Steps in the Conversion of α-Ketosuberate to 7-Mercaptoheptanoic Acid in Methanogenic Bacteria†

Robert H. White

Department of Biochemistry and Nutrition, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061

Received April 11, 1989; Revised Manuscript Received July 17, 1989

ABSTRACT: The biosynthetic steps involved in the conversion of α-ketosuberate to 7-mercaptoheptanoic acid were studied in cell-free extracts of methanogenic bacteria. The pathway was established by measuring the incorporation of stable isotopically labeled precursors into the *S*-methyl ether methyl ester derivative of the enzymatically generated 7-mercaptoheptanoic acid by using gas chromatography–mass spectrometry (GC–MS). Quantitation of the 7-mercaptoheptanoic acid produced in the incubations with the substrates was accomplished by using an internal standard of 6-mercaptohexanoic acid. [4,4,6,6-$^2H_4$]-2-Oxosuberic acid, [7-^{2}H]-7-oxoheptanoic acid, [2-^{2}H]-2(*RS*)-(5-carboxypentyl)thiazolidine-4(*R*)-carboxylic acid, and *S*-(6-carboxyhexyl)cysteine were each shown to be converted to 7-mercaptoheptanoic acid. Incubation of cell extracts with a mixture of 2(*RS*)-(5-carboxypentyl)thiazolidine-4(*R*)-carboxylic acid and [2-^{2}H]-2-(*RS*)-(5-carboxypentyl)-[^{34}S]thiazolidine-4(*R*)-carboxylic acid showed that both ^{34}S and ^{2}H are incorporated into the 7-mercaptoheptanoic acid but only after separation of the cysteine from the [7-^{2}H]-7-oxyheptanoic acid portion of the molecule. Furthermore, the sulfur from the cysteine was incorporated into the thiol only after its elimination from the cysteine and subsequent mixing with an unlabeled sulfur source which had a molecular weight of sufficient size that it was excluded from Sephadex G-25. Hydrogen sulfide was found to supply the sulfur for the production of the 7-mercaptoheptanoic acid in a reaction that was shown to obtain its reducing equivalents from hydrogen via an F_{420}-dependent hydrogenase.

At present, at least three different biosynthetic mechanisms have been described for the generation of C–S bonds in coenzymes, an important group of natural products with a very high proportion of its members containing sulfur. The thiol of coenzyme A is generated by the replacement of an acetate group of *O*-acetylserine with sulfide in a standard pyridoxal phosphate dependent, β-replacement reaction catalyzed by cysteine synthetase (Cook & Wedding, 1976). Decarboxylation of the resulting cysteine generates the cysteamine moiety of coenzyme A. Biotin and lipoic acid may be generated by a radical mechanism that allows for the direct introduction of sulfur at saturated carbons (White, 1980; Frappier et al., 1982), sometimes with inversion of configuration at the carbon as in lipoic acid biosynthesis (Parry & Trainor, 1978; White, 1980) and sometimes with retention of configuration at the site of sulfur introduction as in biotin biosynthesis (Trainor et al., 1980). [Retention of configuration at the site of carbon–sulfur bond formation is also observed during the biosynthesis of the penicillins and appears to proceed by the same type of mechanism as observed for the biosyntheses of biotin and lipoic acid (Baxter et al., 1982).] The mechanism for the introduction of sulfur into the thiazole ring of thiamin is unknown at present, but the sulfur for the reaction originates from cysteine (DeMoll & Shive, 1985), and the mechanism may involve the addition of a thiohydroximic acid to a ketone (White, 1978). The thiol group of coenzyme M (2-mercaptoethanesulfonic acid) is generated by the conversion of the aldehyde group of sulfoacetaldehyde to a thiol (White, 1986, 1988). The present work was undertaken to determine the details of the formation of the thiol group of component B, (7-mercaptoheptanoyl)threonine phosphate, which is proposed to arise from α-ketosuberate by a series of reactions analogous to that described for the production of coenzyme M from sulfopyruvate (White, 1989).

† This material is based upon work supported by the National Science Foundation under Grant DMB-8718216.

Materials and Methods

Materials. Ethyl hydrogen pimelate and ethyl hydrogen suberate were obtained from Lancaster Synthesis Ltd., Windham, NH. 7-Bromoheptanoic acid was obtained from Pfaltz & Bauer, Inc., Waterburg, CT. 6-Mercaptohexanoic acid was prepared from ethyl pimeloyl chloride as previously described for the synthesis of 8-mercaptooctanoic acid (White, 1980). [7,7-$^2H_2$]-7-Hydroxyheptanoic acid was prepared by NaB^2H_4 reduction of ethyl suberoyl chloride (White, 1980).

Bacterial Strains and Growth Conditions. Methanococcus volta strain P.S. (DSM 1537) (Whitman et al., 1982) and rumen isolate 10-16B, as described by Lovley et al. (1984), were used for the work described herein. All bacteria were grown in 2-L bottles pressurized to 30 psi with H_2/CO_2 as previously described (White, 1988). The cells were isolated from the medium by centrifugation and stored at −20 °C.

