



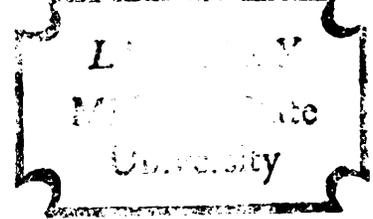
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ACCUMULATION OF SULFITE BY A  
SULFATE-USING REVERTANT OF  
SALMONELLA PULLORUM AND  
BIOCHEMICAL CHARACTERIZATION OF ITS  
CYSTEINE-REQUIRING PARENT

Thesis for the Degree of Ph. D.  
MICHIGAN STATE UNIVERSITY

Bruce C. Kline

1968



This is to certify that the

thesis entitled

ACCUMULATION OF SULFITE BY A SULFATE-USING  
REVERTANT OF SALMONELLA PULLORUM AND  
BIOCHEMICAL CHARACTERIZATION OF  
ITS CYSTEINE-REQUIRING PARENT

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Bruce C. Kline

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Major professor

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## ABSTRACT

### ACCUMULATION OF SULFITE BY A SULFATE-USING REVERTANT OF SALMONELLA PULLORUM AND BIOCHEMICAL CHARACTERIZATION OF ITS CYSTEINE-REQUIRING PARENT

By

Bruce C. Kline

Prototrophic assimilatory sulfate reducing bacteria normally do not accumulate reduced inorganic derivatives of sulfate. Sulfate-using revertants of a natural cysteine-requiring Salmonella pullorum strain cross-feed other cysteine mutants. The feeding compound is not cysteine or sulfide. This rare finding prompted identification of the feeding compound, resolution of the unknown assimilatory pathway used by S. pullorum revertants and biochemical characterization of the cysteine-requiring parent. The nutritional responses of spontaneous revertants of one strain of S. pullorum, strain MS35, indicate that this bacterium is a double cysteine mutant at 37C. All sulfate-using revertants derived from a particular sulfite-using, single revertant cross-fed that revertant. Sulfite was detected in significant amounts in cultures of one sulfate-using revertant. The sulfite was identified on the basis of acid-volatility, oxidation to sulfate and precipitation with BaCl<sub>2</sub>, and the

formation of an authentic S-sulfonate derivative of 5,5'-dithiobis (2-nitrobenzoic acid). The accumulation of sulfite was dependent on the presence of sulfate and the accumulation was inhibited in proportion to either the amount of selenate or L-cysteine added to the culture. It was subsequently shown that the parent organism, MS35, is a double cysteine mutant because of an inability to transport sulfate and an inability to reduce sulfite to sulfide. The double revertant obtained at 37 C cannot use thiosulfate at 37 C but can use thiosulfate at 25 C. The biochemical basis for the temperature-sensitive response to thiosulfate is unknown. This nutritional behavior is believed to be indicative of a new class of cysteine mutant in Salmonella. The mutation that causes the loss of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-sulfite reductase at 37 C does not cause a loss of reduced methyl viologen (MVH)-sulfite reductase. The NADPH-sulfite reductase activity is regained after a shift-down to a growth temperature of 25 C or as the result of a reverse or gain mutation which is expressible at 37 C. In establishing the biochemical nature of the defects, the author encountered considerable difficulty with assays for sulfate activation and sulfite reduction. The presence of a mixture of the 2' and 3' isomers of adenosine monophosphate (2'- and 3'-AMP) was required to synthesize 3'-phosphoadenosine-5'-phosphosulfate (PAPS). The efficacy of the individual mononucleotides was not tested. Without 2'- and



3'-AMP about only 0.05  $\mu$ moles of APS were synthesized, and PAPS was not made. It was observed that NADPH-sulfite reductase was unstable when extracted with low ionic-strength buffer; however, MVH-sulfite reductase was stable. Extraction in high ionic-strength buffer stabilized NADPH-sulfite reductase. Using high ionic-strength extracts, the author observed at 37 C abbreviated periods (less than 5 min) of NADPH-dependent sulfite reduction to sulfide; however, at 25 C reduction to sulfide was linear for at least 30 min. After definition of the sulfate-reducing pathway used by S. pullorum, preliminary evidence was obtained suggesting that the accumulation of sulfite from sulfate occurs because the reduction of sulfate to sulfite is less sensitive to end product control than is the reduction of sulfite to sulfide.



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By  
Bruce C. Kline

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This thesis is dedicated to  
my wife, Mary Ann,  
and to my family.



## ACKNOWLEDGMENTS

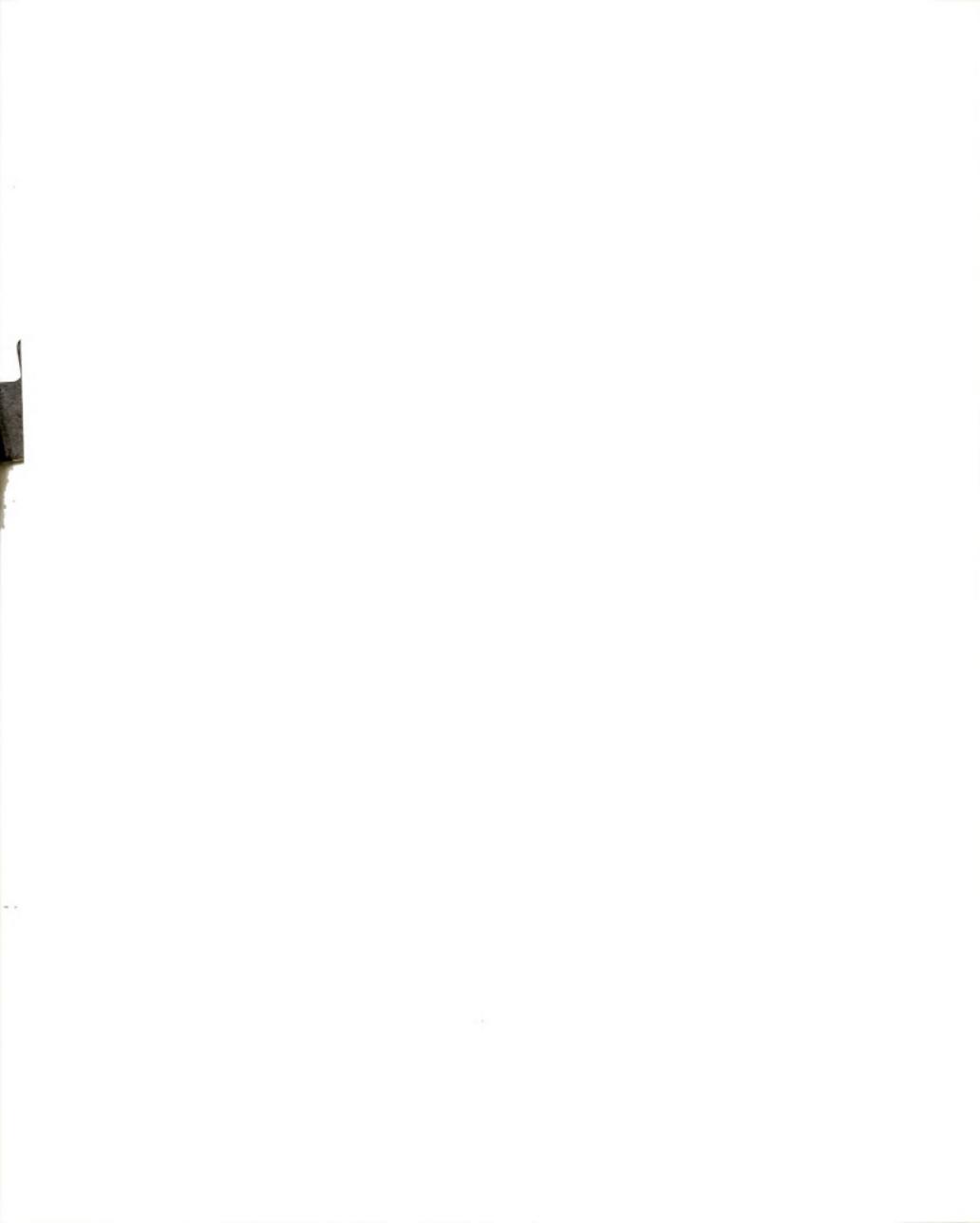
I express my sincerest appreciation to Dr. Delbert E. Schoenhard who has made this work possible. By his daily activity he has presented an outstanding example of interest and concern in this work and in my scientific education. I am grateful on all accounts.

I also warmly acknowledge the very generous gifts of time and facilities which Drs. Robert S. Bandurski and Ralph N. Costilow extended to me so that I might accomplish the goals of this thesis.

I should also like to acknowledge Dr. Otis W. Godfrey for his terse, cryptic, and thoroughly delightful enrichment of the laboratory environment in which this work was performed.

During the course of this study, I was supported in part by a departmental assistantship and my wife, Mary Ann.

\* \* \* \*



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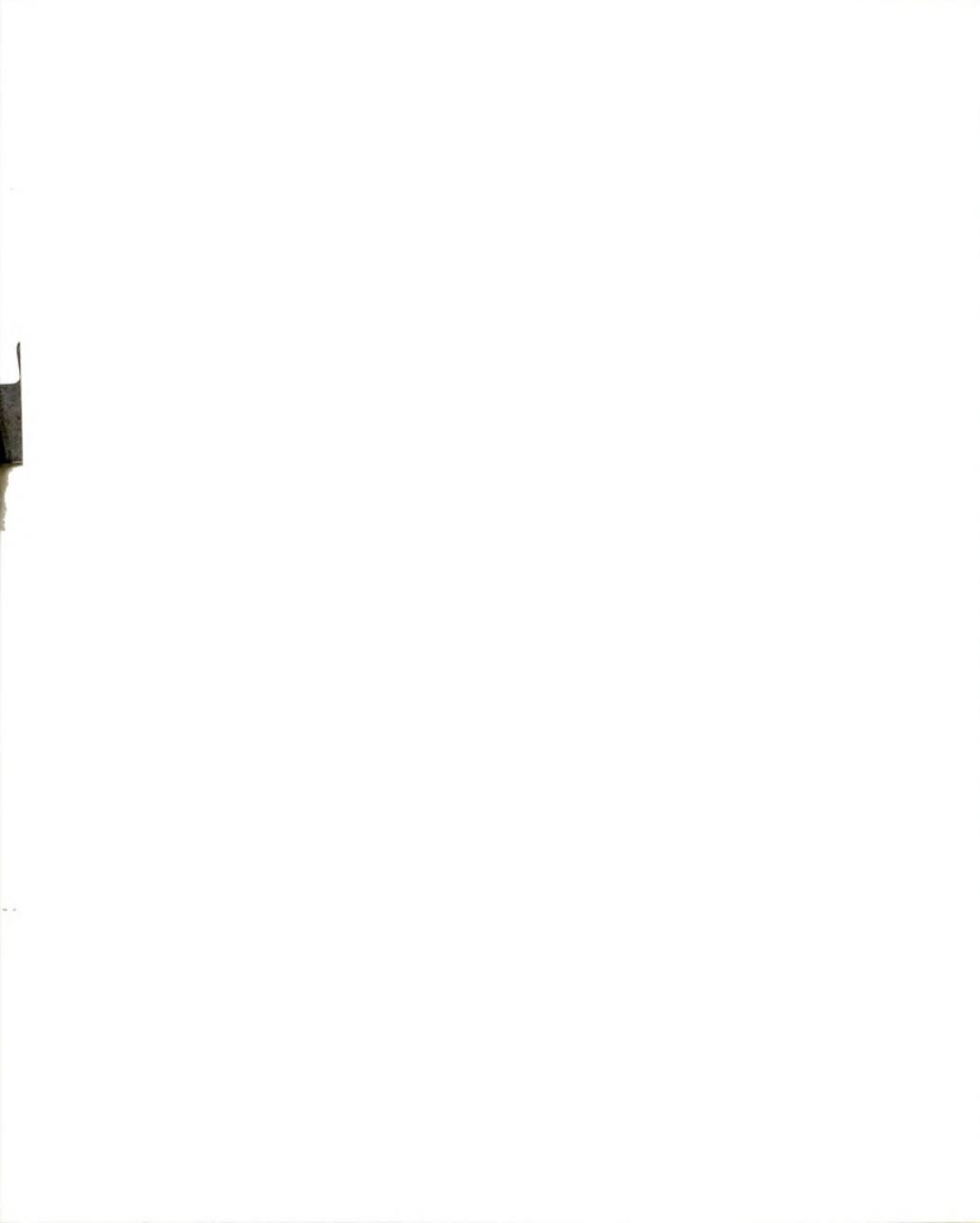
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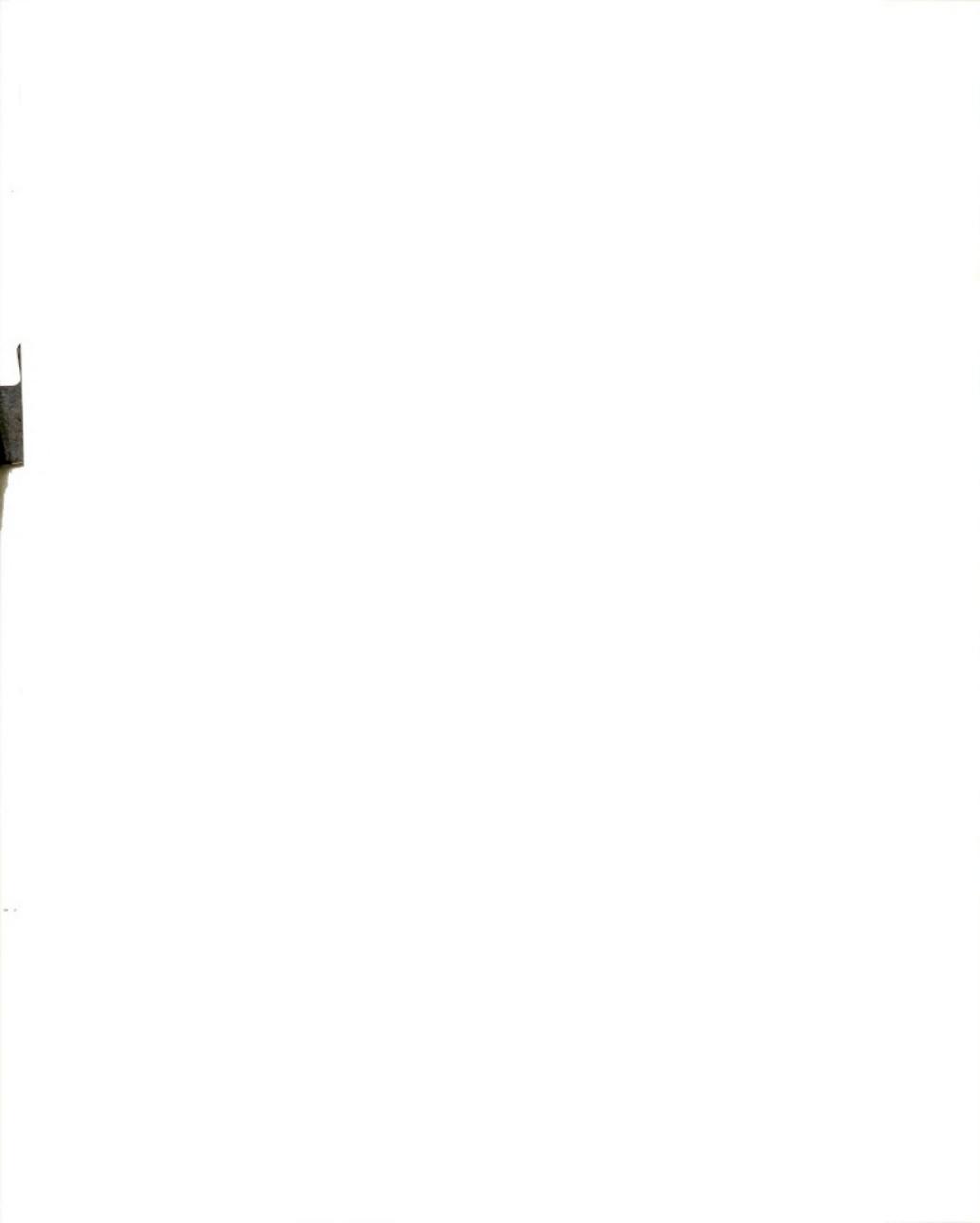
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## INTRODUCTION

There exists a group of nutritionally fastidious Salmonellae, Salmonella pullorum, S. anatum, S. oranienburg, S. meleagridis, S. bareilly, S. typhisuis, S. abortusovis and others, that has a common cysteine requirement because of an inability to use sulfate as a sulfur source (54). The normal metabolism of sulfate has not been investigated in any of these organisms; likewise, the genetic and physiological bases for the cysteine requirements have not been investigated. Salmonella pullorum was chosen for study because previous work (B. C. Kline, M.S. thesis, 1966) had shown that it was transducible; and, therefore, biochemical genetic experiments are possible.

From initial studies of the nutritional responses by spontaneous revertants, the author concluded that a wild type strain of S. pullorum, MS35, contained two mutations preventing the biosynthesis of cysteine from inorganic sulfur compounds. Moreover, when sulfate-using revertants were obtained, an unusual phenomenon occurred; the revertants cross-fed the parent organism. The spectrum of cysteine mutants fed indicated the feeding compound was more oxidized than sulfide. Since the accumulation of intermediates



(sulfide may be an exception) of the assimilatory sulfate reducing pathway by prototrophic microbes is a rare event, a serious effort was made to identify both the feeding compound and its precursors. Identification of the precursor sulfur compound is desirable to eliminate the possibility that accumulation of the feeding compound occurs because of the oxidation of a reduced sulfur compound. In a preliminary paper (B. C. Kline and D. E. Schoenhard, Bacteriol. Proc., p. 118, 1967) sulfite accumulation (ca. 0.5  $\mu$ moles of sulfite per ml of culture) was reported for the sulfate-using revertant. A full description of the efforts to identify the feeding compound (sulfite) and its precursors constitutes the first part of this thesis.

Determination of the pathway of sulfate reduction used by the MS35 prototrophic revertant has provided the basis for identifying the metabolic defects that prevent the synthesis of cysteine in the wild type MS35. A description of these efforts constitutes the second part of this thesis.

