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INCORPORATION OF SULPHATE-SULPHUR INTO ORGANIC MATTER EXTRACTS OF LITTER AND SOIL: INVOLVEMENT OF ATP SULPHURYLASE

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Summary—Organic matter extracts from the 01, 02 and A1 horizons of a hardwood forest rapidly incorporated [^{35}S]sulphate into organic sulphur components larger than 12,000 daltons in molecular weight. In the 02 layer extract, the ^{35}S-label was incorporated into amino acid-S, sulphonate-S and ester sulphate linkages. This process was stimulated by adenosine 5′-triphosphate (ATP), cellulose, cellobiose, glucose, succinate and pyruvate. The involvement of ATP sulphurylase, elaborated by bacteria present in this extract, is suggested by observations that incorporation was also stimulated by Mg^{2+} and inhibited by erythromycin, tetracycline, sodium azide, selenate, molybdate and chlorate. ^{35}S-labelled adenosine 5′-phosphosulphate (APS) or 3′-phosphoadenosine-5′-phosphosulphate (PAPS) also served as S donors for organic-S formation but the rate of organic-S formation was much lower than that observed when [^{35}S]sulphate was the S donor.

INTRODUCTION

The role of soil microorganisms in cycling nutrients from organic to inorganic forms has long been considered to be of great importance. More recently, microbial involvement in nutrient immobilization, thus increasing soil nutrient retention capacity, has been the subject of increasing interest. In temperate soils, the microbially mediated incorporation of sulphate-S into organic matter has been documented (Freney *et al.*, 1971; Saggar *et al.*, 1981; Fitzgerald *et al.*, 1983; Strick *et al.*, 1982). Watersheds at the Coweeta Hydrologic Laboratory, North Carolina were estimated to have a SO_4^{2-} flux into organic S of up to 30 kg S $ha^{-1}\,yr^{-1}$ (Swank *et al.*, 1984). The incorporation of S into an insoluble organic form increases the negative charge associated with the soil colloid fraction and therefore, cation-exchange capacity. This latter property is one of the primary criteria determining soil productivity. In addition to serving as a possible S storage mechanism (Fitzgerald, 1978), the immobilization of SO_4^{2-} may thus play an indirect but important role in soil nutrient status by increasing the retention of cations.

Using organic matter extracts of samples as opposed to parent material, we have attempted to determine factors which regulate SO_4^{2-} incorporation into organic matter under conditions in which SO_4^{2-} adsorption does not occur. The use of such extracts also enables an evaluation of the influence of charged effectors, such as cations, anions and nucleotides which might otherwise become adsorbed by parent material.

MATERIALS AND METHODS

Samples were collected from two sites on watershed 18 at the Coweeta Hydrologic Laboratory, located near Otto, North Carolina. The watershed is a mixed mature hardwood forest and soils are of the sandy loam Ashe series, a Typic Dystrochrept. The two sites are situated on a permanent transect (Swank *et al.*, 1984) where S transformations have been studied extensively since 1982. Samples from the 02 horizon were collected in August 1982 from a site adjacent to a stream having an oak–hickory overstory and a rhododendron understory. These samples were used for all determinations except for studies of the effect of genetic horizon on organic-S formation. To determine the effect of horizon, samples of the 01, 02, A1 (0–10 cm), B (30–40 cm) and C (70–90 cm) horizons were collected in May 1983 from an excavated pit on a site located at mid-slope on the catchment. This site is characterized by an oak–hickory overstory and an understory of dogwood, mountain laurel and flame azaleas. Samples from the 02 and mineral horizons were sieved (< 1 cm) and all roots and stones removed. Samples were kept in sealed polyethylene bags at 10°C before extraction.

Preparation of organic extracts

Step 1, undialyzed extract. Samples from each horizon were shaken for 18 h in a 0.1 M $Na_4P_2O_7$–0.1 N NaOH solution which had been adjusted to pH 8.0 with NaH_2PO_4 crystals (pyrophosphate buffer). The sample to buffer ratio was 1:5. This extraction was shown by Fitzgerald *et al.* (1985) to recover organic-S components with minimum damage to S linkages. The mixture was then centrifuged for 20 min in a Sorvall GSA rotor at 3000 rev min^{-1} to remove particulate debris. The supernatant was decanted and filtered (2.0 μm retention) to remove floating debris.

