THE BIOCHEMICAL BASIS FOR SULFITE AND RIBOFLAVIN ACCUMULATION BY SALMONELLA PULLORUM

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ABSTRACT

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The accumulation of sulfite by prototrophic assimilatory sulfate reducing bacteria has only been reported in Salmonella pullorum. The purpose of this study was to define the genetic and biochemical basis for sulfite accumulation by sulfate-using revertants of S. pullorum.

Several approaches were used to define the reasons for sulfite accumulation. Numerous sulfate-using revertants of S. pullorum strains MS35, MS38, and MS53 were isolated to determine whether all sulfate-using revertants accumulated sulfite. The enzymes serine transacetylase (STA) and O-acetyl-L-serine sulfhydrylase (OASS) were assayed for in extracts of S. pullorum, and the control of these enzymes by end product was determined. Since reduced nicotinamide adenine dinucleotide phosphate (NADPH) dependent sulfite reductase (H_2S-NADP oxidoreductase, EC 1.8.1.2) specific activities were higher in extracts from cells grown at 25 C than from cells grown at 37 C, sulfite accumulation

as a function of temperature was determined. The *in vivo* stability of NADPH and reduced methyl viologen (MVH)-dependent sulfite reductase (H_2S-MV oxidoreductase) was determined by assaying for these enzymes at various stages of growth at both 25 C and 37 C. The *in vitro* stability of NADPH sulfite reductase from 25 C and 37 C grown cells was also determined to see if the *in vivo* responses could be confirmed by *in vitro* observations. The ability of exogenous cysteine to inhibit sulfite accumulation under conditions in which protein synthesis was arrested by chloramphenicol (CAP) was done to determine whether cysteine *per se.* exerted a feedback inhibition on the uptake or activation of sulfate. Finally, the identification of a yellow substance appearing in the growth medium concurrently with sulfite was undertaken.

It was observed that all sulfate-using revertants of *S. pullorum* accumulated sulfite, and this accumulation occurred only when the cells were grown at 37 C. Significant levels of sulfite did not accumulate when the cells were grown at 25 C. This finding suggested that the accumulation of sulfite was due to a thermolabile sulfite reductase. This hypothesis was confirmed by observing that both NADPH and MVH sulfite reductase specific activities were lost during the growth of *S. pullorum* at 37 C, but not at 25 C. NADPH sulfite reductase from cells

of S. pullorum grown at 37 C was more labile in vitro than NADPH sulfite reductase from cells of S. pullorum grown at 25 C or cells of S. typhimurium grown at 37 C. The yellow compound accumulating concurrently with sulfite was riboflavin.

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This thesis is dedicated to

my wife, Janice,

and to my family.

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INTRODUCTION

Salmonella pullorum is an unusual enteric bacterium that cannot synthesize cysteine from sulfate. Furthermore, this requirement is extremely stable. Since wild type Escherichia coli and Salmonella typhimurium do not normally require cysteine to grow in a synthetic medium, Kline and Schoenhard (34) investigated the nature of the biochemical deficiency in the sulfate assimilatory reductive pathway of S. pullorum. Their investigation established that wild type S. pullorum has two genetic blocks preventing the utilization of sulfate as a sole sulfur source. The blocks were the inability to reduce sulfite to sulfide at 37 C, and failure to transport sulphate into the cell. This double biochemical block explained the genetic stability of the cysteine requirement.

An unusual finding reported by Kline and Schoenhard (33) was the accumulation of sulfite by cysteine prototrophs of S. pullorum isolated from the parental auxotroph. The accumulation occurred only if prototrophs of S. pullorum were grown on sulfate, and it was proportional to the amount of sulfate provided in the medium, suggesting that sulfate was the precursor of the accumulation compound. The release of sulfate into the medium was not observed when E. coli or

S. typhimurium cells were grown in media containing sulfate as the sole sulfur source. These data suggested further genetic blocks and/or regulatory anomalies in prototrophs of S. pullorum, which allowed sulfite accumulation. The purpose of this study was to define the genetic and biochemical basis for the accumulation of sulfite.

The study consisted of several approaches. The first was the demonstration of serine transacetylase (STA) and O-acetyl-L-serine sulfhydrylase (OASS) in extracts of S. pullorum. STA is involved in the regulation of cysteine biosynthetic pathway enzymes (29), and its presence and regulation by cysteine provided an insight into control of the cysteine biosynthetic pathway in S. pullorum. The second approach was the development of a non-sulfite accumulating system. A non-sulfite accumulator allowed a comparative approach to the mechanisms responsible for sulfite accumulation. And finally, the identification was accomplished of a yellow compound that accumulated concurrently with sulfite. This compound was shown to be unrelated, directly, to sulfite accumulation.

LITERATURE REVIEW

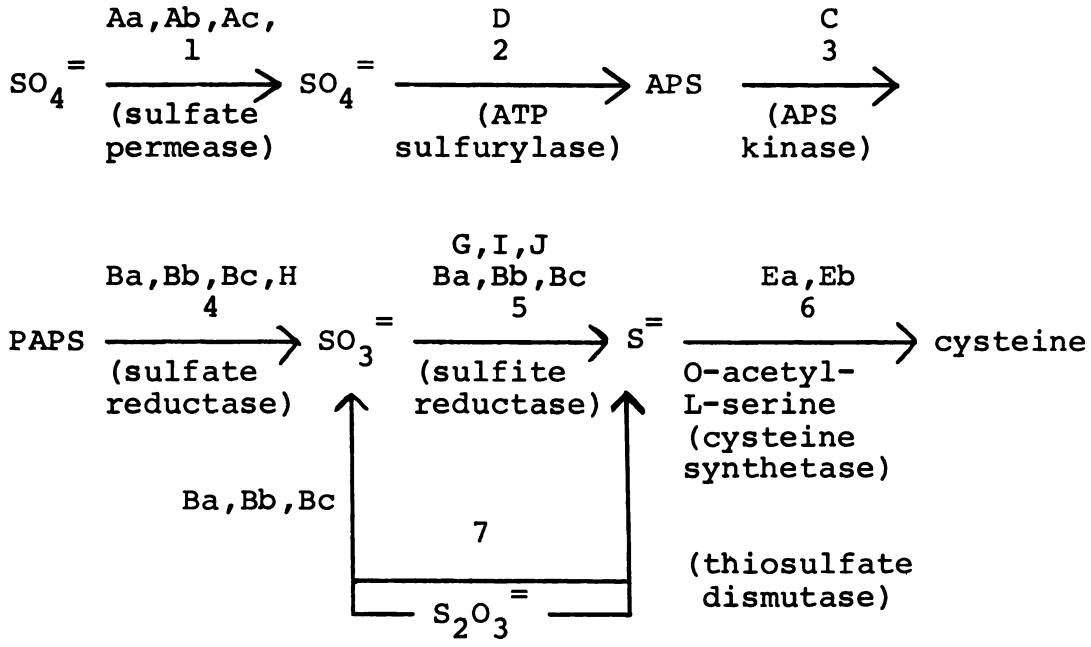
Part I

The Biosynthesis of Cysteine

Assimilatory sulfate reduction and dissimilatory sulfate reduction.--The distinction given here is that of Postgate (54). Assimilatory sulfate reduction is small scale reduction of sulfate to the sulfur containing amino acids cysteine, cystine, and methionine; while dissimilatory sulfate reduction is large scale reduction of sulfate to sulfide, which is involved with energy generation. Thus, the distinction is primarily on the basis of whether the reduction of sulfate is for the synthesis of cell constituents or for energy generation. Dissimilatory sulfate reduction appears to be found in only two genera of strictly anerobic bacteria, Desulphovibrio and Desulphotomaculum (76), while an assimilatory sulfate reduction pathway is widespread in bacteria and the plant kingdom. Further discussion of cysteine biosynthesis will thus refer specifically to the assimilatory sulfate reductive pathway seen on the next page.

Uptake of sulfate into the cell.--The transport of sulfate in S. typhimurium is an active process and is

dependent upon energy, temperature, and the functional integrity of several cistrons (18). Active transport of



sulfate appears to be common to numerous microorganisms (76). The uptake of sulfate into S. typhimurium also involves a sulfate-binding substance located at the cell surface (48). This was demonstrated by the fact that some cells which were unable to transport sulfate were, nevertheless, found to possess sulfate-binding capacity. This sulfate-binding ability was lost upon osmotic shock or conversion to spheroplasts. Transduction analysis (48) of mutants which had lost both binding and transport ability suggested that a single lesion may have been responsible for loss of both activities. Pardee (49, 50) has purified, crystallized, and studied the sulfate-binding protein from

S. typhimurium. The protein has a molecular weight of about 32,000, and is a typical protein in amino acid composition, but is devoid of sulfur containing amino acids. The binding protein may be a chelator for the sulfate anion with calcium at the active site providing the necessary positive charge.

Activation and reduction of sulfate.--Once sulfate has been taken into the cell, it reacts with two molecules of ATP in successive steps before reduction to sulfite. The intermediates formed are adenosine-5'-phosphosulfate (APS) and 3'-phosphoadenosine-5'-phosphosulfate (PAPS), respectively. These intermediates and the enzymes catalyzing their formation have been demonstrated in studies of mammalian tissues (57, 59) and of yeast (58, 5, 60, 61). Subsequently, APS, PAPS, ATP-sulfurylase, and APS-kinase have been demonstrated in bacteria (16, 17). The activation process overcomes the positive free energy change in the direct reduction of sulfate to sulfite. The formation of APS from $SO_4^=$ and ATP (reaction 1, p. 6) is also thermodynamically unfavorable (Keq = $1 \times 10^{-8} - 4 \times 10^{-8}$). The overall reaction occurs because the phosphorylation of APS is favorable, and because of the hydrolysis of pyrophosphate (reaction 3, p. 6), has a large negative free energy (6).

$$ATP + SO_4^{=} \rightleftharpoons APS + PPi \quad \text{Positive } \Delta F \quad (1)$$

$$ATP + APS \rightleftharpoons PAPS + ADP \quad \text{Negative } \Delta F \quad (2)$$

$$PPi \rightleftharpoons 2\ Pi \quad \text{Negative } \Delta F \quad (3)$$

$$2\ ATP + SO_4^{=} \rightleftharpoons PAPS + ADP + 2\ Pi \quad \text{Negative } \Delta F \quad (4)$$

The reduction of active sulfate (PAPS) to sulfite has been shown to involve three proteins (83): enzymes A and B, which are heat labile, and a heat stable, nondialyzable, low molecular weight protein designated fraction C. The entire sulfate reducing system from yeast is soluble as evidenced by the observation that supernatant solutions from a two hour centrifugation at 110,000 x g, retained the total activity observed in crude extracts. Subsequently these authors (4) purified fraction A and presented evidence indicating that fraction A in the presence of NADPH reduced the disulfide group of fraction C to a sulfhydryl group. The reduction was specific for the disulfide of fraction C. Reduced fraction C could then serve to reduce active sulfate to sulfite, but all components involved in this reduction were not identified. Hilz and Kitller (25) reported that reduced lipoic acid could bring about sulfite formation from PAPS while most other sulfhydryl containing compounds were ineffective.

Torii and Bandurski (74, 75) determined that the product of PAPS reduction was not free sulfite. Indications

were that the bound sulfite was attached to a protein sulfhydryl group (SH) because a sulfhydryl appeared when enzymes A and B were incubated with dithionitrobenzoate and NADPH, but the appearance of SH was diminished if PAPS were present in the incubation mixture. The sulfite-binding substance is at least partially protein based on its susceptibility to protease. Because its molecular weight was in the range of 4000-8000, these authors suggested that it may be fraction C.

The reduction of sulfite to sulfide.--The reduction of sulfite to sulfide is a six electron reduction and has been studied in a wide variety of microorganisms and plants. In all cases studied, sulfide was the end product of the reaction, and no free intermediates between sulfite and sulfide have been detected. Siegel et al. (64), studying the sulfite reductase of S. typhimurium, indicated that an intermediate electron carrier functioned in the reduction of sulfite by NADPH sulfite reductase. The intermediate carrier received electrons from NADPH and could pass them directly to flavine adenine dinucleotide (FAD) or cytochrome C, and indirectly to sulfite. In a later communication, Siegel and Monty (65) presented kinetic evidence in support of this hypothesis. They observed that sulfite did not significantly inhibit cytochrome C reduction, while sulfite did competitively inhibit the reduction of

hydroxylamine. They suggested that sulfite and hydroxylamine were reduced at a common enzyme binding site, while cytochrome C reduction occurred at a separate site. Although all activities appear to be co-purifiable, it was suggested that S. typhimurium sulfite reductase is a multi-site, multi-component system.

Wainwright (79), studying the sulfite reductase of yeast, reported the enzyme was composed of six protein fractions, two of which were identical to fractions A and C of the PAPS reducing system. Naiki (45) purified yeast sulfite reductase nearly 200 fold. The purified enzyme could reduce nitrite and hydroxylamine as well as sulfite, and it was shown to have a diaphorase activity. The enzyme could utilize either NADPH or reduced methyl viologen (MVH) as an electron donor. NADPH sulfite reducing activity was sensitive to low ionic strength, heat, and p-chloromercuribenzoate, while MVH sulfite reducing activity was relatively stable to these treatments. Both activities were inhibited by cyanide. These observations, along with the fact that some NADPH sulfite reductase mutants still maintained a MVH sulfite reducing activity (the reciprocal has never been observed), led Naiki to present a model in which MVH linked sulfite reduction is catalyzed by a part of the NADPH sulfite reductase molecule.

The yeast system was further studied in detail by Yoshimoto and Sato (87, 88). They determined a molecular

weight of 350,000 for the purified enzyme. Per mole of enzyme there was one mole of flavin mononucleotide (FMN) and FAD, about five atoms of non-heme iron, and two to three moles of acid labile sulfide per mole of FMN or FAD. In addition, non-flavin chromophores with absorption peaks at 386, 587, and 710 nm were shown. In a study with the enzyme of mutants which lacked the NADPH linked activity, but maintained the MVH linked activity, these authors found that the sedimentation coefficient (S) decreased in the mutant enzymes. Values of 14.8 S were obtained for the wild type enzyme compared to 6.6 S and 5.1 S for two of the mutant enzymes. Mutant enzymes were shown to have lost the FAD or FAD and FMN components. Both wild type and mutant enzymes maintained the 587 nm chromophore. This led Yoshimoto and Sato to conclude that the yeast enzyme is a multi-component flavoprotein with the flavin required for transfer of electrons from NADPH to sulfite.

Prabhakararao and Nicholas (56), also studying the sulfite reductase from bakers' yeast determined that the purified enzyme was stimulated by riboflavin, FMN, and FAD. No gaseous sulfur intermediates (SO_2, SO, or S_2O) were detected in the mass spectrophotometer during the enzymatic reduction of sulfite. A stoichiometry of 3:1 and 1:1 was observed for NADPH : $S^=$ and $SO_3^=$: $S^=$ respectively.

Siegel and Kamin (L. M. Siegel and H. Kamin: Conference on Flavins and Flavin Enzymes. Nagoya, Japan,

August 1967) and Siegel *et al.* (68) purified and studied the NADPH sulfite reductases of *S. typhimurium* and *E. coli*. The molecular weight of the enzymes was about 700,000 and component analysis indicated that, like the yeast enzyme, the *E. coli* sulfite reductase is a hemoflavoprotein. They suggested that the flavin components served to receive and transfer electrons from NADPH. The activity of the enzyme was controlled by at least four genes; the cys G and I genes were required for MVH sulfite reductase activity, the cys J gene was necessary for the NADPH diaphorase (and cytochrome C reductase) activity, and the cys B region was required for both activities.

