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Mechanisms of H_2S Production from Cysteine and Cystine by Microorganisms Isolated from Soil by Selective Enrichment

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Hydrogen sulfide (H_2S) is a major component of biogenic gaseous sulfur emissions from terrestrial environments. However, little is known concerning the pathways for H_2S production from the likely substrates, cysteine and cystine. A mixed microbial culture obtained from cystine-enriched soils was used in assays (50 min, 37°C) with 0.05 M Tris-HCl (pH 8.5), 25 μmol of L-cysteine, 25 μmol of L-cystine, and 0.04 μmol of pyridoxal 5′-phosphate. Sulfide was trapped in a center well containing zinc acetate, while pyruvate was measured by derivatization with 2,4-dinitrophenylhydrazine. Sulfide and total pyruvate production were 17.6 and 17.2 nmol mg of protein^{-1} min^{-1}, respectively. Dithiothreitol did not alter reaction stoichiometry or the amount of H_2S and total pyruvate, whereas *N*-ethylmaleimide reduced both H_2S and total pyruvate production equally. The amount of H_2S produced was reduced by 96% when only L-cystine was included as the substrate in the assay and by 15% with the addition of propargylglycine, a specific suicide inhibitor of cystathionine γ-lyase. These data indicate that the substrate for the reaction was cysteine and the enzyme responsible for H_2S and pyruvate production was cysteine desulfhydrase (EC 4.4.1.1). The enzyme had a K_m of 1.32 mM and was inactivated by temperatures greater than 60°C. Because cysteine is present in soil and cysteine desulfhydrase is an inducible enzyme, the potential for H_2S production by this mechanism exists in terrestrial environments. The relative importance of this mechanism compared with other processes involved in H_2S production from soil is unknown.

Field studies have demonstrated that hydrogen sulfide (H_2S) is a major component of biogenic gaseous sulfur emissions from terrestrial environments (1, 2, 12). Hydrogen sulfide production in nonwaterlogged soils most likely occurs during the aerobic mineralization of organic sulfur compounds (33). Little is known concerning specific degradation pathways in soils, but the substrates are thought to be sulfur-containing proteins and amino acids (4). Cysteine- and cystine-amended soils have been shown to produce H_2S as a result of substrate mineralization and not sulfate reduction (27, 28, 36).

The amino acids cysteine (cystine) and methionine comprise 21 to 31% of the total soil organic S in two Australian soils (17) and from 11 to 15% of the organic sulfur in mineral soils from Scotland (32), with an approximate 2:1 ratio of cysteine (cystine) to methionine. Cysteic acid and cystine have also been shown to occur in root exudates (9) and therefore would be present at higher concentrations in rhizosphere soil, where free amino acid concentrations are 10- to 26-fold greater than in nonrhizosphere soil (22). Free amino acids are also produced through residue degradation, with concentrations in soil being dependent on environmental variables such as temperature, moisture status, type and amount of organic residues, and cultural practices (34).

Two enzymes are most likely responsible for H_2S production from cysteine or cystine in soil. Cysteine desulfhydrase catalyzes the degradation of cysteine to produce H_2S, NH_3, and pyruvate. Cystathionine γ-lyase uses the oxidized form of cysteine, cystine, as a substrate to produce pyruvate, NH_3, and thiocysteine (systematic name, 2-amino-2-carboxyethylhydrodisulfide). Thiocysteine then reacts nonenzymatically with cysteine or other sulfhydryl-containing compounds to yield H_2S and cystine. Cysteine desulfhydrase activity was found to be widespread in bacteria and present in 1 of 27 fungal strains, but not present in 14 strains of actinomycetes and 30 strains of yeasts (26, 31). In contrast, cystathionine γ-lyase activity has been found mainly in actinomycetes and eucaryotic microorganisms (11, 30).