Step 2, dialyzed extract. The filtered extract was dialyzed at 10°C for 6 days using a 4.8 μm pore dia cellulose membrane against 5 mM pyrophosphate buffer, pH 8.0. The buffer:extract ratio was 20:1, and the buffer was changed twice daily. This dialysis removes unlabelled SO_4^{2-} and other ions from the

preparation but retains organic components ≥12,000 daltons in molecular weight.

Step 3, incubation of extract. Stock solutions of glucose and 5′-adenosine triphosphate (ATP), pH 7.0, were sterilized by filtration (0.22 μm) and then added to the dialyzed extract to final concentrations of 20 and 50 mM, respectively. The supplemented extract was shaken at 30°C for 24 h to stimulate microbial activity.

Purification of commercially-available [^{35}S]sulphate

The sodium sulphate preparation received from Amersham was contaminated with [^{35}S]organic sulphate. To remove organic S, the preparation was made 3 N with respect to HCl and autoclaved for 2 h. This treatment hydrolyzes sulphate ester linkages in organic S to yield inorganic sulphate (Fitzgerald, 1978).

Electrophoresis

Electrophoresis was performed on Whatman 3MM chromatography paper in 0.1 M KH_2PO_4–K_2HPO_4 buffer, pH 8.0, for 2 h at 200 V. The strips were dried at 35°C and ^{35}S-labelled components were located by scanning in a Packard 7220/21 radiochromatogram scanner. Inorganic $^{35}SO_4^{2-}$ was identified by comparison to a known standard. The presence of any component migrating at a rate less than $^{35}SO_4^{2-}$ ($R_{SO_4} < 1.0$) indicates that the component contains ^{35}S covalently linked to some larger moiety.

Preparation of ^{35}S-labelled adenosine 5′-phosphosulphate ($AP^{35}S$) and 3′-phosphoadenosine 5′-phosphosulphate ($PAP^{35}S$)

To prepare AP^{35}S, inorganic pyrophosphatase (Sigma, 100 units) and ATP sulphurylase (Sigma, 10 units) were dissolved separately in 1 ml of 1.0 M Tris–HCl buffer, pH 7.6. One ml of each enzyme solution was mixed with 1 ml of 0.1 M ATP, 1 ml of 0.2 M $MgCl_2$, 0.6 ml of 1.0 M Tris–HCl buffer, pH 7.6 and 400 μl containing 10 mCi of $^{35}SO_4^{2-}$ (8.4 nmol ^{35}S, 3.7×10^8 Bq). This mixture was incubated at 30°C for 2 h. In such a reaction, ATP sulphurylase reversibly catalyzes the formation of APS from ATP and SO_4^{2-} with pyrophosphate as an end product. The pyrophosphatase hydrolyses the pyrophosphate to drive the reaction in favor of APS formation (Renosto and Segel, 1977). To denature the enzymes, the mixture was placed in a boiling water bath for 2 min and then cooled in ice. The AP^{35}S preparation was diluted with 0.1 M pyrophosphate buffer, pH 8.0, to a final radioactivity of about 10^4 Bq ml^{-1} and maintained at −20°C.

^{35}S-labelled PAPS was prepared by the method of Robbins (1962) as modified by Renosto and Segel (1977). A PAPS generating system was partially purified from bakers' yeast and a mixture was prepared containing 1.2 ml of this preparation, 0.5 ml of 0.1 M ATP (pH 7.0), 0.5 ml of 0.2 M $MgCl_2$, 0.3 ml of 1.0 M Tris–HCl buffer (pH 8.5), 10 mCi of $^{35}SO_4^{2-}$ (8.4 nmol ^{35}S, 3.7×10^8 Bq) and water to a total volume of 3.0 ml. After 4 h at 37°C, the reaction was terminated by boiling as above and denatured protein was removed by centrifugation. After electrophoresis of the supernatant, PAP^{35}S (located by radiochromatogram scanning) was eluted from paper strips with cold 0.1 M pyrophosphate buffer, pH 8.0, and the eluate (about 10^4 Bq ml^{-1}) was maintained at −20°C. The identity of PAP^{35}S was confirmed by co-electrophoresis with a known unlabelled standard (Sigma) which was located by its absorption of ultraviolet radiation.

