Studies on the Mechanism of Inhibition of *Salmonella typhimurium* by 1,2,4-Triazole*

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The inhibition of *Salmonella typhimurium* by 1,2,4-triazole appears to be mediated through an effect on L-cysteine biosynthesis. O-Acetylserine sulfhydrylase A, the final enzyme in the L-cysteine biosynthetic pathway, was found to catalyze a reaction (triazolylase) between O-acetyl-L-serine and 1,2,4-triazole, giving 1,2,4-triazole-1-alanine as a product. In wild type *S. typhimurium* grown on 4 mM 1,2,4-triazole, 97% of the total O-acetyl-L-serine synthesized *in vivo* is incorporated into 1,2,4-triazole-1-alanine. 1,2,4-Triazole also significantly lowers the levels of several of the enzymes necessary for sulfate reduction. This effect is presumably due to the ability of the inhibitor to lower intracellular concentrations of O-acetyl-L-serine, an inducer of these enzymes. Inhibition of growth is probably caused by L-cysteine starvation, arising from the decreased availability of the L-cysteine precursors, sulfide and O-acetyl-L-serine.

Two 1,2,4-triazole-resistant strains bearing mutations in cysK, the structural gene for O-acetylserine sulfhydrylase A, incorporate only small quantities of O-acetyl-L-serine into 1,2,4-triazole-1-alanine *in vivo*. *In vitro* studies, using purified preparations of O-acetylserine sulfhydrylase A, revealed greater losses of triazolylase activity than sulfhydrylase activity in the enzymes from both cysK mutants. Resistance to 1,2,4-triazole apparently can arise from mutations leading to a preferential loss of triazolylase activity or from mutations which diminish both activities to the extent that high concentrations of O-acetyl-L-serine and sulfide accumulate behind the sulfhydrylase reaction.

The growth of *Salmonella typhimurium* is inhibited by 1,2,4-triazole (1). Although the exact mechanism by which this inhibition occurs has not been established, previous studies have shown that the effect of 1,2,4-triazole can be overcome by the single addition of L-cysteine, sulfate, sulfide, L-serine, L-methionine, or O-acetyl-L-serine to the growth medium (1, 2). One obvious, common denominator to all of these compounds is L-cysteine itself, for sulfite and sulfide are readily utilized precursors of the sulfur moiety of this amino acid (3). L-serine contributes its carbon skeleton to that of L-cysteine (4), and L-methionine spares the L-cysteine requirements of cysteine auxotrophs by about 50% (5). O-Acetyl-L-serine is not only a direct precursor of L-cysteine in *S. typhimurium* (4, 6, 7), but also acts as an inducer of the enzymes responsible for the synthesis of sulfide from inorganic sulfate (8-10).

Evidence suggesting that 1,2,4-triazole may interfere with one or both of these functions of O-acetyl-L-serine originates from the observation that mutants partially defective in their ability to synthesize the latter are unusually sensitive to the action of 1,2,4-triazole (2). Furthermore, in cells grown on sulfate as the sole sulfur source, 1,2,4-triazole prevents the induction of sulfate permease, ATP sulfurylase, and adenosine 5'-phosphosulfate kinase by O-acetyl-L-serine (2). 1,2,4-Triazole-resistant, mutant strains of the types trzA and trzB have been shown to be insensitive to this anti-inducer property of 1,2,4-triazole, and when grown on sulfate in the absence of this inhibitor, such strains are slightly more depressed than wild type for certain of the cysteine biosynthetic enzymes (11).

We have recently demonstrated that trzA strains carry mutations in the structural gene for O-acetylserine sulfhydrylase A, and have proposed that this gene locus be designated cysK (12). The prototrophic phenotype of cysK strains is thought to be due to the presence in *S. typhimurium* of a second sulfhydrylase, O-acetylserine sulfhydrylase B (13). *TrzB* strains also have very low levels of O-acetylserine sulfhydrylase A and have genetic characteristics which suggest that they bear the cysK locus on a plasmid (11, 12, 14). The fact that resistance to 1,2,4-triazole results from a loss of O-acetylserine sulfhydrylase A activity lends additional support to the notion that 1,2,4-triazole inhibits growth either by interfering with the production or utilization of O-acetyl-L-serine or by otherwise preventing the induction of the enzymes of the cysteine biosynthetic pathway by O-acetyl-L-serine.

In this report, we present our finding that in the presence of O-acetylserine sulfhydrylase A, 1,2,4-triazole reacts with O-
acetyl-L-serine to form a 1,2,4-triazole-substituted derivative of alanine. Evidence is presented which indicates that this reaction occurs to a significant extent in vivo, and is probably responsible for the ability of 1,2,4-triazole to inhibit the growth of wild type S. typhimurium.

**Experimental Procedure**

Materials—O-Acetyl-L-serine was synthesized by the method of Sakami and Toennies (15). 1,2,4-Triazole and adenosine 5-phosphosulfate were purchased from Sigma, and 1,2,4-triazole-3-alanine from Fox Chemical Co. was a gift from Dr. A. Ames. 1,2,4-Triazole and HgCl2 were prepared from New England Nuclear. Purified serine transacylase (as cysteine synthetase) and O-acetylserine sulfhydrylase A from wild type and mutant strains were prepared as previously described (6, 7, 12). For all enzymes described in this communication, 1 unit of activity is defined as that amount catalyzing the reaction of 1 mmol/min of substrate under the stated assay conditions.