Knowledge of the pathway of sulfate reduction used by S. pullorum also has provided a basis for studying the control process which permits the accumulation of sulfite. A description of this study constitutes the third part of this thesis.



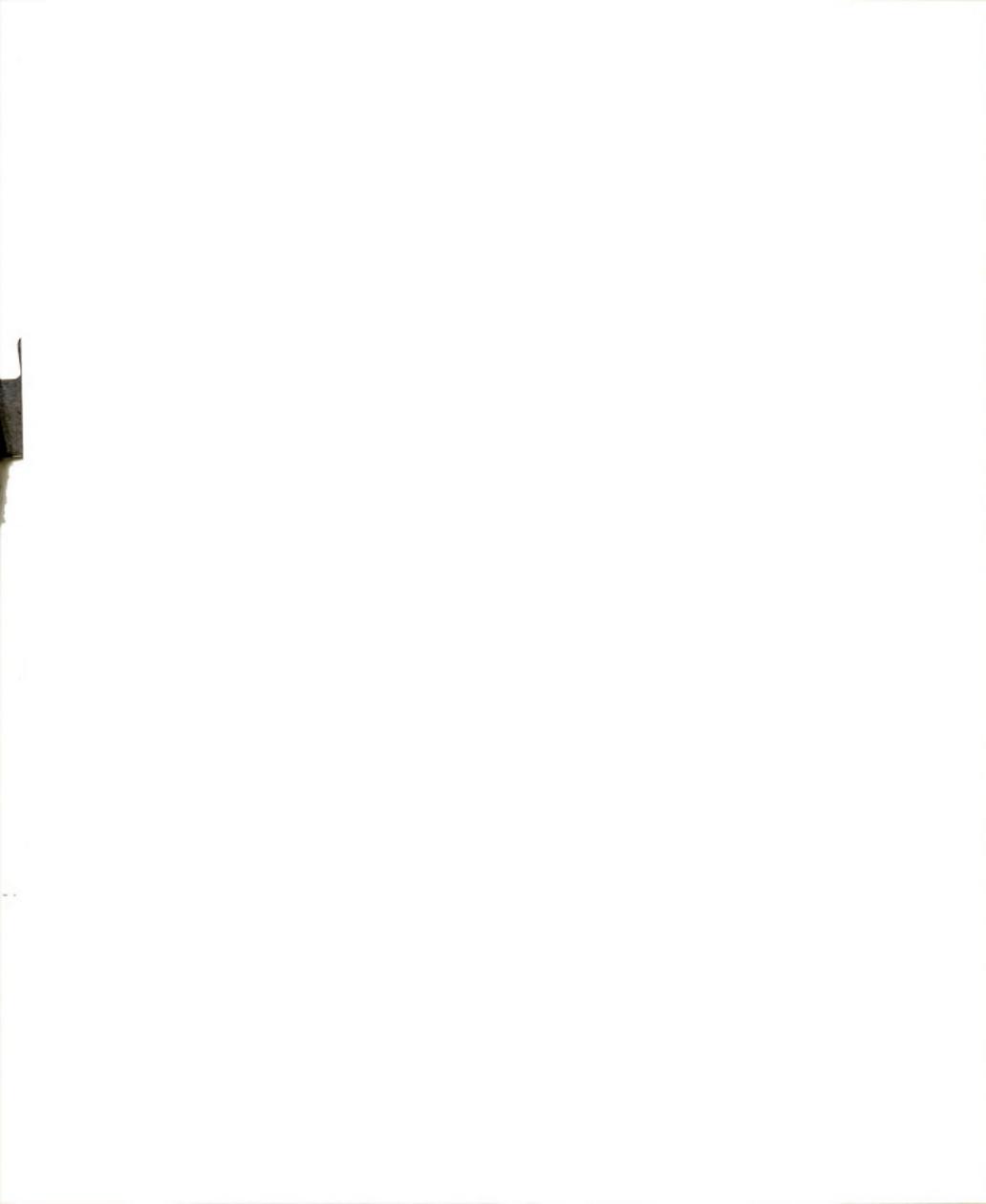
## LITERATURE REVIEW

### Part I

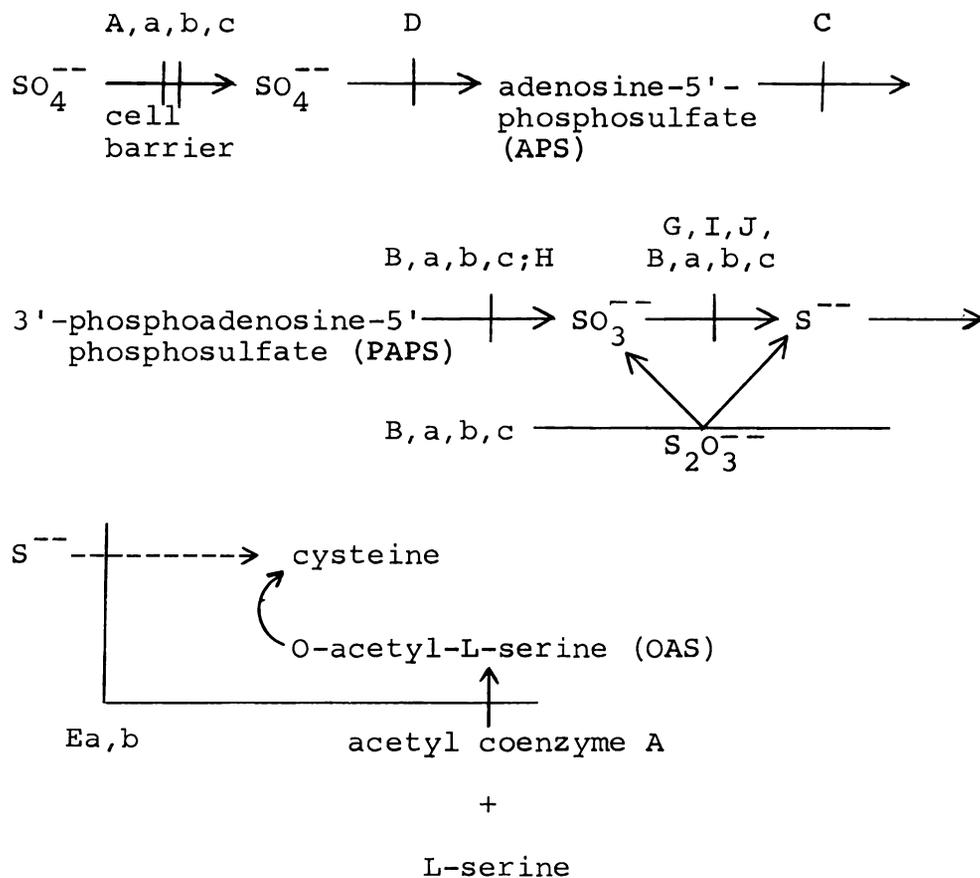
#### Accumulation of Intermediates of Assimilatory Sulfate Reduction

Accumulation with prototrophs. From the results of syntrophism studies made with S. typhimurium cysteine mutants and their prototrophic parent, Clowes (10) concluded the prototrophic parent accumulated none of the intermediates of the sulfate pathway. Roberts et al. (46) determined that  $S^{35}$ -intermediates do not accumulate during growth of prototrophic Escherichia coli in a medium containing a mixture of ( $S^{35}$ )-sulfate and reduced inorganic sulfur compounds. The author has been unable to find any reports of sulfate intermediate accumulation by prototrophic microorganisms except for the accumulation of sulfide (9). However, the sulfide that accumulated probably resulted from the desulfuration of cysteine rather than the reduction of sulfate (44).

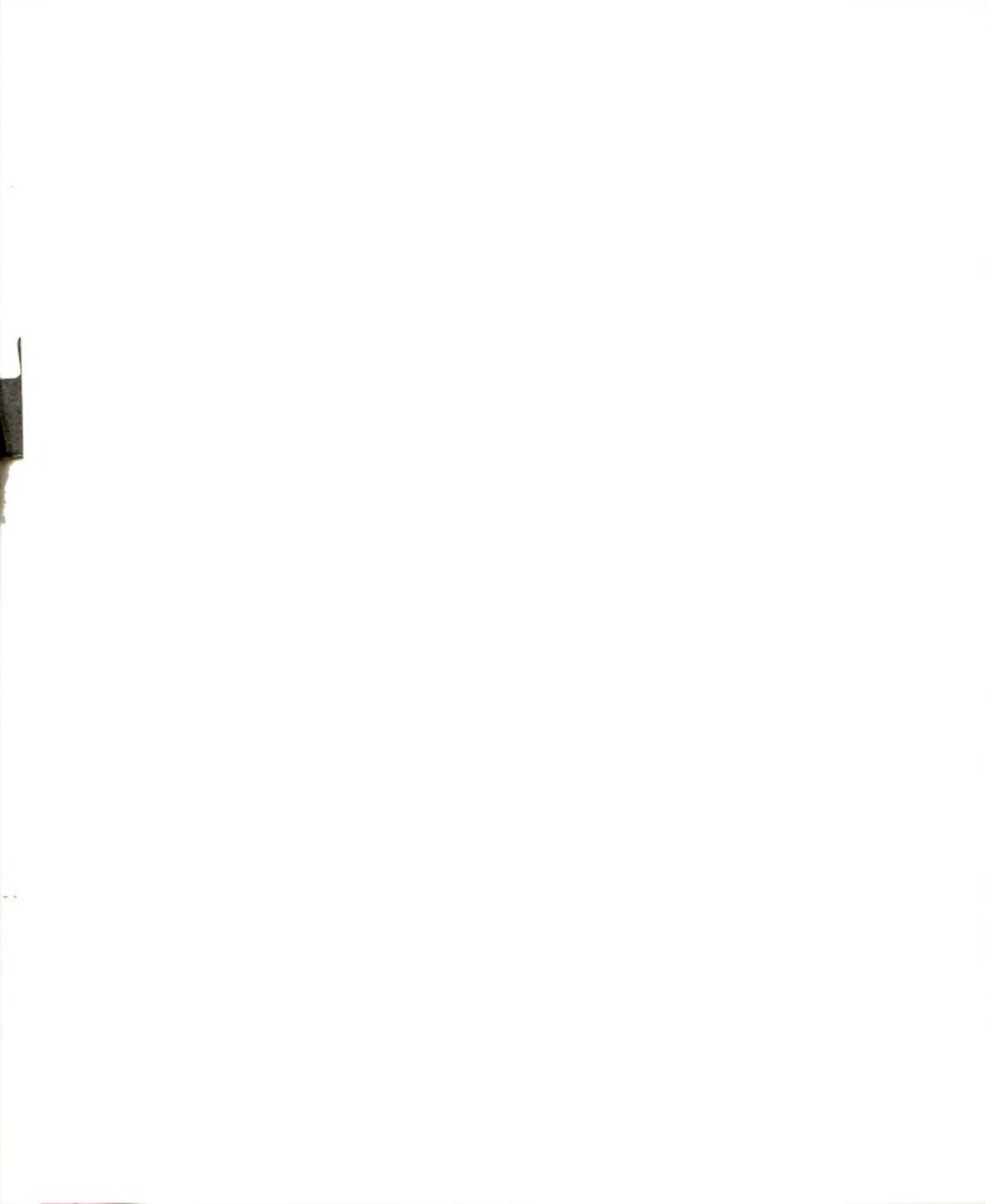
Accumulation with auxotrophs. In contrast to the results of accumulation studies made with prototrophs, cross-feeding by sulfideless cysteine mutants of S. typhimurium (35) and E. coli (29) showed that accumulation did occur.



At the time these studies were made, the accumulations were not identified because the pathway of sulfate reduction was not fully resolved. Since then the pathway has been resolved (8,15,28,32,34,42):



Later, using some of the S. typhimurium mutants, Dreyfus and Monty (15) identified an accumulated sulfur compound as sulfite. The accumulation of APS at high concentrations is not expected in view of the unfavorable equilibrium constant ( $10^{-8}$ ) for its formation. Also the accumulation of a limited amount of PAPS in S. typhimurium mutants (13)



prevents the further accumulation of PAPS since PAPS, itself, inhibits sulfate transport. Thus, potentially, only sulfite or sulfide can accumulate in significant amounts.

Accumulation in higher forms. Sulfite has been found to accumulate in tomato plants (39) and bull semen (30). The accumulation in tomato plants was not the result of a mutation since the plant grew on sulfate as a sole sulfur source. The physiological pathways involved in sulfite accumulation in tomato plants and bull semen are unknown. It is known that mammals oxidize cysteine to sulfite (18) and to sulfate (17) but do not reduce sulfate to cysteine.

Accumulation by oxidation. The oxidation of reduced sulfur compounds by heterotrophic microorganism is poorly understood. The subject has been reviewed briefly by Peck (44), Vishniac and Santer (59), and Fromageot and Senez (18). Essentially the reductive sulfate intermediate, sulfite, is also an oxidation intermediate. Likewise, sulfur compounds that potentially supply intermediates to the reductive pathway [such as thiosulfate and cysteine sulfinic acid (CSA)] are intermediates in the oxidative formation of sulfate.

## Part II

Biochemical Characterization of Natural  
Cysteine Mutants and Reactions of  
Assimilatory Sulfate Reduction

Natural mutants. The biochemical characterization of natural or spontaneous cysteine mutants has been accomplished only to a limited degree. Itikawa and Demerec (23) have noted that spontaneous secondary mutations occur in other cysteine loci in cysteine mutants of S. typhimurium. Gillespie et al. (P. Gillespie, M. Demerec, and H. Itikawa; in press) have identified the secondary effect as either a mutation affecting sulfate transport or "activation" in a primary mutant unable to reduce PAPS-sulfur to sulfite. Secondary mutations which prevent the metabolism of sulfate also have been discovered in cysteine mutants of Neurospora crassa (36) that lack sulfite reductase (32) as the result of the primary mutation. The accumulation of secondary mutations in the loci for sulfite reductase also occurs in mutants that initially did not metabolize sulfate per se. Neurospora crassa uses the same inorganic pathway of sulfate reduction described for S. typhimurium (32). Apparently, double cysteine mutants have a selective advantage over the singly mutated strains. Mitchell and Mitchell (34) have reported a similar situation for adenine mutants of Neurospora.

The metabolic nature of the natural cysteine mutants of the *Salmonellae* listed in the Introduction cannot be ascertained because little nutritional and no biochemical information is available. Thiosulfate, sulfite, and cysteine stimulate the growth of *Salmonella eastbourne* and *Salmonella typhi* whereas *S. anatum* and *S. oranienburg* and *S. pullorum* are only stimulated by cysteine (54).

Mutant-biochemical methodology (5) has been applied successfully to the resolution of the assimilatory pathway of sulfate reduction in the enterobacteria, *E. coli* and *S. typhimurium*. Since the pathway of sulfate metabolism in the enterobacterium *S. pullorum* is unknown, a review of the pertinent information for the resolved pathway of *E. coli* and *S. typhimurium* is presented in the following paragraphs. The schematic diagram of the pathway has been given in Part I of this review.

Transport of sulfate. The first step in the metabolism of sulfate by *S. typhimurium* (15) and *E. coli* (25) is the active transport of sulfate into the cell. This entry process in *S. typhimurium* is mediated by a sulfate-binding protein and a permease (41). The binding protein probably is external to the cell membrane since it is lost by osmotic shock or conversion of cells to spheroplasts. Structurally similar molecules, such as, thiosulfate, sulfite, and group VI anions, inhibit binding of sulfate, and permeable cells become impermeable upon loss of binding protein (41).

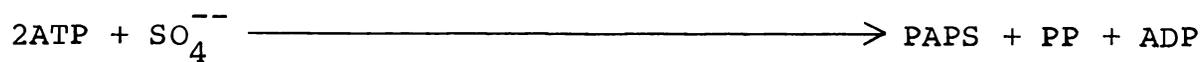
Binding protein has a molecular weight of 32,000 and is of typical amino acid composition except that it lacks sulfur-containing amino acids. No cofactor or energy requirements were found for binding sulfate (40). The locus for the binding protein has not been mapped, but the locus (cys A) for the permease function has been mapped (35). Permeaseless mutants also lose the ability to transport thiosulfate. This finding and the results of competition studies indicate that sulfate and thiosulfate are transported by the same permease system (12).

Sulfate activation. DeMeio et al. (11) were among the first to show that sulfate activating and transferring systems are necessary, but separable, for the formation of phenyl sulfate. Bernstein and McGilvery (6) studied the kinetics of m-aminophenyl sulfate formation with liver homogenates and deduced that an intermediate, activated sulfate, was formed and that at least two enzymes were involved. Hilz and Lippman (22) isolated the sulfuryl group carrier by paper electrophoresis after reacting ATP, sulfate, and  $Mg^{++}$ , with extract from either Neurospora sitophila or liver. Robbins and Lippman (47,48) isolated the compound by Dowex-1 chromatography. They characterized the compound by chemical and enzymic methods as 3'-phosphoadenosine-5'-phosphosulfate (PAPS). Subsequently, Robbins and Lipmann (49) and Bandurski et al. (4) showed that sulfate activation in yeast requires two enzymes whose functions are given below:

ATP-sulfate  
adenyltransferase (E.C.2.7.7.4)  
(ATP sulfurylase)



adenylsulfate 3'-phospho  
transferase (E.C.2.7.1.25)  
(APS kinase)

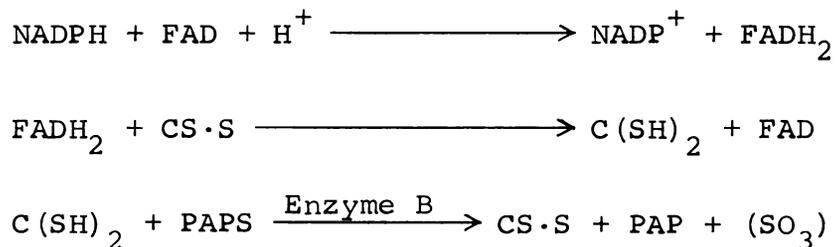


The apparent equilibrium constant measured with the yeast enzyme for the formation of APS is between  $1 \times 10^{-8}$  and  $4 \times 10^{-8}$ . The reaction proceeds by the hydrolysis of pyrophosphate and the phosphorylation of APS so that the sum of the free energies is negative.