While the yeast, bacterial, and *Neurospora crassa* (67) sulfite reductase can utilize NADPH as an electron donor, the purified spinach enzyme is unable to do so, and requires a non-physiological electron donor such as MVH (2). Furthermore, the spinach enzyme could be cleaved into two non-dialyzable fractions (1), one of which could replaced with low efficiency by bovine serum albumin, thiols, or disulfides (3). The spinach enzyme contains no flavins and appears to be an unusual hemoprotein with a molecular weight of about 85,000, a value considerably less than that of yeast and bacterial sulfite reductases (3). Asada *et al.*(3) suggested that the active site of the spinach sulfite reductase may be the same as that of the *E. coli* enzyme. They also indicated that the spinach

enzyme had been cleaved from the flavin and NADPH coupling moieties. In support of this hypothesis, Tamura et al. (73) reported that spinach sulfite reductase could reduce $SO_3^=$ to $S^=$. They used an NADPH generating system and a reconstitution system containing ferredoxin NADP reductase, ferredoxin, an unidentified heat labile and non-dialyzable substance (Cf), and the sulfite reductase.

Aspergillus nidulans sulfite reductase has been purified and studied by Yoshimoto et al. (86). The enzyme requires MVH as an electron donor and cannot utilize NADPH or NADH for this function. A mutant unable to assimilate sulfate or sulfite into cysteine was isolated in which the MVH linked sulfite reductase was absent, indicating that this activity is involved in sulfur assimilation. Since crude extracts of A. nidulans could catalyze a slow reduction of sulfite using NADPH as an electron donor, it is likely that this is the physiological electron donor, and the factor(s) responsible for the utilization of NADPH as an electron donor are lost upon purification. The sedimentation coefficient of the purified enzyme is 4.2 S, which is considerably smaller than that reported for yeast sulfite reductase (14.8 S).

The sulfite reductase of the plant Allium odorum has been purified and studied by Tamura (71, 72). Like the spinach enzyme, the A. odorum purified enzyme appears not to have an NADPH linked activity. However, some

activity in crude preparations was observed. The enzyme of A. odorum does not reduce nitrite as the enzyme of E. coli does, and is more heat stable than the yeast enzyme. Like all sulfite reductases examined, the spectrum was shown to have a peak near 590 nm which may indicate a functional group(s) common to all sulfite reductases.

A mutation in any of six cistrons (Ba,Bb,Bc,G,I,J) of S. typhimurium causes the loss of NADPH sulfite reductase activity. This raised the question as to whether one complex or several enzymes were specified by the six cistrons. Henderson and Loughlin (24) mixed extracts of mutants in each of the six cistrons and found very little in vitro complementation, which led them to conclude that two or more enzymes were not involved. These data were consistent with the view that sulfite reductase is a single complex with a multi-site, multi-component nature.

The assimilation of sulfide into cysteine.--It seems quite certain that sulfide is the form of sulfur which is assimilated into cysteine. This is based on the observations that the product of sulfite reduction is sulfide, and mutants lacking the ability to grow on sulfide as sole sulfur source lack the ability to grow on more oxidized forms of sulfur (16).

Essentially two mechanisms for the assimilation of sulfide into cysteine have been postulated. Schlossmann and Lynen (62) purified a pyridoxal phosphate dependent

enzyme from Saccharomyces cerevisiae, serine sulfhydrase, which catalyzed the following reaction. A pyridoxal phosphate requiring enzyme catalyzing the same reaction has been purified from Pasteurella multocida (13) and given the trivial name cysteine synthase (L-serine hydro-lyase EC 4.2.1.22).

$$\text{Serine} + H_2S \longrightarrow \text{Cysteine} + H_2O$$

Kredich and Tomkins (35) demonstrated a two step mechanism for the conversion of serine and sulfide into cysteine using extracts from E. coli and S. typhimurium. These reactions are shown below.

$$\text{L serine} + \text{acetyl CoA} \xrightarrow[\text{transacetylase}]{\text{serine}} \text{O-acetyl-L-serine} + \text{CoA}$$

$$\text{O-acetyl-L-serine} + H_2S \xrightarrow[\text{sulfhydrylase}]{\text{O-acetylserine}} \text{L-cysteine} + H_2O + \text{acetate}$$

Kredich et al. (36) subsequently showed that STA and OASS formed a complex in S. typhimurium to which they gave the trivial name cysteine synthetase. O-acetyl-L-serine at concentrations of 10^{-4} to 10^{-3}M caused the reversible dissociation of the complex into one molecule of STA and two molecules of OASS, while sulfide inhibited this dissociation. These authors proposed that the ability of OAS to induce the synthesis of cysteine pathway enzymes was related to its effect of causing dissociation of cysteine synthetase.

In E. coli there are several arguments which favor the scheme of Kredich and Tompkins over that of Schlossman and Lynen. Pasternak et al. (52) reported a specific activity for serine sulfhydrase of 0.81 nmoles/mg protein/min, and showed that this enzyme was not repressed by cysteine, while Jones-Mortimer et al. (29) reported an activity of 1.8×10^4 nmoles/mg protein/min for OASS in the same organism, and OASS was repressed by cysteine. This latter group also demonstrated the induction of the sulfate to sulfide enzymes by OAS, which strongly supports the significance of the STA catalyzed reaction. STA is not repressed by cysteine, but is feedback inhibited by cysteine, which is clearly advantageous for a regulatory mechanism.

The genetics of cysteine biosynthesis.--Mitzobuchi et al. (43) established by transduction that the genes controlling cysteine biosynthesis in S. typhimurium were located in five separate linkage groups. Conjugation experiments supported the transduction data, and further mutant analysis indicated that the five regions consisted of fourteen cistrons. Dreyfuss and Monty (16) arrived at the same conclusion in finding that their cysteine requiring mutants of S. typhimurium represented damage to fourteen separate cistrons mapping in five distinct regions of the chromosome. In a study (14) of one of the five independently transducing clusters designated region cys C, it was found that this region contained five cistrons in

the order cys C-D-H-I-J. Between D and H there was a long silent region. The origin of genetic silent regions is unknown, but it was suggested that the material is non-genic. The silent region may contain a partial duplication of genetic material to the right of the cys J cistron (26). This would cause a partial duplication or redundancy in the DNA, and may explain the high occurrence of ditto deletions in the cys C region. The occurrence of the cysteine genes in five separate regions (linkage groups) on the chromosome, rather than one operon, allows for and is in support of differential control. Although each of the five units may have just one operator and are expressed coordinately, the five or more operators may show different sensitivities to repressor, and thus the pathway as a whole would be under differential control.

Part II

Control

Repression and feedback inhibition.--Control mechanisms enable living organisms to make the most efficient use of their enzymes and the raw materials available in the medium. Control mechanisms prevent over-synthesis of compounds such as amino acids, and prevent excess breakdown of catabolites such as glucose, and conserve important intermediates. The net result of control mechanisms is,

therefore, keeping the metabolic machinery of the cell functioning as efficiently as possible. There are primarily two major control mechanisms: feedback inhibition and repression. Feedback inhibition was first demonstrated in 1956 (77, 85) and usually refers to the inhibition of catalytic action of the target enzyme by an end product of a biosynthetic pathway. This inhibition usually occurs on the first enzyme of the biosynthetic pathway or a branch-point, with the result that the pathway as a whole is shut off. Feedback inhibition is operative in biosynthetic pathways, but has not been demonstrated in catabolic pathways (42). Repression was first demonstrated in 1953 (82, 44), and refers to the shutting off of the synthesis of the target enzymes. Repression does not affect preformed enzyme molecules in the cell, and is thus referred to as a coarse control mechanism, with feedback inhibition being the fine control mechanism. Repression has been demonstrated in both biosynthetic and catabolic pathways. Induction is the opposite side of repression, and is thus the same regulatory mechanism acting in an opposite way.

Positive and negative control.--Control in a biosynthetic or catabolic pathway may be one of two major types, positive or negative. If the product of a regulator gene functions to shut off enzymatic synthesis, and in the absence of this regulator gene or its product there is constitutive enzyme synthesis, control is negative. The

lactose operon of E. coli is under negative control (27). If however, a product of the regulator gene is required for synthesis of the structural genes to occur, and if in the absence of this regulator gene a pleiotrophic negative phenotype occurs, then control is positive. The L-arabinose operon (63) is under positive control. The distinction between the two mechanisms is thus based on asking the question, "Is the primary function of a regulator gene to turn on or turn off the structural genes it controls?"

Repression in the cysteine biosynthetic pathway.-- Control in the cysteine biosynthetic pathway is complex and many of the intricacies are not understood in detail. Pasternak (51) and Wheldrake and Pasternak (80) demonstrated the sulfate activating enzymes in extracts of E. coli and Bacillus subtilis, and they showed that these enzymes were repressed by cysteine. Glutathione also repressed these activities in B. subtilis, but not in E. coli. This repression by glutathione may be due to the fact that cysteine is made faster in B. subtilis than it is in E. coli. Dreyfuss and Monty (17) demonstrated PAPS, sulfite, and thiosulfate reducing activity by S. typhimurium grown on djenkolate. These activities were absent when S. typhimurium was grown on saturating amounts of cysteine. Thus, the three activities showed coincident repression. Pasternak et al. (52) postulated that coordinate repression of the cysteine biosynthetic pathway enzymes would not be efficient for the

cell. A coordinate type repression is when all pathway enzymes are repressed to the same extent by a specified amount of end product. Instead, Pasternak postulated a differential repression whereby it would take successively larger quantities of end product to repress successive enzymes along the pathway. Clearly this is most advantageous to the cell as it prevents needless formation of the sulfate-activating enzymes when the organism is grown on sulfite or sulfide. Pasternak *et al.* (52), Ellis *et al.* (21), and later Jones-Mortimer *et al.* (29) provided evidence for differential control. The evidence was based on the observation that a decrease in specific activity of the sulfate-activating enzymes occurred when the organisms were grown on sulfide as compared to sulfate. A prediction of these data and model is that growth on successive intermediates of the pathway would give rise to successively higher intracellular cysteine concentrations, and this has been substantiated by Wheldrake (81). Recent work has indicated that all cysteine biosynthetic pathway enzymes in *S. typhimurium* with the exception of serine transacetylase are subject to repression by cysteine (69).

Repression of an early enzyme in a biosynthetic pathway during growth on an intermediate of the pathway is most likely due to the conversion of that intermediate into end product. The repression of sulfate-activating enzymes observed when *E. coli* is grown on sulfite compared to

sulfate or glutathione has been shown to be due to the conversion of sulfite into cysteine, and not by sulfite *per se.* (21). It is interesting to note therefore that Mager (41) showed that the sulfite reductase of *E. coli* was about 60% repressed by the presence of 2.5×10^{-4}M L-methionine in the growth medium. Whether this repression is mediated directly by methionine, or represents a conversion of methionine into cysteine is unknown.

Feedback inhibition and the role of O-acetyl-L-serine (OAS).--There have been reports of feedback inhibition occurring at two distinct points in the cysteine pathway. Kredich and Tompkins (35) identified a two step pathway from L-serine to L-cysteine in *S. typhimurium*. The first step was the formation of OAS from L-serine and acetyl coenzyme A, catalyzed by serine transacetylase (STA). Cysteine at 1.1 uM was shown to feedback inhibit this enzyme 50%. The enzyme was not repressed by cysteine. Subsequently, these authors and co-workers (7, 36) have purified both the STA and the other enzyme of the two step pathway, O-acetylserine sulfhydrylase. O-acetyl-L-serine, the product of the reaction catalyzed by STA, has a dual function in cysteine biosynthesis. It is the acceptor of sulfide, and serves as an inducer of the enzymes of the pathway (29). Mutants lacking STA are pleiotrophic negative for previous enzymes in the pathway (29, 36). How

OAS induces the enzymes of the pathway is not known, nor is any information available on the interaction between OAS and the regulator gene of the cysteine pathway.

The second point of feedback inhibition appears to be at the uptake of sulfate into the cell. This is most probably not mediated by cysteine, but some controversy exists. Ellis and co-workers (21) have provided some *in vivo* evidence for feedback inhibition of sulfate incorporation by cysteine, but no *in vitro* confirmation could be attained. Dreyfuss (18) utilized a mutant lacking the activating enzymes (cys CD mutant) to show neither cysteine nor sulfide significantly inhibited the uptake of sulfate from the medium. However, both sulfite and thiosulfate were inhibitors of the transport system. In the case of thiosulfate, this may have been due to competition for a common transport system. Dreyfuss and Pardee (19) reported that the transport of sulfate into *S. typhimurium* was inhibited in mutants which accumulated the pathway intermediate PAPS. They suggested that the transport of sulfate was feedback inhibited by PAPS.

Positive control in the cysteine pathway.--Jones-Mortimer (28, 30, 31) reported cysteine biosynthesis was under positive control in *E. coli*. This was based on the finding that mutants in the cysteine B gene (regulator gene) were pleiotrophic negative for most of the other enzymes

of the pathway. The wild type allele was dominant to the mutant allele in partial diploids. Perhaps the most crucial test for positive control would be the isolation of deletion mutants in the cysteine B gene, and then examine the effect of this deletion on the rest of the pathway enzymes. This has not as yet been reported.

Part III

The Accumulation of Sulfite

Accumulation of sulfite by prototrophic S. pullorum.--Sulfate-utilizing revertants of S. pullorum MS35 accumulate sulfite in the growth medium when sulfate is the sulfur source (33). The same phenomenon was observed with sulfate-utilizing revertants of S. pullorum MS38 and MS53, (W. D. Hoeksema, unpublished data), and since sulfite accumulation was observed in all sulfate-utilizing revertants of S. pullorum, this phenomenon appears to be inherent.

The precursor of the accumulated sulfite appeared to be sulfate. The evidence presented by Kline and Schoenhard (33) for this was the observation that sulfite accumulated only when sulfate was present. The amount of sulfite accumulated was reduced as the amount of exogenous cysteine was increased, and a mixture of sulfate, selenate, and cysteine (1:11 ratio of sulfate to selenate) reduced sulfite accumulation by 88%.

It was observed that up to 93% of the sulfur in excess of that required for growth accumulated as fuchsin reactive material. Theoretically, only 50% of the sulfur contained in thiosulfate or cysteine-S-sulfonate can be converted to sulfite by acid volatization. Polythionates also would give a maximum of about 67% conversion to sulfite.

Accumulation of sulfite by other microorganisms.-- To the best of our knowledge there have been no reports of sulfite accumulation by microorganisms which are prototrophic for cysteine. However, microorganisms such as S. typhimurium can be made to accumulate sulfite by selecting for mutants which lack the ability to reduce sulfite to sulfide (16). Although these cells lack the ability to grow on sulfate as a sole sulfur source, they can be made to reduce sulfate to sulfite, which then accumulates in the medium, by first growing the cells on djenkolate to derepress the sulfate-activating and sulfate-reducing enzymes. When thiosulfate is used as the sulfur source, these mutants have the ability to grow, presumably because thiosulfate is broken down into $SO_3^=$ and $S^=$, and $S^=$ is beyond the block. Cells grown on thiosulfate also show an accumulation of sulfite in the medium. These phenomena did not occur with wild type cells or cells which could utilize sulfite as a sulfur source. This specifically implicates a defective sulfite reductase as being responsible for

sulfite accumulation in any organism using the assimilatory reductive pathway. It tends to rule out lesions in the PAPS reductase as being potentially responsible for sulfite accumulation. Mutants in the PAPS reductase would probably not take up sulfate from the medium as PAPS has been suggested to feedback inhibit the active transport of sulfate into the cell (19).

