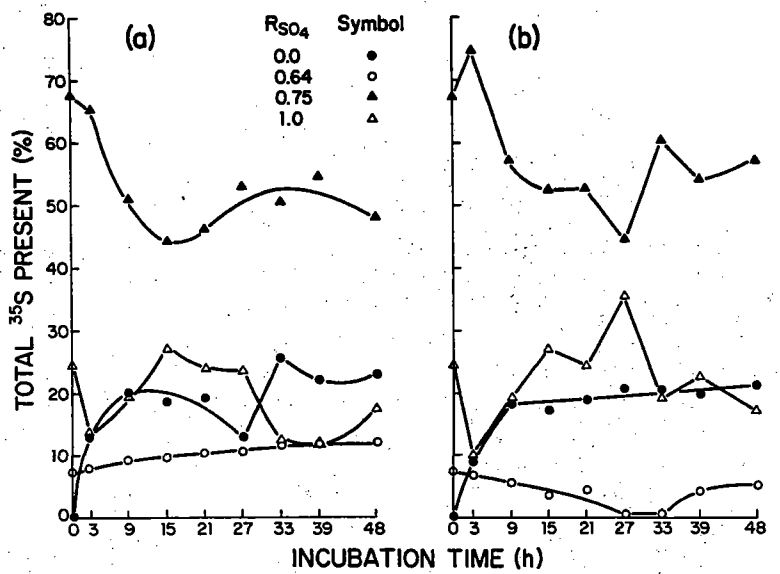

Fig. 3. Fate of ^{35}S from PAP^{35}S during incubation of isolate in (a) glucose and ATP amended extract medium; and (b) minimal salts medium. (▲)—PAP^{35}S, (△)—^{35}S-sulphate, (●)—^{35}S-labelled origin component, (○)—unknown ^{35}S-labelled component ($R_{SO4} = 0.64$, possibly AP^{35}S).

^{35}S remaining. In the glucose- and ATP-amended extract medium, ^{35}S was incorporated into organic matter when SO_4^{2-}, APS or PAPS were utilized as the source of S (Fig. 1a–c). The formation of an organic-S component which exhibited zero electrophoretic mobility (origin component, $R_{SO4} = 0.0$) was observed with all three sulphur sources, although at different rates. Incubation of the isolate with $^{35}SO_4^{2-}$ resulted in rapid incorporation of ^{35}S into this component as well as into a component which exhibited an $R_{SO4} = 0.75$ (Fig. 2a). This component exhibited an electrophoretic mobility identical to that of authentic PAPS (cf. Figs 1a and d). The same rapid incorporation rate was observed with cells grown in minimal salts medium (Fig. 2b). Incubation of the bacterium with PAP^{35}S (Figs 3a and b) resulted in much lower rates of S incorporation into the origin component, as compared to $^{35}SO_4^{2-}$ as the S source, irrespective of whether the isolate was cultured in the extract or minimal salts medium. During growth of the bacterium, ^{35}S-labelled SO_4^{2-} was released from PAP^{35}S and persisted in both incubation media. Under these conditions, a ^{35}S-labelled metabolite having an electrophoretic mobility identical to authentic APS was also formed in the medium ($R_{SO4} = 0.64$, Fig. 1d). This observation was not unexpected since many bacteria are known to produce PAPS 3′-nucleotidase, the enzyme catalyzing the loss of a 3′-phosphate grouping from PAPS to form APS (Dodgson

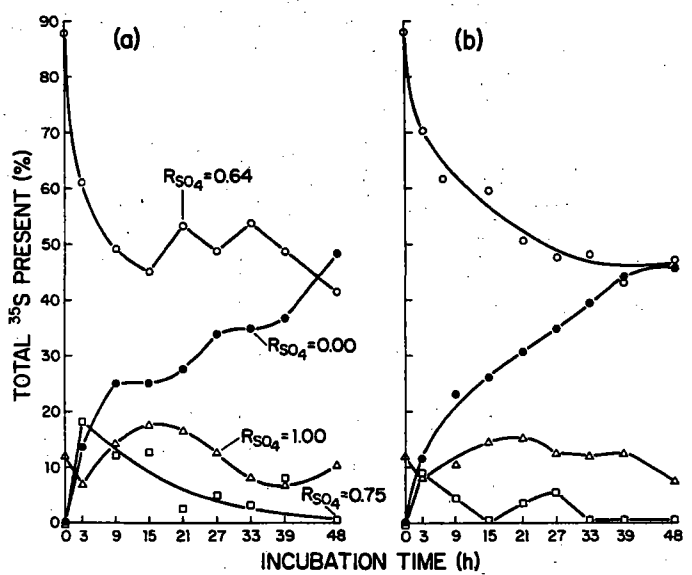

Fig. 4. Fate of ^{35}S from AP^{35}S during incubation of isolate in (a) glucose plus ATP amended extract medium; and (b) minimal salts medium. (○)—AP^{35}S, (●)—^{35}S-labelled origin component, (△)—^{35}S-sulphate, (□)—^{35}S-labelled electrophoretic component with $R_{SO4} = 0.75$.