Preparation of Cell Extracts and Enzymatic Incubations. As previously described (White, 1988), these procedures involved sonication of cell suspensions under nitrogen in an anaerobic buffer (pH 7.5) consisting of 50 mM 2-[[tris(hydroxymethyl)methyl]amino]ethanesulfonic acid (TES), 10 mM $MgCl_2$, and 2 mM mercaptoethanol. The proteins were separated from the cell extracts, under anaerobic conditions at 3 °C, by passing 2 mL of the cell extract through a Sephadex G-25 (1.5 × 24.5 cm) column equilibrated with the same anaerobic buffer that was used in the formation of the extracts.

Preparation of a Derivative of the Enzymatically Generated 7-Mercaptoheptanoic Acid Suitable for Gas Chromatography–Mass Spectrometry (GC–MS). After the incubation times indicated in Tables I and II, a known amount of 6-mercaptohexanoic acid, typically 2 μg (13.5 nmol), was added to the enzymatic incubation mixtures, which were then heated at 100 °C for 10 min and centrifuged to remove the insoluble material. After washing (2×) the insoluble pellet with 1 mL of water, the combined extracts were concentrated to 1 mL with a stream of nitrogen gas at 90 °C. To the resulting yellow

0006-2960/89/0428-9417$01.50/0

solution were added 1 mL of methanol, 0.3 mL of 7 M aqueous ammonia, ~10 mg of $NaBH_4$, and, at 5-min intervals, three 15-μL portions of methyl iodide. The bottles were sealed with Teflon-lined, screw-top caps and vented at frequent intervals to reduce the hydrogen pressure. The bottles remained capped to prevent evaporation of the methyl iodide. After 30 min at room temperature, the samples were evaporated to 1 mL with a stream of nitrogen gas and processed to yield the methyl ester of 7-methylthioheptanoic acid as previously described (White, 1988).

Quantitation of the 7-Mercaptoheptanoic Acid Generated in the Enzymatic Incubations. Quantitation was accomplished by adding 6-mercaptohexanoic acid to the enzymatic reaction mixture as an internal standard at the time of derivative formation. GC–MS data on the resulting derivatized 7-mercaptoheptanoic acid, isolated from each enzymatic reaction mixture, contained an additional peak for the 6-mercaptohexanoic acid derivative. Since the amount of 6-mercaptohexanoic acid added is known and since the ratio of the two compounds can be determined from the data, the exact amount of 7-mercaptoheptanoic acid in the sample can be readily calculated. The ratios of the two compounds were determined by single-ion monitoring of the areas of molecular ions in the mass spectra of these two compounds as they eluted from the column. For the 7-mercaptoheptanoic acid derivative, the sums of the areas for the labeled and unlabeled ions were used. The compounds were separated on a Hewlett-Packard HP-5 capillary column (25 m × 0.32 mm) coated with 0.17 μm of cross-linked 5% phenyl methyl silicone gum phase and programmed from 75 °C at 10 °C/min. Under these chromatographic conditions, the 6-mercaptohexanoic acid and 7-mercaptoheptanoic acid derivatives had retention times of 6.1 and 7.5 min, respectively.

Synthesis of 7-Oxoheptanoic Acid and [7-^2H]-7-Oxoheptanoic Acid. The synthesis of ethyl 7-oxoheptanoate closely follows the procedure described by Burgstahler et al. (1976) for the synthesis of aldehydes from acids via their acid chlorides by using a modification of the Rosenmund reduction. Thus, ethyl hydrogen pimelate (0.94 g, 10 mmol) was dissolved in 5 mL of benzene and 0.92 g of oxalyl chloride (7.2 mmol), and 10 μL of dimethylformamide was added. After 30 min at room temperature, the benzene was evaporated under vacuum and the resulting yellow oil was dissolved in 5 mL of tetrahydrofuran. The resulting solution of ethyl pimeloyl chloride was then added to 40 mL of tetrahydrofuran containing 230 mg of 10% Pd on carbon and 0.5 mL of 2,6-lutidine. The suspension was stirred and degassed under a vacuum and hydrogenated with either hydrogen or deuterium (98 atom % ^2H) for 2 h at 30 psi. After completion of the hydrogenation, the solvent was removed in vacuo and the resulting residue was extracted with diethyl ether and evaporated to yield 1.09 g of ethyl 7-oxoheptanoate. This compound, determined to be >98% pure by GC, displayed an aldehyde stretching frequency of 2745 cm^{-1} for the protiated aldehyde and 2080 cm^{-1} for the deuteriated aldehyde. The ethyl 7-oxoheptanoate was heated at 60 °C for 1.5 h with 3 g of triethyl orthoformate and 60 mg of *p*-toluenesulfonic acid, and the resulting mixture was dissolved in ether, washed with saturated sodium bicarbonate, dried with Na_2CO_3, and evaporated to give 1.5 g of ethyl 7,7-diethoxyheptanoate. Analysis, using GC–MS, showed the product to be >90% pure with the diethyl ester of pimelic acid being the only major contaminant.

