The Stoichiometry and Kinetics of the Inducible Cysteine Desulphhydrase from *Salmonella typhimurium*

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**SUMMARY**

Studies using highly purified cysteine desulphhydrase from *Salmonella typhimurium* reveal that only a small fraction of the cysteine utilized by the enzyme appears as pyruvate. The isolation of 2-methyl-2,4-thiazolidinedicarboxylic acid from reaction mixtures offers an explanation for this unusual stoichiometry. The relative amounts of pyruvate and thiazolidine produced during a reaction depend upon the cysteine concentration, pH, and the presence of a protein termed Fraction B, which prevents the formation of the thiazolidine. We propose that 2-aminoacrylate may be an intermediate in the formation of 2-methyl-2,4-thiazolidinedicarboxylic acid. Substrate velocity curves for cysteine desulphhydrase reveal positive cooperativity with an n value of 1.9 and a K_m for L-cysteine of 0.17 to 0.21 mm. The product, sulfide, inhibits the reaction with a K_i of 0.010 mm. Sulphide inhibition is of the linear competitive type at high cysteine concentrations, but it becomes nonlinear and more pronounced at low cysteine concentrations.

The desulfuration of cysteine to hydrogen sulfide by extracts of bacteria and animal tissues has most often been attributed to the action of the enzyme cysteine desulphhydrase (L-cysteine hydrogen sulfide lyase deaminating, EC 4.4.1.1) (1-3), although there are data which indicate that cystathionase (4, 5), tryptophan deaminating, EC 4.4.1.1) (l-3), although the action of the enzyme cysteine desulfhydrase reaction is usually given as follows:

\[
\text{L-Cysteine \rightarrow \text{enzymatic} \rightarrow 2\text{-aminoacrylate + sulfide}}
\]

\[
\text{2-Aminoacrylate \rightarrow \text{enzymatic or spontaneous} \rightarrow \text{pyruvate + ammonia}}
\]

\[
\text{(Sum) L-cysteine \rightarrow \text{pyruvate + ammonia + sulfide}}
\]

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The unstable intermediate, 2-aminoacrylate, is assumed to decompose rapidly to ammonia and pyruvate even without enzymatic intervention (2). Since this equation predicts equimolar yields of pyruvate, ammonia, and sulfide, it is puzzling that several studies on the stoichiometry of the reaction have found lesser yields of pyruvate than could be accounted for on the basis of sulfide production or cysteine depletion (8-10). Previous investigators have suggested that this unusual stoichiometry might be due to peculiarities of the pyruvate assay used (8) or the presence of transaminases in the crude extracts in which the enzyme has been studied (11).

We have recently reported the purification of an inducible cysteine desulphhydrase from *Salmonella typhimurium* to a state of near homogeneity (12). Using highly purified enzyme, we find that the yield of pyruvate is still only a fraction of that expected on the basis of sulfide or ammonia production. This report details the isolation and characterization of 2-methyl-2,4-thiazolidinedicarboxylic acid as a product of the cysteine desulphhydrase reaction. The results of studies on certain kinetic properties of the purified enzyme are also presented.

**EXPERIMENTAL PROCEDURE**

**Materials**—Purified cysteine desulphhydrase was prepared from extracts of *S. typhimurium* grown on L-cystine (12). L-Cysteine-HCl was purchased from Sigma and recrystallized from 5 N HCl before use. L-Cysteine (free base), L-cystine, L-cystathionine, L-cysteine sulfinic acid, L-cysteic acid, cysteamine-HCl, cystathionine-diHCl, L-djenkolic acid, lactate dehydrogenase, L-glutamate dehydrogenase, and L-homocysteine thiolactone-HCl were also products of Sigma. The latter compound was converted to the free thiol by mild alkaline hydrolysis before use. D-Cysteine-HCl and β-cyano-L-alanine were obtained from Calbiochem, and D-cystine, D-serine, and meso-lanthionine were purchased from Mann. Azaserine was provided through the courtesy of Dr. Harry B. Wood, Jr., at the National Cancer Institute. O-Acetyl-L-serine was synthesized by the method of Sakami and Toennies (13), and β-mercapto pyruvate was prepared according to the procedure of Kun (14). [L-3H]Glycine and L-[14C]Cysteine were purchased from Schwarz-Mann. The latter compound was reduced to cysteine by incubation with a 5-fold excess of dithiothreitol, pH 9, at 37°C for 30 min. Other materials were obtained as previously described (12).