Factors influencing formation of organic-S

Extracts (10 ml) collected either at step 1, 2 or 3 were placed in sterile plugged 125 ml capacity Erlenmeyer flasks. Approximately 10^4 Bq was added to each extract as ^{35}S-labelled SO_4^{2-}, APS or PAPS, and the mixtures were shaken at 30°C. The extracts were sampled (80 μl) as necessary and electrophoresed. The percent ^{35}S incorporated into organic components was determined after radiochromatogram scanning by an area comparison of representative peaks traced on the chart paper. Details of electrophoretic techniques to identify organic-S were given by Strickland *et al.* (1984).

To determine the effect of carbon and energy on capacities for $^{35}SO_4^{2-}$ incorporation into organic matter, dialyzed extracts from the 02 horizon (Step 2) were amended with $^{35}SO_4^{2-}$ and either ATP, glucose, succinate, pyruvate, cellulose or cellobiose (see Tables 1 and 2 and legend to Fig. 2 for concentrations). Dialyzed (step 2) extracts containing ATP (final concentration 20 mM) were also used to determine the influence of Mg^{2+} (20 mM) on SO_4^{2-} incorporation. The effect of antimicrobial agents and anions known to inhibit ATP sulphurylase (selenate, chlorate and molybdate) was determined by incubating dialyzed extracts as indicated in step 3 together with the antibiotic or the anion (see legend to Fig. 4 for concentrations). $^{35}SO_4^{2-}$ was then added and the mixture was incubated again. Prior to $^{35}SO_4^{2-}$ addition, the extracts amended with the appropriate anion were incubated only for 1 h. ^{35}S incorporation was monitored for up to 25 h.

Characterization of organic-^{35}S linkage groups

Organic-S components formed from $^{35}SO_4^{2-}$ after 24 h incubation of the anion with a step 3 extract of 02 layer material were separated by electrophoresis and eluted separately from dried strips with 0.1 M pyrophosphate buffer, pH 8. The amount of ^{35}S present in each as amino acid-S was determined by Raney nickel reduction (Freney *et al.*, 1970). The [^{35}S]ester sulphate and total ^{35}S content of each component was determined by the hydriodic acid reduction method of Johnson and Nishita (1952) and by the alkaline oxidation method of Tabatabai and Bremner (1970), respectively. Sulphonate-^{35}S was estimated as the difference between ^{35}S not reducible by hydriodic acid (all C-S linked S) and the amount of C-S linked S present as amino acid-^{35}S.

RESULTS

Amendment of an undialyzed step 1 extract of 02 layer material with ^{35}S-labelled SO_4^{2-} followed by incubation for 10 days at 30°C resulted in the formation of at least two electrophoretically-distinct organic-^{35}S components (Fig. 1a). These were retained after dialysis (Fig. 1b) showing that both components were greater than 12,000 daltons in

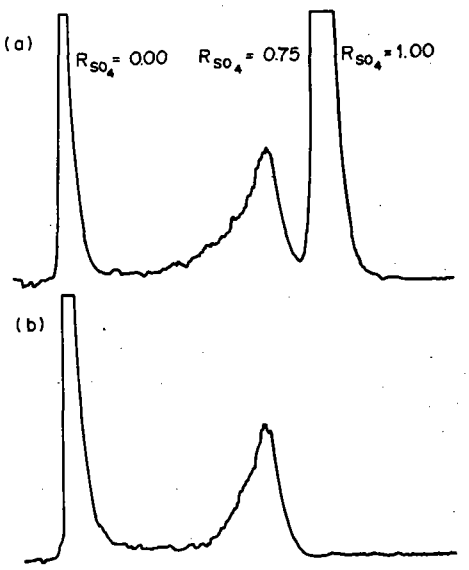

Fig. 1. Electrophoretogram scan showing components present in organic sulphur after incubation of 02 layer extract with ^{35}S-labelled sulphate. (a) Organic ^{35}S before dialysis; (b) organic ^{35}S after dialysis. Components are identified by mobility relative to SO_4^{2-} ($R_{SO_4} = 1.0$).