7-Oxoheptanoic acid was prepared from this material by saponification of the ester and subsequent acid hydrolysis of the diethyl acetal protecting group. Thus, ethyl 7,7-diethoxyheptanoate (494 μL, 2 mM) was mixed with 4 mL of 1 M sodium hydroxide and 1 mL of ethanol and heated at 100 °C for 20 min. The solution was concentrated to one-third the original volume and extracted with hexane to remove a small amount of insoluble oil. The resulting solution, after the addition of 2 mL of 3 M HCl, was heated under N_2 at 100 °C for 6 min, cooled to room temperature, and neutralized by the addition of 2 M NaOH. (The conversion of the ethyl 7-oxoheptanoate to ethyl 7,7-diethoxyheptanoate before saponification of the ester was required because of the intramolecular aldol condensation of the ethyl 7-oxoheptanoate during the saponification.)

Synthesis of 2(RS)-(5-Carboxypentyl)thiazolidine-4(R)-carboxylic Acid and [2-^2H]-2(RS)-(5-Carboxypentyl)[^{34}S]-thiazolidine-4(R)-carboxylic Acid. L-Cysteine (242 mg) and sodium acetate (280 mg) were dissolved in the above-described solution of 7-oxoheptanoic acid, and the pH was adjusted to 4.0. After concentration to half its original volume, the solution was cooled to 3 °C in order to induce crystallization. The resulting white crystals were collected by filtration, washed with cold water and ethanol, and recrystallized (2×) from hot water. A total of 100 mg of crystals was obtained by this procedure. The 270-MHz ^1H NMR of this thiazolidine in D_2O showed the presence of an equal mixture of the cis (2*R*,4*R*) and the trans (2*S*,4*R*) thiazolidine isomers. The chemical shifts and coupling constants were in agreement with those previously reported for thiazolidines derived from L-cysteine (Parthasarathy et al., 1976; Szilagyi & Gyorgydeak, 1979). The FAB mass spectrum of the material, suspended in glycerol, showed an MH^+ ion at *m/z* 248. Reaction of a portion of the material, which was dissolved in methanol containing a trace of HCl with diazomethane, generated the dimethyl ester. The mass spectrum of this dimethyl ester showed a molecular ion at M^+ *m/z* 275 (1%), $M^+ - 59$ *m/z* 216 (11.5%), *m/z* 160 (34%), and *m/z* 146 (100%). Acetylation of the compound with acetic anhydride in pyridine gave the *N*-acetyl derivative, which was converted to the dimethyl ester by reaction with HCl in methanol. This *N*-acetyl dimethyl ester derivative showed a molecular ion at M^+ *m/z* 317 (0.3%) and fragment ions at $M^+ - 59$ *m/z* 274 (1%), $M^+ - CH_3OOC(CH_2)_5$ *m/z* 188 (20%), and *m/z* 146 (100%).

Performing the same synthesis, but with [7-^2H]-7-oxoheptanoic acid and [^{34}S]cysteine prepared as previously described (White, 1988), yielded [2-^2H]-2(*RS*)-(5-carboxypentyl)-[^{34}S]-thiazolidine-4(*R*)-carboxylic acid. This labeled material was mixed with unlabeled material and recrystallized from hot (100 °C) water to generate the labeled compound used in the biosynthetic work. The final material was found to have the following isotopic distribution as measured from the *m/z* 231 ion of the acetate of the dimethyl ester derivative: 55.9% with no label, 3.3% with ^2H, 11.0% with ^{34}S, and 29.8% with both ^2H and ^{34}S. The isotopic distribution of the MH^+ measured by FAB mass spectrometry of the sample suspended in glycerol was found to be 57.1% with no label, 2.2% with ^2H, 9.9% with ^{34}S, and 30.8% with both ^2H and ^{34}S. The presence of molecules containing either a single ^2H or a single ^{34}S results from an exchange between the labeled and unlabeled 7-oxoheptanoic acid and the labeled and unlabeled cysteine of the thiazolidine, which occurred during the recrystallization from hot water. This exchange was confirmed by measuring the isotopic distribution of the labeled thiazolidine which had been dissolved in water and heated at 60 °C for 3.5 h. The resulting thiazolidine was found to be 63.5% with no label, 11.1% with ^2H, 13.9% with ^{34}S, and 11.4% with

Table I: Production of 7-Mercaptoheptanoic Acid from Precursors by Extracts of *M. volta*[a]

expt no.	substrates and concns used	incubation time (h)	protein concn (mg/mL)	nmol of 7-mercapto-heptanoic acid/mg of protein[b]
1	[7-^{2}H]-7-oxoheptanoic acid (12.4 mM)	3	25.2	17.4[c]
2	[7-^{2}H]-7-oxoheptanoic acid (12.4 mM), L-cysteine (30 mM)	3	25.2	12.3[c]
3	*S*-(6-carboxyhexyl)cysteine (1 mM)	3	25.2	16.3
4	7-oxoheptanoic acid (9.5 mM), H_2S (9.1 mM)	4	17.0	13.4
5	[2-^{2}H]-2(*RS*)-(5-carboxypentyl)thiazolidine-4(*R*)-carboxylic acid (10 mM)	4	25.2	4.0[c,d]
6	[7,7-$^2H_2$]-7-hydroxyheptanoic acid (10 mM), cysteine (30 mM)	3	9.3	<0.03