Accumulation of sulfite in higher organisms.--Nightengale et al. (47) reported that sulfite accumulation occurred in tomato plants, and Larson and Salisbury (38) observed sulfite accumulation in bull semen. No explanation for the accumulation of sulfite in these organisms was put forth.

Part IV

Temperature Sensitivity

The contribution of temperature sensitive (ts) mutants to microbiology.--Temperature sensitive mutants have allowed investigators to examine cellular function by the mutational approach in systems previously inaccessible due to the lethal nature of the mutation. Thus a DNA replication mutant would be lethal to the host harboring it, and could not be selected for. However, a ts DNA replication mutant can be selected for and utilized as an investigational tool. Temperature sensitive mutations in

non-lethal functions, as an enzyme involved in the biosynthesis of an amino acid, provide a natural isogenic system in which to investigate cellular function.

It is not the purpose of this review to give a comprehensive discussion of the numerous types of ts mutants isolated and their functions. However, temperature sensitivity does occur in the cysteine biosynthetic pathway of S. pullorum (34), albeit in a natural rather than an induced way, and therefore a discussion of representative types of ts mutants is included.

A temperature sensitive fructose-1, 6-diphosphate aldolase.--Bock and Neidhardt (8, 9) isolated a mutant of E. coli which was able to grow in rich medium at 30 C, but not at 40 C. It was determined after further characterization that the ts mutation was in the gene for aldolase. No aldolase activity in vitro was detected in extracts from cells grown at 30 C or 40 C, but the glucose oxidation pattern suggested that the enzyme was active in vivo at 30 C. Temperature resistant revertants were shown to possess fully restored aldolase activity in vitro. This mutant enabled these authors to study the physiological effect of a specific block in the glycolytic pathway.

Temperature sensitive activating enzymes.--Perhaps the activating enzymes occupy one of the largest classes of ts mutations with ts synthetases for valine, alanine, and phenylanaline reported (46). No in vitro synthetase

activity of these mutants could be observed in extracts grown at either the permissive or the restrictive temperature (20). These types of mutants have helped to establish that the ability of certain amino acids, such as valine (10), to repress valine biosynthetic enzymes, is dependent upon their being charged to their specific transfer-RNA and is not accomplished by the free amino acid. Also, an RNA control (RC) stringent strain of E. coli harboring a ts valyl synthetase (10) fails to overproduce RNA at the restrictive temperature, while an RC relaxed derivative of this same strain will overproduce RNA at the restrictive temperature. Their data suggested that the biochemical mechanisms for RNA regulation do not directly involve the charging level of tRNA.

Mutants which are ts in DNA replication.--There have been many reports of bacterial mutants which are ts in DNA synthesis (22, 37, 70). This is because a ts mutation in DNA replication is easily screened for as compared to other ts mutations. Until recently, no bacterial mutants had been found which were affected in DNA polymerase activity (11). However, in contrast to the bacterial system, ts mutants of coliphage T4 DNA polymerase have been isolated (70). Work with ts DNA polymerase mutants of T4 led these authors (70) to suggest that the enzyme was directly involved in replication and did not function solely as a recombinase or a repair enzyme.

A temperature sensitive sulfite reductase.--Kline and Schoenhard (34) observed that S. pullorum MS35 lacked the ability to grow on $SO_3^=$ or CSA as sole sulfur source at 37 C, but could grow on these sulfur sources at 25 C. The same phenomenon was observed with S. pullorum MS38 and MS53 (W. D. Hoeksema, unpublished data). Unlike the ts synthetases and aldolase previously described, the sulfite reductase of S. pullorum was shown to be active in vitro from extracts grown at the permissive temperature (25 C). No NADPH linked activity was detected in vitro from 37 C grown extracts, but some MVH linked activity was observed.

MATERIALS AND METHODS

Bacteria.--Salmonella pullorum strains MS35, MS38, and MS53 were from our stock collection. All share a common cysteine requirement which can be replaced by sulfide. One distinguishing feature of the strains is that S. pullorum MS35 can be made stably lysogenic for bacteriophage P35, while S. pullorum MS53 is not stably lysogenic, and S. pullorum MS38, although stably lysogenic, releases large numbers of phage P35 into the growth medium.

Salmonella typhimurium LT-2 was supplied by P. E. Hartman from the collection of M. Demerec. Escherichia coli B/r was originally obtained from C. E. Helmstetter. Lactobacillus casei (ATCC 7469) was purchased from the American type culture collection.

Chemicals.--In general all chemicals employed were of reagent grade, and were purchased from standard commercial sources. Methionine-free leucine was used exclusively and was purchased from Nutritional Biochemicals Corporation of Cleveland, Ohio. Lyophilized glucose-6-phosphate dehydrogenase (yeast), L-cysteine sulfinic acid (CSA), O-acetyl-L-serine (OAS), and acetyl coenzyme A (COA) were purchased from Calbiochem, Los Angeles, California. N'-methyl-N'-nitro-N-nitrosoguanidine (NTG) was purchased from

Aldrich Chemical Co., Milwaukee, Wisconsin. Djenkolic acid was purchased from the Alfred Bader Division of Aldrich Chemical Co., Milwaukee, Wisconsin. Ammonium sulfamate and N-1-naphthylethylenediamine dihydrochloride were purchased from Matheson, Coleman, and Bell of Cincinnati, Ohio.

In view of the known instability of sulfite (55), fresh potassium sulfite was purchased at the beginning of this investigation from Matheson, Coleman, and Bell, Cincinnati, Ohio. Solid potassium sulfite was kept tightly stoppered and stored in a cool place. Reagents employing sulfite were prepared fresh daily.

Growth and cultivation of bacteria.--The E salts minimal medium of Vogel and Bonner (78) was used except where noted. Agar at 1.5% (w/v) was added when solid medium was desired. When alternate sulfur sources were required, the $MgSO_4 \cdot 7H_2O$ in the E salts was replaced by equimolar $MgCl_2 \cdot 6H_2O$. Sterile D-glucose was added at 0.4% and L-leucine at 1.5×10^{-4}M. Other amino acid supplementation was at 20 ug/ml. In some experiments, nutritional pools A and B (34) were added to the basal medium. Growth of bacteria was with shaking unless otherwise noted. Kline and Schoenhard (34) routinely added L-methionine to the growth medium to overcome a sulfate induced growth lag. In this study L-methionine was omitted unless noted because of

its potential action as a repressor of cysteine pathway enzymes (41).

Selection of revertants.--Revertants were selected by plating a 0.1 ml aliquot of an overnight broth culture on the selective medium. A crystal of NTG was placed on the center of the plate, and colonies appearing three to five days after incubation at 37 C were picked, streaked for purification, and checked for the proper phenotype.

Preparation of cell free extracts.--Cell free extracts were prepared essentially as described by Kline and Schoenhard (34). Cells were harvested at 4 C by centrifugation, and washed once with E medium minus sulfate. The cells were resuspended in about 3 ml of buffer in a sonication cup which was then placed in an ice bath and subjected to four separate 15 second periods of sonic oscillation at a frequency of about 20 KC per second by a magneto-strictive oscillator (Measuring and Scientific Equipment, Ltd.). Each period of oscillation was separated from the sucessive one by one minute to allow cooling and prevent possible overheating. If the extract was to be used for the determination of sulfite reductase specific activity, the buffer employed was 0.2M phosphate, pH 7.6, and centrifugation after sonication was for one hour at 34,000 x g at 4 C. The SS34 rotor of a Sorval RC-2 or RC-2B refrigerated centrifuge was used. If the extracts were to be used to determine STA or OASS specific activity,

the buffer was 0.05 M tris (hydroxymethyl) aminomethane (Tris)--hydrochloride buffer, pH 7.6. Centrifugation was for ten minutes at 13,000 x g at 25 C following the procedure of Kredich et al. (36), who reported the enzymes from S. typimurium were extremely cold labile. Upon direct test, it was noted that STA levels were higher in extracts prepared as described for the determination of sulfite reductase specific activity and no cold lability of the S. pullorum enzyme was observed. If assays for STA were performed using the 0.2M phosphate buffer extraction method, it is noted in the individual experiments. Extracts prepared for the determination of sulfite reductase specific activity were routinely dialyzed overnight at 4 C against 0.2M phosphate buffer, pH 7.6. Dialysis tubing was boiled in a deionized, distilled water and disodium ethylenediamine-tetraacetate (EDTA) solution before using. Protein was determined by the method of Lowry et al. (40) using bovine serum albumin as standard. Extracts were stored at -20 C.

Derepression of cysteine pathway enzymes.--Maximum derepression of pathway enzymes has been obtained by growth on djenkolic acid as sole sulfur source (16, 17), and is believed to be due to the very slow breakdown of this compound into cysteine. When maximum derepression of S. pullorum cysteine pathway enzymes was desired, djenkolate at 5×10^{-4}M was employed as the sulfur source. Djenkolate

was solubilized in water by the dropwise addition of 1 N KOH, and was filter sterilized before its addition to the medium.

Measurement of the growth response of S. pullorum to various sulfur sources.--The sulfur free E medium was supplemented with the desired sulfur source at a concentration indicated in the individual experiment, and pools A and B at 10 ml of each per liter of medium. If the test sulfur source was sulfide, screw cap test tubes were used with parafilm wrapped around the caps. The medium was inoculated with an overnight broth culture of the test organism which had been centrifuged, washed with E medium minus sulfate, and resuspended in E medium minus sulfate. The inoculation resulted in a cell density of 5×10^6 to 1×10^7 organisms per ml. Incubation was with shaking except in the case of sulfide, and was at the temperature indicated in the individual experiments. Growth was measured as an increase in OD at 420 nm, and was recorded between 24 and 48 hours. A sulfur source which failed to support growth to a density of 1×10^8 cells/ml (OD_{420} of 0.10) after 24 hours under these conditions was considered insufficient.

Assay for sulfite.--Sulfite was assayed by the method of Grant (23) as modified by Dreyfuss and Monty (16). To two ml of the test sample were added 0.5 ml of 1% KOH in 95% ethanol, and 1.0 ml of saturated $HgCl_2$ in 95% ethanol. The solution was centrifuged and one ml of the

clear supernatant was added to four ml of the fuchsin reagent of Grant. The Grant reagent was decolorized with Norit (2 mg/ml) before use, and was made fresh daily. Sulfite standards were carried through the entire procedure. Optical density at an OD_{580}nm was used as a measure of sulfite.

Assay for sulfite reductase.--NADPH linked sulfite reductase activity was assayed as described by Kline and Schoenhard (34). Sulfide was determined by the method of Siegel (66). Glucose-6-phosphate dehydrogenase, when required, was added at a concentration of one enzymatic unit per ml where an enzymatic unit corresponds to that amount of enzyme catalyzing the turnover of one micromole of substrate per minute. The reaction mixture in a final volume of 1 ml contained 0.5 umoles potassium sulfite, 3.0 umoles glucose-6-phosphate, 0.05 umoles NADP, 4.0 umoles $MgCl_2$, 34 umoles phosphate (pH 7.6), and water. The reaction was started by the addition of extract (1.0 to 3.0 mg of protein), and incubation was in 13 x 100 mm tubes which were tightly stoppered by corks wrapped in parafilm. Incubation was at 25 C for 20 min. A blank was run simultaneously containing all components but sulfite. At the end of the 20 minute incubation time, 0.1 ml of 0.02M N, N-dimethyl-p-phenylenediamine sulfate (Eastman Organic Chemicals, Rochester, New York) in 7.2N hydrochloric acid (DPD reagent) was added and was rapidly followed by 0.1 ml of

0.03M ferric chloride in 1.2N hydrochloric acid. This mixture, still tightly stoppered, was shaken vigorously and allowed to stand in the dark for 20 minutes, after which it was centrifuged and the OD_{650}nm of the supernatant was recorded. Specific activities were determined from assays in which the production of sulfide was linear with respect to time and protein concentration.

MVH linked sulfite reductase was measured using the procedure and apparatus described by Asada (2). The reaction mixture in a total volume of 1.65 ml contained potassium phosphate buffer, pH 7.75 (150 umoles), potassium sulfite (1.5 umoles), bovine serum albumin (2 mg), MVH (400 nmoles), water, and extract (0.5 to 1.0 mg of protein). The reaction was started by tipping in sulfite from the side arm, and activity was measured as the decrease in OD_{604}nm. The blank consisted of measuring the endogenous bleaching of MVH for three minutes before tipping in the sulfite. The reaction was run at 25 C.

Assay for serine transacetylase.--The assays employed were those described by Kredich and Tompkins (35). The first assay, and the one routinely used, is based on the disulfide interchange between CoA and DTN [5,5'-dithiobis (2-nitrobenzoic acid)], and the production of thionitrobenzoic acid is followed spectrophotometrically by the increase in OD_{412}nm. The reaction mixture in a final volume of 0.5 ml contained tris-HCl, pH 7.6, (25 umoles),

DTN (0.5 umoles), sodium EDTA (0.5 umole), acetyl CoA (0.05 umole), L-serine (0.5 umole), and extract (30 to 150 ug of protein). The reaction was run at 25 C, and started by the addition of enzyme. A blank contained all components but L-serine.

The second assay for STA was used only if the reaction mixture contained compounds with free SH groups, such as cysteine, which react with DTN. This assay followed the decrease in OD_{232}nm which occurs when the thioester bond of acetyl-CoA is cleaved. The reaction mixture was the same as that described for the DTN procedure except that DTN was omitted. Unless specifically stated to the contrary, the first described assay was used.

Assay for O-acetylserine sulfhydrylase.--OASS was assayed as described by Becker, Kredich, and Tompkins (7). The assay is based on the colorimetric determination of cysteine formed in the reaction. The reaction mixture at a final pH of 7.2 to 7.4 in a volume of 0.2 ml contained tris-HCl (32 umoles), O-acetyl-L-serine (20 umoles), sodium sulfide (0.64 umoles), sodium EDTA (0.16 umole), and extract (1 to 4 ug of protein). Reactions were run at 25 C in capped 13 x 100 mm glass tubes, and were started by the addition of enzyme. Blanks contained everything but OAS. After four minutes of incubation, 1 ml of 1 mM nitrous acid was added to the reaction mixture which was

then vigorously shaken. Six minutes later 0.1 ml of a 2% ammonium sulfamate solution was added, and the mixture was shaken. Two minutes later, 1.6 ml of a solution composed of 1 part 2% $HgCl_2$ in 0.4 N HCl, 4 parts 6.88% sulfanilamide in 0.4 N HCl, and 2 parts of 0.2% N-1-naphthylethylenediamine dihydrochloride in 0.4 N HCl was added. After 5 minutes, absorbancy was read at an OD_{540}nm.

Assay for flavins.--The biological assay for riboflavin was done using the standard procedure given in the Difco manual (15). Lactobacillus casei (ATCC 7469) was used as the test organism.

The fluorimetric assay for flavin was performed using the procedure of Burch (12). The absorbancy wavelength was 265 nm and the emission wavelength was 517 nm. Slit widths were 4 mm.

Chromatographic identification of riboflavin was by the method of Burch (12).