The enzyme, ATP sulfurylase, is specific for ATP but will accept a variety of group VI anions of which  $\text{MoO}_4^{--}$  is the most active (62). The  $\text{AMP-MoO}_4^{--}$  product is unstable and immediately hydrolyzes to AMP and molybdate. Selenate forms  $\text{AMP-SeO}_4^{--}$ , but the molecule is stable. The group VI anions can prevent growth of the cells, but toxicity is prevented by an excess of sulfate or cysteine (42,61). These observations suggest sulfate activation is of physiological significance. S. typhimurium and E. coli mutants defective in ATP-sulfurylase or APS-kinase cannot grow with sulfate as the sole sulfur source (15,68). This strongly indicates APS

and PAPS are obligate intermediates in the reduction of sulfate for cysteine biosynthesis.

Sulfate reduction to sulfite. Sulfate reduction is catalyzed by an enzyme complex given the trivial name, PAPS reductase. The most extensive studies on the reduction mechanism have been performed with an enzyme complex derived from yeast (3,63,64). The complex has been purified and resolved into at least three components, enzymes A and B and Fraction C. The proposed electron flow through this complex is given below:



The sulfite is written in parenthesis because evidence indicates that it is bound and not free in the cytoplasm. The working hypothesis is that the sulfite is bound to Fraction C (57). As shown in the equations, Fraction C contains a reducible disulfide which functions as an electron acceptor. Hilz et al. (21) have suggested that PAPS undergoes thioly-sis followed by reductive cleavage of the R(SH)(SSO<sub>3</sub>) compound. They suggest that R is a lipoyl moiety. However, the data of Wilson et al. (63) suggest the dithiol is not lipoate but Fraction C.

Sulfite reduction to sulfide. Sulfite reductase (hydrogen sulfide-NADP oxidoreductase, E.C.1.8.1.2) catalyzes the reduction of sulfite to sulfide, a six electron reduction. The enzyme is characteristic of the inorganic sulfate pathway and is found in microorganisms such as E. coli (43), S. typhimurium (15), yeast (65,66), N. crassa (32) and several plants (55) including Allium (onion family) (56) and spinach (2). The enzyme has been extensively purified after extraction from E. coli, yeast, A. nidulans, Allium and spinach. Reduced ferredoxin (1) or NADPH is the physiological electron donor, but the reduced, low-potential dyes, methylviologen (MVH) and benzoyl viologen, can also serve as electron donors in vitro.

Although the enzyme appears as a single protein after purification recent studies (37,69,70) indicate that it is composed of several subunits. As a single species a molecular weight of 350,000 was obtained with yeast sulfite reductase (69); a molecular weight of 700,000 is reported for the enzyme from E. coli (L. M. Siegel, H. Kamin, and Q. H. Gibson, Abstr. 7th Inter. Congr. Biochem. Tokyo, p. 187, 1967). The purified MVH-sulfite reductases from Allium (56) and spinach (K. Asada, G. Tamura, and R. S. Bandurski, in press), which no longer accept electrons from a physiological electron donor, have molecular weights in the range of 60,000 to 85,000. Naiki (37) has found that heat, low ionic-strength, and p-chloromercurobenzoate (PCMB)

inactivates yeast NADPH-sulfite reductase, but not yeast MVH-sulfite reductase. Since the NADPH- and MVH-activities copurified over a 200-fold range, Naiki proposed yeast NADPH-sulfite reductase is a two component system. In more extensive studies Yashimoto and Sato (69) have physically and chemically characterized the NADPH-sulfite reductase from yeast. They have also described the incomplete enzyme from certain cysteine requiring mutants of yeast. They concluded that NADPH-sulfite reductase is composed of three subunits (Figure 1) (70).

A brief description of the chemical properties of NADPH-sulfite reductases provides a basis for classifying the components isolated by Yashimoto and Sato. The enzyme contains one mole each of flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), five moles of iron, and two moles of acid-labile sulfur per mole of enzyme. The enzymes from S. typhimurium and E. coli are identical by physical and kinetic parameters. The enzyme from E. coli contains four moles each of FAD and FMN and 12-16 moles each of iron and acid-labile sulfide per mole of enzyme (L. M. Siegel and H. Kamin, in press). Yeast enzyme also contains a 587 m $\mu$  chromophore of unknown chemical nature (69). E. coli enzyme contains chromophoric groups absorbing at 587 m $\mu$  and 620 m $\mu$ . The third component of the yeast enzyme, which contains the 587 m $\mu$  chromophore, also contains the sulfite-binding site. However, Siegel and Kamin report that the 620 m $\mu$  chromophore,

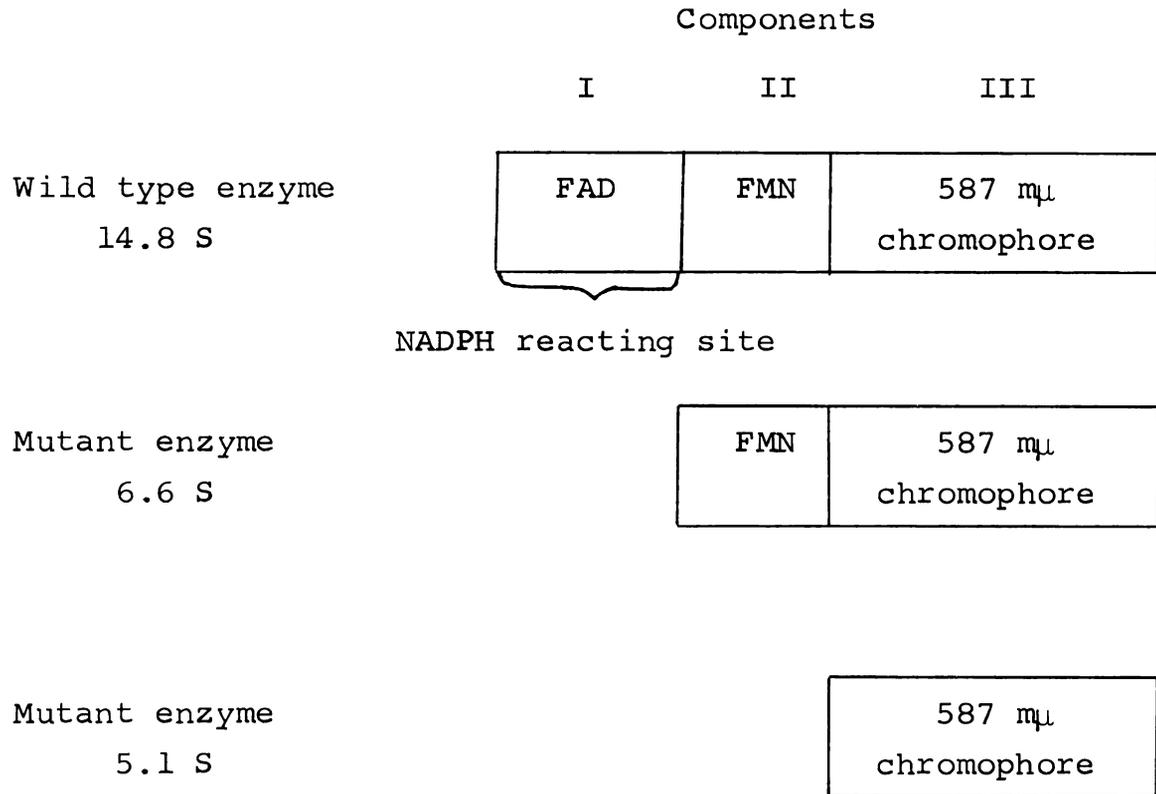
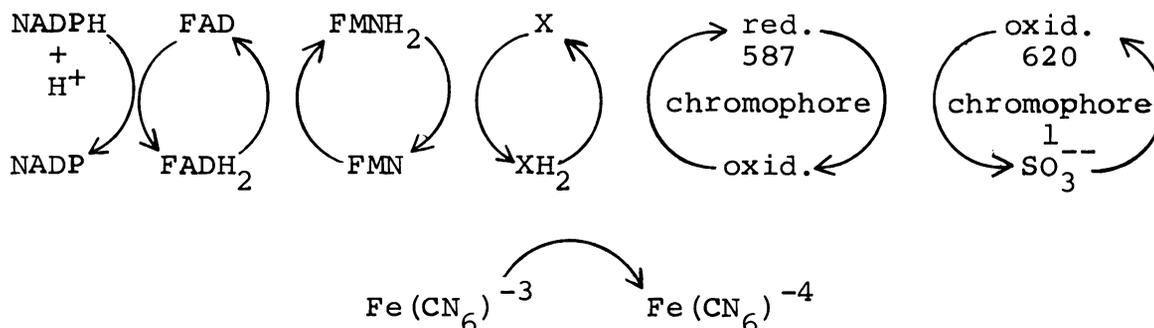


Fig. 1. Schematic illustration of the component nature of yeast sulfite reductase purified from wild type and mutant strains (70).

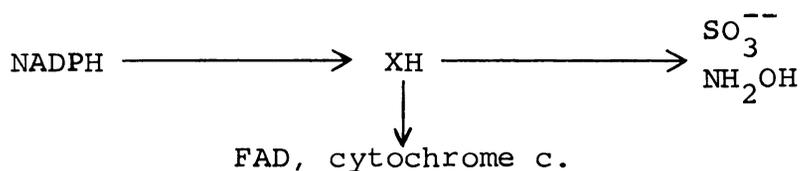


a heme group, is the sulfite binding site for the enzyme from E. coli.

The proposed electron flow in the E. coli sulfite reductase complex studied by Siegel and Kamin is presented below:



The inclusion of the "X" coupling factor is this author's interpretation of their negative finding for a direct interaction between  $\text{FMNH}_2$  and the 587  $\text{m}\mu$  chromophore. This interpretation is consistent with the two step electron flow postulated earlier (52) for S. typhimurium enzyme:



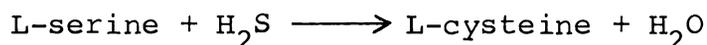
From the above information and this latter scheme it is evident sulfite reductase apparently catalyzes several reactions. Sulfite reductase from yeast catalyzes the reduction not only of sulfite, but of hydroxylamine, nitrate, ferricyanide, FAD, cytochrome c, quinones and 2,6-dichlorophenolindolphenol (DCIP) (69). The diaphorase function of

yeast sulfite reductase is not sensitive to  $\text{CN}^-$  inhibition whereas sulfite-, nitrite-, and hydroxylamine-reduction activities are sensitive to  $\text{CN}^-$  inhibition. This indicates that the binding site for sulfite is different from the binding site for the dyes that act as electron acceptors. The kinetics of  $\text{CN}^-$  inhibition indicate that the same site on the E. coli enzyme binds  $\text{CN}^-$  and  $\text{SO}_3^{--}$  and  $\text{NO}_2^-$  (27). Spectral studies and metal analysis of spinach MVH-sulfite reductase (K. Asada, G. Tamura, and R. S. Bandurski, in press) led to the original conclusion that the enzyme contained an unusual heme protein. The nature of this heme group has not yet been established.

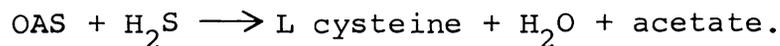
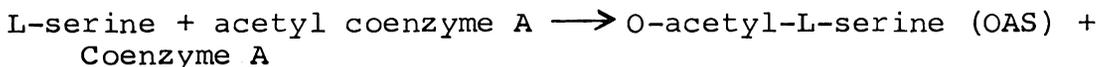
The picture presented here of NADPH-sulfite reductase is incomplete. Important facts, such as the total characterization of each component, the physical relationship between components, the actual mechanism of electron transfer to sulfite, and the relationship of Fraction C bound sulfite, are still unknown. Also it is difficult to understand the apparently fortuitous involvement of so many different enzymic functions. Kemp et al. (27) have demonstrated that the physiological function of E. coli sulfite reductase is probably the reduction of sulfite to sulfide since the Michaleis-Menten constant is lowest for sulfite and since cysteine co-represses both sulfite and nitrite reductase activities. Also, Siegel et al. (52) concluded that the diaphorase activities of S. typhimurium are without

physiological significance since diaphorase, hydroxylamine-, and sulfite-reductase are coordinately repressed by different sulfur sources or lost in certain cysteine mutants.

Formation of cysteine. Schlossman and Lynen (50) purified a pyridoxal-dependent enzyme, serine sulfhydrylase, from yeast which catalyzes the following reaction:



However, the physiological significance of this enzyme was questioned because of kinetic deficiencies [R. S. Bandurski, personal communication; (25)]. The overall correctness of the reaction was demonstrated by isotopic studies that established serine as the precursor of the cysteine carbon skeleton and by mutational studies (46) that suggested sulfide is the incorporated form of sulfur. Recently, Kredich and Tomkins (28) have shown that the cysteine formation in extracts of E. coli or S. typhimurium is a two-step process:



The enzyme catalyzing formation of OAS has been given the trivial name, serine transacetylase; the enzyme catalyzing the second step has been given the trivial name, O-acetyl-serine sulfhydrylase.

## Part III

## Control of Assimilatory Sulfate Reduction

The enzymes of sulfate reduction in S. typhimurium and E. coli are controlled by the intracellular levels of OAS and cysteine. Derepression occurs when the intracellular level of cysteine drops below a critical value (67), but only if OAS is present [H. T. Spencer, J. Collins, and K. J. Monty, Fed. Proc. 26:677, 1967; (25)]. Kredich and Tomkins (28) have demonstrated that cysteine regulates the intracellular level of OAS by end product inhibition of the enzyme, serine transacetylase, which catalyzes the synthesis of OAS. As expected, this enzyme is not repressed by cysteine. Cysteine also affects another receptor since OAS is not inductive if the cysteine level is too high (25). In S. typhimurium most of the enzymes of the sulfate pathway are repressed simultaneously with the addition of a fixed level of cysteine. This phenomenon has been termed coincident repression (14). It is not known if repression is coordinate. In E. coli repression is "differential" and coordinate (43). "Differential" means that the initial reactions of the pathway are more sensitive to the level of repressor than are subsequent reactions. The enzyme of sulfate "activation" are under coordinate control (derepressed in a fixed ratio) but taken as a block the enzymes of sulfate activation are differentially controlled in relation to the entire pathway.

Thus far, the only control mutants that have been noted in S. typhimurium and E. coli (15,25) are pleiotropic negative mutants of the cys E and cys B loci. The cys E mutants cannot synthesize OAS, and, therefore, they are not derepressible. The cys B mutants also lack all enzymes of the pathway (K. J. Monty, personal communication), but it is not known if the cys B locus produces protein with only regulatory or regulatory and catalytic properties.

Jones-Mortimer (24) has presented preliminary evidence that the control of sulfate reduction is "positive." She found that the cys B<sup>+</sup> allele is dominant over the cys B allele; that is, induction (derepression) takes place when the functional product of the B<sup>+</sup> gene is present for activation by OAS. Since the 9 gene loci (concerned with sulfite reduction and cysteine biosynthesis) map in 5 or 6 separate chromosomal sites on the S. typhimurium chromosome (35), the activated initiator complex must affect multiple targets (operator sites). An accurate, detailed accounting of this process should enlighten our understanding of biosynthetic control.

## MATERIALS AND METHODS

### Part I

#### Accumulation of Sulfite

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

Bacterium. Salmonella pullorum strain MS35 was selected as the prototype organism from the stock collection of Dr. D. E. Schoenhard; it was designated wild type. The organism is a natural cysteine and leucine auxotroph; sulfide 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 (60) except that equimolar  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  replaced  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . Sterile D-glucose and L-leucine were added to final concentrations of 0.4% and  $1.5 \times 10^{-5}$  M respectively. Various sulfur sources

were added at levels specified in each experiment. The sulfur compounds were handled with proper consideration for their stability (45). L-methionine ( $1.34 \times 10^{-4}$  M), unless indicated otherwise, was added to all cultures containing sulfate to prevent a sulfate-induced initial growth lag. The lag induced by sulfate and the reasons for its alleviation by methionine are unknown. Broth cultures were grown aerobically at 37 C on a rotary shaker. Limiting oxygen conditions were achieved by use of screw cap bottles sealed with tape. When sulfur-free agar medium was required for plates, 20 g of washed Noble agar was added to one liter of E-broth. Noble agar was washed with deionized, distilled water (25 water:1 agar v/w) by mixing with the water, allowing the agar to settle, and decanting the liquid. This procedure was repeated a total of three times. Enriched minimal agar was formed with 98.75 ml of E medium and 1.25 ml of reconstituted Difco nutrient broth.

Selection of revertants. Spontaneous revertants of MS35 that use sulfate at 37 C have never been observed. Spontaneous revertants that use cysteine sulfinic acid (CSA) at 37 C occur at a frequency of about  $10^{-7}$ . Revertants selected for use of CSA also use sulfite and vice versa. One spontaneous revertant, selected for its use of CSA, was designated revertant 6. Also it can use sulfite, sulfide, and cysteine individually, but not sulfate, as the sole source of sulfur. When a population of revertant 6 was

screened for spontaneous sulfate-using revertants, these revertants were detected at a frequency of  $10^{-8}$  to  $10^{-9}$ . To obtain sulfate-using revertants for this study, plates of enriched minimal agar were spread with  $4 \times 10^7$  cells of revertant 6 and a drop (2  $\mu$ g) of NTG was placed in the center of the agar surface. The plates were incubated at 37 C for 72 hrs. One of the NTG-induced revertants, designated 6-18, was selected and purified for use in this study. Revertants, depending on their sulfur requirements, were stored on sulfur-free E minimal agar plates supplemented either with sulfate ( $4 \times 10^{-4}$ M) or CSA (20  $\mu$ g/ml). MS35 was stored on E agar medium supplemented with L-cysteine (20  $\mu$ g/ml). All cultures were subcultured every two months and stored at 4 C.

Routine determination of sulfite. The routine determination of sulfite was by the fuchsin-formaldehyde technique described by Grant (19) and modified by Dreyfuss and Monty (15). This technique is specific for sulfite in the presence of thiosulfate, CSA, and sulfhydryl-containing compounds.