[a] All experiments were performed at 39 °C under an atmosphere of hydrogen using crude cell extracts. Experiments 1–3 were conducted simultaneously by using the same extract; all other experiments, except experiment 5, were conducted with an independently derived extract. All experiments used 2 mL of extract except experiment 6, which used 1 mL of extract. [b] The calculation of nanomoles of product formed was based on the amount of product produced. [c] The 7-mercaptoheptanoic acid was labeled with ^{2}H at C-7 to the extent of >90%. [d] This extract was the same as that used in experiments 1–3, but the extract was exposed to air while frozen for 30 days before the analysis was performed.

both ^{2}H and ^{34}S, as measured from the m/z 231 ion of the acetate of the dimethyl ester derivative. The decrease in the proportion of molecules with both ^{2}H and ^{34}S, and the increase in the number of molecules with ^{2}H or ^{34}S, proves that the reaction between 7-oxoheptanoic acid and the cysteine is an equilibrium reaction.

In order to establish if the exchange would be slow enough so that the labeled thiazolidine could be used for the required experiments, the labeled thiazolidine was incubated in the assay buffer at 39 °C for 3 h followed by quenching of the exchange by the addition of acid. (The exchange was found to be completely blocked when the thiazolidine was placed in 0.1 M HCl.) The mass spectrum of the resulting thiazolidine as the acetate of the dimethyl ester derivative showed it to be 46.8% with no label, 12.5% with ^{2}H, 17.5% with ^{34}S, and 23.1% with both ^{2}H and ^{34}S as measured from the m/z 231 ion. Although this labeling pattern indicated that exchange occurred under the enzyme assay conditions, it was not enough to prevent the desired experiments from being undertaken.

Synthesis of S-(6-Carboxyhexyl)cysteine. 7-Bromoheptanoic acid (1.05 g, 5 mmol) and L-cysteine (0.605 g, 5 mmol) were dissolved in 5 mL of 3.0 M sodium hydroxide. The resulting clear, colorless solution was kept overnight at 65 °C and neutralized with acetic acid. The resulting precipitated, white solid was removed by filtration, washed with water and then ethanol, dried, and dissolved in a minimal volume of ~90 °C 6 M HCl. The addition of aqueous ammonia precipitated the product, which was washed with water and ethanol and dried to give 0.99 g of *S*-(6-carboxyhexyl)-cysteine. Conversion of the *S*-(6-carboxyhexyl)cysteine to the dibutyl *N*-trifluoroacetyl derivative, and subsequent mass spectral analysis, showed the expected ions at M^+ m/z 457, $M^+ - 73$ m/z 384, and $M^+ - 101$ m/z 356.

Analysis of the Cysteine in Extracts. Cell extract (1 mL), prepared as described above, was heated for 10 min at 100 °C under N_2. After centrifugation to remove the insoluble material, the resulting clear solution was concentrated to 0.2 mL and mixed with 0.2 mL of methanol, 0.1 mL of 7 M ammonia, 10 mg of $NaBH_4$, and 20 μL of methyl iodide. After 30 min at room temperature, the sample was evaporated to remove the methanol and ammonia, and the resulting solution of *S*-methylcysteine was placed on a column of Dowex 50W-8X H^+ (2.5 × 5 mm). After washing the column with water, the *S*-methylcysteine was eluted with 3 M aqueous ammonia and, after removal of the ammonia, quantitated by using a Beckman high-pressure liquid chromatograph equipped with an ion-exchange column and a ninhydrin detector system. Recoveries of known amounts of cysteine added to cell extracts were 63% by this procedure.

Analysis of Cysteine Desulfhydrase Activity. Cell extracts, purified by chromatography on Sephadex G-25, were mixed with a 1.5 M solution of L-cysteine to give a final concentration of 9 mM in L-cysteine. The resulting solutions were incubated in sealed vials under N_2 at 39 °C, and the production of H_2S was assayed as described by Lovenberg et al. (1963).

Results and Discussion

Previous work on the biosynthesis of the 7-mercaptoheptanoic acid moiety of component B (White, 1988, 1989) has indicated that this compound is produced from α-ketosuberate. The basic steps of this conversion were postulated to be identical with those described for the biosynthesis of coenzyme M (2-mercaptoethanesulfonic acid) from sulfopyruvate, i.e., nonoxidative decarboxylation of the α-keto acid to an aldehyde, conversion of the aldehyde to a thiazolidine adduct by reaction with L-cysteine, reduction of the C–N bond of the thiazolidine, and elimination of a dehydroalanine from the product to generate the thiol (White, 1986). For the case of coenzyme M biosynthesis, it was postulated that the reduction of the C–N bond of the thiazolidine adduct [2-(sulfomethyl)thiazolidine-4(*R*)-carboxylic acid] would generate (2-sulfoethyl)cysteine, which would undergo a β-elimination to produce coenzyme M. That the pathway for the biosynthesis of 7-mercaptoheptanoic acid is in some ways similar to that of coenzyme M biosynthesis is supported by the data in Table I, which show that cell-free extracts of methanogenic bacteria are able to catalyze the conversion of [7-^{2}H]-7-oxoheptanoic acid, [2-^{2}H]-2(*RS*)-2-(5-carboxypentyl)thiazolidine-4(*R*)-carboxylic acid, and *S*-(6-carboxyhexyl)cysteine to 7-mercaptoheptanoic acid. (Control experiments, using bovine serum albumin as a protein source, showed no synthesis of 7-mercaptoheptanoic acid.) Also, as previously observed in coenzyme M biosynthesis, the reduced substrate [7-$^2H_2$]-7-hydroxyheptanoic acid was not found to be converted to 7-mercaptoheptanoic acid. The thiazolidine substrate was also shown to be converted to 7-mercaptoheptanoic acid by a protein fraction which was separated from small, freely dissociating, low molecular weight cofactors by use of Sephadex G-25 chromatography of a cell extract of *M. volta* (experiment 1, Table II). In addition, only extracts that were incubated under hydrogen, as opposed to nitrogen (experiments 1 and 2, Table II), produced 7-mercaptoheptanoic acid. The ability of the extracts to convert the thiazolidine to 7-mercaptoheptanoic acid was also reduced by exposing the frozen extracts to air over time (experiment 5, Table I). The conversion of [4,4,6,6-$^2H_4$]-2-oxosuberic acid to 7-mercaptoheptanoic acid was not observed in extracts of *M. volta* but was observed in extracts of strain 10-16B, where 0.1 nmol of [$^2H_4$]-7-

