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The Uptake and Assimilation of Sulphate by *Thiobacillus ferrooxidans*

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Abstract. Sulphate was rapidly bound by cell suspensions of *Thiobacillus ferrooxidans*. The binding was depressed by tetrathionate but was unaffected by Group VI anions, cysteine or methionine. Increasing uptake of sulphate was observed in cell suspensions incubated in the presence of ferrous iron. The bulk of ^{35}S-sulphate was removed from the organisms by washing with dilute sulphuric acid and the remaining label was incorporated into cold trichloroacetic acid-soluble compounds. ^{35}S-labelled adenosine 5′-sulphatophosphate was produced from ATP and ^{35}S-sulphate by cell suspensions and in cell-free extracts. There was no evidence for the production of adenosine 3′-phosphate 5′-sulphatophosphate assayed by a very sensitive bioluminescence method.

Key words: *Thiobacillus ferrooxidans* – Sulphate Assimilation – Sulphur Nucleotides.

Sulphate uptake and transport systems have been studied in a number of heterotrophic microorganisms. The assimilatory reduction of sulphate involves its activation to APS by ATP-sulphurylase (E.C. 2.7.7.4) and, in most microorganisms, the phosphorylation of APS to PAPS by APS-kinase (E.C. 2.7.1.25) (Roy and Trudinger, 1970; Schiff and Hodson, 1973). Sulphite is then produced from APS or PAPS by reductase enzymes. During the oxidation of inorganic sulphur compounds by chemolithotrophic thiobacilli, intermediate APS may be formed by APS-reductase (E.C. 1.8.99.2). Sulphate is then produced from APS by ADP-sulphurylase (E.C. 2.7.7.5) or ATP-sulphurylase (Peck, 1968; Suzuki, 1974). In *Thiobacillus ferrooxidans*, the oxidation of inorganic sulphur compounds is mediated by an AMP-independent sulphite oxidase (E.C. 1.8.3.1) which is not enhanced by 5′-AMP (Vestal and Lundgren, 1971), suggesting that APS may not be formed. In the sulphur-oxidizing thiobacilli, reduced sulphur for cellular synthesis is probably derived from sulphide, which is an intermediate in the oxidation of inorganic sulphur compounds. It has been found that the adaptation to heterotrophy by *Thiobacillus intermedius* can only be accomplished when reduced organic sulphur compounds are included in the culture medium (Smith and Rittenberg, 1974). This suggests that this bacterium is lacking one or more of the enzymes which mediate the activation and reduction of sulphate.

In contrast, *Thiobacillus ferrooxidans* exhibits no requirement for reduced sulphur compounds during its growth on ferrous iron or an organic compound such as glucose. We have studied the uptake, assimilation and activation of sulphate in a strain of *Thiobacillus ferrooxidans* continually maintained on ferrous iron. There was no evidence for PAPS production although we used a very sensitive bioluminescence method (Stanley *et al.*, 1975) which detects picomole amounts of this nucleotide.

Abbreviations. APS = adenosine 5′-sulphatophosphate; PAPS = adenosine 3′-phosphate 5′-sulphatophosphate; PAP = adenosine 3′-phosphate 5′-phosphate.

Materials and Methods

Organism. Cultures of *Thiobacillus ferrooxidans* were grown on ferrous iron in 55 l carboys containing 50 l of a medium described previously (Tuovinen and Kelly, 1973). The cultures were aerated with 4 l of sterilized air/min. The bacteria, at the late exponential growth phase, were harvested and the washed cell suspensions were dispensed into a sulphate-free medium containing $MgCl_2$, 10 mM; $(NH_4)_2HPO_4$, 10 mM, in 0.01 N HCl. Cell-free extracts (S_{10}) were prepared in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM Na_2-EDTA, using an Aminco French Pressure Cell at 20000 lbs/in^2 and then centrifuging at $10000 \times g$ for 30 min. The supernatant fraction (S_{10}) was used for the experiments. The cell suspensions and cell-free extracts were standardized by dry weight determinations (Tuovinen and Kelly, 1973) or protein estimations (Lowry *et al.*, 1951) respectively.