Concentration and collection of the accumulated sulfur product. A typical cell-free supernatant of a stationary phase broth culture of revertant 6-18 supplemented with sulfate ( $4 - 8 \times 10^{-4}$ M) and methionine ( $1.34 \times 10^{-4}$ M) was concentrated about tenfold in vacuo at 80 C. The

concentrate was acidified (pH 1.0-2.0) and the acid-volatile gas was removed from it by bubbling 0.15 M  $\text{AgNO}_3$ -washed nitrogen gas through it for one hr at 37 C. The evolved gas was passed through a 0.15 M NaOH-0.001 M ethylene-diamine tetraacetate (EDTA) trap to dissolve  $\text{SO}_2$  and convert it to stable  $\text{SO}_3^{--}$ . 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 5,5'-dithiobis (2-nitro benzoic acid) (DTNB) reacts with sulfite at neutral and slightly alkaline pH to form the yellow-colored thionitrobenzoate anion and the colorless S-sulfonate derivative (16). At high pH 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 pH 7.0 was made  $2.5 \times 10^{-3}$  M with respect to DTNB. An aliquot containing 1  $\mu$ mole of DTNB was reacted at a final pH of 7.2 with 0.4  $\mu$ moles of putative or authentic sulfite for 10 min at room temperature and then electrophoresed at 4 C for 1.5 hrs at 15 v/cm on a Whatmann #1 paper soaked in 0.05 M acetate buffer (pH 4.8). Also, a sample of the reaction mixture was chromatographed in a descending manner on Whatmann #1 paper using absolute ethanol-0.1 M ammonium acetate (7.5:3, v/v). The position of the separated S-sulfonate derivative at the termination of electrophoresis or at the termination of chromatography was made visible by

spraying with a dilute solution of mercaptoethanol to liberate the yellow-colored thionitrobenzoate anion.

## Part II

### Biochemical Characterization of a Natural Cysteine Mutant, S. pullorum MS35

Chemicals. The 2'-and 3'-isomeric mixture of adenylic acid (2'- and 3'-AMP) used in this work was purchased from Calbiochem, Los Angeles, California. All other chemicals were as described in Part I of this section.

Bacteria. Salmonella pullorum, strain MS35, and revertants derived from it are described in Table 2. Salmonella typhimurium prototrophic strain, LT-2, was also used in this work.

Media and growth of derepressed bacteria. The sulfur-free basal E medium used in this work is the same as described in Part I. Salmonella typhimurium was cultured aerobically in E medium supplemented with djenkolic acid ( $2 \times 10^{-4}$  M) to derepress the synthesis of sulfate-reducing enzymes (15). Salmonella pullorum was cultured similarly except that the E medium which contained djenkolic acid was enriched with 3.2 ml of each of the following solutions of nutrients to increase the growth rate. Solution A contains the following compounds dissolved in deionized, distilled water: L-amino acids each at 2 mg/ml: alanine, arginine, asparagine, aspartic acid, glutamine, glutamic acid,

histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, and threonine; the DL-amino acid at 4 mg/ml, valine; the optically inactive amino acid, glycine, at 2 mg/ml; and the vitamins each at 2 mg/ml, calcium pantothenate and thiamine-HCl. Nutritional "pool" B contains the following compounds dissolved in 1 N KOH:L-amino acids each at 2 mg/ml, tryptophan and tyrosine; and nucleic acid precursors each at 0.2 mg/ml: guanine, adenine, cytosine, and uracil.

Nutritional responses. The auxanographic technique of Beijerinck, described by Lederberg (31), was used to test the nutritional responses to all sulfur compounds except sulfide. A sterile filter paper disc, impregnated with a sulfur compound (50  $\mu\text{g}$  with respect to S), was placed on the surface of a seeded, sulfur-free E medium agar plate. The plate was incubated 24 hrs before scoring the response to the sulfur compound (thiosulfate excepted). The response to thiosulfate was scored after 5 days. The response to sulfide ( $1 \times 10^{-4}\text{M}$ ), tested in minimal broth and contained in a screw cap tube, was scored in 18 to 24 hrs.

When S. pullorum was tested using ordinary E medium (with sulfate), a positive growth response to CSA was inhibited by sulfate. Thus, special precautions which were described in Part I were taken to ensure that the final medium was sulfate-free. Appropriate precautions were also

observed in the preparation of sterile solutions of sulfur sources (45).

Cell-free preparation. Salmonella typhimurium extracts were prepared in 0.05 M potassium phosphate buffer, pH 7.6; S. pullorum extracts were prepared in the same species of buffer and at the same pH but at a concentration of 0.2 M phosphate. All cultures were verified as Salmonella by serology, and mutant cultures were tested for the extent of reversion by plating on appropriate media. The cells (ca., 2 g wet weight) used for a typical extract were collected at 4 C by centrifugation, washed once with buffer, and resuspended in 3 ml of buffer. The bacterial suspension was placed in an ice bath and subjected to four separate 15 sec periods of sonic oscillation at a frequency of about 20 kc per sec by a Measuring and Scientific Equipment, Ltd. magnetostrictive oscillator. Each period of oscillation was separated one min from the preceding period to prevent overheating. The disrupted preparation was centrifuged at 34,000 x g for one hr at 4 C. The supernatant fluid was removed, frozen at -20 C, and stored at this temperature. The amount of protein in the extract was determined by the procedure of Lowry et al. (33) with bovine serum albumin as a standard.

Transport of sulfate. The transport of sulfate was determined by two methods: (a) by measuring the dilution of

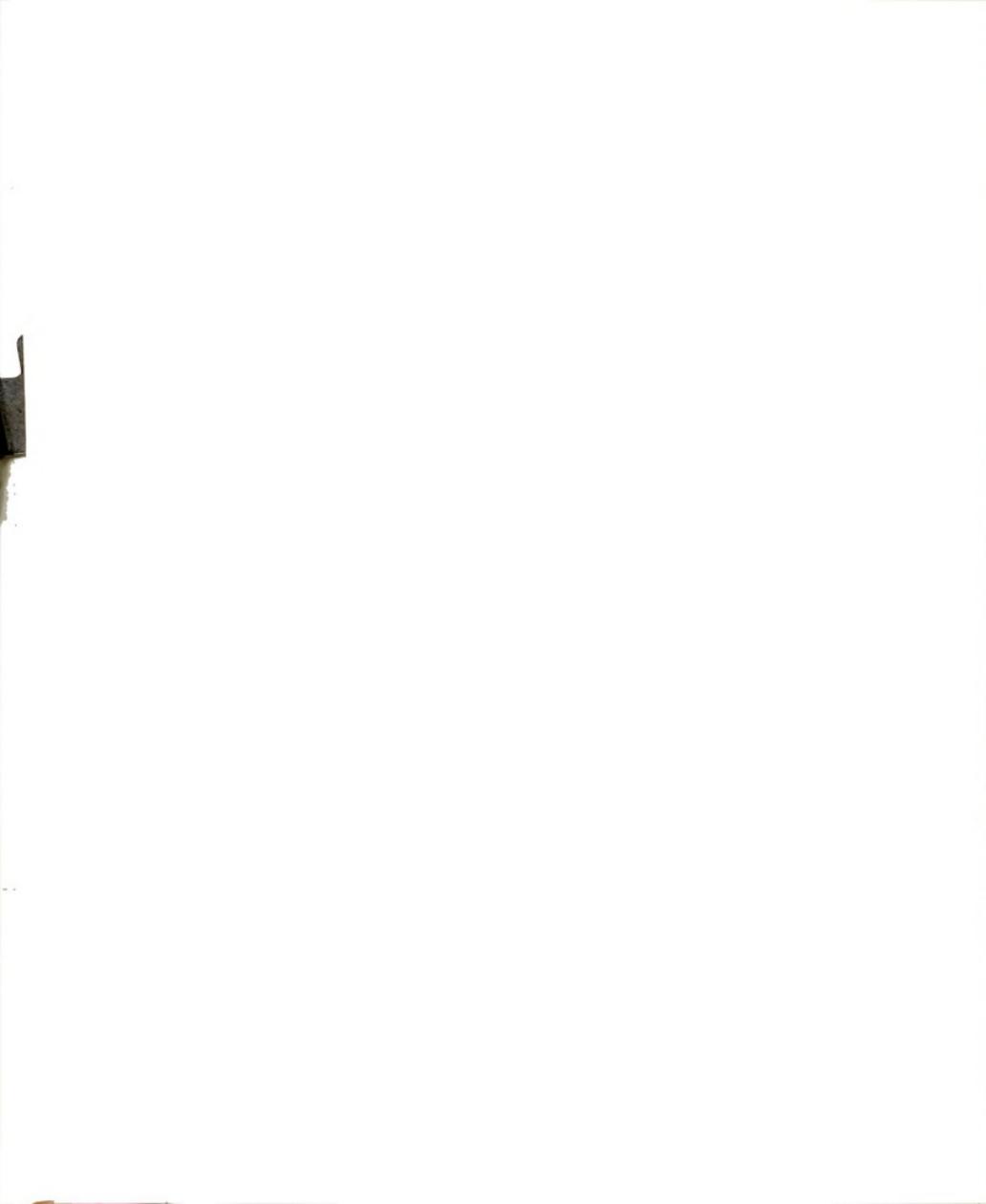


(S<sup>35</sup>)-sulfate in the medium exposed to a high number of bacteria (12) and (b) by measuring the ability of dilute cultures of bacteria to trap radioactivity after exposure to (S<sup>35</sup>)-sulfate (13). Bacteria for both types of assay were grown at 37 C with djenkolic acid as the sulfur source to derepress the enzymes of the sulfate pathway. In the first method the measurement of sulfate transport was performed by mixing 1 ml of E medium containing glucose (0.2%) and bacteria (17 to 83 mg of protein) and 1 ml of E medium containing glucose (0.2%), about 10<sup>6</sup> c.p.m. of (S<sup>35</sup>)-sulfate (1 x 10<sup>-4</sup>M) and chloramphenicol (CM) (200 µg). The mixture was incubated for 5 min at 25 C and then centrifuged at 10,000 x g at 4 C to pellet the bacteria. The supernatant fluid was then diluted 1:10, 0.05 ml of the diluted material was added to a glass vial containing 10 ml of scintillation fluid (7), and the radioactivity present was counted overnight in a Packard scintillation spectrophotometer. A reaction mixture without cells was used for a control (C). In theory, reaction mixtures (D) that contain impermeable cells have more radioactivity per aliquot of supernatant fluid than the control tube so that the deviation,  $\frac{D-C}{C} \times 100$ , is positive. For permeable cells the deviation is negative if sulfate is metabolized or if transport is active (12). In the second method the measurement of sulfate transport was performed by mixing 1.4 ml of E medium containing derepressed bacteria (7 x 10<sup>8</sup>), glucose (0.2%), and CM (100 µg) with



0.60 ml of E medium containing glucose (0.2%) and  $6 \times 10^5$  d.p.m. of ( $S^{35}$ )-sulfate ( $3.3 \times 10^{-5} M$ ). The reaction mixture was incubated at room temperature, and at given intervals a 0.5 ml aliquot was removed, filtered through a  $0.45 \mu$  membrane filter, and rinsed 3 times with 5 ml amounts of ice-cold E medium containing glucose (0.2%) but not sulfate. The membranes were glued to planchets, dried, and counted in a Nuclear Chicago gas-flow counter. No attempt was made to correct for the amount of sulfate that binds nonspecifically to the membrane.

Synthesis of APS and PAPS. The reaction mixture for the synthesis of APS contained in a volume of water (0.5 ml): ATP (3  $\mu$ moles),  $MgCl_2$  (3.7  $\mu$ moles),  $K_2SO_4$  (0.3  $\mu$ moles), carrier free  $H_2^{35}SO_4$  (5.0-100.0  $\mu$ curies) tris-HCl buffer, pH 8.8 (25  $\mu$ moles of tris), and 0.1 ml of extract (1.0-3.0 mg of protein). Incubation was at 37 C. The reaction was stopped by immersion of the reaction tube in a boiling-water bath for 2 min, protein was removed by centrifugation, and the  $APS^{35}$  was separated by electrophoresis (21 v/cm) at 15 C on Whatmann 3 mm paper soaked in 0.03 M citrate buffer, pH 5.8. After drying, the radioactive area was eluted with water, and an aliquot was counted in a liquid scintillation spectrophotometer. At this temperature and pH sulfate nucleotides are partially lost during electrophoresis. Consequently minimal values result. The same type of reaction mixture and procedures was used for the synthesis of PAPS



except that extra ATP (3.0  $\mu$ moles) and a mixture of 2'- and 3'-AMP (0.8  $\mu$ moles total AMP) were added to the reaction mixture. In some initial experiments the extract was dialyzed against 0.1 tris-HCl buffer, pH 8.0, to remove phosphate ion.

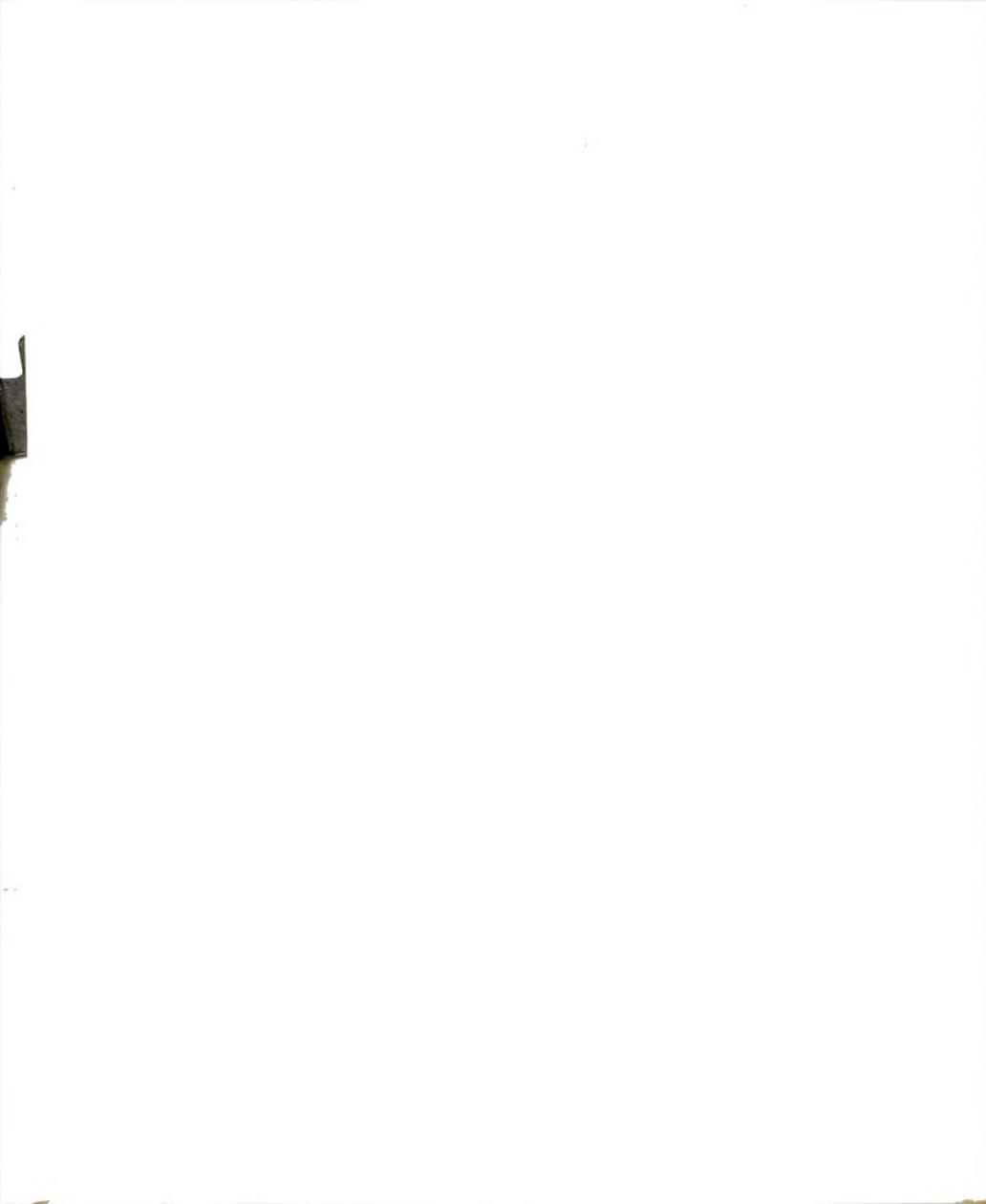
Characterization of APS and PAPS. Extracts of Salmonella typhimurium wild type, strain LT-2, were used to produce putative APS<sup>35</sup>, and PAPS<sup>35</sup> (15) from <sup>35</sup>SO<sub>4</sub><sup>2-</sup> and ATP. The putative nucleotides were characterized as ultraviolet-light-absorbing, charcoal-adsorbable, and acid-labile (90% hydrolysis in 0.01 N HCl at 37 C in 30 min). The APS<sup>35</sup> was further characterized by two dimensional chromatography according to the procedure of Wilson and Bandurski (62). The results were identical to those reported by these authors for synthetic APS<sup>35</sup>. The putative PAPS<sup>35</sup> moved farther than APS and ATP (22) during electrophoresis but moved slower than APS<sup>35</sup> during ascending chromatography using a n-propanol:NH<sub>3</sub>:H<sub>2</sub>O (6:3:1) solvent system. An R<sub>f</sub> value of 0.11 was determined for PAPS<sup>35</sup> in this solvent system.