Assignment of enzyme activities to different groups of microorganisms is clouded by confusion surrounding enzyme identification. Cavallini et al. (5, 6) suggested that cysteine and cystine desulfuration is catalyzed by a single enzyme, cystathionine γ-lyase. The actual substrate for the enzyme, cystine, is rapidly produced by cysteine autooxidation. They argue that because of this oxidation, enzyme assays performed with cysteine as the original substrate cannot be used to infer cysteine desulfhydrase activity. Other investigators claim that two distinct enzymes with different substrate specificities exist (8, 19, 25). In spite of this confusion, the Enzyme Commission has assigned both enzymes the same classification number (EC 4.4.1.1). This seems inappropriate, given that mammalian sources were used by the investigators implicating a single enzyme (5, 6, 10, 20), while microbial sources were used by those showing two distinct enzyme activities (8, 15, 19, 25).

Although the existence of both enzymes in soils has been proposed (16), little work has been done beyond that with purified enzymes and specific microbial isolates. Morra and Dick (28) reported that thiocysteine was produced by cystathionine γ-lyase in cysteine-amended soil. Since then it has been demonstrated that cystathionine γ-lyase is most likely responsible for the H_2S production observed to occur from cystine-amended soil (29). The recovery and quantification of pathway intermediates and products were hampered

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because of interactions with soil constituents. In addition, relatively long (24 h) incubations were required to obtain sufficient activity for measurement. The objectives of the present work were to overcome these problems and determine the possible biochemical pathway(s) responsible for H_2S production from cysteine or cystine by mixed microbial cultures obtained from soil.

MATERIALS AND METHODS

Soils. The soils used were surface (0 to 15 cm deep) samples brought into the laboratory, air-dried, and crushed to pass through a 2-mm sieve (29). The analyses were applied to the air-dried samples by the following methods: pH by glass electrode (1:1 soil-to-water ratio), organic C by the Walkley-Black method (3), total S by sodium hypobromite (NaOBr) oxidation (38), and particle size distribution by pipette analysis (24).

Enrichment cultures. An enrichment culture of the microorganisms responsible for H_2S production was obtained by using a medium similar to that of Kumagai et al. (26). The medium contained 0.2% L-cystine, 0.5% beef extract, 0.5% Bacto-peptone (Difco), 0.3% yeast extract, 0.2% NaCl, 0.2% $CaCl_2 \cdot 2H_2O$, and 0.1% glycerol.

Four grams of air-dried soil were separately amended with 50 μmol of L-cystine, supplied with 1 ml of H_2O, and incubated at 37°C for 24 h. Each of the cystine-amended soils was added to a different 100-ml aliquot of the enrichment medium and shaken at room temperature for 24 h. Five milliliters of each enrichment culture was transferred to a separate 100-ml aliquot of the medium and incubated for an additional 24 h. The 5-ml transfer and 24-h incubation steps were repeated, and the three enrichment cultures were stored at 4°C.

Centrifuging the enrichment cultures at 38 × *g* for 20 min removed insoluble L-cystine but left the cells in suspension. Of the 100 ml of suspension, 80 ml was removed and centrifuged at 3,840 × *g* for 25 min. The liquid was decanted, and the cell pellets were resuspended in 100 ml of 0.05 M Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$. Cells were again centrifuged, and the liquid was decanted. A second wash was performed, and the cells from each of the three enrichment cultures were resuspended in separate 20-ml aliquots of the buffer. The 20-ml buffered cell suspensions from the three soils were combined to obtain sufficient cells to perform all of the assays and stored at 4°C on ice.

Assay methods. Protein was measured by the Bio-Rad method of dye binding and a bovine plasma albumin standard (Bio-Rad Laboratories, Richmond, Calif.). Acidification or sonication of the cell suspension yielded similar results with respect to soluble protein content.

Since H_2S is rapidly oxidized and adsorbed to surfaces, periodic analysis of headspace samples by gas chromatography is unacceptable. Hydrogen sulfide must be trapped immediately upon its release from solution. Preliminary tests indicated that a zinc acetate-NaOH solution (35) and trapping on lead acetate paper (36) did not quantitatively measure H_2S production and suffered from a lack of stability. Instead, H_2S was measured by placing a small center well containing 0.5 ml of 1 M zinc acetate and a piece of pleated filter paper (Whatman 42; 4.0 by 2.5 cm) inside a 25-ml Erlenmeyer flask containing a sidearm with a rubber septum (Kontes, Vineland, N.J.). Upon termination of H_2S collection, the center well and its contents were transferred to a 50-ml Erlenmeyer flask containing 40 ml of deionized water. Methylene blue was developed by addition of one-half the reagent amount prescribed by Tabatabai (37), and the final volume was adjusted to 50 ml. Recovery tests involving $Na_2S \cdot 9H_2O$ standards prepared in an antioxidant buffer according to recommended methods (Orion Research, Cambridge, Mass.) were conducted. Hydrogen sulfide was produced through acidification of the standard solutions by injection of 3 ml of HCl (1.0 M) through the rubber septum of the 25-ml incubation flask. Recoveries ranged from 85 to 93% when total amounts of H_2S-S from 2 to 45 μg were used (29).