Binding and Uptake of ^{35}S-Sulphate. Incubations were carried out at 30°C in a reciprocating water bath. Details of the incubation mixtures are listed in the Results section. The stock solution of sulphate (pH 2.3), which was used in

combination with carrier-free ^{35}S-sulphate, was prepared by mixing 0.5 M K_2SO_4, 0.5 M Na_2SO_4 and the 0.5 M H_2SO_4 in the ratio 1:1:0.86. The cell suspensions were incubated for 2 min prior to adding the radioactive sulphate. Samples taken at intervals were mixed with an equal volume of cold 0.4 N HCl, and after 30 min on ice, were centrifuged at $10000 \times g$ for 10 min, then washed twice with cold incubation medium containing non-labelled sulphate at an equal concentration. Aliquots of the resuspended cells were applied on glass fibre filter discs (Whatman GF/A) into scintillation vials.

Fractionation of Organisms Supplied with ^{35}S-Sulphate. Samples (0.5 ml) of cell suspensions incubated with labelled sulphate for 8 hrs were fractionated as described previously (Tuovinen and Kelly, 1974). Duplicate 100 µl aliquots from each fraction were applied on glass fibre filter discs into scintillation vials.

Determination of [^{35}S]APS and [^{35}S]PAPS. Intact organisms were washed twice with cold non-radioactive medium and resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 2 mM Na_2-EDTA. The cells were disrupted using an MSE ultrasonic probe at 20 kilocycles/sec for 5 min. Aliquots of cell-free extracts obtained after centrifuging at $10000 \times g$ for 10 min were applied directly onto electrophoretograms (Whatman 3 MM).

The formation of [^{35}S]APS and [^{35}S]PAPS in cell-free extracts (S_{10}) was studied in the following incubation mixtures: cell-extract (S_{10}), 10.6 mg; Tris-HCl buffer (pH 7.5), 21.5 µmoles; Na_2-EDTA, 0.86 µmoles; $MgCl_2$, 20 µmoles; Na_2SO_4, 0.2–40 µmoles; ^{35}S-sulphate, 5 µCi; ATP, 20 µmoles, in a total volume of 1.0 ml. Incubations were also carried out in the presence of inorganic pyrophosphatase (from baker's yeast), 1–10 units; creatine phosphate, 50 µmoles; creatine phosphokinase, 0.175 units. APS-kinase activity was tested by radiotracer techniques in the following incubation mixture: Tris-HCl buffer, 25 µmoles; Na_2-EDTA, 1 µmole; $MgCl_2$, 20 µmoles; [^{35}S]APS, 11 nmoles (0.003 µCi); cell extract, 10.0 mg, in a total volume of 0.7 ml. Aliquots of the incubation mixtures, after 0.5, 15, 30 and 45 min incubation, were applied to Whatman 3 MM paper. The labelled sulphur compounds were separated by high voltage electrophoresis (Tate, 1968; Adams *et al.*, 1971).

Bioluminescence Methods for Determining PAPS and ATP. Levels of PAPS in incubation mixtures were determined at intervals of up to 45 min employing the luciferin-luciferase system of *Renilla reniformis*, as described previously (Stanley *et al.*, 1975). This method is much more sensitive than the radiotracer techniques since it detects about 5 pmoles PAPS. The formation of PAPS was studied in two incubation mixtures: A (APS-kinase activity); Tris-HCl buffer (pH 7.5), 25 µmoles; Na_2-EDTA, 1 µmole; ATP, 20 µmoles; $MgCl_2$, 20 µmoles; APS, 4.5 nmoles; cell-extract, 10.0 mg in a total volume of 0.625 ml. A range of APS concentration (1–50 nmoles) ± 20 µmoles ATP was also tested; B (combined ATP-sulphurylase and APS-kinase activity); assay conditions have been described previously (Stanley *et al.*, 1975). The cell-free extract (5.8 mg) was incubated for periods of up to 30 min, after which samples were withdrawn for PAPS determination.

The luciferin-luciferase system of the firefly *(Photinus pyralis)* was used for the continuous assay of ATP (Balharry and Nicholas, 1971). This method was employed to determine the ATP-sulphurylase activity in the cell-free extracts. The production of ATP was initially tested at a range of protein concentrations to ensure a linear response over the assay period (2 min). The reactions were carried out in 20 ml scintillation vials which contained luciferin-luciferase and phosphate-arsenate buffer (Balharry and Nicholas, 1971), and the following additions: Tris-HCl buffer (pH 7.5), 17.5 µmoles; Na_2-EDTA, 0.7 µmoles; APS, 250 nmoles; PP_i, 30 nmoles; cell-extract, 1.1 mg.