Sulfite reductase. Reduced methyl viologen (MVH)-sulfite reductase was assayed using the procedure and apparatus described by Asada (2). The reaction mixture contained phosphate, pH 7.75, (150  $\mu$ moles), potassium sulfite (1  $\mu$ mole), MVH (0.27  $\mu$ moles) and extract (0.5-1.0 mg protein)



in a final volume of 1.5 ml. The endogenous rate of MVH bleaching was determined at 25 C, then the reaction was started by tipping in  $\text{SO}_3^{--}$  from a sidearm. Six reduced methyl viologen (MVH) molecules were oxidized per molecule of sulfide produced. Activity was measured as the  $\mu\text{moles}$  of MVH oxidized, but activity is expressed as equivalents of  $\text{H}_2\text{S}$  produced. When a reaction mixture that continuously generated NADPH was used, sulfite reductase activity was determined as the sulfite-dependent production of sulfide. The reaction mixture contained in a volume of 1 ml: potassium sulfite ( $0.5 \mu\text{moles}$ ), glucose-6-phosphate ( $3 \mu\text{moles}$ ), NADP ( $0.06 \mu\text{moles}$ ),  $\text{MgCl}_2$  ( $4 \mu\text{moles}$ ), phosphate, pH 7.6 ( $34 \mu\text{moles}$ ), and extract (1.5-3.0 mg of protein). Incubation was for 20 min at 25 C in a test tube sealed by a cork wrapped in Parafilm. Sulfide was determined according to the procedure of Siegel (51). Specific activities were calculated from values obtained in tests where the production of sulfide was linear with respect to time and protein concentration.

Determination of sulfite. Sulfite was determined by the modified Grant procedure described in Part I.

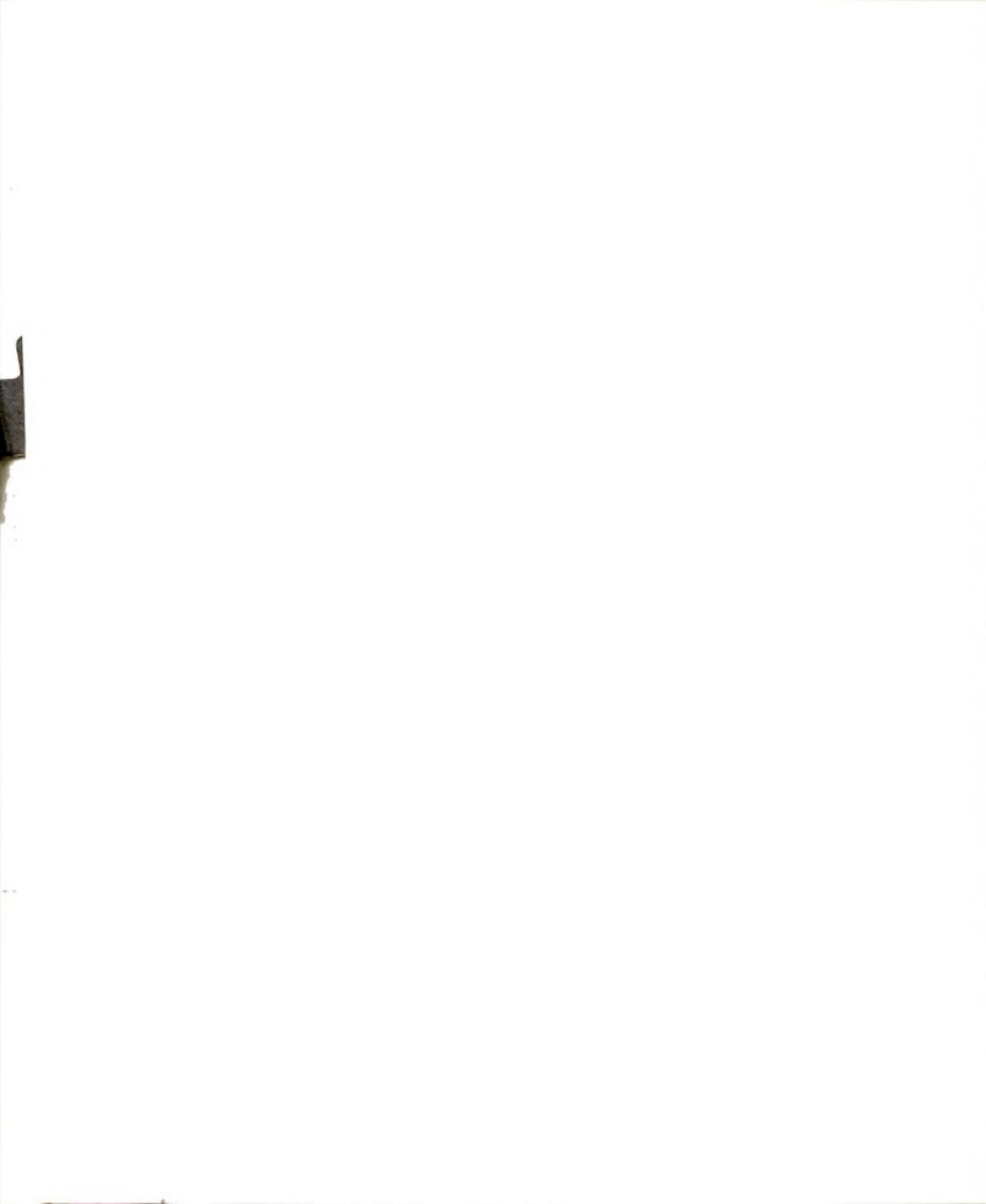


## Part III

## Control of Sulfate Reduction

General. The organism, S. pullorum revertant 6-18, media, and procedures are described in Parts I and II of this section excluding the procedure for the production of sulfite by dense suspensions of bacteria.

Production of sulfite by dense suspensions of bacteria. The bacteria used in these tests were grown in E medium supplemented with sulfate ( $2 \times 10^{-4} \text{M}$ ) and either L-methionine ( $1.34 \times 10^{-4} \text{M}$ ) or 1 ml each of nutrient solutions A and B per 0.1 liter. The reaction mixture for the production of sulfite contained  $\text{K}_2\text{SO}_4$  (5  $\mu\text{moles}$ ), where indicated, L-cysteine (5.0  $\mu\text{moles}$ ), glucose (1.6%), CM (250  $\mu\text{g}$ ), bacteria, washed once with sulfur-free E salts and concentrated ( $4 \times 10^{11}$  per ml) by centrifugation (0.25 ml), and sufficient E salts containing nutrient solutions A and B (1 ml each per 0.1 liter of the salts) to bring the final volume to 5 ml. The mixture was incubated in a 37 C water bath with aeration for 30 min. Sulfite was determined by the modified Grant technique described in Part I of this section. The accumulation of sulfite was found to be proportional to the number of cells added and to the period of incubation.



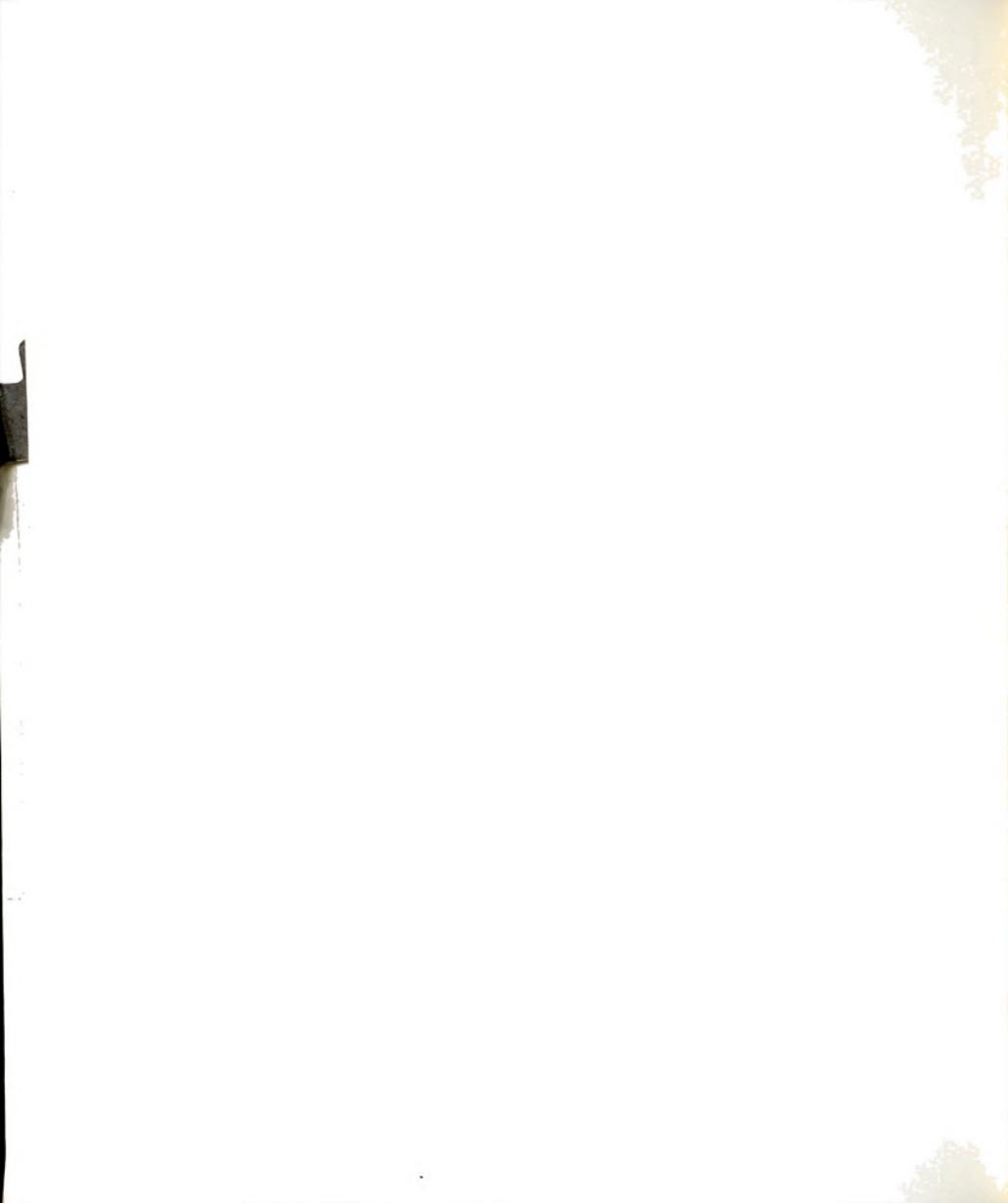
## RESULTS

### Part I

#### Accumulation of Sulfite

An unusual feature, noticed around all spontaneous and induced sulfate-using colonies of S. pullorum, was a zone of growth in the parent, revertant 6, lawn. Subsequent tests showed that one sulfate-using revertant, 6-18, fed only revertant 6, not the wild type organism, MS35. This finding indicated neither sulfide nor cysteine was the secreted compound; otherwise, MS35 would have grown. Thio-sulfate was also eliminated as a feeding compound since revertant 6 cannot use thiosulfate for growth. The results suggested that sulfite, CSA, or some nutritionally equivalent compound had accumulated.

Identification of sulfite. When broth cultures of revertant 6-18 in the stationary phase of growth were concentrated and tested by the modified Grant technique, they were found to contain fuchsin-reactive material equivalent to 93% of the sulfur in excess of that required for growth. When ( $S^{35}$ )-sulfate was used as the sulfur source for growth of 6-18, the acid-volatilized, fuchsin-reactive material



that was trapped had a specific activity 6% less than the value of the sulfate substrate. This finding indicated that, within experimental error, the fuchsin-reactive material of the trap is a sulfur compound. When an aliquot of the NaOH-EDTA trap, containing fuchsin-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 identity of the putative and authentic sulfite derivatives of DTNB is additional evidence that the trap material is sulfite (Figs. 2 and 3).

Precursor of accumulated sulfite. The determination that sulfite accumulated in cultures of revertant 6-18 in the presence of a mixture of sulfate and methionine raised a question concerning the physiological precursor of sulfite. Based on the hypothesis that methionine sulfur can be oxidized to sulfite, methionine should be a sufficient sulfur source for growth; however, it is not. Table 1 shows sulfite only accumulates if 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. Moreover, Table 1 shows that the final level of accumulated sulfite is reduced as the concentration of exogenous cysteine is increased.

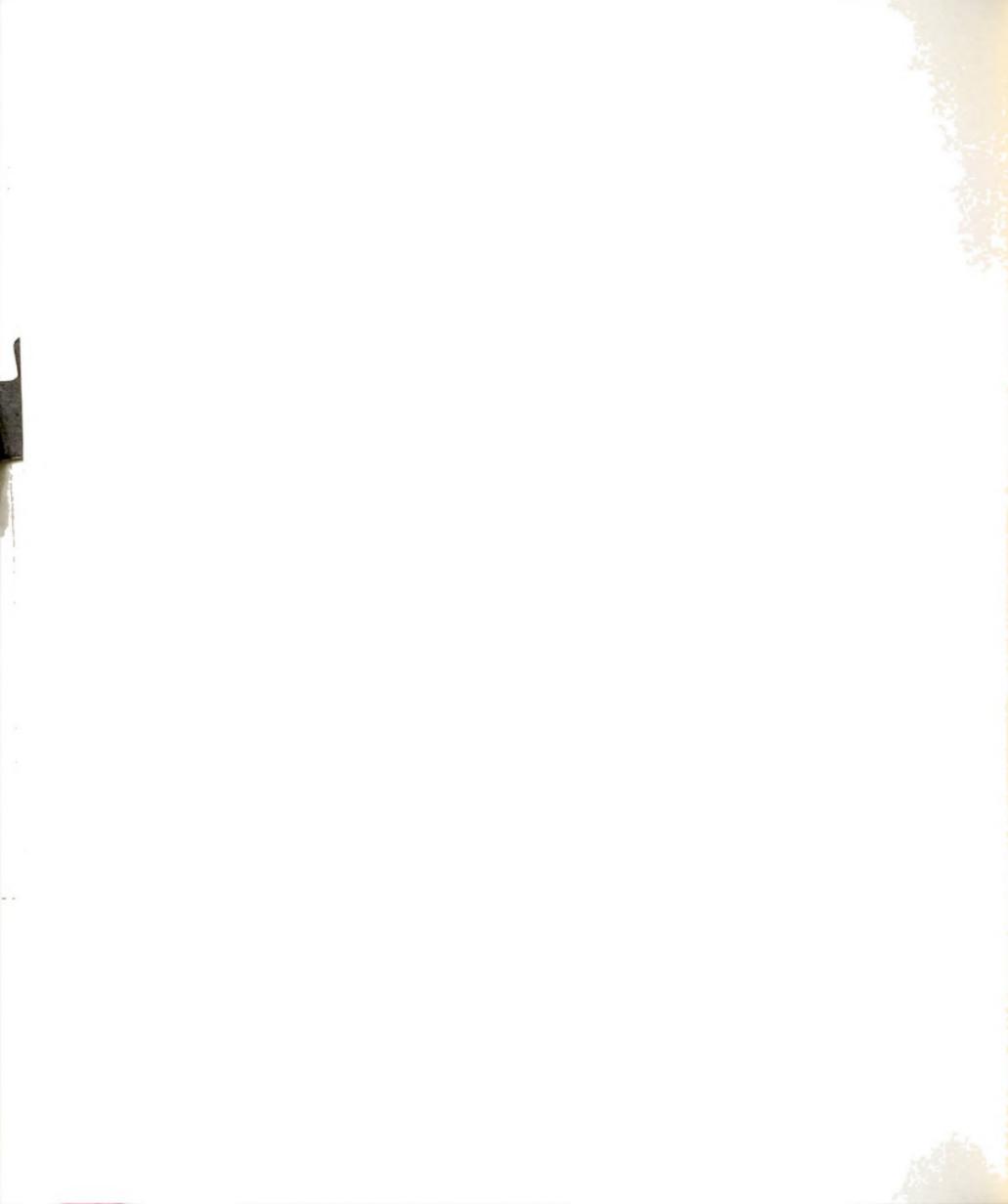


Fig. 2. Chromatographic identification of S-sulfonylthionitrobenzoate. The solid black areas represent the yellow-colored thionitrobenzoate anion. The outlined areas represent the yellow thionitrobenzoate anion that arises after spraying with dilute mercaptoethanol. Chromatographic conditions were ethanol-0.1M ammonium acetate (7.5:3, v/v).

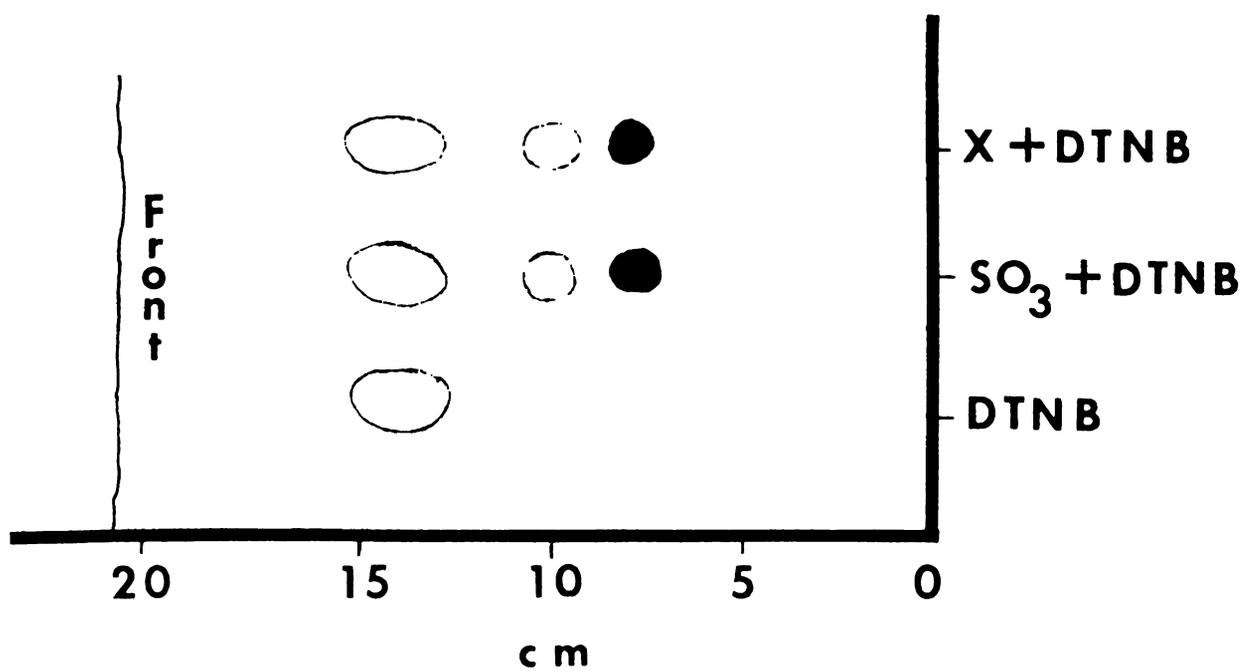


Fig. 2



Fig. 3. Electrophoretic identification of S-sulfonylthionitrobenzoate. The solid black areas and outlined areas are described under Figure 2. Electrophoretic conditions were 0.05M acetate buffer, pH 4.8, with 15 v/cm for 1.5 hrs at 4 C.