**Bacterial Methods—**Wild type Salmonella typhimurium 172 and the previously described cynK mutant strains DW130 (cynK751) and DW132 (trpA160, pyrFl46, cynK752) (12) were grown in a modified minimal salts medium (10) containing 0.6 mM sulfate as the sole sulfur source and 5 g of glucose/liter. Uracil, 10 mg/ml, and L-tryptophan, 50 mg/ml, were added for growth of strain DW132, and solid medium was prepared by the addition of 15 g of Difco agar/liter of minimal medium. Resistance to 1,2,4-triazole was determined by scoring for growth on minimal plates containing this inhibitor at a concentration of 10 mm (11). Cells were grown, harvested, and assayed for the enzymes of cysteine biosynthesis as previously described (10).

**Assay for O-Acetyl-L-serine—**O-Acetyl-L-serine was determined by incubating samples with an excess of sulfide in the presence of a large amount of O-acetylserine sulfhydrylase A, and measuring the amount of cysteine formed. A solution containing 10 to 100 nmol of O-acetyl-L-serine was added to a 13 x 100 mm test tube containing 2 units of purified O-acetylserine sulfhydrylase A, and the volume was adjusted to 0.1 ml with water. Following the addition of 0.10 ml of 0.2 M Tris-HCl, pH 7.2, containing 1 mM Na2EDTA and 6 mM Na2SO4, the tube was capped and incubated at 23° for 15 min. The reaction was terminated by the addition of the reagents used to assay for aliphatic thiols in the routine assay for O-acetylserine sulfhydrylase A activity (6), and the amount of cysteine formed was determined spectrophotometrically. Using gravimetrically standardized solutions of O-acetyl-L-serine, the amounts of cysteine formed were found to be 92% of those expected theoretically, and were linearly proportional to the amounts of added substrate. 1,2,4-Triazole at concentrations as high as 30 mm did not interfere with this assay.

**Synthesis of Radiolabeled Compound TS—**A mixture was prepared containing 75 mmol of Tris-HCl, pH 7.6; 0.09 mmol of L-[U-14C]serine (115 Ci/mol); 0.5 mmol of acetyl-CoA; 20 mmol of 1,2,4-triazole; 0.7 unit of purified serine transacylase (as cysteine synthetase), and 28 units of purified wild type O-acetylserine sulfhydrylase A in a final volume of 0.75 ml. The reaction was allowed to proceed for 30 min at 23°, at which time the solution was titrated to pH 11 with 1 N NaOH to convert any remaining O-acetyl-L-serine to N-acetylserine. After 10 min, the solution was diluted to 10 ml of 0.01 N HCl, and sufficient 6 N HCl was added to give a final pH of 2. This material was applied to a 5-ml column of AG 50W-X8-H+, 200 to 400 mesh resin, which was then washed with 20 ml of water. The absorbed radiolabel was eluted with 1 N NH4OH, concentrated to a small volume, and purified by high voltage electrophoresis for 4 hours on Whatman No. 3MM paper in 0.04 M pyridine acetate, pH 3.6, at 50 volts/cm. Compound TS was localized by autoradiography, eluted from the paper with water, and concentrated to a small volume in a rotary evaporator. The final product contained 34% of the original radiolabel, and was free of detectable serine, O-acetylserine, N-acetylserine, and 1,2,4-triazole.

**Assay of Culture Media for Compound TS—**Overnight cultures of bacteria were inoculated at a cell density of 1 x 10^8 cells/ml into fresh, prewarmed minimal salts medium containing 0.8 mM sulfate as the sole sulfur source and 0.5% glucose. After a period of 2 hours, when the bacteria were growing at a rate of 1 x 4.5 to 5.0 fold, 1,2,4-triazole was added at a final concentration of 4 mM. Bacterial growth was monitored by measuring turbidity at 650 nm, and samples of culture fluid were removed at different times for the determination of Compound TS.

Following the removal of cells from the medium by centrifugation, a 10- to 30-ml portion of each supernatant was diluted with 4 volumes of water, and 1 nmol of 14C-labeled Compound TS was added for subsequent isotope dilution measurements. Each solution was then adjusted to pH 2 with 6 N HCl and passed through a column (25 cm x 0.4 cm) of AG 50W-X8-H+, 200 to 400 mesh resin. After washing the resin with 4 volumes of water, the absorbed radiolabel was eluted with 1 N NH4OH, concentrated to dryness in a rotary evaporator, and dissolved in 0.5 ml of water. The recovery of added radiolabel Compound TS averaged about 70% through these steps.

A sample of each medium, containing a known fraction of the originally added radiolabel, was assayed for Compound TS on a Beckman model 110 automatic amino acid analyzer, using a modification (17) of the conventional procedure for protein hydrolysates (18) in which Compound TS was found to elute with aspartic acid. High voltage electrophoresis of these samples at pH 1.9 revealed no detectable quantities of aspartic acid. After the appropriate calculations for isotope dilution, the results were expressed in terms of the concentration of Compound TS present in the original culture medium.