molecular weight. The amount and rate of S incorporation was dependent upon the level of energy present, because dialyzed (step 2) extracts of this horizon failed to form organic-^{35}S, even after 23 days incubation, unless an energy source such as ATP was provided. In this case, increasing levels of organic-S were formed as ATP concentrations were increased from 5 to 50 mM (Table 1). The form in which energy was supplied also had a marked effect upon the rate of S incorporation into organic material. Of the three compounds examined (Fig. 2), glucose amendments resulted in the highest rate of incorporation. Succinate and pyruvate yielded lower rates of incorporation, but the addition of either carbon source nevertheless resulted in maximum SO_4^{2-} incorporation after 36 h (as opposed to 24 h for glucose at the same concentration). The much less readily utilizable but possibly more environmentally relevant carbon sources, cellobiose and cellulose, also stimulated incorporation with dialyzed (step 2) extracts (Table 2) although at a much lower rate than that observed with glucose, succinate, pyruvate, or undialyzed extracts containing no carbon additions. Amendments with cellobiose or cellulose resulted in a net incorporation of 35 and 14% respectively, of the available SO_4^{2-} after 23 days whereas an undialyzed extract incorporated 41% of the available SO_4^{2-} after 10 days.

When dialyzed (step 2) extracts from the 02 horizon contained ATP (20 mM), incorporation was stimulated about 1.75-fold by the additional presence of 20 mM $MgCl_2$ (Fig. 3). While incorporation was stimulated by Mg^{2+} and by increased energy supply, the process was inhibited by the addition of selenate, chlorate or molybdate to step 3-02 horizon extracts (Fig. 4). The substantial inhibition caused by amendment with the respiratory uncoupler, sodium azide, or the antibacterial antibiotics, erythromycin or tetracycline, is also shown in Fig. 4. However, neither bacitracin, an antibiotic specific for Gram-positive bacteria, nor candicidin which is effective against

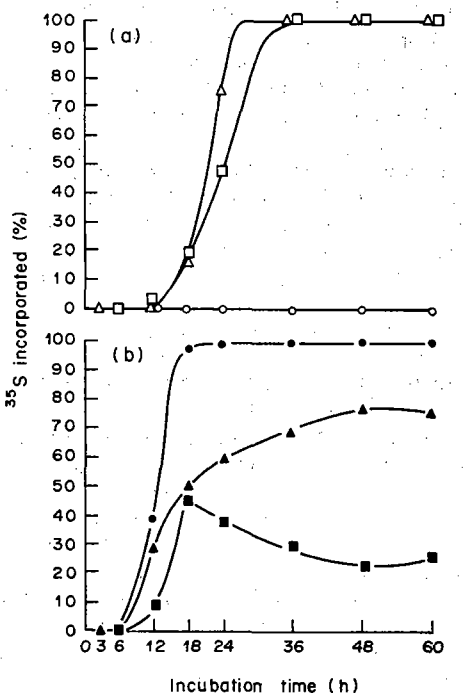

Fig. 2. Effect of carbon and energy source on incorporation of [^{35}S]sulphate by a step 2, dialyzed extract of the 02 layer. (a) Unamended control (○), supplemented with succinate 15 mM (△), supplemented with pyruvate 15 mM (□); (b) supplemented with glucose 15 mM; total ^{35}S incorporated (●), component with $R_{SO_4} = 0.00$ (▲), component with $R_{SO_4} = 0.75$ (■). See Fig. 1 for representative electrophoretic components.