**Assays for Cysteine Desulphhydrase**—This enzyme may be assayed by measuring the rate of production of any of its products, sulfide, pyruvate, or ammonium ion. We have found it most
convenient to measure sulfide production when purifying the enzyme and for kinetic studies. Reactions are carried out at 23° in capped test tubes (10 × 75 mm) containing 2.0 ml of a given concentration of L-cysteine in 0.1 m Tris-HCl, pH 8.6. The reaction is started by the addition of a small volume of enzyme dilute in 0.1 m Tris-HCl, pH 7.6, containing 0.5 mg per ml of bovine serum albumin, and terminated by the addition of 0.2 ml of 0.02 M N,N'-dimethyl-p-phenylenediamine sulfate in 7.2 N HCl followed immediately by 0.2 ml of 0.03 M FeCl₃ in 1.2 N HCl (15). The tube is then recapped, vigorously shaken for a few seconds, and, after storage in the dark for 15 to 20 min, the absorbance at 650 nm is determined in a spectrophotometer. The apparent ε₄₅₀ for sulfide is dependent upon the cysteine concentration, and for kinetic studies in which cysteine concentrations were varied, the curve shown in Fig. 1 was used to calculate the amounts of sulfide formed. At the 2.0 mM cysteine concentration used for enzyme purification and other routine assays, the apparent ε₄₅₀ for sulfide is 1.56 × 10⁴ M⁻¹ cm⁻¹. Due to inhibition of the enzyme by the product sulfide, a plot of sulfide production per given period of time versus enzyme concentration is not linear. We have previously shown (12) that the initial velocity of the reaction, Vᵢ, can be calculated from the expression

\[ Vᵢ = \frac{Q}{1 + \frac{KₐQ^2}{2Kₐ(Kₐ + A)}} \]

where Q is the sulfide concentration at time t, A is the initial cysteine concentration, which is assumed not to vary significantly during the course of the reaction, Kₐ is the Michaelis constant for cysteine, Kₐ₂ is an inhibition constant for sulfide, and Q = 0 at t = 0. At 2.0 mM L-cysteine Kₐ₂/Kₐ(Kₐ + A) is equal to 5 mM⁻¹. One unit of enzyme is defined as that amount which gives a Vᵢ of 1 μmole of sulfide per min under these standard conditions.

For the determination of pyruvate 1.0 ml of reaction mixture is incubated in an uncapped test tube (13 × 100 mm), and the reaction is terminated by the addition of 0.5 ml of 1.0 N H₂SO₄. After 5 min, 0.5 ml of 3 mM 2,4-dinitrophenylhydrazine in 1.5 N HCl is added, followed 15 min later by 0.5 ml of 7.1 M KOH. After an additional 10 min the absorbance of the 2,4-dinitrophenylhydrazone at 540 nm is measured.

Early in the course of this work it was found that one of the products of the cysteine desulfhydrase reaction is a derivative of pyruvate, which gives no appreciable color reaction with the 2,4-dinitrophenylhydrazine reagent unless pretreated with an acidic solution of mercuric ion. We refer to the pyruvate detectable in the absence of mercuric ion as free pyruvate, while the pyruvate which is measured after treatment with mercuric ion is referred to as total pyruvate. Total pyruvate is the sum of free pyruvate and the pyruvate present as the derivative. To measure the total pyruvate produced in a reaction, 0.01 M HgSO₄ in 1.0 N H₂SO₄ is substituted for the 1.0 N H₂SO₄ used in the free pyruvate assay. This results in the formation of a precipitate after the addition of the KOH reagent, which must be removed by centrifugation before determining the absorbance at 540 nm. Using sodium pyruvate solutions standardized by the lactate dehydrogenase method (16), we find that this modified 2,4-dinitrophenylhydrazine assay gives an ε₄₅₀ of 4.4 × 10⁴ M⁻¹ cm⁻¹ without HgSO₄ (free pyruvate) and 4.6 × 10⁴ M⁻¹ cm⁻¹ with HgSO₄ (total pyruvate).

The rate of pyruvate production can also be determined in a continuous spectrophotometric assay, utilizing NADH and a large excess of lactate dehydrogenase. For this purpose the basic reaction mixture is supplemented with 0.2 mM NADH and 5 units per ml of lactate dehydrogenase, and the loss of absorbance at 340 nm is followed with time. Initial reaction velocities are measured in a recording spectrophotometer, and are linearly proportional to the amount of cysteine desulfhydrase added. This procedure measures only the rate of free pyruvate production, since lactate dehydrogenase does not react with the pyruvate derivative.

Stoichiometry—In experiments designed to determine the stoichiometry of the cysteine desulfhydrase reaction, 5 ml of a solution containing 2.0 mM L-cysteine in 0.1 M Tris-HCl, pH 8.4, in a Thunberg tube (150 × 18 mm) were deaerated by bubbling nitrogen for 5 min through an aperture specially fitted to the bottom of the tube. Approximately 0.3 unit of purified cysteine desulfhydrase in a small volume of solution was then added to start the reaction, which was carried out for 15 min at 23° while nitrogen was continuously bubbled through the incubation mixture. Hydrogen sulfide was collected by directing the gas outlet stream through 70 ml of a solution containing 0.5 gram of zinc acetate and 0.75 gram of sodium acetate in a 100-ml volumetric flask. The reaction was terminated by the addition of 1.0 ml of 1.0 N H₂SO₄, and the remaining hydrogen sulfide was collected for an additional 10 min. Control experiments, using standard solutions of sodium sulfide, showed that 95 to 100% of the added sulfide could be collected in this manner.