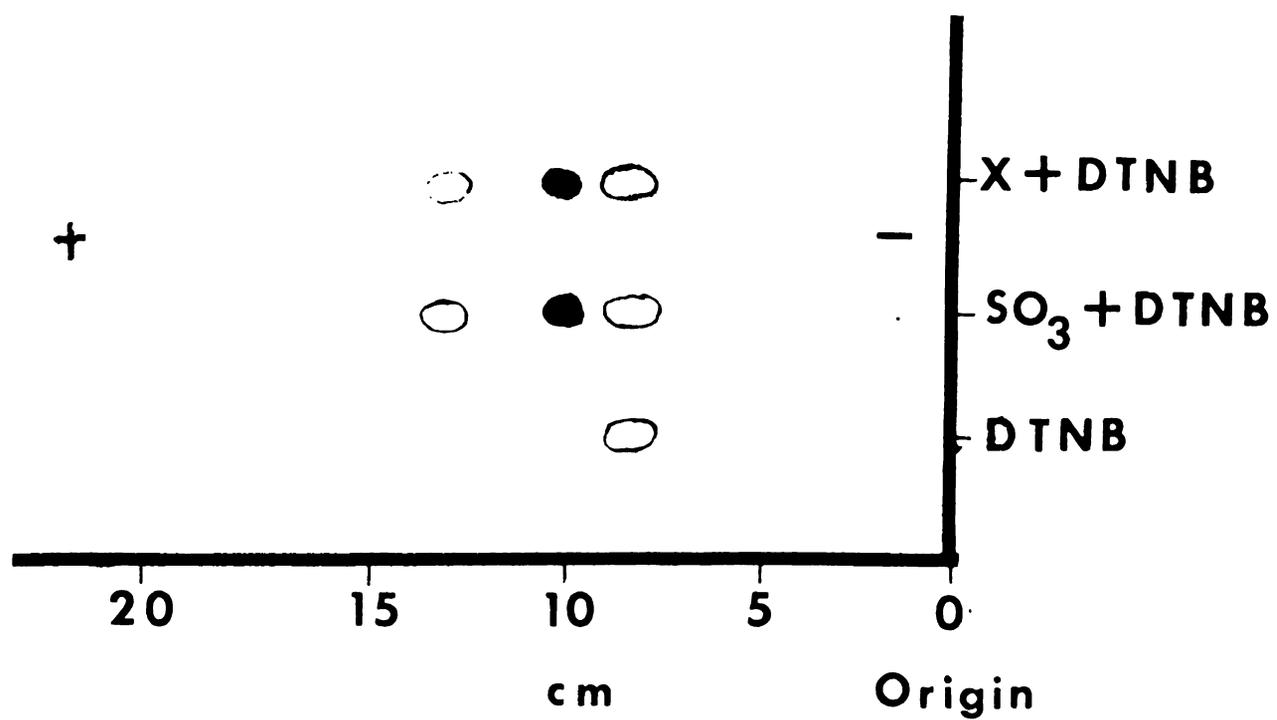


Fig. 3



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

Sulfur substrate	Accumulated sulfite <sup>b</sup> 10 <sup>-4</sup> moles/ liter	Sulfide evolution <sup>c</sup>
SO <sub>4</sub> <sup>2-</sup> (0.8 mM) <sup>a</sup>	4.20	-
SO <sub>4</sub> <sup>2-</sup> (0.8 mM) + L-methionine (0.134 mM)	6.00	-
SO <sub>4</sub> <sup>2-</sup> (0.4 mM) + L-methionine (0.134 mM): <sup>d</sup>	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	+

<sup>a</sup>A concentration of  $1.8 \times 10^{-4}$  M sulfur is required to give full growth in the synthetic E medium used for growth.

<sup>b</sup>Level of sulfite was determined by the Grant technique 12 hours after the culture had reached the stationary phase of growth.

<sup>c</sup>The evolution of sulfide was detected by blackening a strip of lead acetate paper.

<sup>d</sup>In this test four identical cultures were used, each containing SO<sub>4</sub><sup>2-</sup> and L-methionine, and to three flasks L-cysteine was added at the concentration indicated.



If the accumulated sulfite came from sulfate, then group VI anions should compete with sulfate. Three group VI anions: molybdate, chromate, and selenate, each at a concentration of  $4 \times 10^{-3}$  M, were tested in the presence of  $4 \times 10^{-4}$  M sulfate. Figure 4 shows that a mixture of selenate and sulfate allows a little cell growth but totally inhibits accumulation of sulfite. Cysteine at an initial level of  $1.4 \times 10^{-4}$  M does not completely inhibit sulfite accumulation and also overcomes the growth inhibition caused by selenate but not by chromate (not shown). When revertant 6-18 is grown in medium containing selenate (91% of the group VI anions), sulfate, and cysteine, sulfite accumulation is 12% of the amount produced in the presence of only sulfate and cysteine. Also when selenate constituted 36% of the group VI anions, sulfite only accumulated to 40% of the control level (data not shown). It was also found in other experiments that molybdate did not inhibit cell growth or sulfite accumulation whereas chromate totally inhibited cell growth and sulfite accumulation.

## Part II

### Biochemical Characterization of a Natural Cysteine Mutant, S. pullorum MS35

Nutritional data. The nutritional data presented in Table 2 shows three classes of S. pullorum exist at 37 C. The first class is represented by the parent MS35, which can grow only on sulfide or cysteine. The second class is

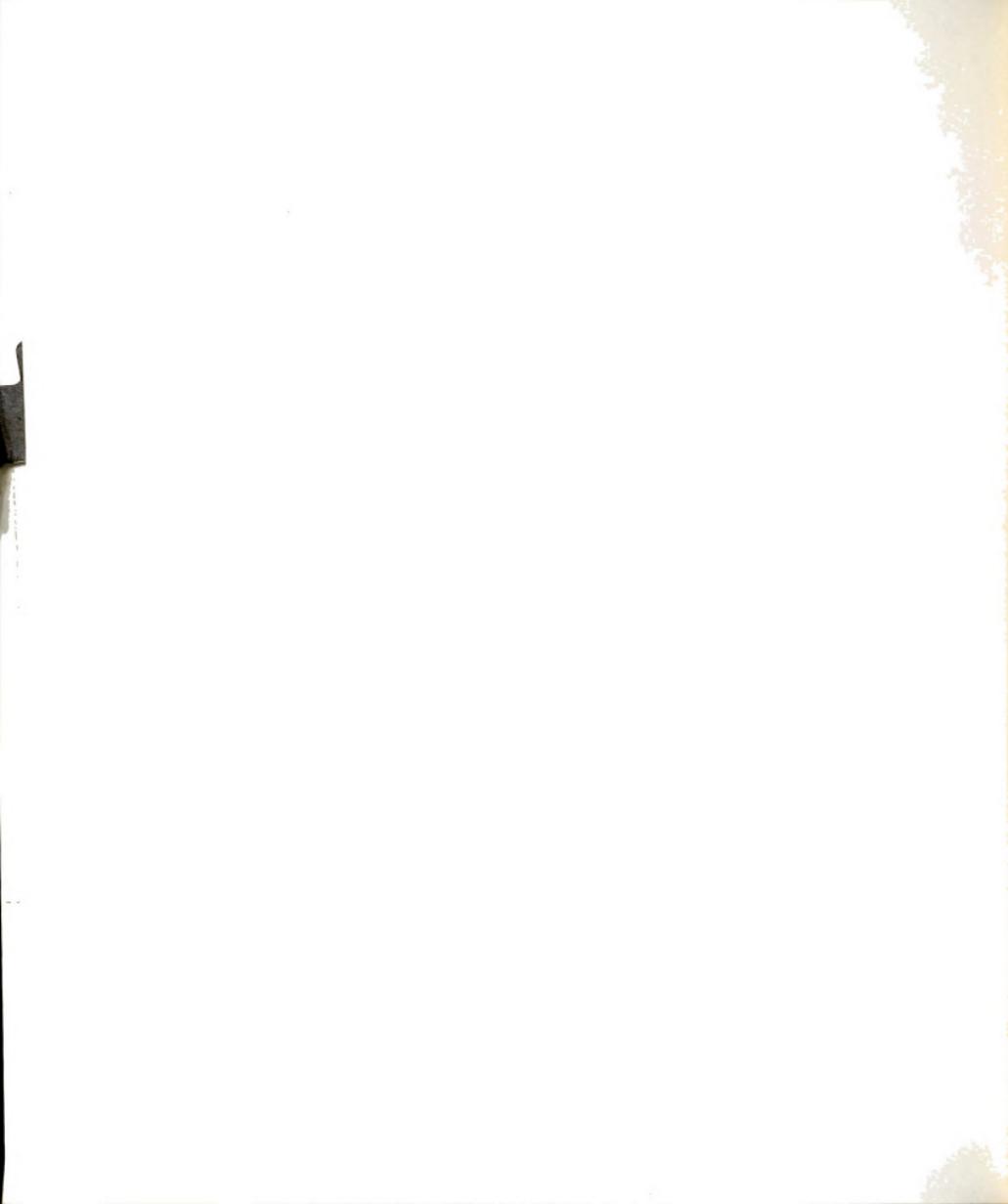


Fig. 4. Effect of selenate on the accumulation of sulfite by S. pullorum revertant 6-18. The organism was cultured aerobically in E medium supplemented with L-methionine ( $1.34 \times 10^{-4}M$ ) and: sulfate ( $4 \times 10^{-4}M$ ) and selenate ( $4 \times 10^{-3}M$ ) (O); or sulfate and L-cysteine ( $1.14 \times 10^{-4}M$ ) ( $\Delta$ ); or sulfate, cysteine, and selenate ( $\square$ ). The amount of growth was followed spectrophotometrically at a wavelength setting of  $420 m\mu$ . A value of  $1.0 OD_{420 m\mu}$  represents  $10^9$  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.

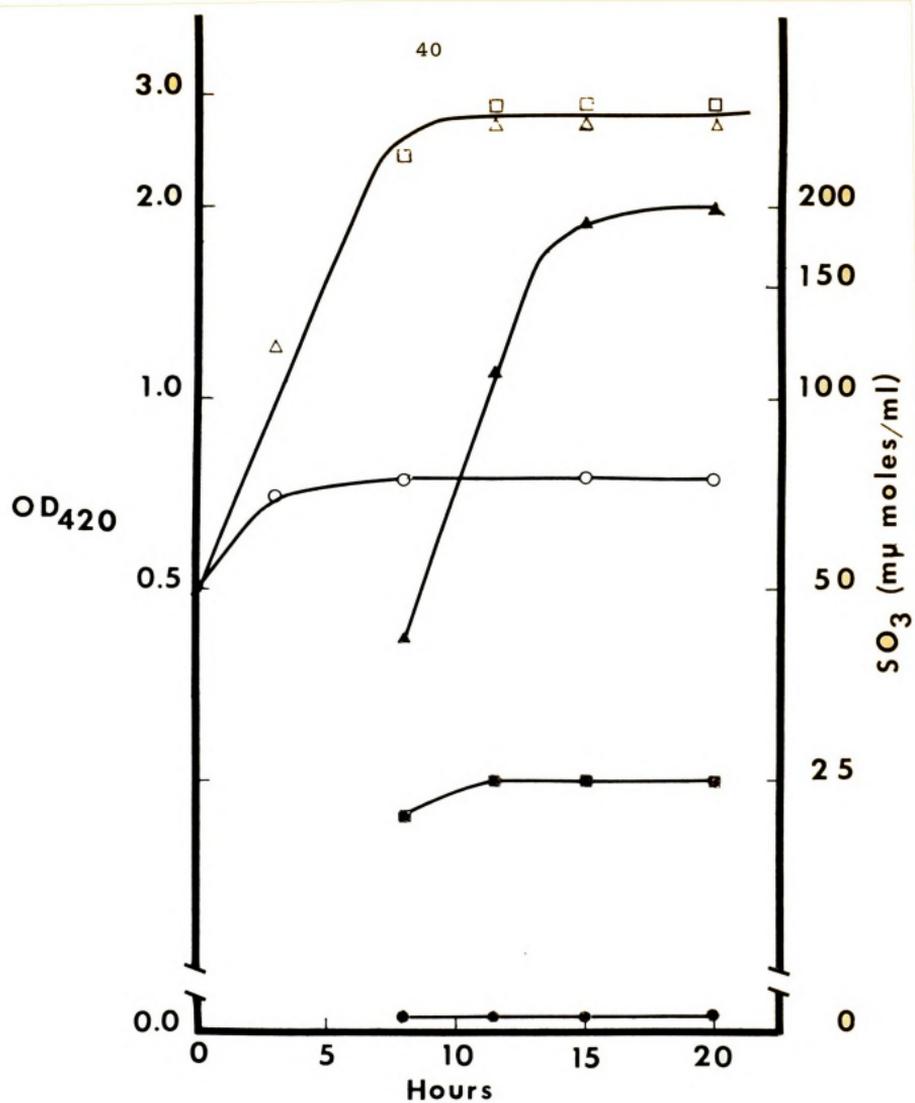


Fig. 4



TABLE 2. Growth responses of S. pullorum as function of sulfur source

Temperature (C)	$\text{SO}_4^{--}$		$\text{S}_2\text{O}_3^{--}$		$\text{SO}_3^{--}$ or CSA	
	25	37	25	37	25	37
<u>S. pullorum</u> <sup>a</sup>						
parent MS35	-	-	-	-	+	-
revertant 6	-	-	-	-	+	(+) <sup>b</sup>
revertant 6-18	+	(+)	+	-	+	+
revertant 20	(+)	-	+	-	+	-

<sup>a</sup>All bacteria grew on sulfide or L-cysteine at 25 and 37 C.

<sup>b</sup>Represents temperature and S-source on which revertant was selected.

represented by revertant 6, which gained the ability to grow on sulfite or CSA apparently as the result of a single mutation in the parent MS35. The third class is represented by revertant 6-18, which gained the ability to grow on sulfate as the result of a mutation in revertant 6; however, revertant 6-18 did not grow on thiosulfate. Table 2 shows that MS35 does grow on sulfite or CSA at 25 C. This finding suggests that sulfate-using revertants occur and grow at 25 C but cannot grow at 37 C, and, therefore, such organisms represent a fourth class of S. pullorum. In confirmation of this suggestion, 65 spontaneous, sulfate-using revertants were isolated directly from MS35 to 25 C (none have ever been isolated at 37 C). None of the revertants grew on sulfate after subculturing at 37 C. One of the revertants,



revertant 20, was selected for further study. The data of Table 2 show that the metabolism of sulfite and CSA by revertant 20, like the parent MS35, is temperature-sensitive at 37 C.

Table 2 shows that both revertants 6-18 and 20 have gained the unselected ability to grow on thiosulfate at 25 C but not at 37 C. It is not known if a third mutation exists which prevents the metabolism of thiosulfate at 37 C or if the temperature-sensitive response to thiosulfate reflects some peculiarity of the reverted cysteine genes. Based on nutritional patterns obtained with known cys mutants of S. typhimurium (15), it is inferred that MS35 is unable to transport sulfate into the cell or reduce sulfite to sulfide.

Metabolic failures: (a) sulfate permeation. The results given in Figure 5 show that dense suspensions of MS35 and revertant 6 exhibit the dilution property expected of impermeable cells whereas suspensions of revertant 20 exhibit a different dilution property. Revertant 20 is permeable to sulfate when it is cultured at 25 C since growth occurs (Table 2) on sulfate-containing medium. The data of Table 3 show that a culture of revertant 20 accumulates sulfite during growth at 37 C, and the data, therefore, indicate revertant 20 is permeable to sulfate at 37 C. The data of Table 4 show that all S. pullorum organisms used in this study metabolize sulfate to PAPS. Thus, exposure of whole cells to (S<sup>35</sup>)-sulfate should trap radioactivity if the cells



Fig. 5. Transport of sulfate in dense suspensions of S. pullorum. A standard solution of E medium containing radioactive sulfate is diluted with an equal volume of the same medium with and without bacteria (1 mg. bacterial protein equals  $8 \times 10^9$  bacterial). After incubation and centrifugation, the amount of radioactivity in the supernatant is measured. If transport has occurred, the percent dilution will be zero or less; if no transport has occurred, the percent deviation will be positive. Symbols: S. pullorum MS35 (○); revertant 6 (Δ); and revertant 20 (□).