Both H_2S and pyruvate production were measured in the microbial cell assays. The standard assay mix, in a 25-ml incubation flask, contained 1 ml of suspended whole cells, 2 ml of 0.05 M Tris-HCl buffer (pH 8.5), 25 μmol of L-cysteine, 25 μmol of L-cystine, and 0.04 μmol of pyridoxal 5′-phosphate (PLP) in a total volume of 4.5 ml. The incubation was performed at 37°C in a shaking water bath for 50 min, after which the reaction was stopped by the injection of 0.5 ml of 60% trichloroacetic acid through the rubber septum. Controls were prepared as above with the substitution of boiled cells for the viable treatment cells.

Five minutes after trichloroacetic acid addition, the center wells were removed and H_2S was determined as described above. The method of Friedemann and Haugen (18) as modified by Kredich et al. (25) was used for pyruvate analysis. Pyruvate was derivitized with 2,4-dinitrophenylhydrazine (DNPH), and the absorbance of DNPH was recorded. To the stopped assay was added 2.5 ml of 3 mM DNPH. After 15 min, 2.5 ml of 7.0 M KOH was added, and the entire assay solution was centrifuged at 12,000 × *g* for 5 min. The A_{540} of the clear solution was determined and compared with a standard curve produced by using sodium pyruvate.

Free pyruvate is used to refer to that fraction of pyruvate forming DNPH without any addition to the trichloroacetic acid stop solution. Total pyruvate is used to refer to the fraction of pyruvate forming the same colored compound when 25 μmol of $HgSO_4$ was included in the trichloroacetic acid solution to release complexed pyruvate from thiazolidine (25).

For treatments to which other compounds were added or standard assay constituents were deleted, the final assay volume remained 4.5 ml and the volume of buffer was altered to compensate. All components of the assay were prepared in 0.05 M Tris-HCl (pH 8.5). All treatments were performed in duplicate.

RESULTS AND DISCUSSION

The standard assay procedure yielded a linear relationship between the amount of H_2S or free pyruvate produced and time (Fig. 1). The results also indicated that substrate (cysteine) concentrations were not limiting the rate of the reaction when present at the standard assay level of 5.56 mM (Fig. 2). Thus, the reactions observed followed zero-order kinetics and reaction rates were a function of enzyme concentration and not of assay constraints. The assay reaction temperature was approximately 20°C lower than the inactivation temperature (Fig. 3). This temperature was sufficient to provide an adequate reaction rate but not to cause enzyme inactivation. The inactivation temperature for H_2S production from microbial cells was similar to that observed for H_2S production from cystine-amended soils (29).

Only a limited amount of H_2S and free pyruvate were produced when cystine was used as a substrate (cysteine

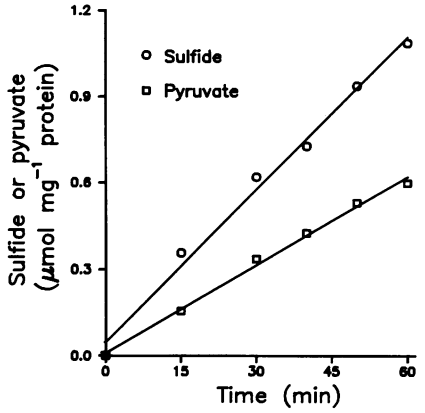

FIG. 1. Free pyruvate and H_2S produced in enrichment culture assays as a function of time of incubation.