To the solution of zinc acetate were then added in rapid succession 10 ml of 0.02 M N,N'-dimethyl-p-phenylenediamine sulfate in 7.2 N HCl and 10 ml of 0.03 M ferric chloride in 1.2 N HCl. The flask was quickly stoppered, shaken vigorously for 1 min, and the total volume was adjusted to 100 ml with water. After storage for 15 min in the dark, the absorbance at 650 nm was determined. The ε₄₅₀ for sulfide under these conditions was found to be 2.67 × 10⁴ M⁻¹ cm⁻¹.

The acidified reaction mixture was then diluted with water to a volume of 25 ml and assayed for cysteine with 5,5'-dithiobis(2-nitrobenzoic acid) (17), for ammonia by the glutamate dehydrogenase method of Su et al. (18), and for free and total pyruvate. No detectable sulfide remained in the reaction mixture.

Other Methods—Thin layer chromatography was carried out on 250-μ thick Silica Gel G plates using the following three solvent systems: 1-butanol-water-glacial acetic acid (4:1:1); 1-butanol-water-pyridine (4:1:1); absolute ethanol-water (67:33). Amino
acids were detected with ninhydrin, and 2,4-dinitrophenylhydrazones were visualized directly.

ORD and ultraviolet spectra were obtained using automatic recording spectrophotometers, Cary models 60 and 15, respectively. Protein determinations were performed by the biuret method (19), and autoradiography was done as previously described (20).

Reaction mixtures to be analyzed for alanine were first oxidized with performic acid (21), lyophilized, and then adsorbed to a column (5 cm \times 1 cm) of Dowex 50W-II+ (X8, 300 to 400 mesh) at pH 2. After washing the column with water to remove cysteic acid, alanine and other amino acids were eluted with 1 N NH₂OH, concentrated by lyophilization, and analyzed on a Beckman model 121 amino acid analyzer. Recoveries subsequent to performic acid oxidation and prior to amino acid analysis were estimated by adding a small amount of [1-¹⁴C]glycine to each sample.

**RESULTS**

Initial attempts to quantify the products of the cysteine desulfhydrase reaction revealed an unusual stoichiometry, which varied with the stage of enzyme purification (see below). The data in Table I show that, using purified enzyme, the molar yields of sulfide and ammonia are equal, while the amount of free pyruvate detected is less than 10% of that expected from the accumulation of the former two products. Furthermore, the disappearance of cysteine from the reaction mixture is greater than can be accounted for by the yield of any one of these three products. As measured after preincubation of the reaction mixture with acidic mercuric ion, the yield of total pyruvate nearly equals that of ammonia or sulfide. In addition, that portion of the total pyruvate which is not detectable as free pyruvate is approximately equal to the amount of cysteine not accounted for by the sum of the total pyruvate formed and the cysteine remaining at the end of the reaction. We have accounted for this unusual stoichiometry by identifying a merccuric ion-labile conjugate of cysteine and pyruvate, provisionally designated Compound CP, as a product of the cysteine desulfhydrase reaction.

*Preparation of Compound CP*—The enzyme was a fraction which had been purified through the first ammonium sulfate step (12) and then desalted at room temperature by gel filtration through a Sephadex G-50 column, equilibrated with 0.1 M Tris-HCl, pH 8.4. The specific activity of this preparation was 1 unit per mg, representing a 4-fold purification from the crude extract.

Three hundred units (12 ml) of cysteine desulfhydrase were added to 380 ml of a solution containing 40 mmoles of L-cysteine (free base) adjusted to pH 8.4 with 5 N NaOH. During the entire course of the reaction, the mixture was stirred vigorously at room temperature in an open beaker, and the pH was kept at 8.0 to 8.4 by the addition of NaOH. An additional 40 mmoles of dry L-cysteine were added after 3 hours of incubation, at which time the total pyruvate concentration was 0.034 M. Four hours later, total pyruvate was 0.054 M and another 176 units of enzyme were added. After an additional 16 hours of incubation, the total pyruvate concentration had reached 0.079 M (32 mmoles), and the free pyruvate concentration was 0.006 M.

The solution was adjusted to pH 7.6 with glacial acetic acid and was filtered through Whatman No. 1 paper. After the addition of 4 volumes of cold absolute ethanol, the filtrate was chilled to -20°C and refrigerated.