**Triazolylase and Sulfhydrylase Assays—**The rate of enzymatic reaction between 1,2,4-triazole and O-acetyl-L-serine was determined by assaying either for the disappearance of O-acetyl-L-serine, or for the production of alkali-resistant, ninhydrin-reactive material. This activity is termed "triazolylase.

Our standard assay mixture contained 2 mm O-acetyl-L-serine; 20 mm 1,2,4-triazole; 0.1 mm Na2EDTA; 0.05 mg/ml of bovine serum albumin; 0.1 M Tris-HCl, pH 7.2; and varying amounts of highly purified O-acetylserine sulfhydrylase A in a final volume of 0.2 ml. The reaction was carried out at 23°, and when substrate disappearance was to be followed, aliquots portions of the incubation mixture were removed at varying times and assayed for remaining O-acetyl-L-serine. For the measurement of alkali-resistant, ninhydrin-reactive material, the reaction was terminated after 10 min of incubation by the addition of 0.1 ml of cold 1.4 M acetic acid, followed by chilling in ice. Five minutes later, 0.2 ml of 1.0 M Na2CO3 in 0.2 N NaOH was added, and the mixture was incubated at 23° for 10 min to convert any remaining O-acetyl-L-serine to its ninhydrin unreaction active isomer, N-acetyl-L-serine. Ninhydrin-reactive product was then determined by adding 0.1 ml of 7 M acetic acid (to neutralize the alkalii) and 2.4 ml of ninhydrin reagent (19), and then heating in a boiling water bath for 20 min. After cooling, the absorbance at 570 nm was measured, and the concentration of product formed was calculated using an extinction coefficient determined for purified product (Compound TS) in an identical assay. Appropriate blanks were used to correct for the amount of color contributed by the Tris buffer and bovine serum albumin in the reaction mixture, and by a small contaminant of L-serine in our O-acetyl-L-serine, 1,2,4-Triazole at concentrations as high as 50 mm neither contributed to nor interfered with color development.

Using this assay, the amount of ninhydrin-reactive material enzymatically produced was linearly proportional to the time of incubation for 35 min, and to the amount of added enzyme up to at least 1.8 mm product formation.

The assay used for the enzymatic synthesis of L-cysteine from O-acetyl-L-serine and sulfide has been described (6).

**Other Methods—**Thin layer chromatography was performed on 250 μm thick plates of Silica Gel G obtained from Analtech Inc. with 10% to 20% Conray B100. Thin layer plates were visualized by the iodide procedure of Rydon and Smith (20), and ninhydrin-reactive compounds were localized by spraying with 0.2% ninhydrin in ethanol. Autoradiography was performed as described previously (4).

Radiolabel was determined by mixing aqueous samples with a toluene-based scintillation fluid containing Triton X-100 (21) and counting in a Mark I scintillation counter at 80% efficiency for both 14C and 35S. All counts were corrected for quench using an external standard. Proton NMR spectroscopy at 100 MHz was performed using a JOELCO model MH-100 spectrometer. Chemical shifts were calculated downward from an internal tetramethylsilane standard.

Protein was determined by the method of Lowry et al. (22) using bovine serum albumin as a standard, and glutathione was assayed by a procedure which detects both the reduced and oxidized forms of this compound (23).

**RESULTS**

Preliminary experiments showed that 1,2,4-triazole at concentrations as high as 50 mM did not inhibit O-acetylserine
sulfhydrylase A activity when assayed at O-acetyl-L-serine concentrations of 1.0 to 100 mM and at sulfide concentrations of 0.5 to 3 mM. However, when 1,2,4-triazole and O-acetyl-L-serine were incubated with enzyme in the absence of sulfide, a time-dependent loss of O-acetyl-L-serine was noted. The rate of consumption of this substrate was found to be constant with time between O-acetyl-L-serine concentrations of 1.0 and 0.3 mM (Fig. 1), and directly proportional to the concentrations of enzyme and 1,2,4-triazole (Fig. 2).

Semiquantitative analyses of such reaction mixtures by thin layer chromatography (Table I) also revealed a time-dependent loss of O-acetyl-L-serine together with the appearance of a ninhydrin-reactive compound distinct from serine and the ninhydrin-unreactive compounds, N-acetylserine and 1,2,4-triazole. In reaction mixtures containing large amounts of enzyme and O-acetyl-L-serine in molar excess over 1,2,4-triazole, a decrease in the latter compound also was noted with time. We inferred from these data that O-acetylserine sulfhydrylase A catalyzes a reaction between O-acetyl-L-serine and 1,2,4-triazole giving a ninhydrin-reactive product, which we provisionally designated Compound TS. The enzymatic synthesis and purification of gram quantities of this material were accomplished as described below.

