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Accumulation of Sulfite by a Sulfate-Using Revertant of *Salmonella pullorum*[1]

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Sulfate-utilizing revertants of a cysteine auxotroph of *Salmonella pullorum* have been found that cause an accumulation in growth medium of a sulfur compound more oxidized than sulfide. The sulfur compound was presumptively identified as sulfite by the formation of a colored complex in the presence of basic fuchsin and formaldehyde, oxidation to sulfate by peroxide, and acid-volatility. The acid-volatile material was identified as sulfite by formation of an *S*-sulfonyl derivative of 5,5'-dithio-bis(2-nitrobenzoic acid) which was chromatographically and electrophoretically identical to an authentic *S*-sulfonyl derivative. The presence of sulfate in minimal medium is required for sulfite accumulation, and both cysteine and selenate inhibit the accumulation. No evidence was obtained to indicate that a reduced sulfur compound was the precursor of the accumulated sulfite.

There is little genetic or physiological information about the inability of *Salmonella pullorum* to synthesize cysteine when sulfate is the sulfur source. Recently, we discovered that one of our stock strains of *S. pullorum* is a double cysteine mutant and that both mutations are spontaneously revertible. While performing the necessary experiments to arrive at this conclusion, we observed incidentally that some sulfate-using revertants cross-fed some cysteine auxotrophs and not others. Sulfide, cysteine, and methionine were eliminated as the cross-fed compound. This implied strongly that an oxidized form of sulfur was accumulating and raised the possibility that either a constitutive revertant had been obtained or that some partially oxidized compound was accumulating from a reduced sulfur compound.

In this paper we present our data for the identification of the feeding compound and evidence that an oxidized rather than a reduced sulfur compound is the physiological precursor of sulfite.

MATERIALS AND METHODS

Chemicals. Radioactive sulfate was obtained from New England Nuclear Corp., Boston, Mass. *N'*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was obtained from Aldrich Chemical Co., Milwaukee Wis. Methionine-free leucine (Nutritional Biochemicals Corp., Cleveland, Ohio) was used in this work. All other chemicals employed were reagent grade.

Bacterium. *S. pullorum* strain MS35 was selected as the prototype organism from our stock collection. This strain requires cysteine and leucine for growth in a synthetic medium. Sulfide but not sulfate, thiosulfate, sulfite, or cysteine sulfinic acid (CSA) can replace cysteine.

Cultivation of bacteria. The sulfur-free minimal E medium used in this study was constructed from the basal salt solution described by Vogel and Bonner (14), except that equimolar $MgCl_2 \cdot 6H_2O$ replaced $MgSO_4 \cdot 7H_2O$. Sterile D-glucose and L-leucine were added to final concentrations of 0.4% and 1.5×10^{-4} M, respectively. Various sulfur sources were added at levels specified in each experiment. The sulfur compounds were handled with proper consideration for their stability (11). L-Methionine (1.34×10^{-4} M), unless indicated otherwise, was added to all cultures containing sulfate to prevent a sulfate-induced initial growth lag. Broth cultures were grown aerobically at 37 C on a rotary shaker. When sulfur-free agar medium was required for plates, 20 g of washed Noble agar was added to 1 liter of E broth. Enriched minimal agar was formed with 98.75 ml of E medium and 1.25 ml of reconstituted nutrient broth (Difco).

Selection of revertants. A 0.1-ml amount of a late logarithmic-phase culture of MS35 was spread upon plates of E minimal agar medium supplemented with glucose, leucine, and CSA (20 μg/ml). After incubation for 72 hr at 37 C, 10 colonies appeared. One colony was purified by three subcultures on the same medium. It was able to use CSA, sulfite, sulfide, and cysteine individually, but not sulfate or thio-

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sulfate, as the sole source of sulfur. This revertant was designated sulfite-utilizer 6. To obtain sulfate-using revertants, plates of enriched minimal agar were spread with 4×10^7 cells of sulfite-utilizer 6, and one drop (2 μg) of NTG was placed in the center of the agar surface. (It was later learned that the use of NTG was unnecessary because spontaneous sulfate-using revertants occur at a frequency of one per 10^8 to 10^9 sulfite-using 6 cells). The plates were incubated at 37 C for 72 hr. One of the NTG-induced revertants, designated sulfate-utilizer 6–18, was selected and purified for use in this study.

Storage of revertants. Revertants, depending on their sulfur requirements, were stored on sulfur-free E minimal agar plates supplemented either with sulfate (4×10^{-4} M) or CSA (20 μg/ml). All cultures were subcultured every 2 months and stored at 4 C.

Routine determination of sulfite. The routine determination of sulfite was by the fuchsin-formaldehyde technique described by Grant (5) and modified by Dreyfuss and Monty (3).