Table 1. Influence of ATP concentration on incorporation of ^{35}S-labelled sulphate into organic matter of extracts from the 02 horizon[a]

ATP concentration (mM)	% ^{35}S present in		
	Origin component	Component 2	Sulphate
0	0	0	100.0
5	9.5	9.1	81.3
10	13.5	16.0	70.5
15	10.6	23.8	65.6
25	20.8	20.0	59.2
50	29.9	26.3	43.8

[a]Dialyzed (step 2) extracts, see Materials and Methods, were incubated with the nucleotide and SO_4^{2-} for 72 h.

Table 2. Stimulation of [^{35}S]sulphate incorporation into organic matter by cellobiose and cellulose[a]

Incubation time (days)	% ^{35}S incorporated after amendment with	
	Cellobiose (1.0% w/v)	Cellulose (1.0% w/v)
2	11.3	5.5
5	7.5	7.8
13	19.0	11.5
23	34.8	14.0

[a]Dialyzed (step 2) extracts of the 02 horizon were incubated with SO_4^{2-} after amendment with each carbon source. Unamended controls did not incorporate S.

fungi and algae (Lampen, 1969) inhibited incorporation at concentrations of 0.5 mg ml^{-1} (data not shown).

In all cases examined, there was a rapid increase in S incorporation into the origin-electrophoretic component of the organic-S followed by a decline in the S content of this component. This decrease was accompanied by a continued increase in levels of S in the electrophoretically mobile component (component 2 see Fig. 2b for an example and Fig. 1 for electrophoretogram scans). Both electrophoretic components contained S as amino acid-S, ester sulphate and sulphonate-S. About 64% of the ^{35}S of the origin component was comprised of sulphonate-S whereas the mobile component consisted mainly of ester sulphate and amino acid-S (Table 3).

The energy requirement for SO_4^{2-} incorporation with extracts from the 01, 02 litter layers and soil is illustrated in Table 4. In contrast to undialyzed step 1 extracts of the A horizon which failed to incorporate S, similar extracts of litter exhibited low rates of incorporation in the absence of added carbon. This activity was abolished after dialysis suggesting that these extracts contained utilizable carbon sufficient to supply energy for this rate of incorporation. In contrast to these results, extracts from all horizons incorporated substantial amounts of S into organic matter when step 3 extracts were incubated for 24 h with 20 mM glucose and 50 mM ATP prior to subsequent incubation in the presence of SO_4^{2-}. No incorporation was detected in extracts from the B or C horizon under these conditions (Table 4).

Higher rates of SO_4^{2-} incorporation were observed when the 02 layer:pyrophosphate buffer ratio was increased from 1:5 to 3:5. Addition of PAP^{35}S to extracts prepared by either means resulted in degradation of this ^{35}S donor to $^{35}SO_4^{2-}$ and possibly AP^{35}S (Table 5). As with SO_4^{2-} incorporation, degradation of PAP^{35}S occurred at a higher rate in the more concentrated extract. Incubation of either extract with ATP and glucose (step 3 extract) prevented the rapid degradation of PAP^{35}S (data not shown), and this observation allowed the determination of whether PAP^{35}S (or AP^{35}S) could serve

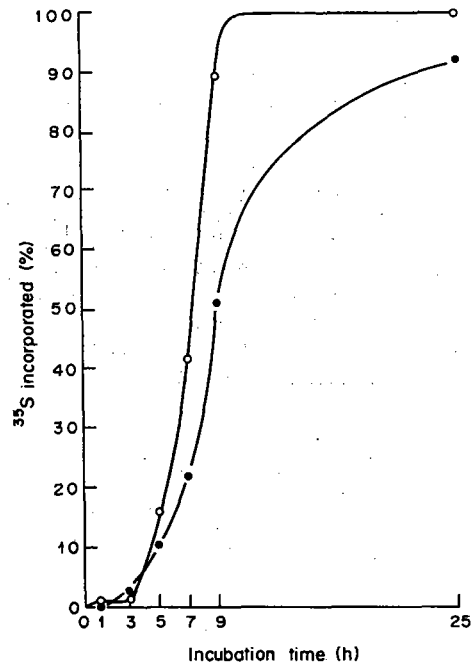

Fig. 3. Effect of 20 mM ATP (●) or 20 mM ATP plus 20 mM $MgCl_2$ (○) on sulphate incorporation by dialyzed (step 2) 02 horizon extracts.