**Preparation and Characterization of Compound TS**—A solution was prepared containing 60 mmol of 1,2,4-triazole and 20 mmol of O-acetyl-L-serine in 100 ml of water, and, after adjustment of the pH to 7.2 with 0.5 M triethylamine, the reaction was started by the addition of 3 mg (3450 units) of highly purified, wild type O-acetylserine sulfhydrylase A in a small volume of 0.1 M Tris-HCl, pH 7.6. This mixture was maintained at pH 7.0 to 7.4 by the frequent addition of 0.5 M triethylamine, and after 1 hour at 23°C, an additional 20 mmol of dry O-acetyl-L-serine and 1 mg of enzyme were added. After another hour of incubation, the reaction was terminated by adjusting the pH to 9.0 with triethylamine. The solution was evaporated to dryness in a rotary evaporator at 30°C, whereupon the residue was extracted with 60 ml of warm water and filtered. Five volumes of ethanol were added to the filtrate, and, after storage for 16 hours at –20°C, the resultant precipitate was collected by filtration. The precipitate was washed with cold ethanol and air dried, giving 3.80 g of white crystalline material. This product was dissolved in 30 ml of warm water and recrystallized by the addition of 2 volumes of cold ethanol, followed by overnight storage at –20°C. The final yield was 2.66 g.

The crystalline product has a melting point of 255–257°C (with decomposition), and, after either thin layer chromatography in several solvent systems, or paper electrophoresis at pH 1.9 and at pH 3.6, gives a single spot reactive to both ninhydrin or by the chlorine/starch/iodide technique (20). Good separations between Compound TS, L-serine, O-acetyl-L-serine, N-acetyl-L-serine, L-cystine, and 1,2,4-triazole can be achieved using the systems noted in Table I.

**Acid-base titration curves at 23°C between pH 1 and pH 12 revealed the presence of titratable groups with pH values of 1.7 and 7.8 for Compound TS, 2.1 and 9.2 for L-serine, and 1.8 and 10.2 for 1,2,4-triazole. The ninhydrin reactivity of Compound TS and the pH of 7.8 indicate that the amino group of the serine-derived portion of this compound is free, a conclusion with which the infrared absorption spectrum is compatible.**

The infrared absorption spectrum of Compound TS also shows an intense, broad band at 1600 cm⁻¹, characteristic of a free carboxylate group, which very likely accounts for the pK of 2.1.
From these data and the fact that Compound TS is formed from O-acetyl-L-serine and 1,2,4-triazole by an enzyme which normally effects a substitution at the β carbon atom of serine, it seems most likely that this product is 1,2,4-triazolylalanine. The elemental analysis agrees quite well with this possibility.

$$C_8H_7N_2O_2$$

Calculated: C 38.46, H 5.16, N 35.88
Found: C 38.3, H 5.26, N 35.7

Three possible 1,2,4-triazolylalanines exist in which the β carbon atom of alanine is attached to either the N-1, the N-4, or the C-3 position of 1,2,4-triazole. The C-3-substituted compound, 1,2,4-triazole-3-alanine, is known to inhibit the growth of S. typhimurium in the presence of 20 mM 3-amino-1,2,4-triazole (24). Compound TS does not inhibit S. typhimurium under these conditions, and can be separated from authentic 1,2,4-triazole-3-alanine by paper electrophoresis (Table I). Furthermore, the infrared absorption spectra of these two compounds were found to differ in many respects.

Proton nuclear magnetic resonance spectroscopy was used to differentiate between the remaining two possible structures. The proton NMR spectrum of Compound TS in D₂O at 100 MHz at 30° is shown in Fig. 3, in which are seen the expected doublet for the methylene protons at δ 66.16 ppm, and a triplet for the α proton at 64.64 ppm. Two singlets at δ 68.39 ppm and δ 68.77 ppm also are noted, which, by exclusion, may be assigned to the C-H protons of the triazole ring. Since the ring C-3 and C-5 positions are asymmetrical, and we conclude, therefore, that Compound TS is 1,2,4-triazole-1-alanine. We assume that the configuration of the alanine portion of this compound is that of L-alanine.

**Kinetic Studies**—Our proposed structure for Compound TS implies that 1,2,4-triazole can serve as a sulfide analogue in a reaction catalyzed by O-acetylserine sulphydrylase A, giving 1,2,4-triazole-1-alanine as a product rather than L-cysteine. Thus, we conclude that O-acetylserine sulphydrylase A also has O-acetylsulhydrylase activity.

Since measuring the rate of consumption of O-acetyl-L-serine is a relatively insensitive and inaccurate method for determining triazolylase activity, a new assay was developed (described in detail under “Experimental Procedure”), in which the appearance of alkali-resistant, ninhydrin-reactive product is measured. This method is based on the fact that the triazole reaction is unaffected by such treatment. Using this assay with wild type O-acetylsulphydrylase A, kinetic experiments confirmed the conclusions drawn from experiments in which substrate disappearance was measured. The rate of the triazolylase reaction was found to be first order with regard to 1,2,4-triazole at concentrations as high as 50 mM (2 mM O-acetyl-L-serine), and zero order or saturated for O-acetyl-L-serine at concentrations as low as 0.5 mM (20 mM 1,2,4-triazole).

Under our standard assay conditions of 2 mM O-acetyl-L-serine and 20 mM 1,2,4-triazole, 1 unit of wild type sulphydrylase activity is equivalent to 0.0063 unit of triazolylase activity (Table II), where a unit of either enzyme activity is defined as that amount which catalyzes the formation of 1 µmol of product/min. It should be noted that the sulphydrylase is assayed under conditions of near saturation for both O-acetyl-L-serine (100 mM with a $K_m$ of 4 mM) and sulfide (2.6 mM with a $K_m < 0.5$ mM), while the triazolylase reaction is saturated only for O-acetyl-L-serine.