RESULTS

Part I

Demonstration of STA and OASS in Extracts of *S. pullorum* and Their Control by Cysteine

The accumulation of sulfite by cultures of *S. pullorum* grown in sulfate could have reflected a repression (or lack of induction) of the genes involved in the production of sulfite reductase. Therefore an effort was made to demonstrate STA and OASS activity in extracts of *S. pullorum* because of their implications in the control mechanisms of cysteine biosynthesis. The sulfate-utilizing revertant of *S. pullorum* MS35, designated 6-18, was the source of the extracts.

Specific activities of STA and OASS as a function of sulfur source.--Table 1 evidences that the specific activity of STA does not vary significantly with the sulfur source. From these data it was evident that STA was not repressed by cysteine. In contrast, the level of OASS does vary, depending on the sulfur source. The level is highest from cells grown on djenkolate, lowest from cells grown on cysteine, and intermediate from cells grown on sulfate. It is interpreted from these data that OASS was repressed by cysteine. No attempt was made to distinguish

between repression mediated by cysteine *per se.*, or by a derivative of cysteine, such as cysteinyl transfer ribonucleic acid (t-RNA).

TABLE 1. STA and OASS specific activities in extracts of *S. pullorum* 6-18 as a function of sulfur source.

Sulfur substrate[a]	STA specific activity[b]	OASS specific activity[c]
Djenkolate	0.035	6.00
Sulfate	0.032	4.00
Cysteine	0.037	0.10

[a]The final concentration of the sulfur sources were djenkolate 5 x 10^{-4}M, sulfate 5 x 10^{-4}M, and cysteine 1.65 x 10^{-4}M.

[b]Specific activity of STA is expressed as umoles of L-serine acetylated per minute per mg of protein.

[c]Specific activity of OASS is expressed as umoles of cysteine formed per minute per mg of protein.

Feedback inhibition of STA by cysteine.--Since the synthesis of STA was not repressed by cysteine, the question arose whether end-product inhibition of this enzyme was operative in *S. pullorum* 6-18. It is seen in Figure 1 that as the cysteine concentration of the reaction mixture increased, a decline in specific activity of STA resulted. The enxyme was 50% inhibited by a 3 uM solution of L-cysteine.

Figure 1. The feedback inhibition of STA from S. pullorum 6-18 by cysteine. The specific activity of STA used in this study was 0.074 umoles of L-serine acetylated/min/mg. This specific activity was obtained by subjecting the crude extract to a protamine sulfate precipitation, and this was followed by an ammonium sulfate precipitation using exactly the procedure of Kredich, Becker, and Tompkins (36). A decrease in OD_{232}nm was used to assay STA specific activity as described in materials and methods.

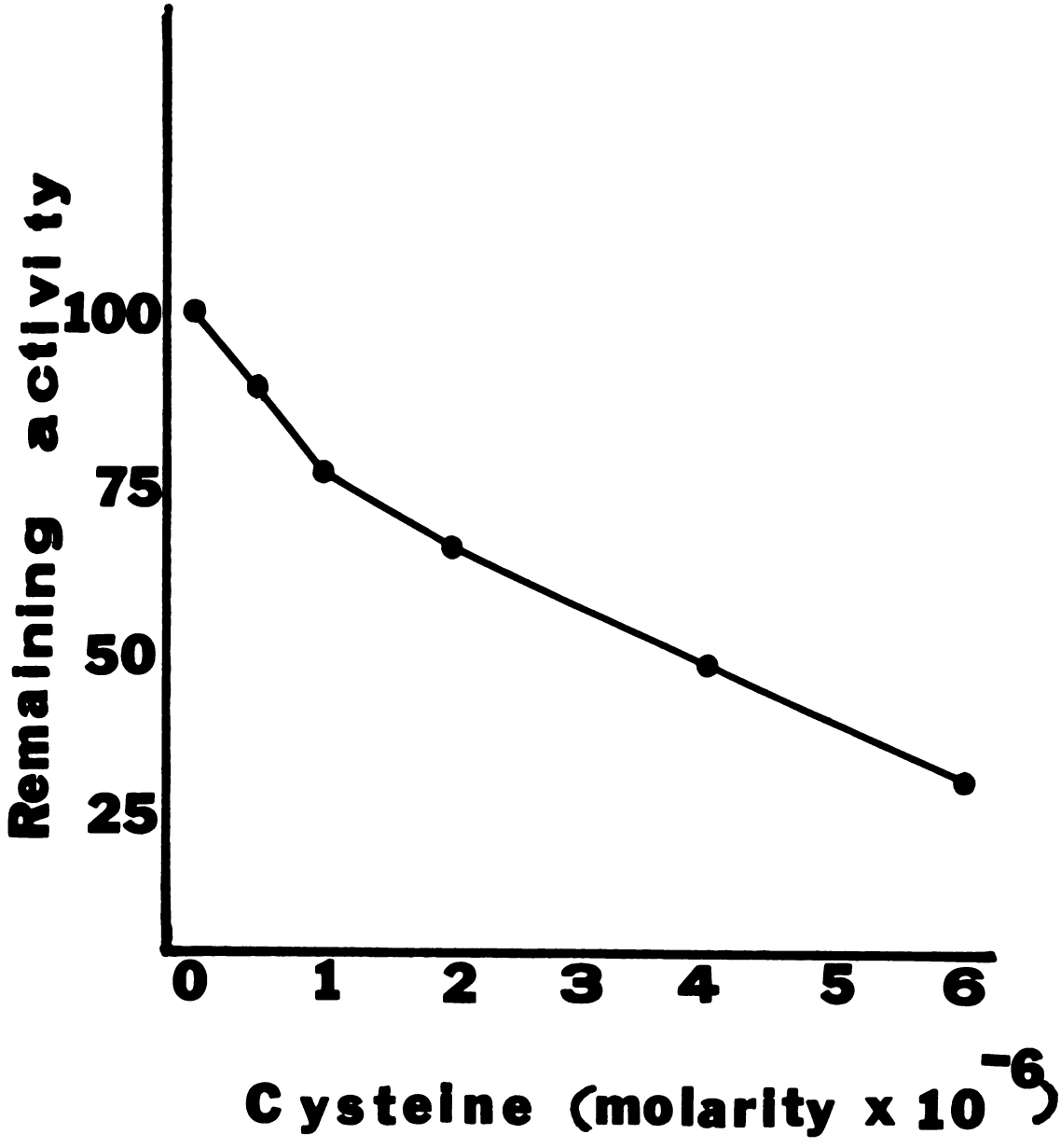

Remaining activity
100
75
50
25
0
1
2
3
4
5
6
Cysteine (molarity x 10^-6)

Figure 1

The linear time course of OASS.--To determine if OASS were subject to end-product inhibition by cysteine in addition to repression, the linear time course of the reaction was investigated. The product of the reaction is cysteine, and if the enzyme were inhibited by cysteine, the reaction would be linear only if cysteine were being actively removed. As shown in Figure 2 the reaction is linear for at least 20 minutes. The amount of cysteine formed at the end of this time was about 0.84 mM. Therefore, there is no significant feedback inhibition of OASS by concentrations of cysteine equal to or less than 0.84 mM.

Part II

The Development of a Non-Sulfite Accumulating System

The analysis of revertants.--The possibility exists that sulfite accumulation does not occur in all sulfate-using revertants of S. pullorum. To test this hypothesis, thirty sulfate-using revertants of S. pullorum MS35 were isolated. All revertants accumulated sulfite when grown on sulfate. In addition to strain MS35 of S. pullorum, we have strains MS38 and MS53 in our stock collection. Both of these strains have unique features which distinguish them from MS35 and from each other. Both MS38 and MS53, like MS35, are double cysteine auxotrophs. Sulfate-using revertants which I designated 38-2 and 53-2,

Figure 2. The linear time course of OASS. The reaction was done in a volume of 0.4 ml. Aliquots of 50 ul were removed at the times indicated, and assayed. The non-enzymic reaction mixture contained everything but enzyme. Symbols: (■) Enzymic Reaction; and (●) Non-Enzymic Reaction.

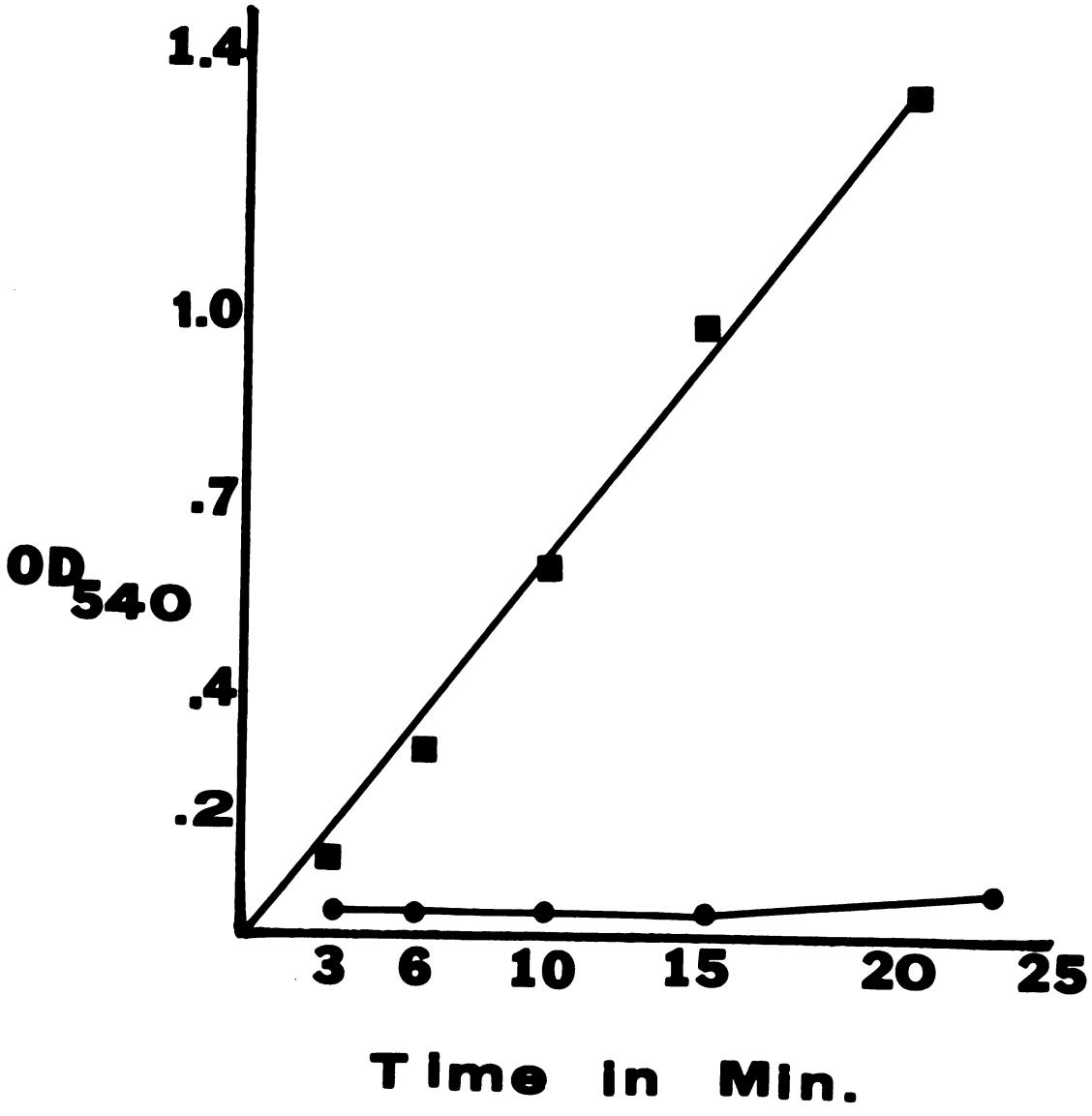


Figure 2

respectively, accumulated sulfite when grown on sulfate. The auxanographic responses are shown in Table 2. It thus appeared that sulfite accumulation was not restricted to some sulfate-using revertants of MS35, but rather appeared to be inherent to S. pullorum.

Table 2. The growth responses of S. pullorum strains MS35, MS38, and MS53 to various sulfur sources.

	$SO_4^=$		CSA or $SO_3^=$		$S^=$		Djenkolate or cysteine	
Temperature (C)	25	37	25	37	25	37	25	37
S. pullorum MS35, MS38, MS53	-	-	+	-	+	+	+	+
Prototrophic revertants[a]	+	+	+	+	+	+	+	+

[a]Prototrophic revertants were selected in two steps at 37 C by NTG mutagenesis. First, revertants were selected which had the ability to grow on $SO_3^=$ or CSA at 37 C, and second, from one of these, revertants which had the ability to grow on $SO_4^=$ were selected.

Sulfite accumulation as a function of temperature.--All the work of Kline and Schoenhard (33) on sulfite accumulation was done at 37 C. Since NADPH sulfite reductase specific activity appeared to be higher in extracts from cells grown at 25 C than in extracts from cells grown at 37 C, sulfite accumulation as a function of temperature

was examined. It was observed that the accumulation of sulfite was strongly temperature-dependent with only a trace of sulfite accumulated at 25 C (Table 3). There was a similar temperature-dependent accumulation of sulfite by revertants 38-2 and 53-2.

Table 3. The accumulation of sulfite by S. pullorum 6-18 as a function of temperature.

Temperature	Maximum sulfite accumulated[a] (nmoles/ml)
37 C	> 200[b]
25 C	< 20

[a]Sulfite was determined by the Grant technique at 10 hours after the culture had reached the stationary phase of growth.

[b]Up to 93% of the sulfur in excess of that required for growth appears as accumulated sulfite.

Part III

The Thermodependent Loss of Sulfite Reductase Activity

The temperature-dependent accumulation of sulfite, and the observation that sulfite accumulation appeared to begin in the late logarithmic phase of growth, together were tentatively interpreted to mean that sulfite accumulation occurred because of a thermolabile sulfite reductase.

The correlation between sulfite reductase activity and sulfite accumulation.--To test the hypothesis of a thermolabile sulfite reductase, cells were harvested at various stages of growth, at which time sulfite accumulation and sulfite reductase specific activity were determined. As shown in Figure 3, sulfite reductase specific activity was lost during the growth of cells at 37 C. Concommitant with the decline of sulfite reductase specific activity was an accumulation of sulfite in the growth medium.

When the same experiment was performed at 25 C (Figure 4), no loss of sulfite reductase activity was observed, and no sulfite was accumulated. Thus the appearance of sulfite in the growth medium probably resulted from reduced sulfite reductase activity *in vivo*.

Sulfite reductase and STA activity at 25 C and 37 C.--The previous assays were for NADPH linked sulfite reductase specific activity. It was desirable to determine whether MVH linked sulfite reductase specific activity was being lost also. Since in yeast it has been reported (88) that MVH sulfite reductase is catalyzed by a portion of the NADPH sulfite reductase molecule, it is entirely possible to retain MVH linked activity while losing NADPH linked activities. To determine the specificity of the loss of sulfite reductase specific activity, STA specific activity was also measured in the same extracts. MVH linked activity was lost as well as NADPH linked activity

Figure 3. The correlation of sulfite reductase activity and sulfite accumulation at 37 C. S. pullorum 6-18 was grown with shaking in E medium supplemented with sulfate (5×10^{-4}M). At the desired points of growth cells were harvested, and sulfite accumulation and sulfite reductase specific activity were determined. Symbols: (■) relative sulfite reductase specific activity; and (●) sulfite accumulated.