Table II: Labeling Patterns of 7-Mercaptoheptanoic Acid Produced in Cell-Free Extracts from [2-^{2}H]-2(*RS*)-(5-Carboxypentyl)[^{34}S]thiazolidine-4(*R*)-carboxylic Acid[a]

expt no.	conditions	protein concn (mg/mL)	distribution of label in M^+ m/z 190[b]				nmol of 7-mercapto-heptanoic acid/mg of protein[c]
			m/z 190 (no ^{2}H or ^{34}S)	m/z 191 (^{2}H)	m/z 192 (^{34}S)	m/z 194 (^{2}H and ^{34}S)	
	Sephadex G-25 purified cell extract						
1	2.4 mM thiazolidine, 3 h under H_2	16.0	52.1 (100)	25.9 (61.3)	15.2 (40.6)	6.8 (19.7)	8.65
2	2.4 mM thiazolidine, 3 h under N_2	16.0	100 (100)	~0.0 (~12)	~0.0 (~6)	~0.0 (ND[e])	<0.01[d]
	non-Sephadex purified cell extract						
3	3.0 mM thiazolidine, 0.5 h under H_2	24.0	71.3 (100)	26.0 (48.1)	2.4 (13.3)	0.3 (3.35)	0.57
4	3.0 mM thiazolidine, 1.0 h under H_2	24.0	75.4 (100)	21.3 (39.8)	2.8 (12.6)	0.2 (2.8)	3.4
5	3.4 mM thiazolidine, 6 h under H_2	38.2	45.9 (100)	32.7 (82.8)	13.2 (42.5)	8.2 (25.7)	13.2

[a] All experiments were performed at 39 °C with extracts of *M. volta*. Experiments 1 and 2 were performed with the same extract split in half. Experiments 3 and 4 were each carried out with a separately prepared extract. Experiments 1 and 2 used 2.5 mL of extract, experiments 3 and 4 used 2 mL of extract, and experiment 5 used 2.4 mL of extract. The labeling pattern in the substrate was 55.9% no label, 3.3% ^{2}H, 11.0% ^{34}S, and 29.8% both ^{2}H and ^{34}S. [b] The numbers in parentheses are the observed normalized ion intensities. The observed normalized ion intensity ratios for the ion, ion + 1 m/z, ion + 2 m/z, and ion + 3 m/z, for unlabeled methyl 7-methylthioheptanoate, were 100, 11.6, 5.6, and 0.5, respectively, for the M^+ m/z 190 ion. [c] The calculated amount of product formed was based on the amount of product produced. [d] Total of 0.14 nmol of the 7-mercaptoheptanoic acid derivative/mg of protein was found; however, no excess of ^{2}H or ^{34}S over the natural abundance was detected. [e] Not detected.

mercaptoheptanoic acid/mg of protein was generated. All these observations are consistent with the thiol group of the 7-mercaptoheptanoic acid being generated via a thiazolidine adduct of an aldehyde as was proposed for the biosynthesis of coenzyme M (White, 1986, 1988).

An unexpected finding (experiments 1 and 2, Table I) concerns the conversion of [7-^{2}H]-7-oxoheptanoic acid to 7-mercaptoheptanoic acid. The addition of cysteine to the incubation mixture resulted in a *decrease* in the amount of 7-mercaptoheptanoic acid produced of from 17.4 to 12.3 nmol/mg of protein. If a cysteine thiazolidine derivative of the 7-oxoheptanoic acid was an intermediate in the biosynthesis, then the addition of cysteine to the incubation mixture would be expected to stimulate the production of the product. This was, in fact, observed during the biosynthesis of coenzyme M, where a 5-fold increase in the amount of coenzyme produced was observed with the addition of cysteine to the reaction (White, 1988). These observations cause one to seriously question the involvement of a thiazolidine adduct of cysteine in the biosynthesis.