The results of similar analyses of the purified O-acetylsulphydrylase A enzymes from two 1,2,4-triazole-resistant cysK strains, DW130 and DW132, are also contained in Table II. Although the mutant protein from DW130 retains 1.6% of the wild type level of sulphydrylase activity, the triazolylase activity of this enzyme is only 0.62% that of wild type. This preferential loss of triazolylase activity is even more dramatically evident in the case of the DW132 enzyme, which has 31% of wild type sulphydrylase activity, but only 1.6% of wild type triazolylase activity. Thus, while the ratio of sulphydrylase to triazolylase activity is 158 in the wild type enzyme, this ratio is 418 for the DW130 protein and 3105 for the enzyme from DW132.

**Dissociation of Cysteine Synthetase**—Cysteine synthetase, a bifunctional protein complex from S. typhimurium, is dissociated by low concentrations of O-acetyl-L-serine into 2 molecules of O-acetylserine sulphydrylase A and 1 molecule of

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**Table II**

<table>
<thead>
<tr>
<th>Strain</th>
<th>O-Acetylserine sulphydrylase</th>
<th>O-Acetylserine triazolylase</th>
<th>Ratio of activities: sulphydrylase to triazolylase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg % wild type</td>
<td>units/mg % wild type</td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td>1153</td>
<td>100</td>
<td>7.3</td>
</tr>
<tr>
<td>DW130</td>
<td>18.8</td>
<td>1.6</td>
<td>0.045</td>
</tr>
<tr>
<td>DW132</td>
<td>354</td>
<td>31</td>
<td>0.114</td>
</tr>
</tbody>
</table>

Assays were performed using highly purified preparations of O-acetylserine sulphydrylase A from each bacterial strain. Sulphydrylase activity was assayed using 100 mM O-acetyl-L-serine and 2.6 mM sulfide, while triazolylase activity was assayed at 2 mM O-acetyl-L-serine and 20 mM 1,2,4-triazole. Specific activities have been calculated from protein concentrations which were estimated using an $K_m$ for purified enzyme of 0.346 ml mg⁻¹ cm⁻¹ (6).
serine transacetylase (7). Since sulfide is known to prevent this dissociation, we investigated whether 1,2,4-triazole might act in a similar manner.

The assay used for these studies has been described (7), and is based on the fact that the O-acetylserine sulphydrylase enzyme activity of the cysteine synthetase complex is only about one-half that expected from its content of O-acetylserine sulphydrylase A protein. The increase in enzyme activity noted after a brief preincubation with O-acetyl-L-serine is taken as a measure of the amount of dissociation of the complex to give fully active O-acetylserine sulphydrylase A. The actual preincubation time is not crucial, because an equilibrium between fully active and partially active enzyme is reached in less than a minute after the addition of O-acetyl-L-serine. Dissociation of cysteine synthetase by the high concentration of substrate used in the actual enzyme assay is prevented by starting the reaction with the simultaneous addition of O-acetyl-L-serine and sulfide.

Although the dissociation of cysteine synthetase appears to be a complicated phenomenon, we find it convenient to analyze our data by a method based on an arbitrary mechanism in which 1 molecule of the enzyme complex reacts with 1 molecule of O-acetyl-L-serine to give an enzyme form with full activity. This activated species of enzyme is not necessarily free, dissociated O-acetylserine sulphydrylase A. This simple scheme may be represented as:

\[ E + A \rightarrow EA \]  

(1)

where \( E \) is nonactivated cysteine synthetase, \( A \) is free O-acetyl-L-serine (always in large excess over total enzyme), and \( EA \) is "activated enzyme" containing 1 mole of bound O-acetyl-L-serine. By analogy with the derivation of the Michaelis-Menten equation (25) and one of its linear transforms (26), the following expression can be derived:

\[ \frac{1}{(EA)} = \frac{K_d}{E_t(A)} + \frac{1}{E_t} \]  

(2)

where \( K_d \) is the dissociation constant for \( EA \) in Equation 1 and \( E_t \) is the sum of \( E \) and \( EA \). A double reciprocal plot of activation data can then be used to obtain apparent values for \( K_d \) (that concentration of O-acetyl-L-serine necessary for half-maximum activation) and \( E_t \) (the maximum amount of activation possible).

The data in Fig. 4 show that 1,2,4-triazole does inhibit the activation of cysteine synthetase. Double reciprocal plots of these data (Fig. 5) give apparent values for \( K_d \) which range from 0.11 mM in the absence of inhibitor to 1.6 mM at 10 mM 1,2,4-triazole, and the intersection of these plots at the same point on the vertical axis suggests that inhibition of activation is competitive with O-acetyl-L-serine. If one assumes that 1,2,4-triazole competes with O-acetyl-L-serine for a binding site on \( E \), our model becomes equivalent to that given for competitive inhibition of a single substrate enzyme reaction (27). The slopes of our double reciprocal plots then would be equal to \( K_d(1 + I/K_c)/E_t \), where \( I \) is the concentration of inhibitor, and \( K_c \) is an inhibition constant for 1,2,4-triazole in the reaction between nonactivated cysteine synthetase and O-acetyl-L-serine. In this case, a replot of slopes versus inhibitor concentration should give a straight line with a horizontal intercept equal to \(-K_c\). The straight line obtained from such a replot of our data gives a value for \( K_c \) of 1.1 mM (Fig. 5).