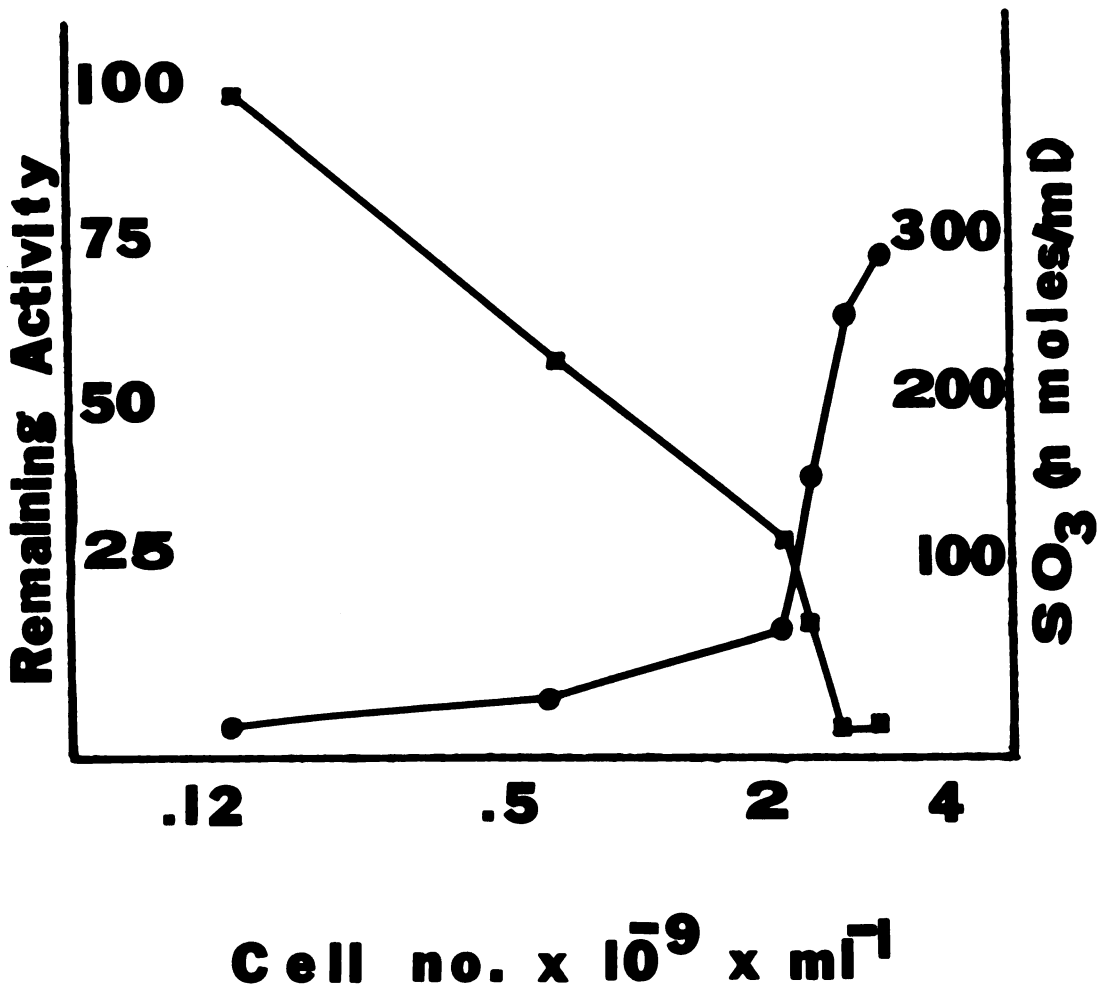

Remaining Activity
100
75
50
25
300
200
100
SO_3 in moles/ml
.12
.5
2
4
Cell no. x 10^{-9} x ml^{-1}

Figure 3

Figure 4. The correlation of sulfite reductase activity and sulfite accumulation at 25 C. S. pullorum 6-18 was grown with shaking in E medium supplemented with sulfate (5×10^{-4} M). At the desired points of growth cells were harvested, and sulfite accumulation and sulfite reductase specific activity were determined. Symbols: (■) relative sulfite reductase specific activity; and (●) sulfite accumulated.

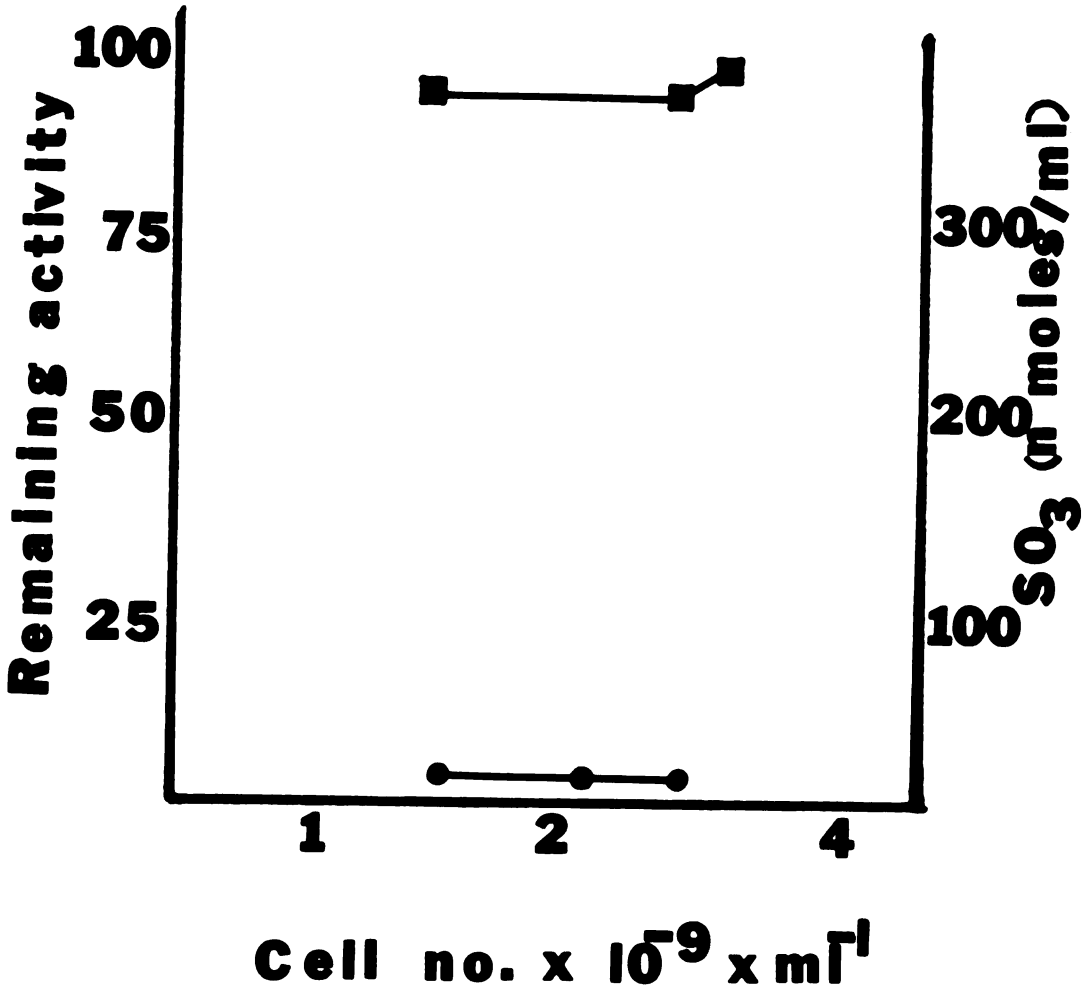

Remaining activity
100
75
50
25
300
200
100
SO_3 (n moles/ml)
1
2
4
Cell no. x 10^{-9} x ml^{-1}

Figure 4

at 37 C (Table 4) and furthermore, both activities were lost to approximately the same extent during the growth of S. pullorum 6-18 at 37 C. STA levels remained essentially constant during the growth of S. pullorum at both 25 C and 37 C. The loss of sulfite reductase activity therefore was a specific response, and most probably did not result from a general metabolic breakdown of S. pullorum at 37 C.

Table 4. NADPH sulfite reductase activity, MVH sulfite reductase activity, and STA activity during the growth of S. pullorum 6-18[a].

	NADPH sulfite reductase		MVH sulfite reductase		STA	
Growth stage	25 C	37 C	25 C	37 C	25 C	37 C
Log cells[b]	100	100	100	100	100	100
Early stationary cells	96	38	117	34	95	95

[a]All activities are expressed as relative activities for comparative purposes. All activities were determined in extracts prepared as described for the determination of sulfite reductase specific activity. All extracts used in this work were dialyzed overnight against 0.2M phosphate buffer pH 7.6.

[b]Log phase cells represent approximately 3×10^8 cells/ml, while early stationary phase cells represent approximately 1.5×10^9 cells/ml. The medium used in this study was E medium supplemented with djenkolate (5×10^{-4}M).

Part IV

Characterization of Sulfite Accumulation

Temperature shift experiments.--To allow a characterization of the temperature sensitive accumulation of sulfite, temperature shift experiments were performed. It was observed (Figure 5) that the accumulation of sulfite was not totally reversible under the conditions stated. Cells grown at 37 C in E medium and shifted to 25 C still accumulated sulfite, while cells grown at 25 C and shifted to 37 C did not accumulate significant sulfite for a period of at least 8 hours. It should be noted that sulfide was generated in both the 25 C → 25 C and the 25 C → 37 C shift cultures. In a 25 C to 41 C temperature shift experiment (Figure 6) some sulfite accumulation occurred. Although the amount accumulated was not large, there was a definite accumulation and therefore some *in vivo* denaturation of sulfite reductase may have occurred.

Growth of S. pullorum at 41 C on various sulfur sources.--Although *S. pullorum* accumulates sulfite at 37 C, it still has the ability to grow on sulfate as a sole sulfur source. A prediction of the thermolabile sulfite reductase hypothesis is that by increasing the temperature above 37 C, a point would be reached which would not allow growth on any intermediate of the pathway previous to the sulfite reductase catalyzed reaction, but

Figure 5. Sulfite accumulated in temperature shifted cultures. Cells of S. pullorum 6-18 were grown in E medium supplemented with djenkolate (5×10^{-4}M) to the stationary phase of growth at 37 C and 25 C. At this time the cells were harvested and resuspended in E medium supplemented with sulfate (5×10^{-4}M), and chloramphenicol (50 ug/ml). The cells were concentrated about two-fold (6×10^{9}/ml) upon resuspension so that small amounts of sulfite accumulated could be detected. After resuspension, one aliquot was maintained at the temperature which the cells were originally grown, and the other aliquot was shifted to a temperature different from that at which the cells had originally grown. Sulfite accumulation was then assayed with time. Symbols: (□) 37 C to 37 C, (○) 37 C to 25 C, (■) 25 C to 25 C; and (●) 25 C to 37 C.

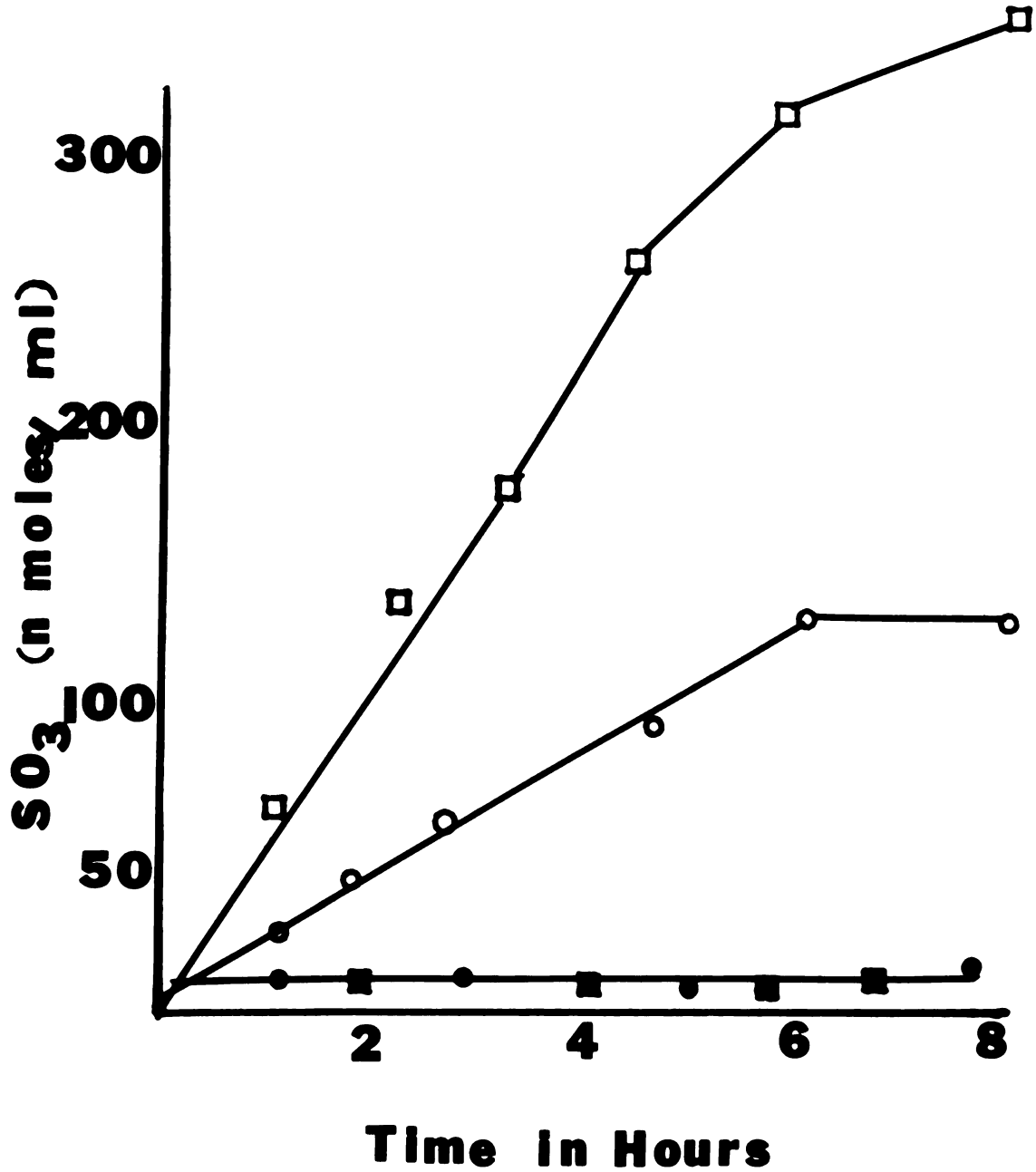


Figure 5

Figure 6. Sulfite accumulated in a 25 C to 41 C temperature shifted culture. Cells of S. pullorum 6-18 were grown in E medium supplemented with djenkolate (5×10^{-4}M) to the stationary phase of growth. At this time the cells were harvested and resuspended in E medium supplemented with sulfate (5×10^{-4}M), pools A and B (10 ml of each per liter of E medium), and chloramphenicol (50 ug/ml). The cells were concentrated about two-fold (6×10^{9} cells/ml) upon resuspension so that small amounts of sulfite accumulated could be detected. After resuspension the cells were incubated with shaking at 41 C, and sulfite accumulated was assayed with time.