**TABLE I**

| Component | No enzyme | Plus enzyme and 15 units of enzyme per ml | Difference between total pyruvate and free pyruvate |
|-----------|-----------|----------------------------------------|---------------------------------|
| Sulfide   | 0.00      | 3.17                                  | 3.28 |
| Ammonia   | 0.10      | 2.60                                  | 3.26 |
| Free pyruvate | 0.00    | 0.30                                  | 2.76 |
| Total pyruvate | 0.00  | 2.90                                  | 3.00 |
| Remaining cysteine | 9.22 | 3.68                                  | 5.55 |
| Sum of total pyruvate and remaining cysteine | 0.02 | 0.58                                  | 0.85 |
| Cysteine not accounted for by total pyruvate plus remaining cysteine | 0.00 | 0.00                                  | 0.00 |
| Difference between total pyruvate and free pyruvate | 0.00 | 0.35                                  | 0.95 |

* By direct assay with 5,5'-dithiobis(2-nitrobenzoic acid) (17).

* This calculation is based on the assumption that the maximal amount of recoverable cysteine is 9.22 mmoles, as in the control where cysteine was incubated in the absence of enzyme. The recovery of only 0.22 mmoles from an incubation originally containing 10 mmoles of cysteine is probably due to small losses occurring during transfers and some oxidation of cysteine to cystine, which is not measured by the cysteine assay used.

45°, the solution was filtered to pH 7.6 and filtered. Four volumes of absolute ethanol were added, and the solution was again filtered and reconverted to a volume of 40 ml. The pH of the concentrate was adjusted to 7.6, and then it was filtered first through Whatman No. 1 paper and then through a Millipore (0.45 μm) membrane filter. The addition of 19 volumes of ice-cold absolute ethanol to this solution resulted in the formation of a white, gel-like precipitate. After storage at -20°C overnight, the precipitate was collected by filtration, washed with cold absolute ethanol, and dried in vacuo. The yield was 5.8 g.

The dried material was dissolved in water at room temperature (200 mg per ml), and, after the addition of 4 volumes of cold absolute ethanol, the turbid, yellow solution was clarified by passage through a Millipore membrane filter. Absolute ethanol was added to a final concentration of 95%, and after 2 hours at -20° the resultant precipitate was collected by filtration and dried in vacuo. The yield was 4.5 g. A second reprecipitation gave Compound CP in 3.1 g yield.

**Characterization of Compound CP**—To 40 ml of a solution containing 500 mg (1.96 mmoles of total pyruvate) of Compound CP were added 20 ml of 0.5 M HgCl₂ in 1 N HCl. A white precipitate formed which, after adjustment of the solution to pH 3.0 with concentrated NH₄OH, was collected by filtration, washed with two 5-ml portions of water, and set aside for further analysis.

Excess mercuric ion was removed from the filtrate and washed by passage through a column (20 cm \times 2.5 cm) of Dowex 50W-I+ X8, following which the column was eluted with water. The eluate was assayed by both the lactate dehydrogenase and 2,4-dinitrophenylhydrazine methods and was found to have a total of 2.3 and 2.0 mmoles of pyruvate, respectively, in a volume.
The compound, 2-methyl-2,4-thiazolidinedicarboxylic acid, is a conjugate of pyruvate and cysteine, which by analogy with other thiazolidines might be predicted to decompose in the presence of mercuric ion, giving as products free pyruvate and the mercuric mercaptide of cysteine (23). The free acid of this thiazolidine derivative was chemically synthesized from pyruvic acid and L-cysteine by the method of Schubert (24), and recrystallized from hot water. A 0.8 M solution was titrated to pH 9 with concentrated NaOH, and the disodium salt was precipitated by the addition of 10 volumes of cold ethanol. After two reprecipitations the dried product was compared with Compound CP.

Both compounds were found to contain negligible amounts of free pyruvate, thiol, sulfide, and ammonium ion, while giving 1 mole of total pyruvate per 245 to 255 g of material. The results of elemental analyses were as follows:

| Compound       | Analysis       |
|----------------|----------------|
| Compound CP    |                |
| Found:         | C 29.3, N 5.49, H 3.32, S 12.7 |
| Disodium 2-methyl-2,4-thiazolidinedicarboxylate |                |
| Calculated:    | C 30.6, N 5.90, H 3.60, S 13.2 |
| Found:         | C 30.2, N 5.70, H 3.50, S 13.2 |

Two moles of sodium were found per mole of total pyruvate, indicating that both compounds are disodium salts. The two compounds cannot be distinguished from each other by thin layer chromatography in three solvent systems, and have identical infrared spectra. A comparison of the ORD spectra from 225 to 400 nm for the disodium salts of both products shows them identical melting points of the free acids, constitute excellent evidence for the stereochemical identity of Compound CP with the chemically synthesized product. Since both syntheses started with L-cysteine as a reactant, and the chemical synthesis utilizes pyruvate, we feel that both are probably equal mixtures of diastereomers at C-2 with the configuration of the α carbon atom of L-cysteine at C-4.