Direct assays of preincubation mixtures revealed that the triazolylase reaction utilized less than 15% of the O-acetyl-L-serine initially added, ruling out O-acetyl-L-serine depletion as a possible explanation for these results. Control studies showed that the inclusion of 20 mM 1,2,4-triazole or 1 mM Compound TS in the enzyme assay itself does not inhibit significantly the O-acetylserine sulphydrylase activity of either nonactivated or previously activated cysteine synthetase, and 1 mM Compound TS neither activates nor inhibits the activation of cysteine synthetase.

In Vivo Synthesis of Compound TS—The addition of 4 mM 1,2,4-triazole to a dilute, exponentially growing culture of wild type S. typhimurium causes a decrease in growth rate, which becomes increasingly pronounced over a period of several
hours. In the experiment depicted in Fig. 6, the generation time gradually rose from 50 min to a value of 750 min within 4 hours after the addition of 1,2,4-triazole. This slow growth rate then remained constant for approximately 18 hours, at which time the culture became overgrown with spontaneously arising, mutant bacteria resistant to this inhibitor.

Analyses of the media from cultures treated with 1,2,4-triazole revealed the presence of a ninhydrin-reactive material which was shown by thin layer and ion exchange chromatography and by high voltage electrophoresis to be identical with Compound TS. This material accumulated at a rate which was approximately linear with time, and eventually reached a concentration of almost 1 mM (Fig. 6). Ninhydrin-reactive material similar to Compound TS could not be detected in control cultures lacking 1,2,4-triazole.

In order to compare the relative rates of cysteine biosynthesis in vitro with those of Compound TS, the total protein and sulfur content of exponentially growing cells were determined. A known number of bacteria grown on $^{35}$S as sole sulfur source were carefully harvested, washed, disrupted by sonic oscillation, and assayed for total protein, glutathione, and radiolabel. The results showed that $10^{12}$ cells have a packed wet weight of 1.82 g and contain 271 mg of protein, 64 $\mu$mol of nondialyzable sulfur, and 11 $\mu$mol of dialyzable sulfur, 7 $\mu$mol of which are in the form of glutathione. Since virtually all of the nondialyzable sulfur in such extracts is present in protein, either as L-methionine, the sulfur moiety of which is derived from L-cysteine in S. typhimurium (28, 29), or as L-cysteine itself, it follows that in sulfate-grown cells, a minimum of 0.236 $\mu$mol of L-cysteine must be synthesized from 0-acetyl-L-serine for L-cysteine biosynthesis rather than for the synthesis of Compound TS.

Using these values and the data shown in Fig. 6, we estimate that during the 18-hour time interval in which the inhibited cells grew with a generation time of 750 min, a total of 16 $\mu$mol of L-cysteine and 593 $\mu$mol of Compound TS were synthesized per liter of culture. Thus, for every mol of O-acetyl-L-serine utilized for L-cysteine biosynthesis, 37 mol were incorporated into Compound TS. We also calculate that the minimum rate of synthesis of O-acetyl-L-serine for 1,2,4-triazole-inhibited cells growing with a generation time of 750 min is 9.1 nmol/min/mg of total protein, while the requirement for this metabolite in nontreated cells growing with a generation time of 50 min is only 3.6 nmol/min/mg of total protein.

The generation times of the 1,2,4-triazole-resistant cysK strains, DW130 and DW132, were increased by less than 10% by 4 mM 1,2,4-triazole. The small quantities of Compound TS found in the culture media of these strains indicated that the ratios of Compound TS synthesis to L-cysteine synthesis are 0.01 for DW130 and 0.5 for DW132. We conclude, therefore, that the cysK mutations present in these strains lead to a 74- to 3700-fold increase over wild type in the in vivo ability to utilize O-acetyl-L-serine for L-cysteine biosynthesis rather than for the synthesis of Compound TS.

Effects of 1,2,4-Triazole on Derepression—The rapid rate of production of Compound TS by wild type bacteria might be expected to lower significantly the intracellular concentration of O-acetyl-L-serine. Since O-acetyl-L-serine is not only a direct precursor of L-cysteine, but also serves as an internal inducer of the enzymes of sulfate reduction in S. typhimurium (10), a reduced concentration of this metabolite should lead to decreases in the rates of synthesis of these enzymes. This hypothesis was tested by assaying 1,2,4-triazole-treated bacteria for the enzymes of cysteine biosynthesis at varying times after the addition of inhibitor.

The data given in Table III show that 1,2,4-triazole causes gradual decreases in the specific activities of several of the cysteine biosynthetic enzymes. When the enzyme activities of inhibitor-treated cultures are compared with those from bacteria grown for the same length of time in the absence of 1,2,4-triazole, the results suggest that a gradient of susceptibility exists in which the first enzymes in the pathway are the earliest and most severely affected. Within 1 hour of the addition of 1,2,4-triazole, the specific activity of ATP sulfurylase was reduced to one-third of that found in the untreated control culture, and fell to 6% of the control value after 4 hours.