Concentration and collection of the accumulated sulfur product. A typical cell-free supernatant fluid of a stationary-phase broth culture of sulfate-utilizer 6-18, supplemented with sulfate (4×10^{-4} to 8×10^{-4} M) and methionine (1.34×10^{-4} M), was concentrated about 10-fold in vacuo at 80 C by evaporation of the water. At the end of the evaporation, 95% of the fuchsin-reactive material remained in the concentrate. The concentrate was acidified (*p*H 1.0 to 2.0), and the acid-volatile gas was removed from it by bubbling 0.15 M $AgNO_3$-washed nitrogen gas through it for 1 hr at 37 C. The evolved gas was passed through a 0.15 M NaOH-0.001 M ethylene diaminetetraacetate (EDTA) trap to dissolve SO_2 and convert it to stable SO_3^{2-}. From 40 to 70% of the fuchsin-reactive material in the concentrate was recovered in the NaOH-EDTA trap.

Characterization of putative sulfite. It is known that the disulfide bond of DTNB [5,5′-dithio-bis(2-nitrobenzoic acid)] reacts with sulfite at neutral and slightly alkaline *p*H to form the yellow-colored thionitrobenzoate anion and the colorless *S*-sulfonate derivative (4). At high *p*H, DTNB is unstable. In this work a buffered solvent was required for the reaction, since the putative sulfite was dissolved in 0.15 M NaOH. Thus, a salt solution of E medium adjusted to *p*H 7.0 was made 2.5×10^{-3} M with respect to DTNB. A sample containing 1 μmole of DTNB was reacted at a final *p*H of 7.2 with 0.4 μmole of putative or authentic sulfite for 10 min at room temperature and then chromatographed (Fig. 1) and electrophoresed (Fig. 2) as described.

RESULTS

An unusual feature noticed when isolating sulfate-using mutants from sulfite-utilizer 6 was a zone of growth around each sulfate-using mutant colony. Subsequent tests showed that one sulfate-using revertant, 6–18, fed only sulfite-utilizer 6, not MS35. This finding indicated neither sulfide nor cysteine was the secreted compound; otherwise, MS35 would have grown. Thiosulfate and methionine were also eliminated as feeding compounds because sulfite-utilizer 6 cannot use these compounds for growth. Together, these results suggested that sulfite, CSA, or some nutritionally equivalent compound has accumulated.

Identification of sulfite. When broth cultures of sulfate-utilizer 6–18 in the stationary phase of growth were concentrated in vacuo and tested by the modified Grant technique, they were found to contain fuchsin-reactive material equivalent in some cases to 93% of the sulfur in excess of that required for growth. The fuchsin-reactive material could be volatilized by acid and subsequently trapped in a solution of NaOH-EDTA. When a sample of the NaOH-EDTA trap, containing fushsin-reactive material, was acidified with a few drops of HCl and then H_2O_2 and $BaCl_2$ were added, there was an immediate formation of a white insoluble precipitate indicative of $BaSO_4$. When the H_2O_2 was omitted, the precipitate did not form. The chromatographic and electrophoretic identity of the putative and authentic sulfite derivatives of DTNB is additional evidence that the trap material is sulfite (Fig. 1 and 2).

Precursor of accumulated sulfite. The finding that sulfite accumulated in cultures of sulfate-utilizer 6–18 in the presence of a mixture of sulfate and methionine raised a question concerning the physiological precursor of sulfite. Recall that methionine was added to the culture medium to prevent a growth lag that occurs in the presence of sulfate alone. Based on the assumption that methionine sulfur can be oxidized to sulfite, methionine should be a sufficient sulfur source for growth; however, it is not. Moreover, when a mixture of (^{35}S)-sulfate and (^{32}S)-methionine was used as the sulfur source for

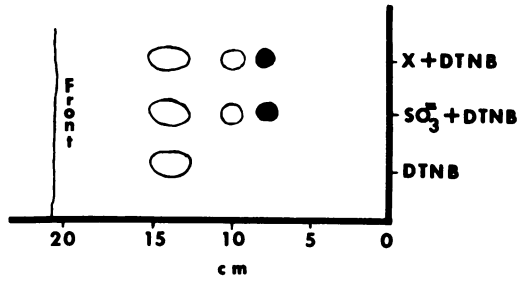

FIG. 1. *Chromatographic identification of S-sulfonylthio-nitrobenzoate. Solid black areas represent the yellow-colored thionitrobenzoate anion. Outlined areas represent the yellow thionitrobenzoate anion that arises after spraying with dilute mercaptoethanol. Chromatographic conditions were ethyl alcohol-0.1 M ammonium acetate (7.5:3, v/v).*

growth of sulfate-using 6-18, the acid-volatilized, fuchsin-reactive material that was trapped in an NaOH-EDTA solution had a specific activity 6% less than the value of the sulfate substrate.