*Fraction B*—Using 2.0 mM L-cysteine at pH 8.6, the portion of enzymatically produced total pyruvate appearing as free pyruvate varies with the stage of enzyme purification from a total pyruvate to free pyruvate ratio of 2, using a crude extract, to a ratio of approximately 6, using highly purified enzyme. Thus, if the progress of enzyme purification is followed using the usual types of pyruvate assays rather than the total pyruvate or sulfide assays, an apparent large loss of activity occurs after the first ammonium sulfate precipitation step (12).

The greater relative yields of free pyruvate noted with crude preparations of cysteine desulphhydrase can be attributed to the presence of a factor which we have designated Fraction B. Preparations of this substance can be obtained which have no appreciable cysteine desulphhydrase activity, but which, when added to reaction mixtures containing pure cysteine desulphhydrase, increase the yields of free pyruvate without affecting the rates of total pyruvate or sulfide production (Table I). Fraction B does not convert purified Compound CP to pyruvate or sulfide, even in the presence of purified cysteine desulphhydrase, but the addition of this factor to a cysteine desulphhydrase reaction mixture, in which Compound CP has already accumulated, results in a decrease in the total pyruvate to free pyruvate ratio of products formed after such addition. Thus the action of Fraction B seems to be to prevent the formation of Compound CP during the cysteine desulphhydrase reaction rather than to degrade it.

In our attempts to devise a quantitative assay for Fraction B we have found that a linear relationship exists between the total pyruvate to Compound CP ratio (where Compound CP is assumed to be the difference between total pyruvate and free pyruvate) and the amount of Fraction B added to a cysteine desulphhydrase reaction mixture (Fig. 3). Thus our standard assay consists of adding Fraction B to 1.0 ml of a standard incubation mixture containing 0.05 unit per ml of purified cysteine desulphhydrase and measuring the total pyruvate to Compound CP ratio after a 5-min incubation. A control in which Fraction B is omitted is also run and the difference in the total pyruvate to
Compound CP ratio is determined. One unit of activity is defined as that amount of Fraction B which causes an increase in the total pyruvate to Compound CP ratio of 1.0 under these standard conditions. The assay is useful between the limits of 0.3 to 5 units of Fraction B activity per ml of reaction mixture.

Fraction B was purified from frozen cells of *S. typhimurium*, LT2, grown in minimal salts-glucose medium containing either 1.0 mM L-cysteine or 0.5 mM L-djenkolate as the sole sulfur source (20). We find that when Fraction B activity are independent of the sulfur source used for growth, and for that reason djenkolate-grown cells were used in the preparation described here to eliminate the possibility of contamination of Fraction B with cysteine desulfhydrase. When assaying extracts from cystine-grown cells, interfering cysteine desulfhydrase activity can be removed without appreciably affecting Fraction B activity by heating at 90° for 1 min.

Frozen cells were thawed and suspended in 2 volumes of 0.1 M Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol at 4° and disrupted by sonication. Following centrifugation at 40,000 × g for 30 min, the supernatant was removed and treated with 0.4 volume of 10% streptomycin sulfate, pH 7.6. After 10 min of standing at room temperature, the precipitate was removed by centrifugation and ammonium sulfate, 210 mg per ml, was slowly added to the supernatant with stirring. Following centrifugation, the supernatant from this step was heated to 90° in a boiling water bath, and, after 1 min at that temperature, cooled in an ice bath. Coagulated protein was removed by centrifugation, and Fraction B activity was precipitated by the addition of an additional 280 mg per ml of ammonium sulfate to the supernatant. This precipitate was dissolved in 0.1 M Tris-HCl, pH 7.6, 0.5 M NaCl, and dialyzed at 4° against the same buffer.

This procedure results in a 25- to 30-fold purification with a 45 to 50% yield (Table II). The ammonium sulfate and heat steps remove all cysteine desulfhydrase activity, whether the cells are grown on djenkolate or cystine as a sole sulfur source. Fraction B activity is resistant to treatment with RNase and DNase but is rapidly inactivated by treatment with small amounts of trypsin, which, after subsequent dialysis, have no effect on the cysteine desulfhydrase assay itself. The purified material is relatively stable when stored frozen, losing approximately 10% of its activity per month at -20°.

### Other Factors Influencing Synthesis of Compound CP

The cysteine desulfhydrase-mediated synthesis of Compound CP is markedly dependent upon pH and cysteine concentration. Using 2.0 mM L-cysteine and purified cysteine desulfhydrase, the total pyruvate to free pyruvate ratio increases from a value of 2 at pH 7.2 to a value of about 6 at pH 8.6 (Fig. 4A). At a constant pH of 8.6, the total pyruvate to free pyruvate ratio is directly but not linearly, proportional to L-cysteine concentration, and extrapolates to a value of 1 at zero cysteine concentration (Fig. 4B).