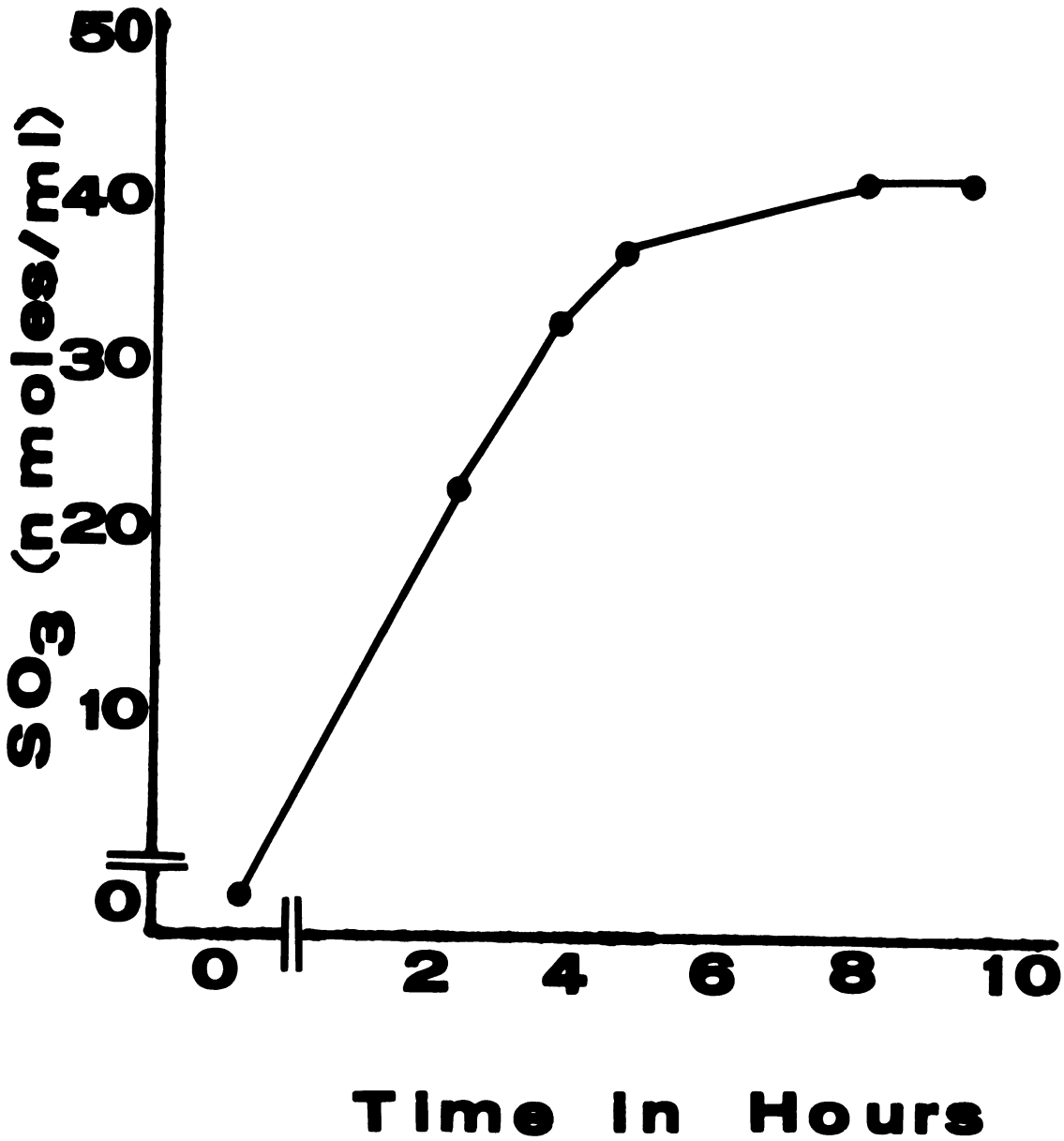


Figure 6

would allow growth on sulfur intermediates past this step. S. pullorum 6-18 at 41 C has the ability to grow on sulfide, cysteine, and djenkolate (Table 5) but lacks the ability to grow on sulfite, CSA, or sulfate. CSA is believed to be broken down in vivo into sulfite (39).

Table 5. The growth of S. pullorum 6-18 as a function of temperature and sulfur source.

Sulfur source[a]	Growth response[b]	
	37 C	41 C
$SO_4^=$	++	-
$SO_3^=$	++	-
CSA	++	-
$S^=$	+	+
Cysteine	++	++
Djenkolate	++	++

[a]Cells were tested for growth in E medium containing one of the following sulfur sources: sulfate (5×10^{-4}M), sulfite (5×10^{-4}M), CSA (1.3×10^{-4}M), sulfide (2×10^{-4}M), cysteine (1.6×10^{-4}M), or djenkolate (5×10^{-4}M). In addition, the medium contained nutritional pools A and B at a concentration of 10 ml of each per liter of medium.

[b]Growth was determined by measuring the OD_{420}nm after 30 hours incubation at the indicated temperature. Incubation was with shaking except when determining the growth response to sulfide. The inoculum was approximately 1×10^7 cells/ml.

- = no growth detected, $< 10^8$ cells/ml
+ = $> 1 \times 10^8$ cells/ml, $< 8 \times 10^8$ cells/ml
++ = $> 8 \times 10^8$ cells/ml.

No growth was obtained at 41 C on any sulfur intermediates or cysteine, unless nutritional pools A and B were present in the medium, therefore enzymes involved in other biosynthetic pathways in S. pullorum may be labile at 41 C. If the tubes were allowed to incubate for long periods of time (48 - 60 hours), occasionally some growth appeared in the tubes containing sulfate, sulfite, or CSA as sulfur sources.

The effect of exogenous cysteine on sulfite accumulation.--Kline and Schoenhard (33) demonstrated that as the concentration of exogenous cysteine in the E medium containing sulfate was increased, sulfite accumulation decreased. To determine if this were due to a feedback inhibition, and/or a repression mechanism, various amounts of cysteine were added to dense suspensions of S. pullorum 6-18 in E medium containing sulfate. Protein synthesis was arrested by the addition of chloramphenicol (CAP); thus, an immediate shut off of sulfite accumulation under these conditions would strongly suggest a feedback inhibition by cysteine. However, cysteine does not appear to inhibit sulfite accumulation under the conditions shown in Figure 7. Concentrations of cysteine as high as 20 ug/ml produced little inhibition of sulfite accumulation compared to the control containing no cysteine. Cysteine at a concentration of 4 ug/ml appeared to stimulate sulfite

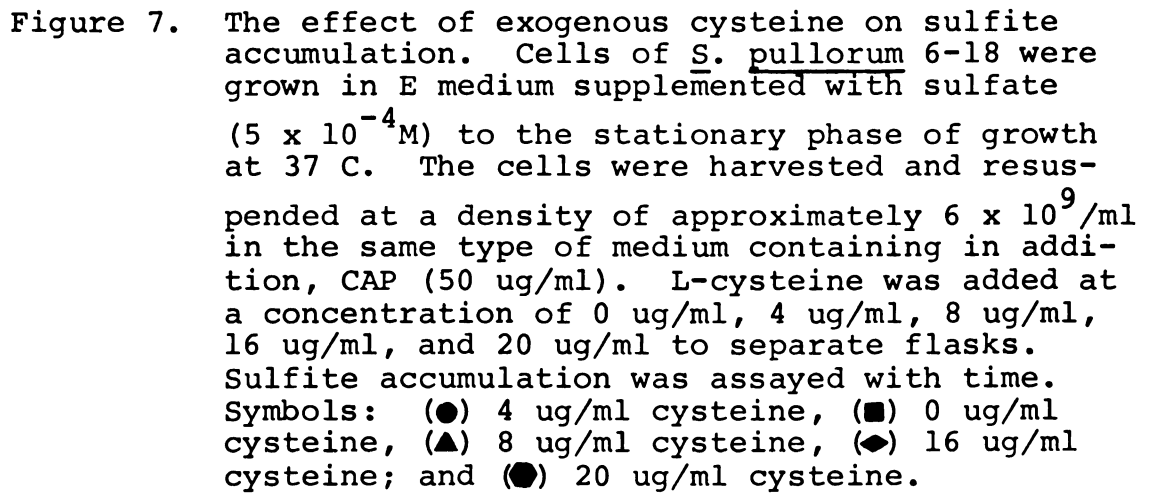


Figure 7. The effect of exogenous cysteine on sulfite accumulation. Cells of S. pullorum 6-18 were grown in E medium supplemented with sulfate (5×10^{-4}M) to the stationary phase of growth at 37 C. The cells were harvested and resuspended at a density of approximately 6×10^{9}/ml in the same type of medium containing in addition, CAP (50 ug/ml). L-cysteine was added at a concentration of 0 ug/ml, 4 ug/ml, 8 ug/ml, 16 ug/ml, and 20 ug/ml to separate flasks. Sulfite accumulation was assayed with time. Symbols: (●) 4 ug/ml cysteine, (■) 0 ug/ml cysteine, (▲) 8 ug/ml cysteine, (◆) 16 ug/ml cysteine; and (⬢) 20 ug/ml cysteine.

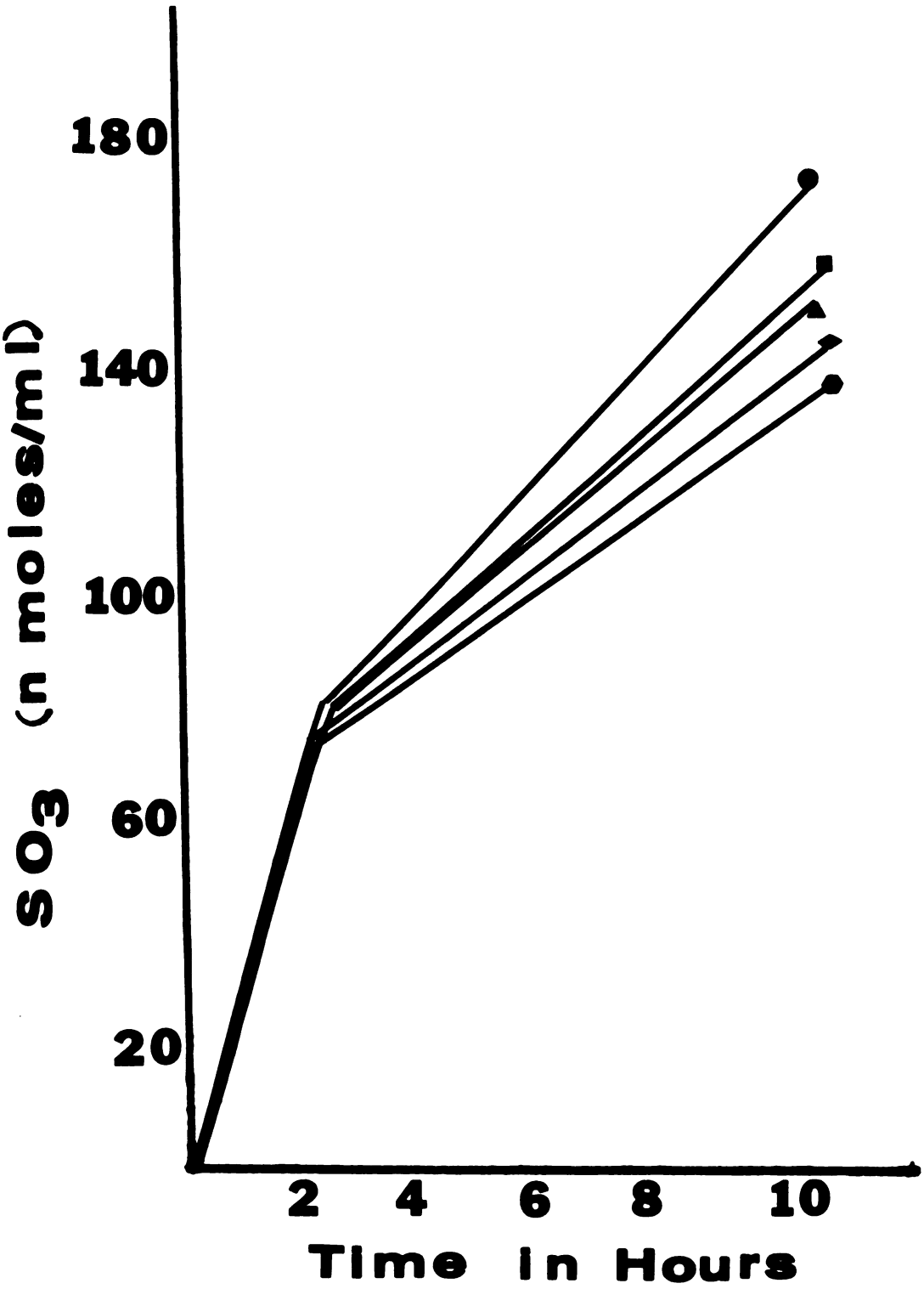


Figure 7

accumulation. This stimulation by low amounts of cysteine was observed in several experiments.

Part V

In Vitro Lability of Sulfite Reductase

To examine *in vitro* lability of the enzyme sulfite reductase, a thermodenaturation experiment was performed. An extract prepared from *S. pullorum* 6-18 grown at 37 C loses NADPH sulfite reductase activity at a considerably more rapid rate than a similarly prepared extract from *S. typhimurium* LT-2 (Figure 8). An extract prepared from *S. pullorum* 6-18 grown at 25 C was more stable than that from *S. pullorum* grown at 37 C. However, it appears that NADPH sulfite reductase from *S. pullorum* grown at either 37 C or 25 C is more labile *in vitro* than the enzyme from *S. typhimurium*. A certain amount of variation occurred in the thermodenaturation profile from extract to extract. Therefore, each point of Figure 8 represents an average of at least four determinations from at least two different extracts. Mixing of untreated and heated (45 C for 10 min) extracts from cells of *S. pullorum* grown at 37 C with *S. typhimurium* extracts did not result in lowering the predicted sulfite reductase specific activities.

Figure 8. The thermolability of NADPH linked sulfite reductase *in vitro*. Extracts were prepared as described in materials and methods, and were dialyzed overnight against 0.2M phosphate buffer, pH 7.6. After initial determination of linearity and specific activity, 1.5 ml of the extract were placed in a prewarmed 13 x 100 mm tube which was then incubated at 45 C in a water bath. At 15, 30, 45, and 60 minutes, duplicate aliquots were removed and added to reaction mixtures as described in materials and methods. All reaction mixtures were incubated at 25 C in a water bath for 20 minutes, and NADPH sulfite reductase was assayed for as described. Cells used as the source of extracts in these experiments were grown in E medium supplemented with djenkolate (5×10^{-4}M). Leucine was omitted in the growth medium of *S. typhimurium*. Symbols: (●) sulfite reductase from cells of *S. typhimurium* grown at 37 C, (■) sulfite reductase from cells of *S. pullorum* grown at 25 C; and (▲) sulfite reductase from cells of *S. pullorum* grown at 37 C.