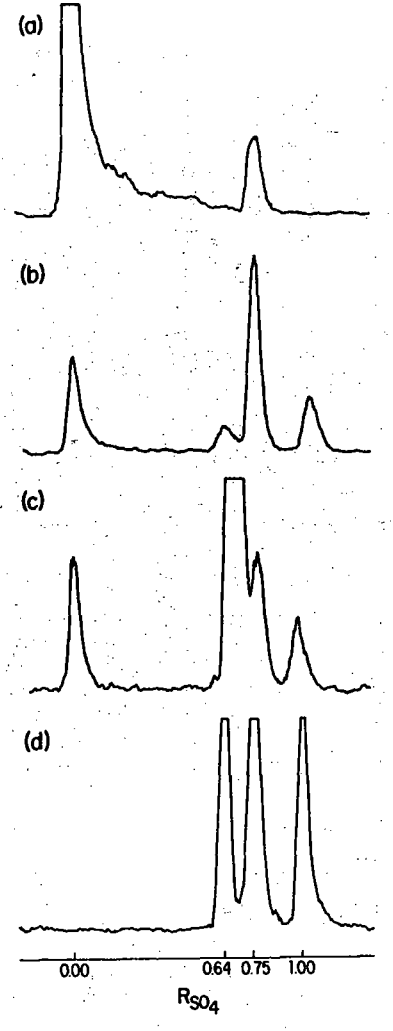

Fig. 1. Electrophoretogram scans showing the formation of organic-S from: (a) ^{35}S-labelled sulphate; (b) PAP^{35}S; and (c) AP^{35}S after 48 h incubation of the isolate in the glucose and ATP amended extract medium. Electrophoresis of a mixture of the sulphur donors as standards produced the scan profile shown in (d); AP^{35}S, PAP^{35}S and $^{35}SO_4^{2-}$ exhibited R_{SO4} values of 0.64, 0.75 and 1.0, respectively.

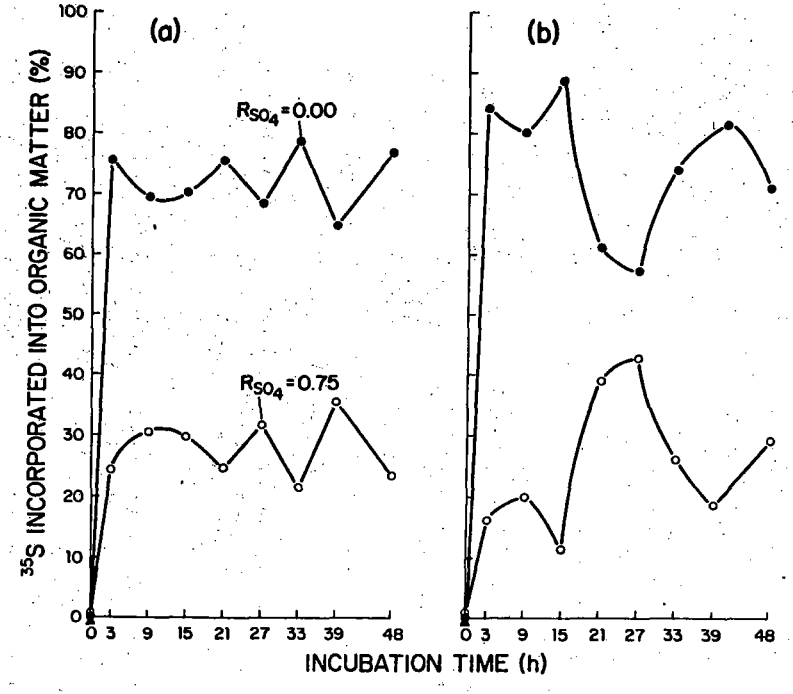

Fig. 2. Formation of organic-S from sulphate during incubation of the isolate in (a) glucose and ATP amended extract medium; and (b) minimal salts medium. (●)—incorporation of ^{35}S into origin electrophoretic component; (○)—incorporation of ^{35}S into component with $R_{SO4} = 0.75$; this component co-electrophoreses with authentic PAPS, see Fig. 1d.