Other investigators have previously postulated (8, 10) and demonstrated (25) the nonenzymatic formation in aqueous solutions of derivatives between cysteine and certain carbonyl compounds. Therefore, studies were performed to evaluate the extent to which the nonenzymatic formation of Compound CP occurs. Fig. 5 shows the results of experiments in which L-cysteine and sodium pyruvate at several different concentrations were incubated in 0.1 M Tris-HCl, pH 8.6, at 23° for varying periods of time. Using 2.0 mM L-cysteine and 0.2 mM pyruvate, no appreciable loss of free pyruvate could be detected even after 90 min of incubation. At higher concentrations of both substrates, however, significant losses of free pyruvate were noted with time, while total pyruvate concentrations remained constant. Under these conditions the half-life of free pyruvate is 60 min at 20 mM L-cysteine, 2.0 mM pyruvate, and 13 min at 100 mM L-cysteine, 10 mM pyruvate. Fraction B has no effect on the rate of nonenzymatic formation of mercuric ion-labile pyruvate.

Although no appreciable nonenzymatic loss of free pyruvate occurs at the cysteine and pyruvate concentrations present in our routine cysteine desulfhydrase assay, all of our analytical data have been obtained on Compound CP which was prepared using 0.1 M L-cysteine as substrate. Therefore it is likely that at least a portion of our enzymatically produced material was formed by a non-enzyme-dependent reaction between L-cysteine and pyruvate. To establish the identity of Compound CP with the mercuric ion-labile pyruvate made in the presence of cysteine desulfhydrase at low cysteine concentrations, reaction mixtures containing 2.0 mM L-[35S]cysteine as substrate were analyzed for radiolabeled Compound CP. Small portions of these reaction mixtures were spotted on Whatman No. 8 MM paper, and, after electrophoresis in 0.025 M sodium citrate, pH 5.8, for 1 hour at 20 volts per cm, the positions of ninhydrin-positive carrier compounds were compared with the locations of radiolabel as detected by autoradiography. The areas on the paper corresponding to cysteine and Compound CP were then cut out and counted.
exactly superimposed over the faintly ninhydrin-positive area and 0.1 incorporated into material with the electrophoretic mobility of cysteine desulfhydrase, only 1.7% of the total radiolabel was the enzymatic reaction was included in a reaction mixture lacking cysteine desulfhydrase, 12.2% of the total radiolabel migrated with Compound CP. Fraction B had no effect on this nonenzymatic reaction. In contrast, after incubation with cysteine desulfhydrase, 12.2% of the total radiolabel migrated with Compound CP, and the addition of Fraction B at a concentration of 20 units per ml decreased this incorporation to a level of 2.2% of the total radiolabel. Autoradiography revealed a radioactive spot which corresponded to added carrier Compound CP. These data substantiate the notion that under our usual assay conditions the formation of 2-methyl-2,4-thiazolidinedicarboxylic acid is dependent upon the cysteine desulfhydrase reaction.

Substrate Specificity and pH Optimum—Among potential substrates thus far tested, purified cysteine desulfhydrase is quite specific for L-cysteine. Incubation of the enzyme with L-cysteine, D-cysteine, L-cystine, L-cysteine sulfenic acid, L-cysteic acid, cysteamine, cystamine, L-cystathionine, L-homocysteine, L-serine, N-serine, 3-mercaptopuruvate, meso-lanthionine, L-djenkolic acid, β-cyano-L-alanine, azaserine, and O-acetyl-L-serine at concentrations of 1.0 to 2.0 mM does not lead to the formation of detectable sulfide or 2,4-dinitrophenylhydrazine-reacting material, even after pretreatment of the reaction mixture with acidic mercuric ion. L-Tryptophan does react at about 0.1% the rate of L-cysteine as judged by the accumulation of pyruvate in an assay coupled with NADH and lactate dehydrogenase (12).

Using both the sulfide and total pyruvate assays with 2.0 mM L-cysteine as substrate, L-cysteine (0.5 mM), D-cysteine (0.5 mM), and L-serine (2.0 mM) all inhibit cysteine desulfhydrase about 20%. The other compounds tested for substrate activity inhibit the enzyme less than 15% at concentrations of 0.5 to 2.0 mM.

The pH optimum of cysteine desulfhydrase in 0.1 M Tris-HCl is 8.6, with a rather sharp decline in activity at pH levels below 8.3. The activity at pH 7.0 is less than 5% that observed at 8.6.

Kinetic Studies—Kinetic studies of cysteine desulfhydrase have been complicated by the potent inhibition of the enzyme by its product, sulfide. One approach to this problem has been to measure rates of pyruvate production in capped reaction tubes, which allows diffusion of hydrogen sulfide from the reaction mixture (9). Due to the quantitatively uncertain extent of sulfide diffusion under such conditions and the lesser sensitivity of the pyruvate assay, we have chosen to measure rates of sulfide production and to analyze our results after correcting for sulfide inhibition.