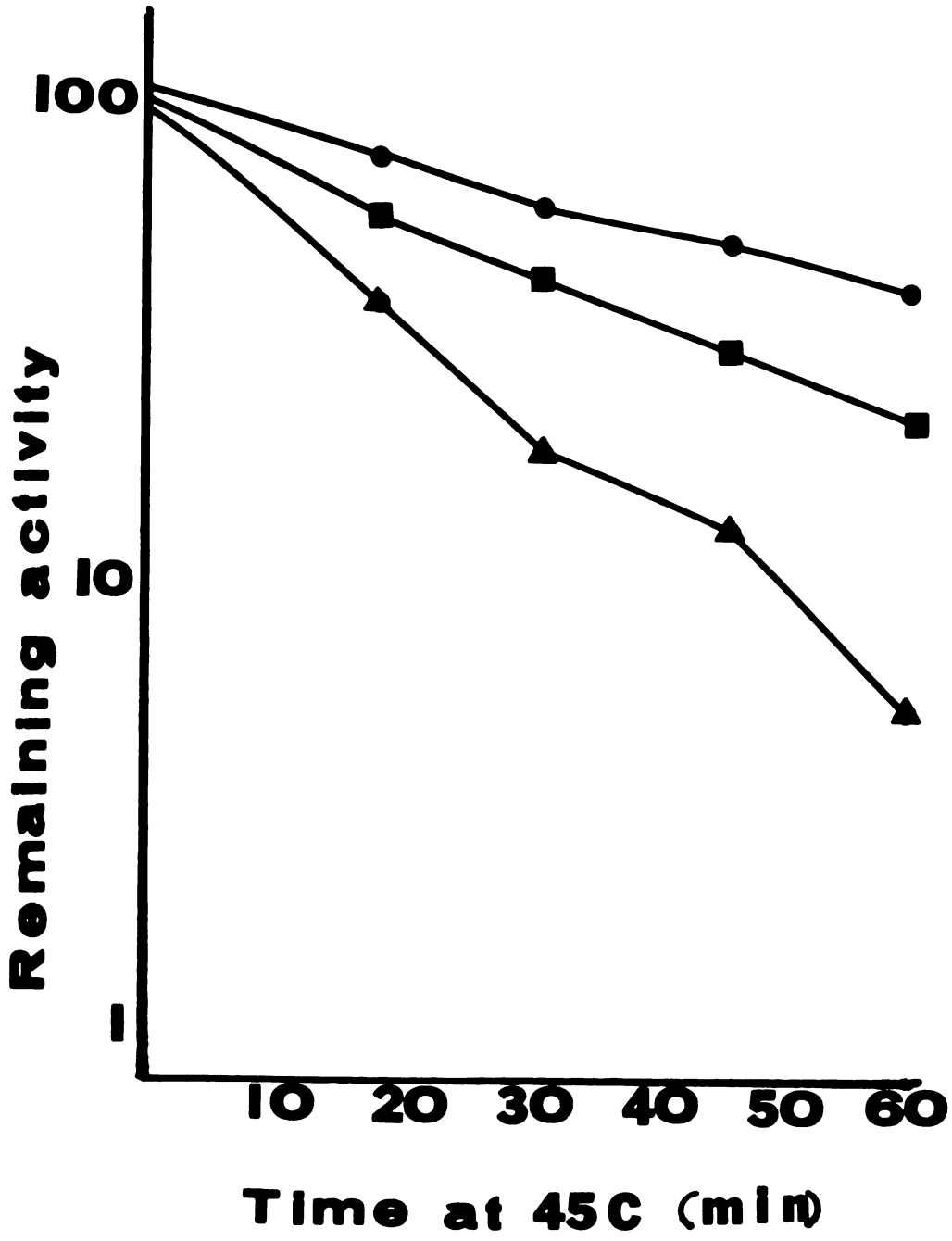


Figure 8

Part VI

The Accumulation of Flavins by S. pullorum

The identification of external flavins.--One aspect which could have helped to understand sulfite accumulation was the identification of other compounds accumulating in larger than normal amounts. During the growth of S. pullorum in minimal medium there was a distinct yellow-green coloration of the medium which did not occur to the same extent when E. coli B/r or S. typhimurium LT-2 prototrophs were grown in the same medium. Removal of the cells by centrifugation and filtration left a yellow-green supernatant fluid of pH 6.2. This supernatant fluid had a distinct fluorescence when viewed under ultraviolet (UV) light. Its fluorescence was similar to that of acidic solutions of riboflavin. These data are summarized in Table 6 and Figures 9 and 10.

The quantitation of external flavins.--It appeared that flavin accumulation was occuring in the growth medium of S. pullorum. Since some flavin overproduction is common among bacteria (53, 84), a quantitation of the external flavins was sought. The first approach was the use of a biological assay using the riboflavin-requiring organism Lactobacillus casei. The addition of increasing amounts of S. pullorum cell-free growth medium to the riboflavin-free medium resulted in an increasing growth response of L. casei

Table 6. Fluorescence of S. pullorum growth medium after removal of the cells.

Treatment[a]	S. pullorum growth medium[b]	Acidic riboflavin solution
Untreated	green fluorescence	green fluorescence
Ultra violet light	blue fluorescence	blue fluorescence
Visible light	blue fluorescence	blue fluorescence

[a]Exposed overnight to the light source indicated.

[b]Cells were grown 10 hours into the stationary phase on E medium supplemented with sulfate (5×10^{-4}M). Cells were removed by centrifugation and the supernatant fluid was then filtered using a millipore apparatus.

as measured by an increase in absorbancy at an OD_{420}nm (Figure 11). The biological activity was destroyed by exposure to visible or UV light. By comparison to a standard curve using known riboflavin, it was estimated that the biological stimulating activity of S. pullorum growth medium supernatant fluid is equivalent to about 2 ug riboflavin/ml of supernatant fluid.

To determine the kind and distribution of external flavins, a fluorimetric assay was performed. The results shown in Tables 7 and 8 indicate that the primary external flavin of S. pullorum is riboflavin, and there was considerably more external flavin present in the growth medium of S. pullorum than there was in the growth medium

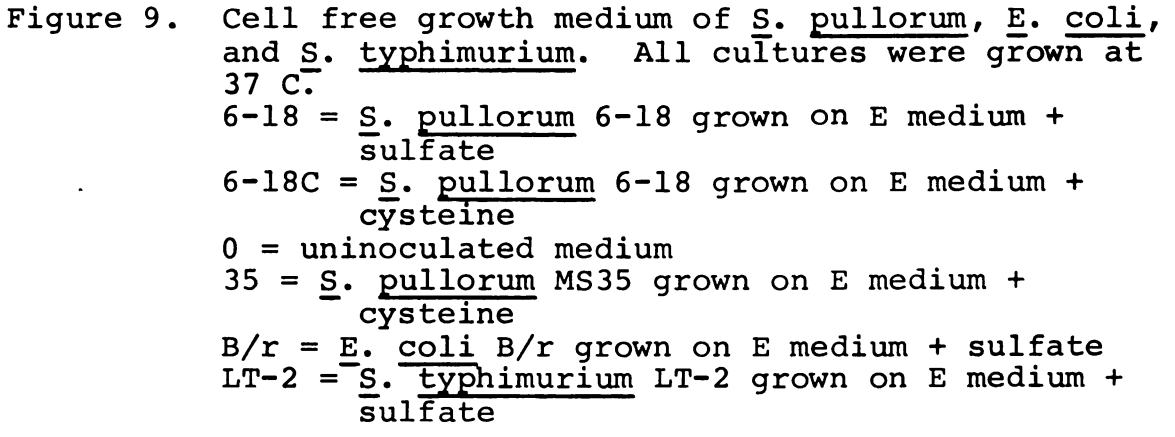


Figure 9. Cell free growth medium of S. pullorum, E. coli, and S. typhimurium. All cultures were grown at 37 C.
6-18 = S. pullorum 6-18 grown on E medium + sulfate
6-18C = S. pullorum 6-18 grown on E medium + cysteine
0 = uninoculated medium
35 = S. pullorum MS35 grown on E medium + cysteine
B/r = E. coli B/r grown on E medium + sulfate
LT-2 = S. typhimurium LT-2 grown on E medium + sulfate

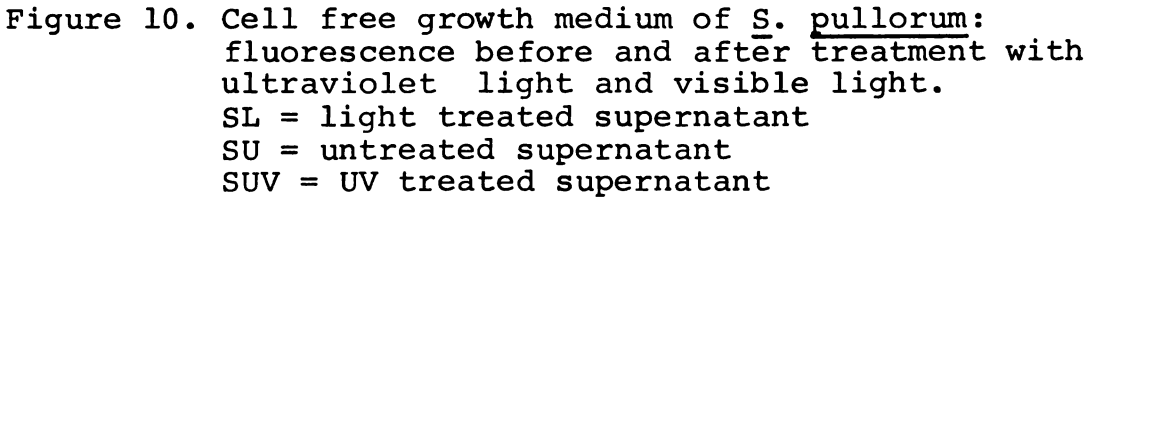


Figure 10. Cell free growth medium of S. pullorum: fluorescence before and after treatment with ultraviolet light and visible light.
SL = light treated supernatant
SU = untreated supernatant
SUV = UV treated supernatant

Figure 9

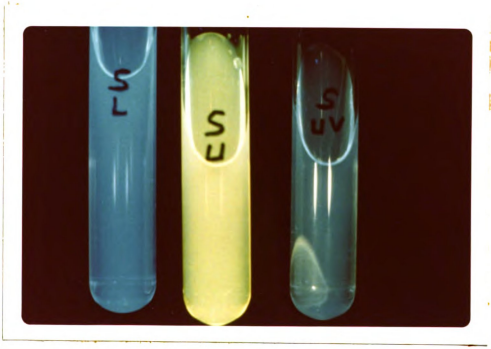


Figure 10

Figure 11. Cell free growth medium of S. pullorum: biological activity. The procedure used was that given in materials and methods. The riboflavin assay medium used in these studies was from Difco Laboratories, Detroit, Michigan. The supernatant fluid in this experiment was diluted one to three. Symbols: (●) untreated supernatant and (■) UV or visible light treated supernatant.

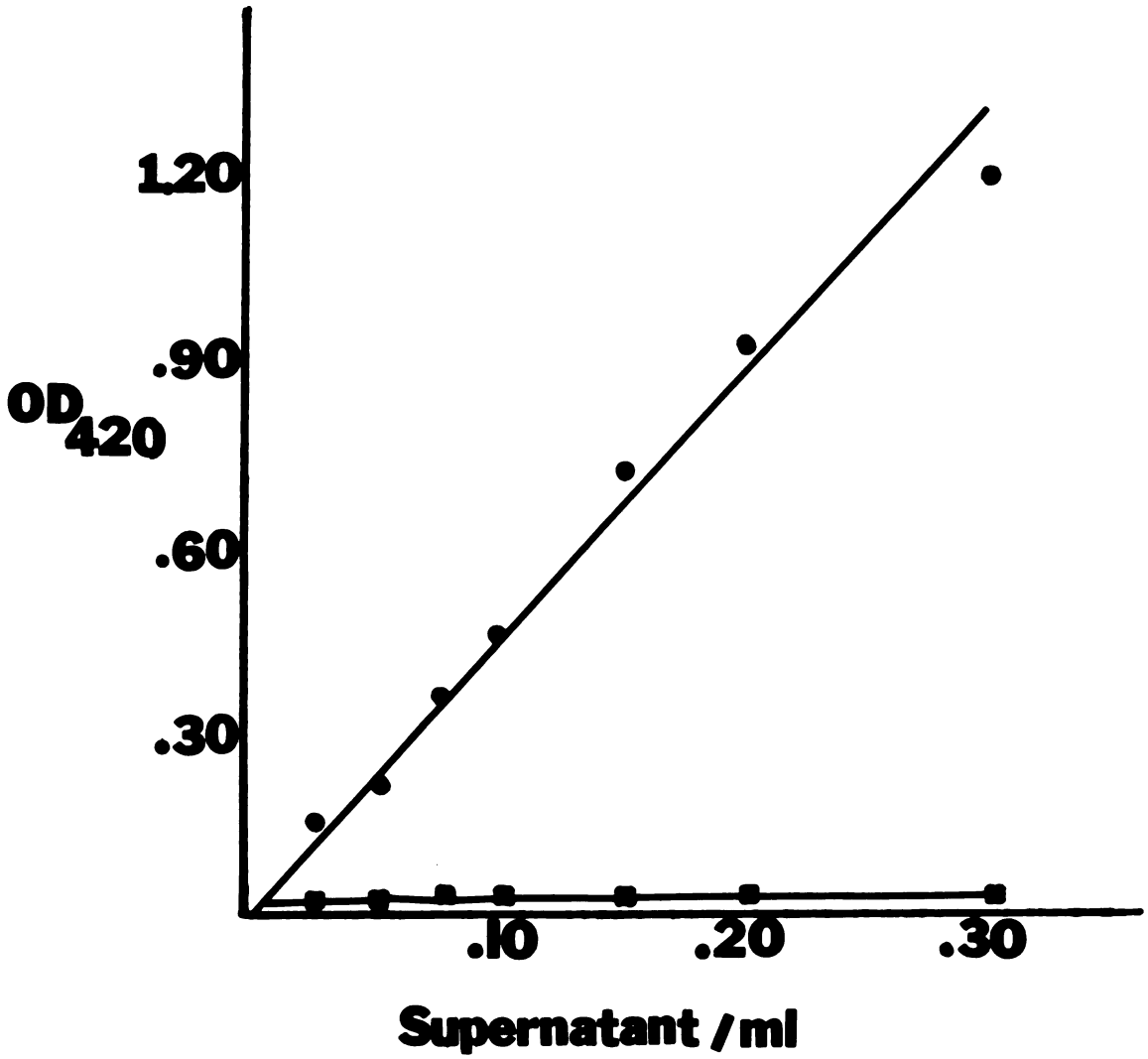


Figure 11

of E. coli or S. typhimurium. The presence of riboflavin in S. pullorum growth medium was also confirmed by paper chromatography.

Table 7. Flavins in culture supernatant fluids.

Organism	Flavin adenine dinucleotide	Flavin mononucleotide	Riboflavin
S. pullorum	1%	9%	90%
S. typhimurium	11%	42%	47%
E. coli B/r	11%	53%	36%

Table 8. Molar quantities of flavins in culture supernatant fluids.

Organism	Nanomoles flavin/mg dry weight[a]
S. pullorum 6-18	8.6
S. typhimurium LT-2	2.8
E. coli B/r	3.8

[a]Dry weights were determined by placing a 1 ml concentrated suspension of cells on a preweighed planchet. This was placed in a drying oven at 110 C for four hours. After drying and cooling, the planchet was reweighed. The dry weight of the suspending medium was subtracted.

DISCUSSION

Part I

Demonstration of STA and OASS in Extracts of S. pullorum and Their Control by Cysteine

The demonstration of STA and OASS activity in S. pullorum extracts further confirmed the hypothesis that the assimilatory reductive pathway defined in S. typhimurium, E. coli (16, 35, 51), and other microorganisms is also used by S. pullorum. These enzymes were present in extracts of both S. pullorum parental strain MS35 and the sulfate-using revertant 6-18, which agreed with the conclusion of Kline and Schoenhard (34) that the inability of S. pullorum MS35 to synthesize cysteine at 37 C was due to its inability to reduce sulfite to sulfide, and to take up sulfate from the growth medium.