Radioassay of Labelled Compounds. The radioactive samples on glass fibre filter discs in vials were dried overnight on silica gel and immersed in scintillation fluid (3.0 g 2,5-diphenyloxazole and 0.3 g 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene per litre toluene) before counting them in a Packard Tri-Carb liquid scintillation spectrometer. The radioactive areas on electrophoretograms were detected in a Model 7201 Packard Radiochromatogram scanner unit. The labelled areas of the electrophoretograms were cut out and counted by liquid scintillation spectrometry.

Chemicals. ^{35}S-sulphite and carrier-free ^{35}S-sulphate were obtained from The Radiochemical Centre (Amersham, U.K.); [^{35}S]PAPS from New England Nuclear (Boston, Mass., U.S.A.). [^{35}S]APS was prepared from ^{35}S-sulphite and AMP using a partially purified APS-reductase from *Thiobacillus denitrificans* (Adams *et al.*, 1971). The biochemical compounds were purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), except for PAP which was obtained from Boehringer Mannheim GmbH (Tutzing, West Germany); creatine phosphate from Nutritional Biochemicals Corporation (Cleveland, Ohio, U.S.A.). The *Renilla* acetone powder (luciferin-luciferase) and luciferyl sulphate were gifts from Dr. M. J. Cormier, University of Georgia, U.S.A.

Results

Binding of ^{35}S-Sulphate by Intact Organisms

Sulphate was rapidly bound by cell suspensions (Fig. 1). The amount of ^{35}S-sulphate bound after 10 sec remained constant for the subsequent 8 min. The binding of ^{35}S-sulphate exhibited typical saturation kinetics with a K_m value of 0.38 mM (Fig. 2). Tetrathionate (10 mM) depressed the amount of sulphate bound over a period of 60 sec, whereas equal amounts of ^{35}S-sulphate were bound in the presence or absence of either 40 mM Fe^{2+} or glucose (Fig. 3). Typical inhibitors of sulphate transport systems, such as $WO_4^{=}$, $SeO_4^{=}$, VO_3^{-}, cysteine and methionine, each at 1 mM concentration, had little or no effect on the amount of ^{35}S-sulphate bound within 60 sec. There was no evidence for the formation of labelled sulphur nucleotides in whole cells exposed to ^{35}S-sulphate for 5 min.

Uptake and Assimilation of ^{35}S-Sulphate by Intact Organisms

Gradually increasing uptake of ^{35}S-sulphate over a period of 8 hrs was observed in cell suspensions incubated with ferrous iron (40 mM). No increase of the initial amount of ^{35}S-sulphate bound was observed in parallel cultures which were either supplied with tetrathionate (10 mM) or given no added source of energy (Fig. 4). More than half of the tracer could

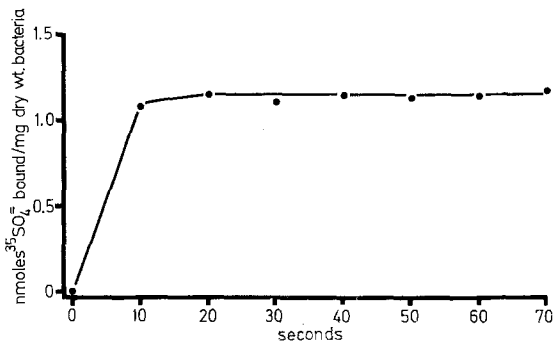

Fig. 1. Binding of ^{35}S-sulphate by whole cells. The incubation medium contained per ml: washed cells, 5.6 mg dry wt.; $FeCl_2$, 40 µmoles; $MgCl_2$, 9 µmoles; $(NH_4)_2HPO_4$, 9 µmoles; ^{35}S-sulphate, 1 µmole (1.8 µCi); in 0.009 N HCl; pH 2.2. The bacteria were incubated for 2 min in the medium prior to starting the experiment by adding the labelled sulphate