The cleavage of *S*-(6-carboxyhexyl)cysteine, an expected intermediate to 7-mercaptoheptanoic acid via a thiazolidine adduct, to 7-mercaptoheptanoic acid is reported in Table I (experiment 3). This cleavage, however, could simply result from the action of β-cystathionase (cysteine desulfhydrase), an enzyme with low specificity for the R group of the cysteine (Delavier-Klutchko & Flavin, 1965; Greenberg et al., 1964). Alternatively, the bacterial enzyme L-methionine γ-lyase is known to catalyze β-elimination reactions of L-cysteine derivatives (Tanaka et al., 1977) and could be responsible for the generation of 7-mercaptoheptanoic acid from this substrate.

If 2(*RS*)-(5-carboxypentyl)thiazolidine-4(*R*)-carboxylic acid was converted to 7-mercaptoheptanoic acid as proposed for coenzyme M biosynthesis, then the C-2 and its attached proton and the sulfur of the thiazolidine should be incorporated as a unit into the 7-mercaptoheptanoic acid. This was tested by measuring the incorporation of 2(*RS*)-(5-carboxypentyl)-thiazolidine-4(*R*)-carboxylic acid, labeled with both ^{34}S and ^{2}H at the C-2 of the thiazolidine, into the 7-mercaptoheptanoic acid. Also, by performing the experiment with the thiazolidine where only a portion of the molecules contain both the ^{2}H and ^{34}S label, any scrambling of the cysteine between different 7-oxoheptanoic acid molecules can be determined. This is necessary since data indicate that the reaction between cysteine and aldehydes is a readily reversible one in which a small concentration of cysteine and aldehyde can be found in solution. This reversibility has been demonstrated by chemical analysis of the cysteine generated in solutions of "pure" thiazolidines (Ratner & Clarke, 1937) and by epimerization of the C-2 of 2-substituted thiazolidine-4-carboxylic acids (Nagasawa et al., 1981; Szilagyi & Gyorgydeak, 1979). The occurrence of this scrambling is made apparent in the data by the reduction in the mole percent of the molecules that contain *both* ^{2}H and ^{34}S.

The isotopic pattern of the 7-mercaptoheptanoic acid derivative generated from the ^{2}H- and ^{34}S-labeled thiazolidine with complete exchange between the L-cysteine and the 7-oxoheptanoic acid will be given by the intensities of the coefficients of the expression $(a + b)(c + d)$, where $b/(a + b)$ is the atom percent enrichment of ^{2}H and $d/(c + d)$ is the atom percent enrichment of ^{34}S. The product ac then equals the mole fraction of the molecules with no label, bc the mole fraction of the molecules with ^{2}H, ad the mole fraction of the molecules with ^{34}S, and bd the mole fraction of the molecules with both ^{2}H and ^{34}S. From the isotopic intensities measured from the m/z 231 ion of the *N*-acetyl dimethyl ester of the synthetic sample of [2-^{2}H]-2(*RS*)-(5-carboxypentyl)[^{34}S]-thiazolidine-4(*R*)-carboxylic acid, these values are calculated to be $a = 0.67$, $b = 0.33$, $c = 0.60$, and $d = 0.40$. Using these values in the above expression, one obtains 0.40, 0.20, 0.27, and 0.13, respectively, for the expected distribution of label in the 7-mercaptoheptanoic acid derived from the cysteine and from the 7-oxoheptanoic acid portions of the thiazolidine, with complete scrambling between these two components. This pattern is clearly different from that of the experimental data (experiment 1, Table II). However, since the ratio of molecules with one ^{2}H to those with no label are about the same for both the calculated and the observed isotope patterns, the difference between the patterns likely results from the isotopic dilution of the ^{34}S that is incorporated into the 7-mercaptoheptanoic acid. If we let $a = 0.67$ and $d = 0.33$, then using the observed isotopic distribution, one can easily calculate the values of c and d that are required to generate the experimental data. These values are found to be $c = 0.78$ and $b = 0.22$. Since the cysteine sulfur was 40% ^{34}S, 0.49 part of this sulfur would have to have mixed with 0.51 part of a sulfur source containing the natural abundance of ^{34}S in order to generate the observed distribution.

There are two possible models that can explain the observed dilution of the sulfur label and the lack of incorporation of ^{34}S at short incubation times, both of which allow for the rapid, reversible dissociation of thiazolidine to cysteine and 7-oxo-

heptanoic acid. In the first model, the cysteine generated from the dissociation of the thiazolidine is mixed with unlabeled cysteine in the cell extract. The resulting thiazolidine, re-formed from this ^{34}S-diluted cysteine, would then be used as the substrate for the formation of 7-mercaptoheptanoic acid. Alternatively, the thiazolidine-derived, ^{34}S-labeled cysteine could exchange its sulfur with an unlabeled sulfur source, catalyzed by cysteine synthetase in the cell extracts, before re-formation of the thiazolidine.

In the second model, the thiazolidine is dissociated into cysteine and 7-oxoheptanoic acid as in the first model, but the resulting cysteine is cleaved over time to release its sulfur, which mixes with an unlabeled sulfur source, which, in turn, serves as the substrate for the synthesis of 7-mercaptoheptanoic acid from 7-oxoheptanoic acid.