Adenosine 5'-phosphosulfate kinase activity exhibited a similar but less rapid decline, and after 4 hours, was approximately 40% of the control. 3'-Phosphoadenosine 5'-phosphosulfate reductase activity was reduced significantly only after 4 hours, while sulfite reductase gradually decreased to about 50% of the control value. Cellular levels of O-acetylserine sulfhydrylase activity were not influenced by growth on 1,2,4-triazole, and the specific activity of serine transacetylase actually doubled in inhibitor-treated bacteria.

In similar studies performed with strains DW130 and DW132, 1,2,4-triazole caused no appreciable changes in the levels of any of the biosynthetic enzymes, including serine transacetylase. None of our assays were significantly affected by either the single or combined addition in vitro of 4 mM 1,2,4-triazole and 1 mM Compound TS, and mixing experiments provided no evidence for the presence of enzyme inhibitors in extracts from 1,2,4-triazole-treated wild type cells.

**DISCUSSION**

Our results show that O-acetylserine sulfhydrylase A catalyzes a reaction between O-acetyl-L-serine and 1,2,4-triazole,

![Fig. 6. Inhibition of wild type Salmonella typhimurium by 1,2,4-triazole. Bacteria were grown with shaking at 37°C in a minimal salts medium containing 0.8 mM sulfate as the sulfur source. At zero time the culture was divided, and to one portion was added 4 mM 1,2,4-triazole. Growth was monitored by measuring turbidity at 650 nm, where an A$_{650}$ of 1.0 is equivalent to 1.0 x 10$^6$ cells/ml. No inhibitor (O--O); 4 mM 1,2,4-triazole (Δ--Δ). Samples of medium from the inhibitor-treated culture were assayed at various times for Compound TS (O---O).](http://www.jbc.org/)

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*Note: The image contains a graph depicting the inhibition of wild type Salmonella typhimurium by 1,2,4-triazole. The x-axis represents time (Hours), and the y-axis represents turbidity at 650 nm. The graph shows the generation time gradually rose from 50 min to 750 min within 4 hours after the addition of 1,2,4-triazole.*
Effects of 1,2,4-triazole on enzymes of cysteine biosynthesis in wild type Salmonella typhimurium

Cell extracts were assayed at the indicated times after the addition of 4 mM 1,2,4-triazole.

<table>
<thead>
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<th>Time 1,2,4-Triazole</th>
<th>ATP硫基酰胺酶</th>
<th>Adenosine 5'-phosphophosphate kinase</th>
<th>3-Phosphoadenosine 5'-phosphophosphate reductase</th>
<th>Sulfite reductase</th>
<th>O-Acetylserine sulfhydrylase</th>
<th>Serine transacetylase</th>
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giving 1,2,4-triazole-1-alanine as a product. 1,2,4-Triazole also acts as an analogue of sulfide in its ability to inhibit the O-acetyl-L-serine-mediated activation of cysteine synthetase. Since cysK mutant strains grown in the presence of 1,2,4-triazole produce only small quantities of 1,2,4-triazole-1-alanine, we assume that the in vitro synthesis of this compound by wild type S. typhimurium is catalyzed largely or entirely by O-acetylserine sulfhydrylase A.

Although in vitro kinetic studies demonstrate a much greater reactivity of O-acetylserine sulfhydrylase A with sulfide as a substrate than with 1,2,4-triazole, the results of our growth experiments reveal that in wild type bacteria grown on sulfate, L-cysteine is synthesized in vitro at less than 3% the rate of 1,2,4-triazole-1-alanine. This apparent contradiction is not surprising since the intracellular concentrations of the substrates, particularly of sulfide and O-acetyl-L-serine, are undoubtedly much lower than those used in our enzyme assays.

The ease with which O-acetyl-L-serine is consumed in vitro by the triazolylase reaction suggests that the growth inhibitory effects of 1,2,4-triazole may be caused by starvation for O-acetyl-L-serine, and hence for L-cysteine. This possibility is supported by the observation that cysE mutants partially deficient in serine transacetylase are especially sensitive to 1,2,4-triazole (2), and by the fact that inhibition is readily overcome by either O-acetyl-L-serine or L-cysteine.

On the basis of previously reported observations and new data presented here, we propose the model depicted in Fig. 7 for the mechanism of 1,2,4-triazole inhibition. The essentials of this regulatory scheme have already been established for Escherichia coli and S. typhimurium, and consist of feedback inhibition of serine transacetylase by L-cysteine (4, 7), and induction of the remaining cysteine biosynthetic enzymes by O-acetyl-L-serine (8-10). Low levels of sulfide and L-cysteine ordinarily lead to high intracellular levels of O-acetyl-L-serine, owing both to a decreased rate of utilization by the sulfide-dependent sulfhydrylase reaction, and to an increased rate of synthesis caused by less feedback inhibition of serine transacetylase. As O-acetyl-L-serine accumulates, it serves as a signal of sulfur starvation and, in its role as an inducer, acts to alleviate that situation.