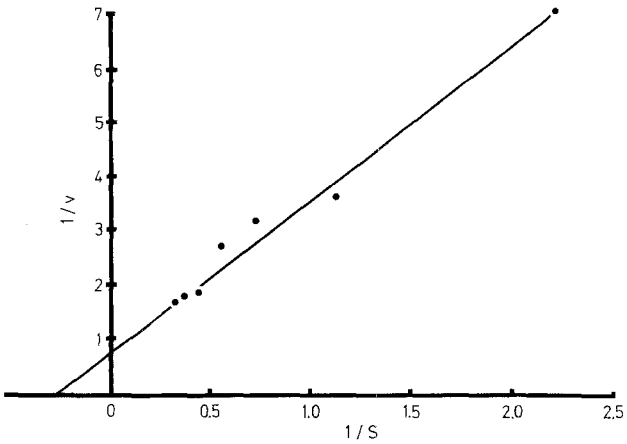

Fig. 2. Effect of sulphate concentration on binding of ^{35}S-sulphate by whole cells. The results are presented as a Lineweaver-Burk plot (K_m = 0.38 mM). The bacteria were incubated with ferrous iron as in the legend to Fig. 1. Specific activity of ^{35}S-sulphate, 16.3 µCi/1 µmole. *S* concentration of ^{35}S-sulphate (mM); *v* nmoles ^{35}S-sulphate bound/30 sec/mg dry wt

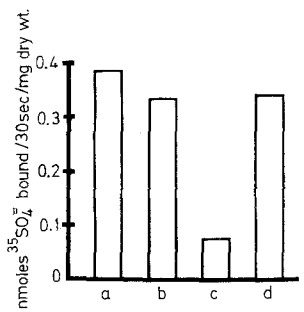

Fig. 3a–d. The effect of the source of energy on binding of 2 mM ^{35}S-sulphate by whole cells. Experimental details are given in the legend to Fig. 1. The cell suspensions in 0.019 N HCl were incubated for 2 min prior to adding ^{35}S-sulphate (specific activity, 3.65 µCi/1 µmole). (a) No added source of energy; (b) 40 mM $FeCl_2$; (c) 10 mM $K_2S_4O_6$; (d) 40 mM glucose

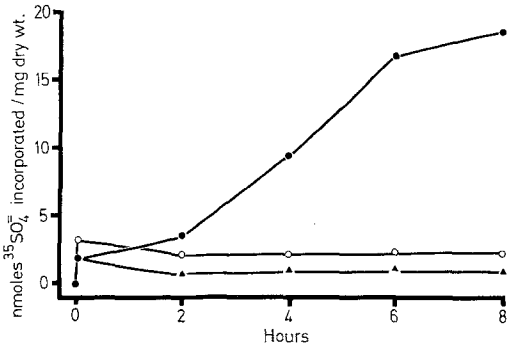

Fig. 4. The incorporation of ^{35}S-sulphate into *Thiobacillus ferrooxidans*. The bacteria (13.4 mg dry wt/ml) were incubated in 0.0087 N HCl containing 8.7 mM $MgCl_2$, 8.7 mM $(NH_4)_2HPO_4$ and carrier-free ^{35}S-sulphate (10 µCi/ml culture) and in addition either 40 mM $FeSO_4$ (●——●), 10 mM $K_2S_4O_6$ (▲——▲), or 40 mM Na_2SO_4 (○——○)

Table 1. Fractionation of ^{35}S incorporated into cells via the assimilation of ^{35}S-sulphate. The cultures, (A) no added source of energy; (B) 40 mM Fe^{2+}; (C) 10 mM $K_2S_4O_6$ were incubated for 8 hrs as described in the legend to Fig. 4