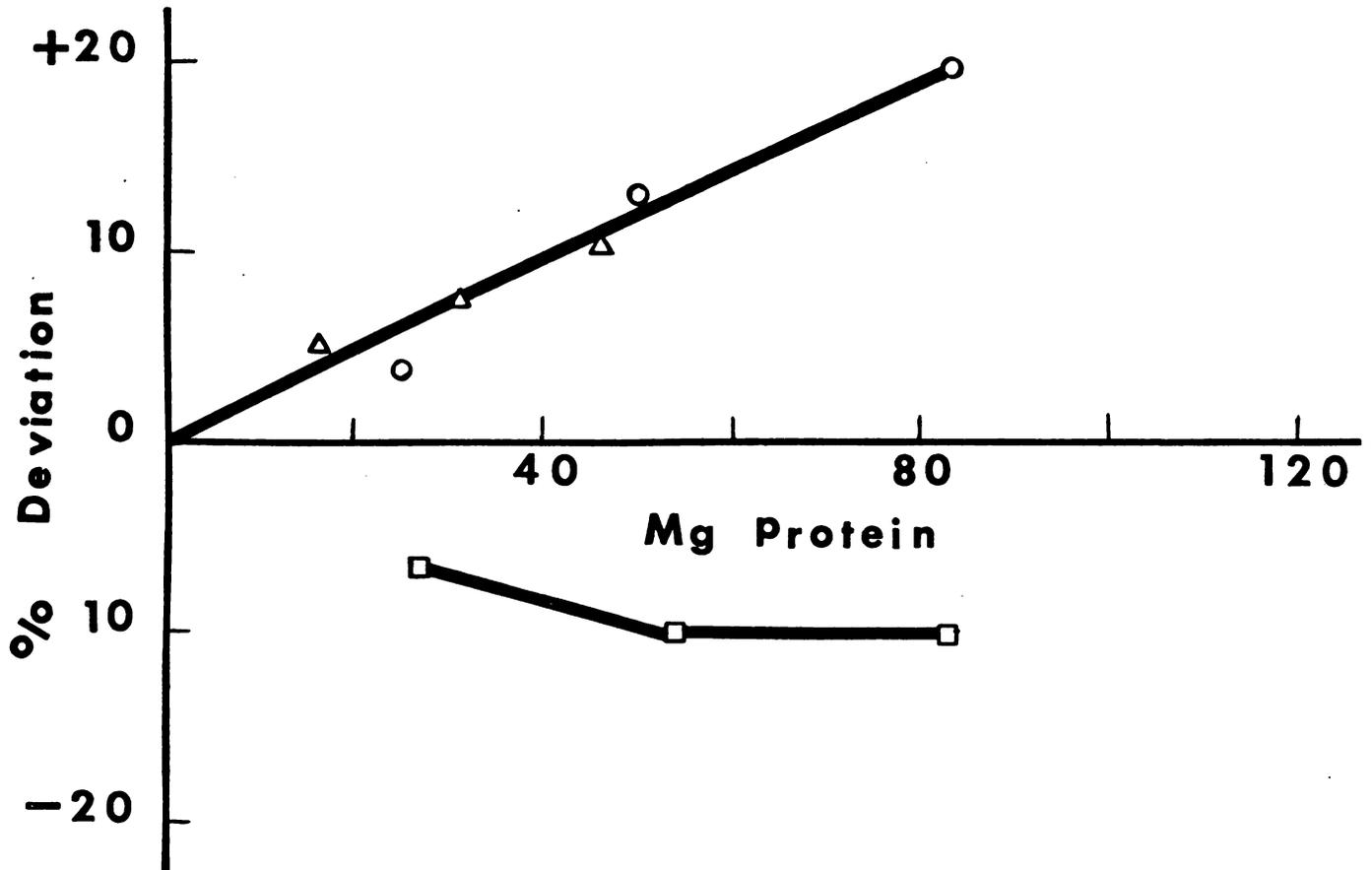


Fig. 5



TABLE 3. Accumulation of sulfite from sulfate by S. pullorum revertant 20 at 37 C

Sulfur source	Hrs. after inoculation	Total mg bacterial protein per ml <sup>a</sup>	μmoles sulfite per ml
L-djenkolic acid (0.2 mM) and methionine (0.134 mM)	24	0.32	0.0
L-djenkolic acid (0.2 mM), L-methionine (0.134 mM), and SO <sub>4</sub> <sup>2-</sup> (0.4 mM)	13 20 24	0.14 0.32 0.32	17.5 50.0 75.0

<sup>a</sup>Each culture was inoculated with an aliquot of a log phase culture of revertant 20 in E medium (supplemented with djenkolic acid) so that the initial concentration of bacteria was  $7 \times 10^7$  per ml (0.009 mg bacterial protein). The bacteria were grown aerobically.

are permeable. The data of Table 5 show that MS35 and revertant 6 do not trap radioactivity but that revertant 20 does trap significant activity. Since MS35 and revertant 6 behave like impermeable organisms in dense suspensions and cannot incorporate (S<sup>35</sup>)-sulfate but are cryptic for sulfate activating enzymes, the author concludes that the physiological defect in the metabolism of sulfate per se is in the transport of sulfate.

Metabolic failures: (b) sulfite reduction. The ability of MS35 and revertant 20 to grow at 37 C on sulfide but not on sulfite or CSA suggested that MS35 and revertant 20 were unable to reduce sulfite or CSA sulfur to sulfide.

TABLE 4. Production of APS and PAPS by extracts of S. pullorum and S. typhimurium

	Minutes incubation <sup>a</sup> in presence of:		Total $\mu$ moles produced	
	ATP	ATP + AMP	APS <sup>b</sup>	PAPS
<u>S. pullorum</u>				
MS35	45	0	(0.010) <sup>c</sup>	0
	0	120		225.0
-----				
revertant 6	45	0	(0.023)	
	0	120	1.0	31.0
-----				
revertant 6-18  (enzyme boiled 5 min)	45	0	(0.115)	0.0
	45	45		(25.0)
	0	120		85.0
	45	45		(0.1)
-----				
revertant 6-20	0	120		49.0
<u>S. typhimurium</u>				
LT-2	45	0	3.3	0.3
	120	0	2.90	2.8
	0	120	8.20	165.0

<sup>a</sup>Extracts of the derepressed bacteria (1.0-3.0 mg protein) were incubated with ATP (3  $\mu$ moles)  $K_2^{35}SO_4$  (0.3  $\mu$ moles; 5.0-100.0  $\mu$ c),  $MgCl_2$  (3.7  $\mu$ moles), tris-HCl<sup>4</sup> buffer (25  $\mu$ moles), pH 8.8, in 0.5 ml for the time indicated and the products analyzed by paper electrophoresis. Extracts of S. pullorum synthesized PAPS only when extra ATP (3.0  $\mu$ moles) and 2'- and 3'-AMP (0.8  $\mu$ moles) were added.

<sup>b</sup>The values for APS were obtained with specific activities in the range of 100-330  $\mu$ c/ $\mu$ mole of sulfate. The normal range of activities employed was 17-30  $\mu$ c/ $\mu$ mole of sulfate.

<sup>c</sup>Values in parentheses were obtained with enzyme dialyzed against 0.1 M tris buffer, pH 8.0, to remove phosphate.

TABLE 5. Retention of radioactivity after exposure<sup>a</sup> of S. pullorum strains to  $^{35}\text{SO}_4^{--}$

Min of exposure	c.p.m. retained by filter		
	Parental strain	Revertants	
	MS35	6	20
2.5	39	50	625
8.0	75	20	2600
25.0	350	60	4220

<sup>a</sup>S. pullorum cultures were grown at 37 C to log phase in sulfate-free E medium with added L-djenkolic acid ( $2 \times 10^{-4}\text{M}$ ), harvested by centrifugation, washed in sulfur-free E medium, and resuspended ( $5.7 \times 10^8$  bacteria/ml) in E medium containing glucose (0.2%) and CM (70  $\mu\text{g/ml}$ ). 1.4 ml of resuspended bacteria were mixed with 0.6 ml of E medium containing glucose (0.2%) and  $\text{K}_2^{35}\text{SO}_4$  (0.033  $\mu\text{moles}$ ;  $1.25 \times 10^5$  c.p.m.), and the mixture was incubated at 25 C. At the indicated times 0.5 ml were removed, filtered, washed on the filter, and the  $^{35}\text{S}$  activity retained by the dried filter was measured by use of a gas-flow counter.

The data of Table 2 show that MS35 and revertant 20 only grow on sulfite or CSA at 25 C; likewise, the data of Table 6 show that NADPH-sulfate reductase is only present in extracts made from these derepressed bacteria cultured at 25 C on djenkolic acid. Also, when MS35 is grown on CSA at 25 C, it contains NADPH-sulfite reductase. Revertant 6, which has gained the ability to grow on sulfite and CSA at 37 C, also has gained the ability to produce NADPH-sulfite reductase at this temperature. Thus, the ability to grow on sulfite or CSA after a shiftdown in temperature (MS35 or revertant 20) or the occurrence of a mutation (revertant 6) is correlated with the enzymatic ability to reduce sulfite to sulfide.

The data of Table 6 also show that all extracts lacking NADPH-sulfite reductase do contain MVH-sulfite reductase. The product of this latter reaction has been identified as sulfide by the methylene blue test (51). The MVH- and NADPH-linked activities appear co-repressible during growth of revertant 6-18 on L-cysteine; however, sufficient evidence has not yet been obtained to conclude both activities are functions of the same enzyme.

TABLE 6. Sulfite-dependent production of sulfide by extracts of *S. pullorum*

Bacteria	Growth temperature <sup>b</sup>	Specific activity <sup>a</sup>	
		Electron donor	
		MVH	NADPH
MS35	37	5.2	0.06
	25	...	1.85
	25 <sup>c</sup>	...	1.20
revertant:			
6	37	4.0	0.80
	25	...	2.00
6-18	37	3.9	1.00
	25	...	5.20
	37 <sup>d</sup>	0.01	0.01
	37 <sup>e</sup>	...	0.06
20	37	4.4	0.07
	25	...	5.50

<sup>a</sup>Reaction mixtures for determination of MVH-sulfite reductase contained in a volume of 1.5 ml:potassium phosphate buffer, pH 7.75 (150  $\mu$ moles),  $K_2SO_3$  (1  $\mu$ mole), MVH (0.27  $\mu$ moles) and crude extract (0.5-1.0 mg protein). Incubation was at 25 C. Specific activity is expressed as  $\mu$ m of  $H_2S$  produced/min/mg protein although the actual value determined was  $\mu$ m of MVH oxidized/min/mg protein. NADPH-sulfite reductase activity was determined after addition to extracts (1.0-3.0 mg of protein) of  $K_2SO_3$  (0.5  $\mu$ moles) glucose-6-phosphate (3.0  $\mu$ moles), NADP (0.06  $\mu$ moles),  $MgCl_2$  (4.0  $\mu$ moles), potassium phosphate (34  $\mu$ moles, pH 7.6) in a volume of 1 ml and incubation at 25 C for 30 min, by measurement of the sulfide produced. Each value obtained was corrected for the amount of sulfide produced by a reaction mixture lacking sulfite. Specific activity is expressed as  $\mu$ moles of  $H_2S$  produced/min/mg protein.

<sup>b</sup>All bacteria were grown on djenkolic acid unless noted otherwise.

<sup>c</sup>Bacteria grown on CSA (20  $\mu$ g per ml).

<sup>d</sup>Bacteria grown on L-cysteine (25  $\mu$ g per ml).

<sup>e</sup>Without complete NADPH generating system (minus glucose-6-phosphate).

## Part III

## Control of Sulfate Reduction

The data of Fig. 6 show that sulfite accumulation during growth is inversely proportional to the cysteine concentration. This data suggests cysteine causes repression of the enzymes that reduce sulfate to sulfite. The data of Table 7 show that under conditions in which protein synthesis is impaired cysteine may also cause an inhibition or a stimulation of sulfite accumulation. Thus, under certain conditions end product inhibition is apparently inoperative. The same results were obtained in several experiments. However, cysteine occasionally did not enhance or inhibit the respective cultures listed in Table 7.

Evidence is presented in Part I of this section that indicates sulfate is the precursor of accumulated sulfite. Normally in other organisms, the metabolic fate of sulfite is reduction to sulfide. The data of Table 6 show that sulfite reductase is fully repressed by a cysteine level ( $1.14 \times 10^{-4}M$ ) that does not fully repress sulfite accumulation. Therefore, the enzymes of sulfate activation and reduction are probably less sensitive to repression than is the enzyme of sulfite reduction. The data of Table 8 show that extracts made from a culture that is actively accumulating sulfite contain the enzymes of PAPS synthesis but not those of sulfite reduction.



Fig. 6. Accumulation of sulfite in cultures of S. pullorum revertant 6-18 as a function of L-cysteine concentration. Fresh, logarithmic cultures, grown in E minimal broth supplemented with L-methionine ( $1.34 \times 10^{-4}M$ ) and sulfate ( $4 \times 10^{-4}M$ ) or L-methionine, and sulfate ( $5 \times 10^{-4}M$ ), and nutritional solutions A and B (1 ml each solution per 100 ml of medium) were diluted into identical medium so that the final cell concentration was about  $10^9$  per ml. Then fresh L-cysteine was added to each culture to a final concentration specified on the abscissa. L-cysteine was omitted from one culture. The cultures were then incubated aerobically at 37 C. Accumulation of sulfite was measured periodically in each culture. The maximum value attained in each culture is plotted on the graph. Symbols: cultures without nutritional solutions A and B (O); cultures with nutritional solutions ( $\Delta$ ).

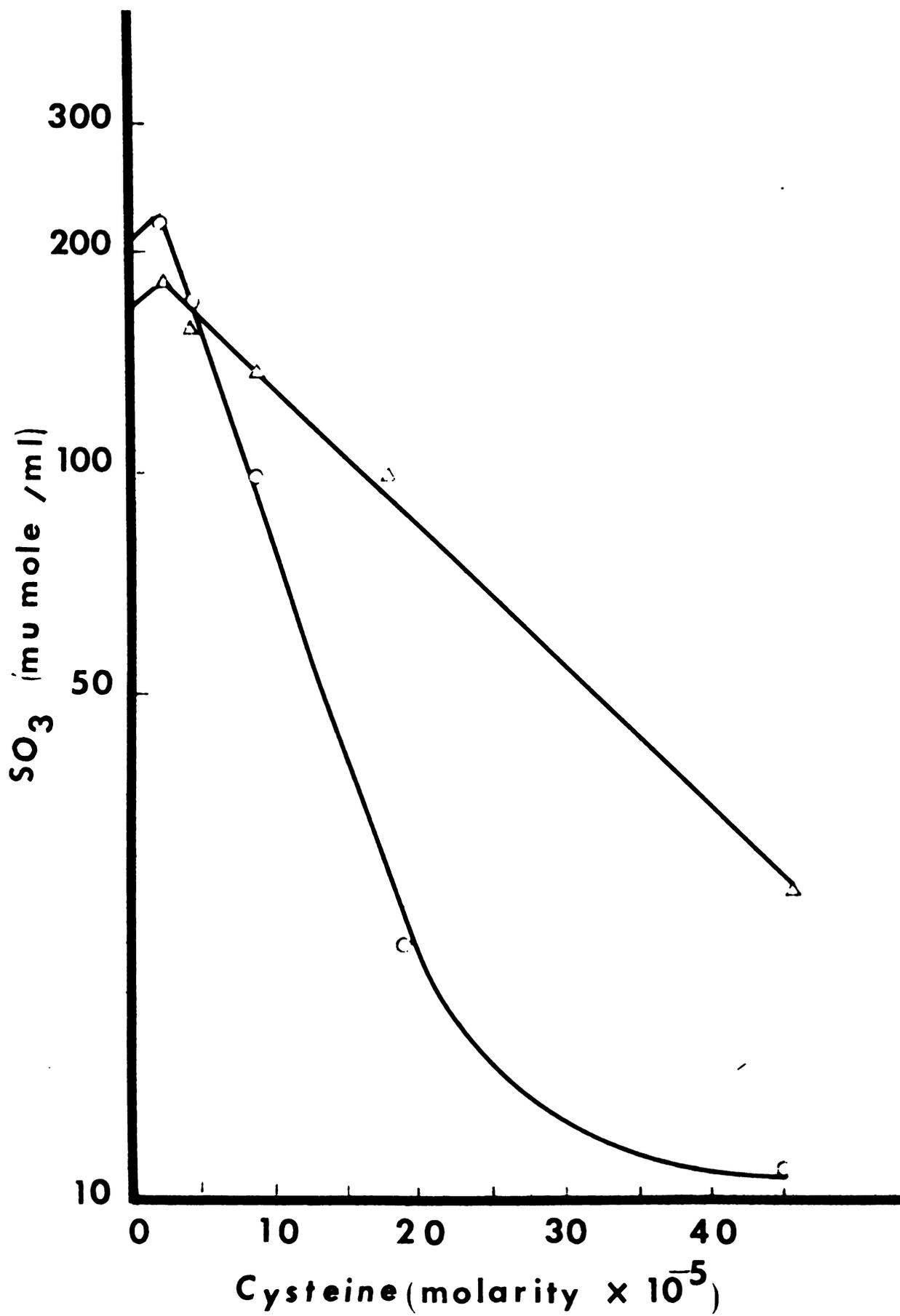


Fig. 6



TABLE 7. Accumulation of sulfite in dense suspensions of S. pullorum revertant 6-18<sup>a</sup>

Sulfur source	μmoles of SO <sub>3</sub> accumulated per ml
<u>Experiment I</u> <sup>b</sup>	
SO <sub>4</sub> <sup>--</sup>	50.0
SO <sub>4</sub> <sup>--</sup> + cysteine	125.0
cysteine	0.0
<u>Experiment II</u> <sup>c</sup>	
SO <sub>4</sub> <sup>--</sup>	35.0
SO <sub>4</sub> <sup>--</sup> + cysteine	5.0

<sup>a</sup>The reaction mixture contained K<sub>2</sub>SO<sub>4</sub> (5 μmoles), where indicated, L-cysteine (5.0 μm), glucose (1.6%), CM (250 μg), bacteria, washed once with sulfure-free E salts and concentrated (4 x 10<sup>11</sup>/ml) by centrifugation (0.25 ml), and sufficient E salts containing nutrient solutions A and B (1 ml each/0.1 liter of the salts) to bring the final volume to 5 ml.

<sup>b</sup>Cells used in Experiment I were grown at 37 C in supplemented medium described under Figure 6.

<sup>c</sup>Cells used in Experiment II were grown at 37 C in unsupplemented medium but tested in medium containing solutions A and B.



TABLE 8. Presence of sulfate pathway enzymes after growth of *S. pullorum* revertant 6-18 in a medium containing sulfate and cysteine

Hours after inoculation	$\mu\text{moles SO}_3^{--}$ per ml accumulated	Specific activity <sup>b</sup> of NADPH-sulfite reductase	Total $\mu\text{moles}$ of PAPS synthesized <sup>c</sup>
24	40	0.00	...
28	100	0.00	4.6

<sup>a</sup>Culture was grown at 37 C in unsupplemented E broth containing sulfate ( $2 \times 10^{-4}\text{M}$ ) and L-cysteine ( $1.14 \times 10^{-4}\text{M}$ ); L-methionine was omitted.

<sup>b</sup>Reaction mixture described and specific activity defined under Table 6.