Our findings indicate that the addition of 1,2,4-triazole to this system would cause an immediate decrease in the rate of L-cysteine biosynthesis, resulting not only from the utilization of O-acetyl-L-serine in the triazolylase reaction, but perhaps also from the ability of the inhibitor to compete for enzyme. Although our in vitro studies have not demonstrated inhibition of the sulfhydrylase reaction by 1,2,4-triazole, it may be argued from kinetic principles that such inhibition should occur at low enough sulfide concentrations. Our in vivo studies leave little doubt that O-acetyl-L-serine is consumed at a rapid rate by the triazolylase reaction.

If sulfide and 1,2,4-triazole do compete with one another for O-acetyl-L-serine and O-acetylserine sulfhydrylase A, one would expect the resultant decrease in the rate of the sulfhydrylase reaction to lead to an accumulation of sulfide, which, in turn, might allow the cell to overcome inhibition. Instead, we find that the degree of inhibition gradually decreases with time, reaching a steady state approximately 4 hours after the addition of inhibitor. It is unlikely that the accumulation of 1,2,4-triazole-1-alanine accounts for this finding, since we have found that concentrations of this compound as high as 20 mM do not inhibit growth.

The gradual decreases in levels of several enzymes in the sulfate reduction pathway which were observed in 1,2,4-triazole-treated cells offer a possible explanation for the kinetics of growth inhibition noted in Fig. 6. During early exponential growth on sulfate, sulfide might be plentiful enough initially to allow the sulfhydrylase reaction to proceed reasonably well with the triazolylase reaction. However, if the increased rate of O-acetyl-L-serine utilization were to lower significantly the intracellular concentration of this inducer, this effect eventually would give rise to decreased levels of the enzymes necessary for sulfide production, and hence to less sulfide, that substrate with which the inhibitor competes in the first place. A gradual slowing of the growth rate would result, owing to dilution and degradation of the sulfate-reducing enzymes, and the eventual extent of inhibition would be due in large part to the ability of 1,2,4-triazole to inhibit sulfide production, as well as to the capacity of this compound to compete with sulfide for O-acetyl-L-serine and enzyme.

The gradient of susceptibility to 1,2,4-triazole noted for the various cysteine biosynthetic enzymes parallels the ease with which these enzymes are repressed by growth on different sulfur sources (10). This finding is consistent with the notion that both phenomena are caused by decreases in the intracellular concentration of O-acetyl-L-serine. The striking reduction in the level of ATP sulfurylase, resulting from a 4-hour exposure to 1,2,4-triazole, is probably sufficient in itself to account for the observed decrease in growth rate. The fact that
sulfite reductase activity was reduced by only 50% would account for the ability of exogenous sulfite to overcome inhibition.

Our data allow us to calculate that the total in vivo rate of O-acetyl-L-serine synthesis per cell mass is 2 to 3 times greater in inhibitor-treated bacteria than in control cultures. If L-serine were a limiting substrate for serine transacetylase, the ability of exogenous L-serine to overcome inhibition could be explained on the basis of an increased rate of O-acetyl-L-serine synthesis.

According to our model, mutations in cysK, the structural gene for O-acetylserine sulfhydrylase A, could lead to 1,2,4-triazole resistance in one or both of two different ways. A mutation such as is present in strain DW122, which results in a marked decrease in triazolylase activity with a relative sparing of sulfhydrylase activity, would prevent excessive O-acetyl-L-serine utilization by 1,2,4-triazole, and allow sulfide production and cysteine biosynthesis to proceed at an adequate rate in the presence of this inhibitor. In DW132, the in vitro measured loss of triazolylase activity is 20 times greater than the loss of sulfhydrylase activity, and a 74-fold decrease in the ratio of Compound TS to cysteine production is noted in vivo.

The second type of cysK mutation leading to 1,2,4-triazole resistance would be one in which both enzymic activities are severely impaired, resulting in the accumulation of O-acetyl-L-serine and sulfide behind a "metabolic block." The presence of a high concentration of sulfide at the time of 1,2,4-triazole addition would inhibit the triazolylase reaction, and the small amount of O-acetyl-L-serine consumed by the inhibitor would not lower an already elevated level of this inducer below that necessary for the synthesis of the enzymes of sulfate reduction. The cysK allele in DW130 appears to be an example of this type of mutation, and the 3700-fold decrease in the in vivo ratio of Compound TS to cysteine synthesis observed in this strain suggests that a "metabolic block" in the sulfhydrylase reaction can be very effective in overcoming 1,2,4-triazole inhibition.

Mutations in loci other than cysK are also capable of leading to 1,2,4-triazole resistance. We have recently isolated and studied a strain of S. typhimurium which appears to contain a mutation in the promoter region of cysE, the structural gene for serine transacetylase. This prototroph is resistant to 1,2,4-triazole and contains 5 times the normal, wild type level of serine transacetylase. The resistance of this strain to 1,2,4-triazole probably results from its ability to synthesize O-acetyl-L-serine at a rapid enough rate to prevent both the depletion of this substrate and the subsequent repression of the enzymes of sulfate reduction.

cysB1352 is a mutant allele of the regulatory gene cysB, which results in the constitutive expression of the cysteine biosynthetic enzymes even in strains unable to synthesize O-acetyl-L-serine (8, 10). The resistance of strains bearing this mutation (11) is easily understood on the basis of our model, and emphasizes the importance of enzyme repression during inhibition by 1,2,4-triazole.

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