Table 1 shows sulfite accumulates only when sulfate is present. Growth in the presence of a mixture of cysteine and methionine does not give sulfite even though sulfide is generated during growth on this combination of amino acids. The final level of accumulated sulfite is reduced as the concentration of exogenous cysteine is increased (Table 1). The latter observation suggests that the sulfate reduction is involved in accumulation of sulfite, since the enzymes of sulfate reduction are known to be repressed by cysteine in *S. typhimurium* (2), *Escherichia coli* (10, 15), and *Bacillus subtilis* (4). Nevertheless, we sought the additional evidence that other group VI anions, which serve as substrates for the sulfate permease (9) and sulfurylase (17), could provide to support our interpretation.

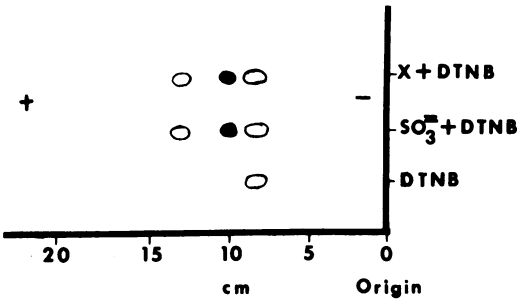

FIG. 2. *Electrophoretic identification of S-sulfonylthionitrobenzoate. Solid black areas and outlined areas are described under Fig. 1. Electrophoretic conditions were 0.05 M acetate buffer, pH 4.8, with 15 v/cm for 1.5 hr at 4 C.*

TABLE 1. *Accumulation of sulfite after aerobic growth at 37 C of revertant 6-18 on various sulfur substrates*

Sulfur substrate	Accumulated sulfite[a] (10^{-4} moles/liter)	Sulfide evolution[b]
SO_4^{2-} (0.8 mM)[c]	4.20	−
SO_4^{2-} (0.8 mM) + L-methionine (0.134 mM)	6.00	−
SO_4^{2-} (0.4 mM) + L-methionine (0.134 mM)[d]	3.40	−
L-Cysteine (0.045 mM)	1.60	+
L-Cysteine (0.09 mM)	1.00	+
L-Cysteine (0.18 mM	0.20	+
L-Cysteine (0.18 mM) + L-methionine (0.134 mM)	0.00	+

[a] Sulfite was determined by the Grant technique at 12 hr after the culture had reached the stationary phase of growth.

[b] The evolution of sulfide was detected by blackening a strip of lead acetate paper.

[c] A concentration of 1.8×10^{-4} M sulfur is required to give full growth in the synthetic E medium used for growth.

[d] In this test, four identical cultures were used, each containing SO_4^{2-} and L-methionine; L-cysteine was added to three flasks at the concentration indicated.

The data of Fig. 3 show that selenate arrests both growth and sulfite accumulation. A control culture lacking selenate, in which growth was arrested by chloramphenicol (100 μg/ml), also resulted in no accumulation of sulfite (data not shown). This finding indicated that growth per se under the conditions of the experiment may be required for sulfite accumulation. Thus, this inhibition of sulfite accumulation is probably the result of indirect effects.

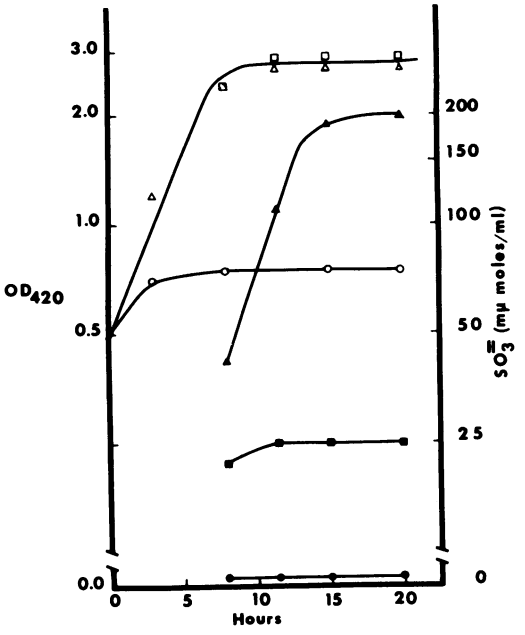

FIG. 3. *Effect of selenate on the accumulation of sulfite by S. pullorum revertant 6-18. The organism was cultured aerobically in E medium supplemented with L-methionine (1.34×10^{-4} M) and: (○) sulfate (4×10^{-4}) M and selenate (4×10^{-3} M), (△) sulfate and L-cysteine (1.14×10^{-4} M), or (□) sulfate, cysteine, and selenate. The amount of growth was followed spectrophotometrically at a wavelength setting of 420 nm. An optical density of 1.0 corresponds to 10^9 bacteria per ml. The routine determination of sulfite accumulation in the growth medium is recorded for each flask, respectively, by the same-shaped symbol (solid) used for recording growth.*