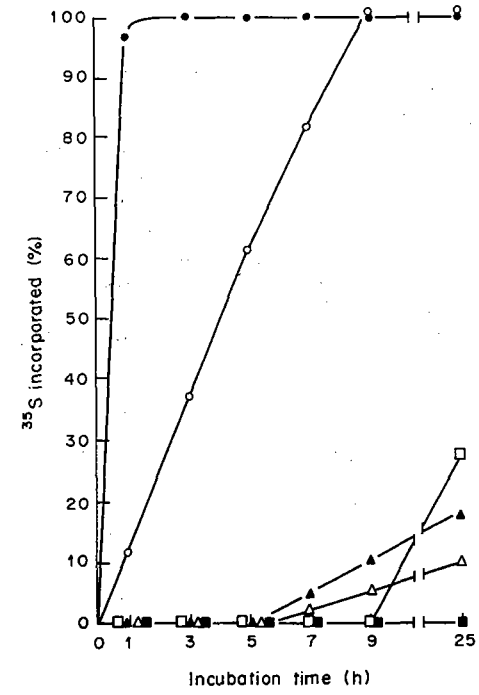

Fig. 4 Influence of antimicrobial agents and inhibitors of ATP sulphurylase on [^{35}S]sulphate incorporation by step 3, 02 horizon extracts: (●) control, (○) 10 μM selenate, (△) 1 mM chlorate, (▲) 1 mM molybdate, (□) 0.5 mg ml^{-1} erythromycin, (■) 0.5 mg ml^{-1} tetracycline or sodium azide.

Table 3. Characterization of electrophoretically separable organic-S components labelled during incubation with [^{35}S]sulphate[a]

Component mobility (R_{SO4})[b]	% of total ^{35}S present as		
	Amino acid-S	Ester sulphate	Sulphonate-S
0	27.2	8.9	63.9
0.75	51.2	43.0	5.8

[a]Step 3, 02 horizon extract (see Materials and Methods) was incubated for 24 h with [^{35}S]sulphate.
[b]Mobility relative to that of $^{35}SO_4^{2-}$ at pH 8.0.

Table 4. Effect of genetic horizon on [^{35}S]sulphate incorporation into organic matter

Type of extract[a]	Incubation time (days)	% ^{35}S incorporated[b]		
		01	02	A
Undialyzed, step 1	3	59.8	3.3	0
	11	70.7	2.4	0
Dialyzed, step 2	23	0	0	0
Incubated, step 3	0.3	100	100	17.5
	1	100	100	71.5

[a]See Materials and Methods for description of extract preparation.
[b]Not detected in step 3 extracts of B or C horizon.

as a S donor for organic-S formation. The addition of PAP^{35}S or AP^{35}S in place of $^{35}SO_4^{2-}$ to these step 3 extracts did not result in incorporation levels comparable to those obtained with $^{35}SO_4^{2-}$ alone. Moreover, the addition of unlabelled SO_4^{2-} (0.1 mM final concentration) essentially eliminated incorporation of S from either PAP^{35}S or AP^{35}S (Fig. 5), suggesting that the anion was preferred as a S donor over the nucleotides by bacteria present in these extracts.

DISCUSSION

The enzyme, ATP sulphurylase, catalyzes the formation of APS from SO_4^{2-} and ATP; a reaction which is usually suggested to represent the first step in the formation of organic-S in all forms of life examined so far (De Meio, 1975). A Mg^{2+}–ATP chelate is required for the activity of this enzyme and SO_4^{2-} analogs such as selenate, molybdate and chlorate competitively inhibit SO_4^{2-} binding by this enzyme (Wilson and Bandurski, 1958). Stimulation of the formation from SO_4^{2-} of organic-S in soil by sources of carbon and energy was demonstrated by Freney *et al.* (1971), Saggar *et al.* (1981), Fitzgerald *et al.* (1983) and by Maynard *et al.* (1985). The use of organic matter extracts as opposed to samples of parent material has enabled a partial elucidation of the mechanism of SO_4^{2-} incorporation. The hydrolysis of PAPS in the absence of energy supplements to these extracts and the stimulation of SO_4^{2-} incorporation by Mg^{2+} and ATP above that of ATP alone indicate that the incorporation process is coupled to the hydrolysis of ATP. These results together with the observed inhibition of incorporation by the above mentioned SO_4^{2-} analogs, suggest the involvement of ATP sulphurylase and thus the reaction sequence, $SO_4^{2-} \rightarrow$APS$\rightarrow$PAPS$\rightarrow$organic S. However, neither APS nor PAPS mediated incorporation of S at rates approaching those observed with SO_4^{2-} alone. This apparent contradiction may be explained on the basis that organic-S formation by bacteria present in organic matter extracts is probably dependent upon