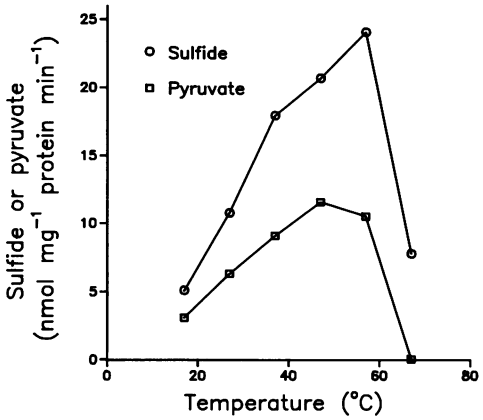

FIG. 3. Free pyruvate and H_2S produced in enrichment culture assays as a function of temperature.

omitted) even though the cells were grown on cystine during enrichment (Table 1). Swaby and Fedel (36) reported similar observations for many genera of both sporeforming and nonsporeforming mesophiles. Isolates which originally produced H_2S from cystine lost this ability upon subculturing, and H_2S production was not restored by soil passage or cultivation on various media. Synergistic pairs of microorganisms were deemed necessary for H_2S production from cystine (36).

When both cysteine and cystine were included as substrates (i.e., the standard assay), the amount of H_2S produced was 25 times greater than in the absence of cysteine (Table 1). A 4.5-fold increase in free pyruvate production also occurred (Table 1). When cystine was omitted, however, the amounts of H_2S produced were slightly higher and those of free pyruvate were slightly lower than in the standard assay. Although autooxidation of cysteine to cystine most likely occurred, producing small amounts of cystine, the results strongly indicate that cysteine is the dominant substrate. Omitting PLP during the assay decreased both H_2S and free pyruvate production (Table 1). Because both cysteine desulfhydrase and cystathionine γ-lyase are PLP-dependent enzymes (14, 23), it appears that at least a portion of the necessary cofactor was present in the cell culture itself.

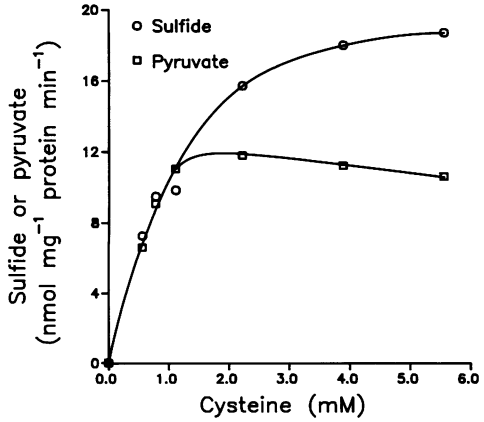

FIG. 2. Free pyruvate and H_2S produced in enrichment culture assays as a function of substrate (cysteine) concentration.

Propargylglycine, a specific suicide inhibitor of cystathionine γ-lyase (35), inhibited H_2S production by the enrichment culture cells by 15% (Table 1). In soils, a similar concentration of propargylglycine inhibited H_2S production by 90% (29). These results suggest that an enzyme other than cystathionine γ-lyase was responsible for producing H_2S in the enrichment culture than in soil.

Experiments involving the addition of hypotaurine were also conducted to determine the level of cystathionine γ-lyase activity in the enrichment culture cells. A reduction in H_2S production would be expected if cystathionine γ-lyase was present because hypotaurine can act as a sulfur acceptor in transsulfuration reactions involving persulfides such as thiocysteine (6): R-SSH (thiocysteine) + R-SO_2H (hypotaurine) → R-SO_2SH + R-SH (cysteine).

TABLE 1. Effect of assay components on production of H_2S and free pyruvate by microbial cells from cystine-enriched soils

Assay mix[a]	Mean production (nmol/mg of protein/min) ± 1 SD	
	H_2S[b]	Free pyruvate[c]
Standrd assay mix	17.8 ± 0.0	9.0 ± 0.3
Without PLP	15.4 ± 0.0	7.6 ± 0.1
Without cystine	19.8 ± 1.1	8.4 ± 0.0
Without cysteine	0.7 ± 0.2	2.0 ± 0.2
DL-Propargylglycine		
5 μmol	15.2 ± 0.6	8.8 ± 0.0
50 μmol	15.2 ± 0.1	8.4 ± 0.1
Hypotaurine		
5 μmol	18.4 ± 0.7	10.0 ± 0.3
50 μmol	13.0 ± 1.0	8.4 ± 0.0
Dithiothreitol (25 μmol)	19.2 ± 0.1	4.2 ± 0.0
N-Ethylmaleimide (25 μmol)	11.2 ± 0.3	11.2 ± 0.6

[a] Standard assay contained 2.0 ml of 0.05 M Tris-HCl buffer (pH 8.5), 25 μmol of L-cysteine, 25 μmol of L-cystine, and 0.04 μmol of PLP in a total volume of 4.5 ml. The reaction was stopped with 0.5 ml of 60% trichloroacetic acid.