Both these models could be used to explain the observed dilution of the sulfur; however, only the second model is consistent with the present experimental data. The first model would require that cysteine be present in the cell extracts at a level equivalent to the cysteine added from the thiazolidine, typically, 200–300 μmol/mg of protein for the experiments reported herein. The level of free cysteine in cell extracts was found to be only 1.6 nmol/mg of protein. Furthermore, the dilution of the sulfur label occurred to the same extent even when a protein fraction purified by Sephadex G-25 chromatography of the cell extract was used as the source of the biosynthetic enzymes (experiment 1, Table II).

The possible exchange of the cysteine sulfur catalyzed by cysteine synthetase was tested by incubating cell extracts (24 mg of protein/mL) with L-cysteine (10 mM) and DL-[2,3,3-$^2H_3$]serine (80 mM) for 6 h under the standard conditions, isolating the cysteine, and measuring for the incorporation of deuterium into the cysteine by using GC–MS (White, 1981). If the cysteine had reversibly dissociated to serine and hydrogen sulfide, then some of the labeled serine would have been incorporated into the cysteine. Performing the experiment showed no label in the cysteine (<0.3%), indicating that this is not a possible explanation for the sulfur dilution.

In addition to the above evidence, it was found that smaller and smaller amounts of ^{34}S are incorporated into the 7-mercaptoheptanoic acid from the thiazolidine with shorter and shorter incubation times (in order of decreasing incubation time: experiments 5, 1, 4, and 3, Table II). Extrapolation of the 1- and 3-h ^{34}S levels to zero time showed, in fact, that no ^{34}S was incorporated into the first 7-mercaptoheptanoic acid produced, thus indicating that the cysteine-derived thiazolidine is not an intermediate in the biosynthesis of 7-mercaptoheptanoic acid.

Since the sulfur from the cysteine in the thiazolidine is clearly incorporated into the 7-mercaptoheptanoic acid after long incubation times, the most rational explanation is that the cysteine is cleaved to either some form of sulfane sulfur or to hydrogen sulfide over time and that the resulting released sulfur mixes with some form of bound, unlabeled sulfur that can supply the sulfur for the biosynthesis of the 7-mercaptoheptanoic acid. Cell extracts of *M. volta* were found to readily cleave L-cysteine when added to the protein solution at a concentration of 8 mM, with the generation of hydrogen sulfide at a rate of ~7.6 nmol/(h·mg of protein). This cleavage reaction could result from cysteine desulfhydrase, an enzyme that is widely distributed in both the eubacteria (Ohkishi et al., 1981) and the methanogenic archaebacteria (Mazumder et al., 1986), and it would explain the increased incorporation of ^{34}S over time since the released ^{34}S can increasingly label an unlabeled sulfur source over time, which then supplies the sulfur for the biosynthesis. The dilution of the incorporated sulfur, even in a Sephadex G-25 purified protein fraction from a cell extract (experiment 1, Table II), suggests that this sulfur source is protein bound. The sulfur source must also be present in the cell extract since the biosynthesis occurs readily even in the absence of an added sulfur source (experiment 1, Table I).

One possible explanation for the source of the sulfur is that the enzyme that catalyzed the reaction is itself the source of the sulfur pool and is responsible for the isotope dilution. This, however, seems very unlikely due to the large size of the observed pool, at least 5 nmol of sulfur/mg of protein. Thus, if each protein contained one sulfur atom and the enzyme had a molecular weight of say 50K, then 25% of the cellular protein would have to be this single protein. Since this is unrealistic, one must then consider that a separate pool of protein-bound sulfur may supply the sulfur for the enzymatic reaction. An obvious choice would be the iron–sulfur proteins present in the cells. Analysis of cell extracts of *M. volta* for acid-labile sulfide, as described by Lovenberg et al. (1963), showed them to contain 50.0 nmol of acid-labile sulfide/mg of protein, which, if mixed with the H_2S generated by the cysteine cleavage, is more than enough to account for the observed isotopic dilution.

This mixing could occur with all of the iron–sulfur centers by either a slow exchange of the cysteine-released sulfur or a rapid mixing of a subpopulation of the iron–sulfur centers or a combination of these two processes. However, on the basis of the tight binding of sulfide in ferredoxin (Malkin & Rabinowitz, 1967) and the absence of exchange of sulfide in the clostridial ferredoxin (Lovenberg et al., 1963; Hong & Rabinowitz, 1970) at neutral pHs, if an exchange is occurring, it must be enzyme mediated. The reaction involved in this exchange could be catalyzed by rhodanese, which appears to facilitate the construction of the iron–sulfur cluster in apoferredoxin (Cerletti, 1986) and is known to transfer sulfur to succinate dehydrogenase (Bonomi et al., 1977b) and ferredoxin (Bonomi et al., 1977a). The exchange reaction could also involve 3-mercaptopyruvate sulfotransferase, an enzyme believed to be involved in the biosynthesis of iron–sulfur proteins (Taniguchi & Kimura, 1974; White, 1983), or a previously undescribed enzymatic activity responsible for the formation of the iron–sulfur cluster of ferredoxin in chloroplasts (Takahashi et al., 1986).