To circumvent the arrest of growth by selenate, we added to the culture a level of cysteine low enough to allow some sulfite accumulation (*see* Table 1) but high enough to overcome the toxicity of selenate (16). When a mixture of selenate, sulfate, and cysteine are used for growth, sulfite accumulation is reduced 88% (Fig. 3). This decrease in sulfite accumulation is in good agreement with the 1:11 ratio of sulfate-to-selenate used. When this experiment was repeated with a 1.9:10 ratio (data not shown), sulfite accumulation was reduced by only 60%, indicating that sulfate is competitive.

It was found in other experiments that a 1:10 ratio of sulfate to molybdate did not inhibit cell growth or sulfite accumulation, but chromate totally inhibited both responses (data not shown). Cysteine was without effect on chromate-containing cultures.

DISCUSSION

All the tests with the sulfur compound that accumulates in cultures of sulfate-utilizer 6–18 and with the trapped acid-volatilized material made from the accumulated sulfur compound are positive for sulfite. Nevertheless, a certain bias is inherent in this identification, since the critical chemical tests were made on the acid-volatile material after release from the cell-free cultural medium by acidification and not on the untreated medium. Possible sources of sulfite by this treatment are cysteine-*S*-sulfonate (CSS), polythionates ($^{-}O_3SS_nSO_3^{-}$), and thiosulfate (11). Thiosulfate is eliminated because *S. pullorum* cannot use it as a sulfur source. We have found that as much as 93% of the sulfate sulfur added in excess of the amount of sulfur required for growth is volatilized. This observation renders unlikely CSS and polythionates as sulfite sources, since, for each mole of sulfite released by acid treatment, an equivalent quantity of sulfur remains unvolatilized ($^{-}O_3SSSO_3^{-}$ is an exception). Thus, 50% (66% for $^{-}O_3SSSO_3^{-}$) of the excess sulfur in the cultural medium is the maximum expected upon acidification of compounds other than sulfite.

Several of our observations suggest that an oxidized rather than a reduced sulfur compound is the physiological precursor of the sulfite. First, sulfite occurs only if sulfate is present during growth. Second, growth in in the presence of a mixture of $^{35}SO_4^{2-}$ and ^{32}S-methionine gives a higher yield of $^{35}SO_3^{2-}$ of undiminished specific activity, suggesting that methionine sulfur does not enter into sulfite and that the greater yield of sulfite occurs because less sulfate is required for cell growth. Third, growth in the presence of cysteine, methionine, and sulfide results in no accumulation of sulfide, and, if sulfate is also present, the sulfite yield is lowered as the concentration of cysteine is increased. Finally, the accumulation of sulfite is inhibited by selenate in the presence of sulfate, cysteine, and methionine. It is expected that selenate, an inhibitor of sulfate transportation and activation, would be without effect if sulfite came from the oxidation of a reduced sulfur compound.

The foregoing observations strongly suggest that sulfite accumulates from sulfate, presumably after activation of sulfate. In a separate publication (Kline and Schoenhard, *in preparation*), we will show that *S. pullorum* does form "active sulfate," and sulfite is an intermediate of sulfate reduction to sulfide.

We are not aware of any reports which show that sulfite accumulates when heterotrophic microbes are grown on sulfate. Assimilatory sulfate reduction has been studied intensively in other *Salmonella* species only with *S. typhimurium* (1, 3, 7). The known intermediates of sulfate reduction do not accumulate during growth of the wild-type strain (1), but sulfite does accumulate in derepressed, chloramphenicol-arrested suspensions of mutants lacking sulfite reductase (3). Likewise, Roberts et al. (12) reported no accumulation of intermediates when wild-type *E. coli* was grown in the presence of sulfate. Torii and Bandurski (13) found that, with the reduction of 3′-phosphoadenosine-5′-phosphosulfate catalyzed by yeast extracts, the product, sulfite, was protein-bound. In contrast, Nightingale et al. (8) reported that sulfite accumulates in starved tomato plants soon after sulfate is introduced, but the peak of accumulation is about 24 hr. Larson and Salisbury (6) also reported that sulfite accumulates in bull semen. The physiological significance of the plant and semen findings is unknown.

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