[b] Least significant difference ($LSD_{0.05}$) value for comparison of H_2S means is 1.3 nmol/mg of protein/min.

[c] Least significant difference ($LSD_{0.05}$) value for comparison of free pyruvate means is 0.5 nmol/mg of protein/min.

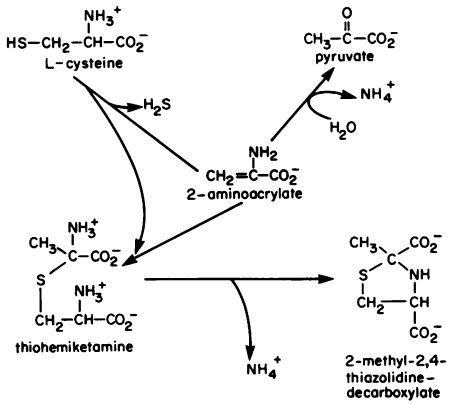

FIG. 4. Pathways for L-cysteine degradation by purified cysteine desulfhydrase from *S. typhimurium* and for production of pyruvate from 2-methyl-2,4-thiazolidinedicarboxylate.

Hypotaurine reduced H_2S at 50 μmol but had no effect at 5 μmol (Table 1). These results also support the hypothesis that cystathionine γ-lyase was not the enzyme primarily responsible for H_2S production by the enrichment culture.

Two enzymes seem to be active in the enrichment cells to produce H_2S, cysteine desulfhydrase and cystathionine γ-lyase, but cysteine desulfhydrase is dominant. Equimolar amounts of H_2S and pyruvate should be produced by this enzyme. However, this was not observed, a result consistent with observations made by Kredich et al. (25) with purified cysteine desulfhydrase from *Salmonella typhimurium*. This is due to interference from 2-aminoacrylate, an intermediate in the degradation of cysteine by cysteine desulfhydrase (Fig. 4). 2-Aminoacrylate is nonenzymatically hydrolyzed to pyruvate or reacts with a molecule of cysteine to produce thiohemiketamine (25). The thioketamine can further react to form 2-methyl-2,4-thiazolidinedicarboxylic acid (Fig. 4).

Addition of dithiothreitol, which maintains the sulfhydryl group in the reduced state (7), would promote the nonenzymatic reaction of cysteine with 2-aminoacrylate, resulting in a decrease in free pyruvate, while the amount of H_2S produced would remain essentially unchanged. Actual experimental values show a 60% decrease in the amount of free pyruvate produced, while H_2S production was slightly elevated (Table 1). In contrast, *N*-ethylmaleimide complexes with sulfhydryl groups, removing them from any further reaction (13). This would cause a decrease in H_2S production because of substrate removal and, because thioketamine formation would also be inhibited, produce equimolar amounts of H_2S and pyruvate. The addition of *N*-ethylmaleimide yielded the expected results (Table 1). Although the dependence of enzyme activity on the presence of sulfhydryl groups is unknown and direct enzyme inhibition by *N*-ethylmaleimide is possible, this mechanism seems unlikely, since Collins and Monty (8) concluded that dithiothreitol does not act as a reducing agent for cysteine desulfhydrase.

Additional evidence that 2-aminoacrylate was being produced and causing interference was obtained by conducting experiments in which $HgSO_4$ was added after incubation. Hg^{2+} decomposes 2-methyl-2,4-thiazolidinedicarboxylic acid to produce additional free pyruvate (25) (Fig. 4). Thus, addition of Hg^{2+} permits measurement of total pyruvate formed during the enzymatic degradation of cysteine, whether it was originally in the free form or complexed as part of thiazolidine.