<sup>c</sup>Reaction mixture described under Table 4. Value probably lower than real value since extract was not dialyzed to remove endogenous unlabeled sulfate. Therefore, the specific activity of PAPS<sup>35</sup> may be less than value of added (<sup>35</sup>S)-sulfate.



## DISCUSSION

### Part I

#### Accumulation of Sulfite

The accumulation compound has been identified as sulfite by reaction with fuchsin in the presence of acid and formaldehyde, by acid-volatility, by oxidation to sulfate under acidic conditions in the presence of  $H_2O_2$ , and by the formation of a S-sulfonate derivative of 5,5'-dithio-bis-(2-nitrobenzoic acid).

The identification of acid-volatile sulfur in the NaOH-EDTA trap solution does not necessarily imply sulfite was the primary source in the concentrate. Several compounds (cysteine-S-sulfonate, thiosulfate, and polythionates) give sulfite as an acid decomposition product, and the compound dithionous acid gives sulfite upon rapid auto-oxidation. Only 50% of the sulfur contained in thiosulfate or cysteine-S-sulfonate can possibly be converted to sulfite by treatment with acid. Since 93% of the excess sulfur that remained after growth was accounted for as fuchsin-reactive material, it follows that thiosulfate and cysteine-S-sulfonate were not the primary source of sulfite. Elimination of dithionous acid as the sulfite source is technically



difficult because of its rapid auto-oxidation to sulfite. However, it seems unlikely that dithionous acid accumulated since it is not a known intermediate of assimilatory sulfate reduction (15).

The data indicate that sulfate was the precursor of the accumulated sulfite. The strongest evidence is that selenate does inhibit the accumulation of sulfite from sulfate even in the presence of the reduced compounds, cysteine and methionine (Table 1). It is not known if selenate competes by inhibition of sulfate transport (41) or of sulfate activation (62).

It is interesting that S. pullorum forms sulfide in the presence of cysteine and methionine, but it still does not accumulate sulfite. This observation indicates that the ability to accumulate sulfite from reduced sulfur compounds is absent. Moreover, the data of Table 1 also show that the end product, cysteine, hinders the accumulation of sulfite rather than enhances it. Enhancement is expected if sulfite comes from a reduced sulfur source, and hindrance is expected by end product inhibition and/or repression when sulfite comes from sulfate (14). No statement can be made about the sensitivity of sulfite accumulation to the level of exogenous cysteine, since a significant amount of cysteine may be lost during growth as the production of  $H_2S$  indicates.

If sulfite accumulation is dependent upon a reductive rather than an oxidative process, a depletion of factors (e.g., ATP) affecting the reductive process should lessen the accumulation of sulfite. Growth of facultative anaerobes in an anaerobic environment normally results in a lower, total ATP yield (20). Therefore, since S. pullorum is a facultative anaerobe, sulfite accumulation is expected to be less when it is derived from sulfate. In accord with this prediction a 40% decrease in cell yield and a 90% decrease in sulfite accumulation were observed in anaerobic cultures of revertant 6-18 when these decreases were contrasted with the results of identical aerobic cultures.

The author is not aware of any reports that show sulfite accumulates when heterotrophic microbes are grown on sulfate. Assimilatory sulfate reduction has been studied intensively in other Salmonella only with S. typhimurium (15,35). The known intermediates of sulfate reduction do not accumulate during growth of the wild type strain (10), but sulfite does accumulate in derepressed, CM-arrested suspensions of mutants lacking sulfite reductase (15). Likewise, Roberts et al. (46) reported no accumulation of intermediates when wild type E. coli was grown in the presence of sulfate. Torii and Bandurski (57) have found that with the reduction of 3'-phosphoadenosine-5'-phosphosulfate (PAPS) catalyzed by yeast extracts the product, sulfite, was protein bound. Thus, the accumulation of sulfite by a

sulfate-using revertant of S. pullorum is an unexpected finding. In contrast, Nightingale et al. (39) have reported that sulfite accumulates in starved tomato plants soon after sulfate is introduced, but the peak of accumulation is about 24 hours. Larson and Salisbury (30) also have reported that sulfite accumulates in bull semen.

Microbes may form sulfite either by reduction of sulfate to sulfite or by oxidation of reduced sulfur compounds (44). The data presented in this section indicate that S. pullorum does not accumulate sulfite from cysteine or sulfide but that sulfate is the precursor of sulfite. The experiments of this section do not reveal to any great extent the cause or causes for sulfite accumulation. At the time these experiments were performed, the author had not yet determined the reactions of sulfate reduction to sulfide. The subsequent determination of these reactions has allowed experimental investigation of sulfite accumulation at the enzyme level. The results of such preliminary investigations are discussed in Part III of this section.

## Part II

### Biochemical Characterization of a Natural Cysteine Mutant, S. pullorum MS35

Salmonella pullorum MS35 is a double cysteine mutant as judged by nutritional responses of revertants. The data presented in this section show that one mutation causes a loss of sulfate transport, and the other mutation,



temperature-dependent, causes a loss of sulfite reductase at 37 C but not at 25 C.

The gene that controls sulfate permeation into S. pullorum also imparts nutritional properties that are similar to those of S. typhimurium (15). For example, transport-positive revertants of S. pullorum selected for growth on sulfate at 25 C or 37 C gain the unselected ability to grow on thiosulfate at 25 C. The change in permeability toward sulfate apparently enables the revertants to grow on thiosulfate at 25 C. This interpretation is also supported by evidence that S. typhimurium has a common transport system for sulfate and thiosulfate (12).

Unlike S. typhimurium prototrophs, S. pullorum cysteine "prototrophs" are temperature sensitive in their growth response on thiosulfate at 37 C. The ability of S. pullorum to cleave thiosulfate to sulfite and sulfide at 25 C but not at 37 C is one possible explanation of this temperature-sensitive response. However, this explanation cannot be substantiated since extracts produced from these prototrophs grown at 25 C do not catalyze (26) the production of sulfide from thiosulfate (unpublished data). The author does not know if this negative finding represents a technical error or the innate inability of the organism to catalyze this reaction. The ability of S. pullorum to transport sulfate but not thiosulfate at 37 C is another

possible explanation of the temperature-sensitive response. This possibility has not been investigated.

The second metabolic defect in S. pullorum MS35 prevents the reduction of sulfite to sulfide. The expression of the mutation is dependent on the temperature of incubation. At 37 C the properties of NADPH-sulfite reductaseless bacteria, MS35 and revertant 20, resemble known properties of S. typhimurium cys J mutants (L. M. Siegel and H. Kamin, in press) in two respects: (a) the S. pullorum mutants contain MVH-sulfite reductase activity and (b) these mutants are not pleiotropically negative for other enzymes of the sulfate pathway. The similarities may be superficial since the gene(s) which control sulfite reduction in S. pullorum are unknown.

The results of this paper are consistent with the hypothesis that S. pullorum uses the inorganic pathway of sulfate reduction. The synthesis of APS and PAPS by S. pullorum extracts is in accord with the conclusion of others (15,42) that APS and PAPS are obligate intermediates. The evidences that sulfite (not CSA) is an intermediate of sulfate reduction are as follows: (a) revertants that grow on sulfate can only be obtained from bacteria that metabolize sulfite or CSA; (b) sulfite is accumulated in the cultures of both a sulfate-using revertant and a mutant blocked in the reduction of sulfite to sulfide; (c) revertants that gain the ability to grow on sulfite or CSA simultaneously



gain the enzyme, sulfite reductase; thus, reversion to sulfite utilization is equivalent to CSA utilization and vice versa; and (d) five mutants of revertant 6-18 that cannot grow on sulfide also cannot grow on any of the inorganic or organic (CSA) sulfur sources used in this work (Miss B. J. Klooster, personal communication). This latter observation not only indicates that an organic pathway of CSA-dependent, cysteine synthesis (53) is absent, but also that sulfide is the final form which sulfate-sulfur assumes before incorporation. Thus, S. pullorum uses essentially the same pathway of sulfate reduction described for several other organisms (15,32,43).

Considerable difficulty was encountered initially with assays for sulfate activation and sulfite reduction. The successful synthesis of PAPS was only demonstrated with S. pullorum extracts after the addition of an isomeric mixture of 2'- and 3'-AMP. The efficacy of the individual isomers has not been tested. Likewise, the addition of 2'- and 3'-AMP to reaction mixtures made with S. typhimurium extract increased fifty-fold the amount of PAPS synthesized. It is not known why the isomeric mixture is required. A reasonable speculation is that it inhibits a 3'-nucleotidase. The presence of enzymes which degrade APS and PAPS in E. coli and S. typhimurium extracts has been reported (68). The S. pullorum and S. typhimurium extracts used in this work were



not tested for nucleotidase activity, but Neu (38) has found 3'-nucleotidase activity in the strain of S. typhimurium used in this study.

The NADPH-sulfite reductase of S. pullorum extracted from revertant 6 grown at 37 C, like the sulfite reductase of yeast (37), is unstable in low ionic-strength buffer (half life at -20 C, ca., 1-2 weeks). Also, the S. pullorum enzyme is inactive in the presence of oxygen but is active in anaerobic reaction mixtures. Like yeast sulfite reductase, S. pullorum extracts, in which the NADPH-sulfite reductase has decayed, still contain MVH-sulfite reductase. The NADPH- and MVH-dependent reductions of sulfite are catalyzed by the same yeast enzyme (69,70). This is also assumed to be true for S. pullorum since both NADPH and MVH-activities are repressed by cysteine.

The effects of high ionic strength buffer on NADPH-activity in extracts prepared from S. pullorum revertants, 6 and 6-18, are marked. The requirement for an anaerobic condition is lost, and the stability of the NADPH-dependent activity is increased at least twenty-fold. The S. pullorum NADPH-sulfite reductase extracted with high ionic-strength buffer has another peculiarity. Extended linear production of sulfide (at least 30 min) can only be achieved at the lower of the two temperatures tested, 25 and 37 C. At 37 C the reaction is complete in less than 5 min. The kinetics



of sulfide production catalyzed by the enzyme extracted in low ionic strength buffer are unknown.

Yashimoto and Sato have purified and characterized yeast NADPH-sulfite reductase (69). They find that the enzyme is composed of at least three components: a flavin adenine dinucleotide (FAD) component, a flavin mononucleotide (FMN) component, and a component containing a 587 m $\mu$  chromophore. Loss of either the FAD or FMN components results in a loss of NADPH- but not MVH-sulfite reductase (70). Nothing is known about the component nature of S. pullorum NADPH- sulfite reductase or about the interrelationship of temperature, ionicity, anaerobiasis, and electron source in the activity of this enzyme. Currently these subjects are under investigation.

### Part III

#### Control of Sulfate Reduction

The results given in this section suggest cysteine can affect control of sulfite accumulation. Cysteine apparently represses sulfite accumulation, but more evidence is needed before this conclusion can be accepted. For example, it must be demonstrated that the level of the enzymes of sulfate reduction vary in proportion to both the amount of cysteine present and of sulfite accumulated. Also, cysteine apparently feedback inhibits sulfite accumulation but not under all circumstances. The metabolic events in S. pullorum



which allow or prevent cysteine feedback inhibition are unknown. However, the observation that sulfite accumulated in a growing culture containing a high enough cysteine level to repress sulfite reductase suggests that feedback inhibition and repression are not significant control processes. The conclusion about feedback inhibition follows from the generalization that a higher end-product level is required to produce repression than to produce feedback inhibition (58). The conclusion about repression was supported when the enzymes of sulfate activation but not sulfite reduction were detected in extracts made from cultures which were actively accumulating sulfite. This repression situation in S. pullorum is completely opposite that found in E. coli since with E. coli the initial enzymes of the sulfate pathway are more sensitive to end-product repression than are the latter enzymes (43). It is not known if the quasi-control situation in S. pullorum is typical of all sulfate-using double revertants of MS35 or is only characteristic of the sulfate-using revertants derived from a particular single revertant, revertant 6.



## SUMMARY

The nutritional responses of spontaneous revertants of one strain of S. pullorum, strain MS35, indicate that this bacterium is a double cysteine mutant at 37 C. All sulfate-using revertants derived from a particular sulfite-using, single revertant cross-fed that revertant. Sulfite was detected in significant amounts in cultures of one sulfate-using revertant. The sulfite was identified on the basis of acid-volatility, oxidation to sulfate and precipitation with  $\text{BaCl}_2$ , and the formation of an authentic S-sulfonate derivative of 5,5'-dithiobis (2-nitrobenzoic acid). The accumulation of sulfite is dependent on the presence of sulfate and the accumulation is inhibited in proportion to either the amount of selenate or L-cysteine added to the culture.

It was subsequently shown that at 37 C the parent organism, MS35, is a double cysteine mutant because of an inability to transport sulfate and an inability to reduce sulfite to sulfide. The double revertant obtained at 37 C cannot use thiosulfate at 37 C but can use thiosulfate at 25 C. The biochemical basis for the temperature-sensitive response to thiosulfate is unknown. This nutritional



behavior is believed to be indicative of a new class of cysteine mutant in Salmonella. The mutation that causes the loss of reduced nicotinamide adenine dinucleotide phosphate (NADPH)-sulfite reductase at 37 C does not cause a loss of reduced methyl viologen (MVH)-sulfite reductase. the NADPH-sulfite reductase activity is regained after a shift-down to a growth temperature of 25 C or as the result of a reverse or gain mutation which is expressible at 37 C.

In establishing the biochemical nature of the defects, the author encountered considerable difficulty with assays for sulfate activation and sulfite reduction. The presence of a mixture of the 2' and 3' isomers of adenosine monophosphate (2'- and 3'-AMP) was required to synthesize 3'-phosphoadenosine-5'-phosphosulfate (PAPS). The efficacy of the individual mononucleotides was not tested. Without 2'- and 3'-AMP about only 0.05  $\mu$ moles of APS were synthesized, and PAPS was not made. It was observed that NADPH-sulfite reductase was unstable when extracted with low ionic-strength buffer; however, reduced methyl viologen (MVH)-sulfite reductase was stable. Extraction in high ionic-strength buffer stabilized NADPH sulfite reductase. Using high ionic-strength extracts, the author observed at 37 C abbreviated periods (less than 5 min) of NADPH-dependent sulfite reduction to sulfide; however, at 25 C reduction to sulfide was linear for at least 30 min.

After definition of the sulfate-reducing pathway used by S. pullorum, preliminary evidence was obtained suggesting that the accumulation of sulfite from sulfate occurs because the reduction of sulfate to sulfite is less sensitive to end product control than is the reduction of sulfite to sulfide.

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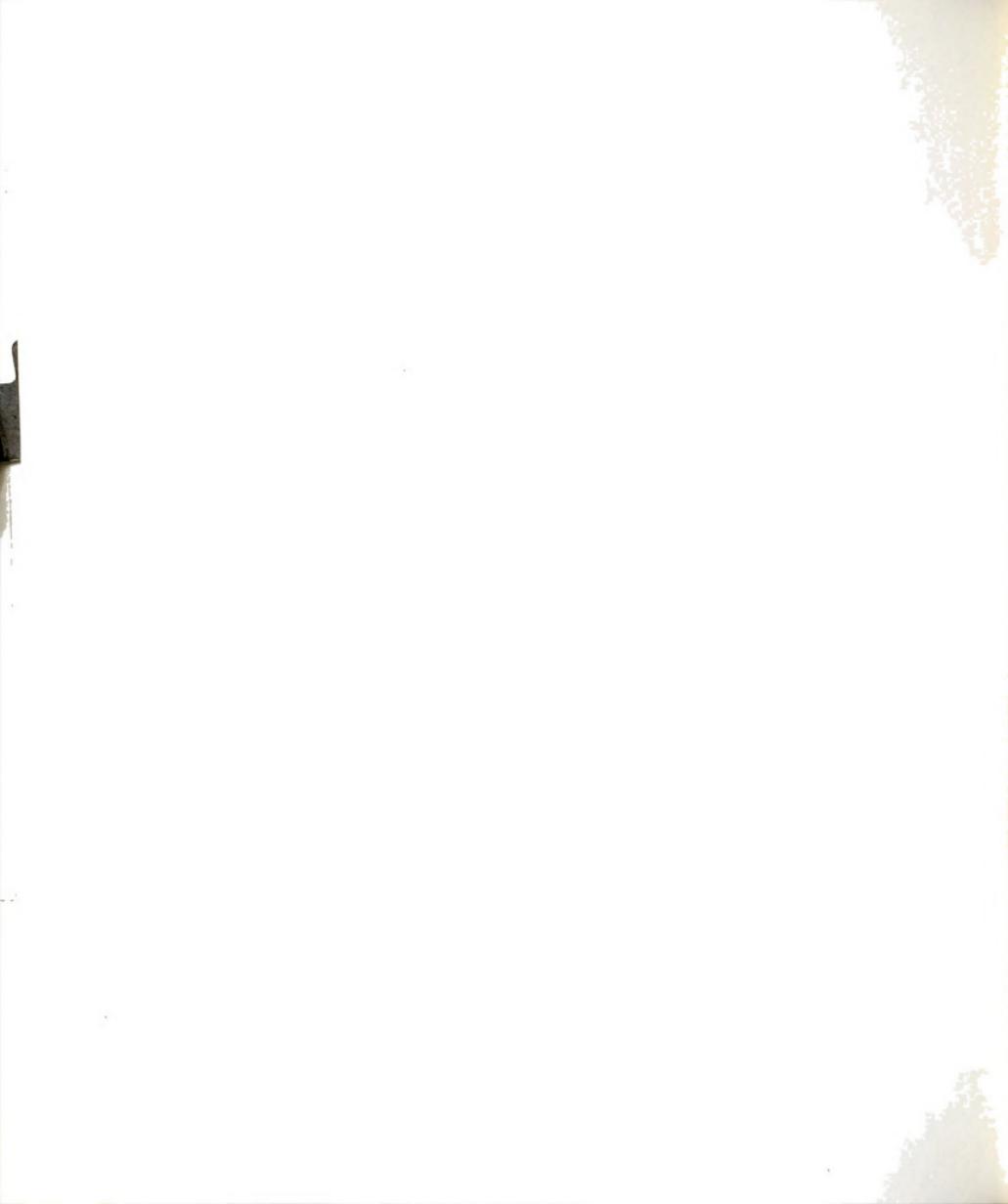
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