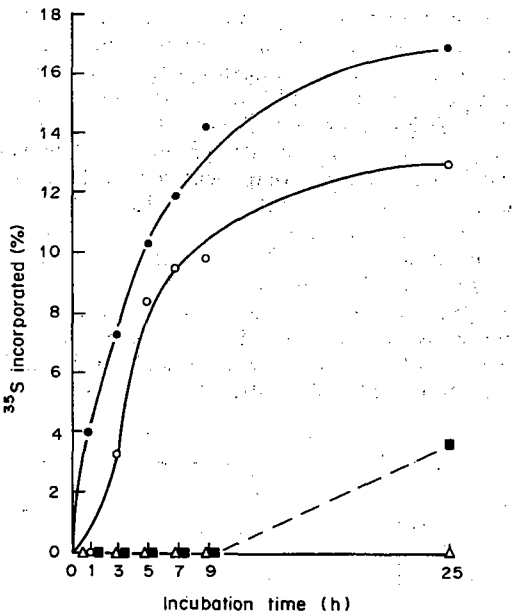

Fig. 5. Incorporation of ^{35}S from ^{35}S-labelled APS or PAPS by step 3, 02 horizon extracts: (●) PAP^{35}S (△) PAP^{35}S plus 0.1 mM unlabelled SO_4^{2-}, (○) AP^{35}S, (■) AP^{35}S plus 0.1 mM unlabelled SO_4^{2-}.

Table 5. Influence of sample:pyrophosphate buffer ratio on sulphur incorporation into organic sulphur components separated by electrophoresis

Sulphur donor and incubation time (h)	1:5				3:5			
	% ^{35}S in component				% ^{35}S in component			
	Origin	2[a]	3[b]	Sulphate	Origin	2[a]	3[b]	Sulphate
[^{35}S)sulphate[c] (+50 mM ATP)								
2	0	0	0	100.0	8.0	0	3.2	88.8
4	0	0	0	100.0	15.2	0	10.0	74.8
6	1.6	0	0	98.4	25.2	0	18.2	56.7
PAP^{35}S								
0	0	6.2	76.3	17.5	0	6.2	76.3	17.5
2	0	11.4	53.6	35.1	0	15.2	13.1	71.8
4	0	16.9	56.9	26.2	2.4	0	0	97.6
6	0	19.7	54.2	26.1	3.3	0	0	96.7

[a]Mobility comparable to APS ($R_{SO4} = 0.67$).
[b]Component with 0.75 R_{SO4} mobility; PAPS co-electrophoreses with this component.
[c]Added to step 2, dialyzed extracts of the 02 horizon.

uptake of the S-donor. Although a well defined transport mechanism for SO_4^{2-} has been described for bacteria (Dreyfuss, 1964), the diffusion of APS and PAPS through the bacterial membrane is unlikely in view of the hydrophillic nature of these nucleotides. Thus, the extracellular degradation of APS or PAPS to SO_4^{2-} may represent a prerequisite for organic-S formation. The observation that organic-^{35}S formation from $AP^{35}S$ or $PAP^{35}S$ was inhibited by excess (0.1 mM) unlabelled SO_4^{2-} supports this explanation. However, it is worth noting that ^{35}S transfer from $AP^{35}S$ began to recover after 9 h under these conditions suggesting that APS may be able to function to some extent as a S donor without prior conversion to SO_4^{2-}.

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