TABLE 2. Effect of assay components on production of H_2S and total pyruvate by microbial cells from cystine-enriched soils

Assay conditions[a]	Mean production (nmol/mg of protein/min) ± 1 SD	
	H_2S[b]	Total pyruvate[c]
Standard assay mix	17.6 ± 1.6	17.2 ± 1.0
Dithiothreitol (25 μmol)	19.2 ± 1.0	17.6 ± 0.4
N-Ethylmaleimide (25 μmol)	14.8 ± 1.3	14.0 ± 0.0

[a] Standard assay mix contained the same components as in Table 1. The reaction was stopped with 0.5 ml of 60% trichloroacetic acid containing 25 μmol of $HgSO_4$.

[b] Least significant difference ($LSD_{0.05}$) value for comparison of H_2S means is 4.1 nmol/mg of protein/min.

[c] Least significant difference ($LSD_{0.05}$) value for comparison of total pyruvate means is 2.0 nmol/mg of protein/min.

Equimolar amounts of H_2S and total pyruvate were produced when Hg^{2+} was included in the assay (Table 2), which agrees with the predicted stoichiometry of cysteine degradation by cysteine desulfhydrase. Dithiothreitol did not alter this 1:1 ratio between H_2S and total pyruvate (Table 2) even though it had previously been shown to enhance thiazolidine formation and decrease free pyruvate production (Table 1). Likewise, *N*-ethylmaleimide addition did not alter the 1:1 stoichiometry (Table 2).

The results discussed above are also consistent with the results obtained from substrate concentration studies. Equimolar amounts of H_2S and free pyruvate were produced when substrate concentrations did not exceed 1.1 mM (Fig. 2). Cysteine concentrations above 1.1 mM increased the formation of the thiohemiketamine intermediate and the respective thiazolidine compound, resulting in decreased free pyruvate.

Plotting the amount of H_2S produced at all substrate concentrations and that of free pyruvate produced when substrate levels were below 1.1 mM resulted in a linear Lineweaver-Burk relationship (Fig. 5). Above 1.1 mM substrate, total pyruvate production provides a better estimate of reaction rate than free pyruvate. An apparent K_m of 1.32 mM was obtained with the above products at the specified substrate concentrations. Purified cysteine desulfhydrase originating from plant tissue has a lower K_m of 0.2 mM (21), and enzyme purified from *S. typhimurium* has K_m values of between 0.17 and 0.21 mM (25).

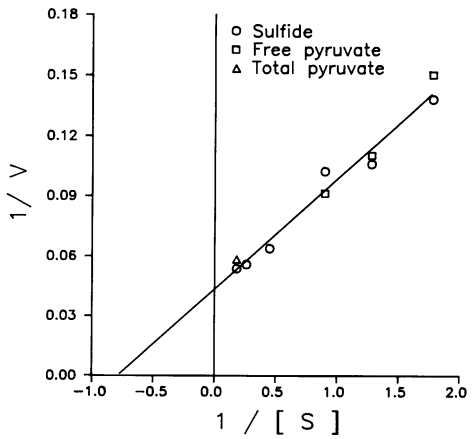

FIG. 5. Lineweaver-Burk plot of amounts of various products formed in enrichment culture assays.

Results obtained with enrichment cultures indicated that cysteine desulfhydrase was the enzyme most responsible for H_2S production. However, previous results with soils amended with cystine had indicated that another enzyme, cystathionine γ-lyase, was most active in promoting H_2S production (28, 29). It is likely that the microorganisms selected for in this work did not represent the spectrum of microorganisms in the same relative proportions as existed in the soil samples. The enzyme cysthathionine γ-lyase appears to be more prevalent in the actinomycetes and fungi (30), whereas cysteine desulfhydrase appears to be mainly of bacterial origin (26, 31). We may have selected a large bacterial component in our enrichment cultures and thus that microbial group primarily responsible for cysteine desulfhydrase production. However, these considerations do not negate the fact that cysteine exists in soil systems and that cysteine desulfhydrase is an inducible enzyme in mixed cultures of soil microorganisms. We have therefore established a potential mechanism by which H_2S may be produced from cysteine in terrestrial environments. The actual amount of H_2S produced by this mechanism in soil is as yet unknown.

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