Substrate-velocity studies were carried out by measuring the accumulation of sulfide as a function of time at different concentrations of L-cysteine. Initial velocities of reaction, Vi, were then estimated graphically using the t050 for sulfide appropriate for each L-cysteine concentration (see Fig. 1). As shown in Fig. 6, a plot of Vi versus L-cysteine concentration gives a sigmoid-shaped curve with a half-maximum Vi at 0.21 mM L-cysteine. A plot of 1/Vi versus 1/S reveals that at L-cysteine concentrations greater than 0.3 mM a straight line is obtained, and that at points corresponding to lower substrate concentrations the slope of the line increases (Fig. 6, inset). The apparent Km for L-cysteine calculated from the linear portion of the double reciprocal plot corresponding to higher cysteine concentrations is 0.17 mM.
FIG. 6. Substrate-velocity curve for purified cysteine desulfhydrase. The $V_i$ at each cysteine concentration was determined as the initial rate of sulfide production as described under "Results." The inset shows the plot of $1/V_i$ versus $1/S$. The $K_s$ estimated from the straight line obtained with lower values of $1/S$ is 0.17 mM.

Treating the data according to the method of Hill (26), a plot of $ln [V_i/(V_m - V_i)]$ versus $ln S$ gives a straight line with a slope of 1.9 (Fig. 7). Thus the dependence of the reaction rate on L-cysteine concentrations shows positive cooperativity with an $n$ value of almost 2 at substrate concentrations less than 0.03 mM.

The rate of sulfide production is unaffected by sodium pyruvate, Compound CP, or ammonium sulfate when added either singly or in various combinations at concentrations of 0.025 mM or 0.5 mM. Preincubation of cysteine desulfhydrase with 0.02 mM sodium sulfide, however, leads to a partial inhibition of activity which is unrelated to the time of preincubation for at least 10 min. Removal of the sulfide by dilution results in a loss of inhibition to a level expected by the lower concentration of sulfide. Since the inhibition appears to be very rapid and reversible, we have endeavored to describe it in terms based on the assumptions of steady state, rapid equilibrium kinetics.

Due to the difficulties involved in estimating initial velocities by the sulfide method in solutions to which exogeneous sulfide has already been added, we have carried out our inhibition studies by measuring the time-dependent accumulation of endogeneously formed sulfide in the absence of added sulfide. Fig. 8 shows product (sulfide) versus time curves at five different concentrations of L-cysteine using a constant amount of enzyme. The shapes of these curves indicate that the percentage of inhibition at a given sulfide concentration is markedly dependent on the cysteine concentration, and is greater at lower substrate concentrations. Fraction B at a concentration of 10 units per ml has no effect on the shape of such curves.

We find that under certain conditions sulfide inhibition appears to be of the linear competitive type (27) which can be described by:

$$Q = \frac{K_m}{V_m} \left( \frac{1}{V} + \frac{Q}{K_q} \right) \frac{1}{A} + \frac{1}{V_m} \quad (2)$$

where $V$ is the instantaneous rate of sulfide formation, $A$ is the cysteine concentration, $Q$ is the inhibitor (in this case the product sulfide) concentration, $V_m$ is the maximum velocity at saturating substrate and zero inhibitor concentrations, $K_m$ is the Michaelis constant for cysteine, and $K_q$ is an inhibition constant for sulfide.

We have previously shown (12) that under conditions where the change in cysteine concentration is very slight during the course of the reaction, $A$ may be considered a constant, and if $V_i = \frac{V_m A}{(K_m + A)}$ and $V = \frac{dQ/dt}$, Equation 2 can be solved to give the integrated rate equation:

$$Q = \frac{2K_q V_m (K_m + A)}{K_m Q} - 2K_q (K_m + A)$$

which holds only when $Q = 0$ at $t = 0$. Thus when the sulfide concentration is measured as a function of time under conditions where the change in cysteine concentration is small (less than 10% in our experiments) and the sulfide concentration is zero at time zero, a plot of $Q$ versus $t/Q$ should give a straight line with a $y$ intercept equal to $-2K_q (K_m + A)/K_m$. If sulfide inhibition is of the linear competitive type.

When the data from Fig. 8 are used to plot $Q$ versus $t/Q$ we find that at a cysteine concentration of 1.7 mM a straight line is
obtained to sulfide concentrations as high as 0.09 mM (Fig. 9). Using lower substrate concentrations a straight line can be drawn through points corresponding to low sulfide concentrations, but at higher sulfide concentrations and longer incubation times the points describe lines which become concave downward. Since preincubation studies show no time-dependent effect of sulfide on the enzyme activity, we conclude that, under the combined conditions of low cysteine concentration (less than 1 mM) and high sulfide concentration (greater than 0.05 mM) the inhibition of cysteine desulphydrase by sulfide is nonlinear.