Sequential treatment	^{35}S-content of the fraction (%)		
	A	B	C
Unfractionated bacteria washed once with 0.01 N H_2SO_4 (CPM)	10451	27246	7226
Cold 0.01 N H_2SO_4 second wash	57.6	75.5	63.3
Cold 10% (w/v) TCA 4° C, 15 hrs	40.5	23.6	35.3
70% (v/v) ethanol 45° C, 30 min	1.4	0.2	0.2
Ethanol:diethyl ether (1:1), 30° C, 30 min	0	0	0
5% (w/v) TCA 90° C, 30 min	0.8	0.7	0.5
2 N HCl 90° C, 45 min	0	0	0
1 N NaOH cell residue	0	0	0
Recovery (%)	101	98.8	80.2

be removed with cold 0.01 N H_2SO_4, while the rest of the ^{35}S-sulphate was incorporated into cold trichloroacetic acid-soluble compounds (Table 1). [^{35}S]-APS was found in cell suspensions incubated with Fe^{2+} for 30 min whereas sulphite was not detected (Table 2). APS was not formed in cell suspensions supplied with glucose or in those not given a source of energy (Table 2). There was no evidence for the incorporation of ^{35}S-sulphate into [^{35}S]PAPS in any of the experiments with cell suspensions of *Thiobacillus ferrooxidans*.

Table 2. Production of [^{35}S]APS in cell suspension supplied with 2 mM ^{35}S-sulphate (3.65 μCi/1 μmole). The bacteria were incubated for 2 min prior to adding ^{35}S-sulphate as described in the legend to Fig. 1. The bacteria were washed and disrupted by ultrasonication. Aliquots of the supernatant fraction obtained by centrifuging the cell-extracts at 10000 × *g* for 30 min, were applied to electrophoretograms

Source of energy (40 mM)	Incubation time (min)	Distribution of ^{35}S on electrophoretogram (%)	
		[^{35}S]APS	^{35}S-sulphate
None	0.5	0	100
	15	0	100
	30	2	98
Fe^{2+}	0.5	0	100
	15	0	100
	30	81	19
Glucose	0.5	0	100
	15	0	100
	30	0.3	99.7

Formation of Sulphur Nucleotides in Cell-Free Extracts

Cell-free extracts (S_{10}) produced very low amounts of [^{35}S]APS from ^{35}S-sulphate. Less than 0.6% of the tracer was found in [^{35}S]APS after a 30 min incubation in the presence of inorganic pyrophosphatase and an ATP-generating system as described in "Materials and Methods". Similarly, ATP-sulphurylase activity was low (230 pmoles ATP produced/min/mg protein obtained by assaying ATP production from APS and PP_i with the firefly luciferin-luciferase system).

Although a sensitive bioluminescence method was used to assay for the formation of PAPS from ATP and $SO_4^=$ or from APS, no activity was recorded. The addition of 5′-AMP (20 μmoles) to the incubation mixture, which is known to protect the degradation of PAPS (Kelley *et al.*, 1975; Stanley *et al.*, 1975), was without effect as was the use of varying amounts of APS in the assay.

Discussion

Sulphate has been shown to be essential for the growth of *Thiobacillus ferrooxidans* on ferrous iron (Tuovinen *et al.*, 1971). The results reported in this paper show that similar amounts of ^{35}S-sulphate were rapidly bound by the bacteria irrespective of the presence of ferrous iron or glucose in the incubation medium. The effect of tetrathionate may be explained as a competition with ^{35}S-sulphate for the binding sites, although the Group VI anions such as $SeO_4^=$ did not produce a similar effect in the short term experiments. Alternatively, the 2-min preincubation with tetrathionate prior to adding ^{35}S-sulphate may have started a sequence of reactions through which sulphide required for the cellular synthesis was derived directly from intermediate products of tetrathionate oxidation and which subsequently altered the affinity of the bacteria to exogenous sulphate. Increasing amounts of ^{35}S-sulphate were incorporated when the cells were incubated with ferrous iron for longer periods. The fact that the label was incorporated into [^{35}S]APS rules out the possibility that erroneously high counts were obtained by the deposition of ^{35}S-sulphate labelled jarosite on the surface of the cells.

Sulphate was activated to APS by cell suspensions and in cell-free extracts as determined by the radioassay and the sensitive bioluminescence system of the firefly. No evidence was obtained for the involvement of PAPS in the assimilatory sulphur metabolism in *Thiobacillus ferrooxidans*. This was confirmed by a very sensitive bioluminescence method used previously to detect picomole amounts of PAPS in extracts of yeast or wheat leaves (Stanley *et al.*, 1975). It has also been shown that exogenously supplied PAPS is degraded in extracts of *Thiobacillus ferrooxidans* at a much slower rate than APS (Kelley *et al.*, 1975), which again supports the view that PAPS is not an important sulphur compound in this bacterium.

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