The control of the above two enzymes by end-product was similar to that determined for these enzymes in S. typhimurium (35), where STA was feedback inhibited by cysteine and OASS was repressed by cysteine. O-Acetyl-L-serine (OAS), the product of the reaction catalyzed by STA, has been shown to be an inducer of cysteine biosynthetic pathway enzymes (29). The feedback inhibition of

STA by cysteine, and the absence of repression of STA synthesis, allows for an immediate response to increased intracellular levels of cysteine, and similarly, for a sharp response to decreased intracellular levels of cysteine. If STA were repressed by cysteine and not feedback inhibited, the turning off and the turning on of the pathway would be extremely slow, and certainly less advantageous to the microorganism.

Conclusive demonstration of the ability of OAS to induce cysteine pathway enzymes in S. pullorum has not been presented. However, I do have a cysteine mutant which lacks the ability to grow on any intermediate of the pathway. This mutant has less than 10% of the wild type STA activity (W. D. Hoeksema, unpublished data). However, if OAS is present in the medium, the mutant will grow on sulfate. Therefore the role of OAS in S. pullorum, like that in E. coli and S. typhimurium, appears to be to accept sulfide, thus forming cysteine, and to act as an inducer of the cysteine pathway enzymes.

The only enzyme of the assimilatory reductive pathway which has not as yet been demonstrated in extracts of S. pullorum is the PAPS (sulfate) reductase. Since the product of sulfate reduction in many microorganisms is sulfite, and since sulfate utilizing revertant 6-18 accumulates sulfite, the existence of the PAPS reductase in S. pullorum is strongly implied.

Part II

The Development of a Non-Sulfite Accumulating System

All sulfate-using revertants of S. pullorum MS35, MS38, and MS53 accumulated sulfite at 37 C. The accumulation in all cases was temperature dependent with very little sulfite accumulated at 25 C. This temperature dependent accumulation may reflect a genetic inability of S. pullorum to form a stable sulfite reductase at 37 C. Parental MS35 lacks a detectable NADPH sulfite reductase in extracts from 37 C grown cells, but these extracts do have detectable MVH sulfite reductase activities. Since both activities are repressed by cysteine (32), they probably represent catalytic properties of the same enzyme.

Sulfate-using revertant 6-18 has NADPH sulfite reductase activity as well as MVH sulfite reductase activity in extracts from 37 C grown cells. However Kline and Schoenhard (34) demonstrated that NADPH sulfite reductase specific activity was higher in extracts from cells of revertant 6-18 grown at 25 C than from cells grown at 37 C. The same phenomenon has been observed in this study. Kline and Schoenhard did not compare MVH sulfite reductase activity in extracts prepared from cells grown at 25 C and 37 C. In this study it was observed that the MVH linked activities, like the NADPH linked activities, are up to four times

higher in extracts from cells grown at 25 C than from cells grown at 37 C. Therefore, the conclusion of Kline and Schoenhard (34) that S. pullorum MS35, while lacking an NADPH sulfite reductase at 37 C maintains a MVH sulfite reductase, should perhaps be modified to state that only some or partial MVH sulfite reductase activity is maintained at 37 C.

S. pullorum is not better adapted to growth at 25 C, since it grows faster at 37 C under all conditions tested in both minimal and rich medium. The activity of other enzymes appears to be higher in extracts from cells grown at 37 C than from cells grown at 25 C, and this would be expected from an organism which grows better at the higher temperature. This author has observed that STA levels are one and a half times higher in extracts from cells grown at 37 C than from cells grown at 25 C.

These factors: the temperature sensitive accumulation of sulfite, higher sulfite reductase levels in extracts from cells grown at 25 C than from cells grown at 37 C, and the fact that S. pullorum does not have optimum growth at 25 C, specifically point to the sulfite reductase (or lack of it) as being directly involved in the phenomenon of sulfite accumulation.

The fact that sulfite accumulation occurs in all sulfate-using revertants of S. pullorum reveals that the phenomenon is not characteristic of an unusual or rare

revertant, but rather that sulfite accumulation at 37 C is an inherent characteristic of sulfate-using revertants of S. pullorum.

Part III

The Thermodependent Loss of Sulfite Reductase Activity

The sulfite reductase of S. pullorum may be extremely labile at 37 C as evidenced from the temperature sensitive accumulation of sulfite and the low sulfite reductase specific activities in extracts from cells grown at 37 C. This lability allows for the accumulation of sulfite. The data shown in Figures 3 and 4 substantiated this. NADPH sulfite reductase specific activity declined during the growth of S. pullorum at 37 C, and concommitant with this decline was an appearance of sulfite in the growth medium. No decline was observed in NADPH sulfite reductase specific activity at 25 C, and no sulfite accumulation occurred.

To confirm that the phenomenon observed was due to an in vivo lability, and not repression or the death of cells, the same type of experiment was performed except that cells were grown on djenkolate as a sulfur source. Growth on djenkolate gives maximum derepression of cysteine pathway enzymes. A loss of sulfite reductase activity at 37 C, much like that when sulfate was the sulfur source,

was observed (Table 4). In addition to a loss in NADPH linked activity at 37 C, MVH linked activity appeared to be lost also, and to approximately the same extent. No significant loss in STA activity was observed at 37 C, which points to a specific loss in sulfite reductase activity.

According to current theory on the mechanism of catalysis by sulfite reductase (45, 56, 88), electrons from MVH can be passed directly to sulfite or at least enter at a different point than electrons from NADPH. Electrons from NADPH are passed to a coupling system consisting of FAD, FMN, and other components of the enzyme. A loss of NADPH activity only, would occur if the labile component was the coupling system, while a loss of both activities as observed here is interpreted to mean that the labile component is common to both activities. Possible hypotheses would be that the labile component was involved with the binding of sulfite or with the transfer of electrons directly to sulfite. Currently, the answer to this question is not known. Since sulfite reductase activity was lost when S. pullorum was grown on sulfate or djenkolate as sulfur source and since STA activity was not lost, repression or lack of induction appeared not to be influential in the accumulation of sulfite.

Kline (32) indicated that the sulfite reductase of S. pullorum was unstable when he reported that high ionic strength extraction (0.2M phosphate) was required for

prolonged stability of NADPH sulfite reductase. Furthermore, he (32) reported that extended linear production of sulfide by NADPH sulfite reductase could only be achieved at 25 C and not at 37 C. At 37 C the reaction was complete in less than 5 minutes.

Part IV

Characterization of Sulfite Accumulation

A thermolabile enzyme can be characterized in several ways. The first question asked was whether the phenomenon was reversible in vivo. As seen from temperature shift experiment (Figure 5) there was no total reversibility of sulfite accumulation over the course of eight hours. Therefore, once the enzymatic activity is lost during growth at 37 C, activity is not restored upon a shift back to 25 C. Similarly, once the enzyme is made in a "stable" form at 25 C, it is not readily denatured by a shift to 37 C. In a 25 C to 41 C shift shown in Figure 6, some sulfite accumulated, indicating that some in vivo denaturation of enzyme made at 25 C did occur. The amount of sulfite accumulated was small compared to either the 37 C to 37 C or the 37 C to 25 C shifts. It appeared that sulfite reductase synthesized at 25 C and sulfite reductase synthesized at 37 C were uniquely different products. Enzyme formed at 37 C was labile and not

restored upon a shift to 25 C. Enzyme made at 25 C was stable and continued functioning upon a shift to higher temperature.

In the cultures involved in the 25 C to 25 C and the 25 C to 37 C shifts (Figure 5), sulfide was generated. In the opinion of this author sulfide generation may result from either of two possible mechanisms. It may have resulted from the production of cysteine, which then feedback inhibited STA with the result that no OAS was available to accept sulfide, or it may have resulted from the desulfurylation of cysteine. Both mechanisms require the production of significant amounts of cysteine, which requires a functional sulfite reductase. Therefore, the generation of sulfide in a culture shifted from 25 C to 37 C also is evidence that the sulfite reductase was not readily denatured.

If the sulfite reductase is labile, then increasing the growth temperature above 37 C would completely block cysteine synthesis from sulfate. At 41 C, S. pullorum 6-18 has the ability to grow on only sulfur compounds and intermediates which enter into the pathway after the sulfite reductase catalyzed step (Table 5). Any intermediate or sulfur compound which enters the pathway previous to this step will not support growth at 41 C. Thus, in the case of sulfite reductase from S. pullorum 6-18, 25 C would be the permissive temperature, 41 C the restrictive temperature, and

37 C would be a partially restrictive temperature. The designation of partially restrictive to 37 C is based on the observation that, although the enzyme is labile at this temperature, growth on sulfite, CSA, or sulfate as sole sulfur sources is possible.

Sulfite accumulation at 37 C from sulfate was a tool with which to determine whether cysteine exerted a feedback inhibition on the uptake or activation of sulfate. Either inhibition would result in a decrease in the sulfite accumulated. Cysteine in amounts as high as 20 ug/ml did not significantly inhibit sulfite accumulation (Figure 7) and therefore there is presumably no feedback inhibition by cysteine on the uptake or activation of sulfate. This result would tend to support the data of Dreyfuss and Pardee (19) who demonstrated that PAPS, not cysteine, inhibited the uptake of sulfate in S. typhimurium. These data would contradict with the conclusion of Ellis et al. (21) that cysteine does exert a feedback inhibition on the uptake or activation of sulfate. The feedback inhibition observed by Ellis et al. may have been due to the feedback inhibition of STA by cysteine, which therefore lowered the OAS levels, thus preventing $^{35}SO_4^=$ incorporation. Ellis et al. conclusions were drawn from an in vivo experiment, and they were unable to obtain in vitro feedback inhibition of sulfate activation. The stimulation of sulfite

accumulation by low amounts of cysteine (4 ug/ml) was seen in several experiments, and was also observed by Kline (32). It is interesting to note in this respect that Ellis *et al.* (21) and Pasternak (51) both reported that the presence of L-cysteine in reaction mixtures stimulated the production of PAPS by extracts of *E. coli*.

Viable count experiments revealed that sulfite accumulation was *not* associated with a death phase and growth of *S. pullorum* on glycerol (0.5% V/V) as a carbon source also allowed sulfite accumulation to approximately the same levels observed when glucose was used. This provided evidence against catabolite repression being involved in sulfite accumulation.

Part V

Sulfite Reductase Lability *in Vitro*

The thermolabile sulfite reductase of *S. pullorum* 6-18 is active *in vitro*. In this sense it is unlike many other temperature sensitive enzymes which are inactive *in vitro* (8, 10, 46), even when they are prepared from extracts grown at permissive temperatures.

The thermostability of the sulfite reductase in extracts from cells grown at both 25 C and 37 C was examined. Sulfite reductase from cells of *S. pullorum* grown at 37 C is considerably more labile than the sulfite

reductase from cells of S. pullorum grown at 25 C or cells of S. typhimurium grown at 37 C (Figure 8). Some variation in these experiments appeared from one extract to the next, and therefore a certain amount of caution must be exercised in the interpretation of these data. However, the sulfite reductase from cells of S. pullorum grown at 25 C appears to be more stable than the sulfite reductase from cells of S. pullorum grown at 37 C, and therefore certain, as yet unidentified, bonds may be formed when the enzyme is made at 25 C that are absent when the enzyme is made at 37 C. The absence of these bond(s) leads directly or indirectly to the thermolability of 37 C sulfite reductase in vitro and in vivo.

The model this author favors is that there are at least two conformations a sulfite reductase molecule can assume upon being made. One leads to an extremely thermolabile sulfite reductase, and one leads to a relatively stable sulfite reductase. At 25 C primarily stable sulfite reductase is formed, thus almost no sulfite accumulates in a culture shifted from 25 C to 37 C, only a small amount of sulfite accumulates in a culture shifted from 25 C to 41 C, and the enzyme is more stable in vitro than sulfite reductase from cells grown at 37 C. At 37 C primarily thermolabile sulfite reductase is made. Thus, sulfite accumulates during growth at 37 C, sulfite accumulates in a culture shifted from 37 C to 25 C, and the

enzyme has a strong thermolability *in vitro*. At 41 C only thermolabile sulfite reductase is produced, and because of the increase in temperature (37 C vs. 41 C) which decreases the functional half life *in vivo*, no growth is possible on sulfur intermediates prior to the sulfite reductase catalyzed step.

Part VI

The Accumulation of Flavins by *S. pullorum*

This study came out of the observation that during growth of *S. pullorum* a distinct yellow discoloration of the growth medium occurs. Since sulfite had already been identified as an accumulation product, the identification of a second accumulation product could potentially clarify the mechanisms responsible for sulfite accumulation. As the data indicate, this substance appears to be primarily riboflavin. There does not appear to be a relatedness between sulfite accumulation and riboflavin accumulation. Therefore, riboflavin accumulation may reflect the fact that enzymes determining flavin biosynthesis in *S. pullorum* are very poorly regulated, which results in an extreme flavin overproduction.

SUMMARY

S. pullorum strains MS35, MS38, and MS53 are cystine auxotrophs. At 37 C there appeared to be double blocks in the assimilatory reductive pathway preventing the utilization of sulfate as a sole sulfur source while there was a single block at 25 C. Sulfate-using revertants of all three strains accumulate large amounts of sulfite in the growth medium when the organism is grown on sulfate at 37 C, but this accumulation is negligible at 25 C. This supported the hypothesis that *S. pullorum* accumulated sulfite because of a thermolabile sulfite reductase. This thermolability was substantiated by demonstrating that both NADPH and MVH sulfite reductase specific activities are lost during the growth of *S. pullorum* at 37 C but not at 25 C. As the sulfite reductase activity is lost, there is an appearance of sulfite in the growth medium. Sulfite reductase appeared to be the only enzyme which showed this extreme loss during growth at 37 C, as STA levels remained essentially constant. At 41 C sulfate-using revertants of *S. pullorum* lose the ability to grow on sulfate, sulfite, and CSA, while retaining the ability to grow on sulfide, cysteine, and djenkolate. Thus, phenotypically they appear as sulfite reductaseless mutants at 41 C. The *in vitro* lability of

NADPH sulfite reductase from cells of S. pullorum grown at 37 C appeared to be considerably greater than the lability of this enzyme from S. pullorum grown at 25 C or from S. typhimurium LT-2 grown at 37 C. S. pullorum appears therefore to form a thermolabile sulfite reductase when grown at 37 C.

Feedback inhibition appears to be operative in the assimilatory reductive pathway in S. pullorum. STA was feedback inhibited in S. pullorum as it was shown to be in S. typhimurium. Cysteine feedback inhibition on the uptake or activation of sulfate was not demonstrated in S. pullorum, as evidenced by the fact that exogenous cysteine added to the growth medium of S. pullorum, in which protein synthesis was arrested by CAP, did not significantly inhibit sulfite accumulation. However, this lack of feedback inhibition on the uptake or activation was not necessarily unique to S. pullorum, and may well point to the fact that feedback inhibition at this point is mediated by a compound other than cysteine.

The cysteine biosynthetic pathway is not the only pathway which shows unusual genetic blocks in S. pullorum. Flavins, primarily in the form of riboflavin, also accumulate in the growth medium in larger than expected amounts. The biochemical mechanisms which allow for the accumulation of riboflavin by S. pullorum are unknown.

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