If one assumes that the linear portions of the curves obtained by plotting \( Q \) versus \( t/Q \) reflect conditions where sulfide inhibition is of the linear competitive type, estimates for \( K_s \) can be made at several different sulfide concentrations. If at various concentrations of \( J \):

\[
y = \frac{-2K_s(K_s + A)}{K_m}
\]

then:

\[
-y = \frac{2K_s A}{K_m} + 2K_s
\]

and a replot of \(-y\) intercepts versus \( A \) should give a straight line with a new \( y \) intercept of \( 2K_s \) and an \( x \) intercept of \(-K_m\). Using the data obtained from experiments run at six different cysteine concentrations, such a plot gives a straight line (Fig. 10) with a value for \( K_s \) of 0.010 mM and a value for \( K_m \) of 0.19 mM.

**Discussion**

Our data indicate that during the cysteine desulphydrase reaction, a portion of the total desulfurated cysteine, as measured by the production of sulfide, reacts with additional cysteine to give 2-methyl-2,4-thiazolidinedicarboxylic acid. Thus for every mole of thiazolidine formed, 2 moles of cysteine are consumed, releasing 1 mole of sulfide, 1 mole of ammonia, and no free pyruvate. This scheme fits well with the stoichiometric data presented in Table 1 and accounts for the low yields of free pyruvate observed by us and others (8–10).

Dugaiczky et al. (25) have demonstrated the nonenzymatic reaction of dihydroxyacetone phosphate with cysteine to give a product tentatively identified as either a thiohemiketal or a thiazolidine. They also showed that the incubation of pyruvate with 0.025 mM cysteine results in a time-dependent loss of lactate dehydrogenase assayable pyruvate. Although these authors made no attempt to isolate the product of the pyruvate-cysteine reaction, their finding that \( N \)-acetylcysteine does not trap pyruvate suggests that ring closure of the thiobemiketal to give the thiazolidine is necessary for the effect. Our data on the nonenzymatic formation of mercier ion-labile pyruvate from cysteine and free pyruvate confirm these earlier observations and favor the notion that the product of this reaction is the thiazolidine.

Several investigators have suggested that the immediate products of the cysteine desulphydrase reaction are sulfide and 2-aminoacrylate, and that the latter compound, being unstable in aqueous solution, is spontaneously and rapidly hydrolyzed to ammonia and pyruvate (2, 3). Thus, while the nonenzymatic formation of mercier ion-labile pyruvate from free pyruvate and cysteine is low to undetectable under the conditions of our assay, it is possible that a reaction between 2-aminoacrylate and cysteine might occur readily. The immediate product would be a thiocemiketamine, which could then cyclize to the thiazolidine, liberating ammonia in the process (Fig. 11). Our data showing that the total pyruvate to free pyruvate ratio extrapolates to a value of 1.0 at zero cysteine concentration (Fig. 4B) are consistent with the prediction of this model that the amount of Compound CP formed should be directly proportional to the cysteine concentration.

In an experiment designed to demonstrate the existence of 2-aminoacrylate as an intermediate in the cysteine desulphydrase reaction, sodium borohydride was added to reaction mixtures on the assumption that any 2-aminoacrylate present would be reduced to alanine. The data presented in Table III show that when a complete reaction mixture was treated with borohydride, the amount of alanine recovered was considerably more than that found in control mixtures lacking enzyme or cysteine. The lesser yield of alanine noted with the higher concentration of borohydride is probably explained by the fact that while cysteine desulphydrase retains approximately 50% of its activity in the presence of 0.2 mM borohydride, the enzyme is rapidly and completely inactivated by 10 mM borohydride. Therefore the ala-
which reacts catalytically with an intermediate in the reaction to give free pyruvate. If 2-aminoacrylate is in fact a precursor of the thiazolidine, Fraction B might facilitate its hydrolysis to pyruvate and ammonia, either directly or by catalyzing an enamine tautomerization to give that tautomer which is less reactive with cysteine or more readily hydrolyzed. Alternatively, Fraction B might hydrolyze the thiohemiketamine before cyclization takes place or perhaps even interact with cysteine desulfhydrase itself in such a way as to enable it to release pyruvate and ammonia directly. The exact role of Fraction B in cellular metabolism is also unclear, since it is found at the same concentrations in cells either grown on cysteine or starved for sulfur by growth on djenkolate. Perhaps Fraction B is an enzyme of general usefulness to the cell rather than being limited to a single function related to cysteine catabolism.

The results of our kinetic studies are essentially in agreement with those of Collins (9), who, using a partially purified preparation of enzyme and a different assay, found cooperative kinetics for cysteine with a $K_0$ of 0.32 mM and an $n$ value of 1.9. Collins also studied the inhibition of the enzyme by sulfide and found evidence for mixed inhibition kinetics with a $K_i$ ($K_0$ in our terminology) for sulfide of 0.007 mM.

It may be of some significance that sulfide inhibition shows a greater deviation from linearity at lower cysteine concentrations where the dependence of the reaction rate upon cysteine concentration is positively cooperative. The exaggeration of product inhibition at these low cysteine concentrations may be related to the ability of sulfide to interfere with cooperativity either by competing with cysteine for an allosteric site or by otherwise preventing the enhancement of enzyme activity related to an allosteric event.

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