The sulfur source could also be in the form of a sulfane-containing polysulfide. Enzyme-bound polysulfides have been either proven or implied to be involved in the oxidative metabolism of S_8 in *Thiobacillus* (Aminuddin & Nicholas, 1973; Moriarty & Nicholas, 1970) and in the reductive metabolism of S_8 in methanogenic bacteria (Stetter & Gaag, 1983; Daniels et al., 1986; Fischer et al., 1983), in reducing eubacteria (Zophel et al., 1988), and in thermophiles (Belkin et al., 1985). The possible involvement of polysulfides in the biosynthesis of 7-mercaptoheptanoic acid in *M. volta* is based on the finding that extracts of these cells contained 20 nmol of sulfane sulfur/mg of protein, as assayed by the cold cyanolysis method of Wood (1987). This sulfane sulfur could be formed by the action of 3-mercaptopyruvate sulfotransferase, an enzyme known to form polysulfides as well as sulfur (Hylin & Wood, 1959; Jarabak & Westley, 1980) from 3-mercaptopyruvate, a substrate readily derived from L-cysteine. Both these products could serve as nontoxic reserve sources of sulfur for the generation of not only the 7-mercaptoheptanoic acid but also the iron–sulfur proteins and other sulfur-containing metabolites as well. One problem with sulfane sulfur as the sulfur

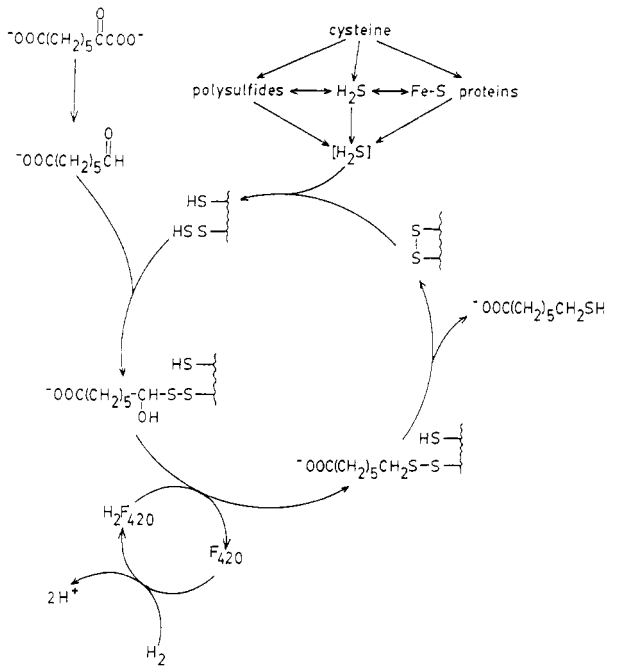

FIGURE 1: Proposed pathway for the biosynthesis of 7-mercaptoheptanoic acid.

source is that it must be reduced before or during its incorporation into the 7-oxoheptanoic acid. As shown below, this reduction could readily occur as part of the mechanism for the formation of the thiol, with the sulfane sulfur being incorporated into the enzyme as the reaction proceeds.

A general model for the biosynthesis of 7-mercaptoheptanoic acid from 2-oxosuberate is shown in Figure 1. The figure takes into consideration the nonoxidative decarboxylation of the 2-oxosuberate to 7-oxoheptanoic acid, the cleavage of the cysteine to H_2S and its subsequent mixing with a pool of unlabeled sulfur in the cells, and its subsequent reaction with a disulfide to generate a persulfide. The combination of the 7-oxoheptanoic acid with this bound persulfide produces an intermediate, which, upon reduction, is positioned in such a manner that formation of the original disulfide releases the 7-mercaptoheptanoic acid. The use of F_{420} as the reductant is supported by the fact the *M. volta* contains only an F_{420}-dependent hydrogenase (Muth et al., 1987), and by the recent demonstration that F_{420} can supply the electrons for the required reduction (R. H. White, unpublished results). Thus, in some ways, the chemistry of this reaction is analogous to the F_{420}-dependent reduction of methylenetetrahydromethanopterin (Hartzell et al., 1985). The proposal of a persulfide sulfur as the nucleophile in the reaction is based on the increased nucleophilicity of the persulfide sulfur due to the α effect (Grekov & Veselov, 1978), and on the occurrence of persulfide residues in enzymes such as rhodanese, xanthine oxidase, and aldehyde oxidase (Wood, 1982).

The enzymatic reaction described herein is not without its organic models. Aldehydes and ketones are known to react with H_2S to generate disulfides both in the absence (Cairns et al., 1952) and in the presence of catalysts (Cohen, 1976). Even polysulfides react to generate thiols (Yukawa & Kishi, 1951). Reduction of aldehydes with sulfurated $NaBH_4$ also leads to thiols, but by a mechanism that appears to involve sulfur displacement of a borate ester of the reduced aldehyde (Lalancette & Freche, 1969).

ACKNOWLEDGMENTS

I thank Kim Harich for running the mass spectra, Hwei-Yiing Li for assistance in growing the bacteria, and Linda D. White for editing the manuscript.

Registry No. H_2S, 7783-06-4; α-ketosuberate, 96406-05-2; 7-mercaptoheptanoic acid, 52000-32-5; 7-oxoheptanoic acid, 17126-90-8; 2-(5-carboxypentyl)thiazolidine-4-carboxylic acid, 123330-47-2; *S*-(6-carboxyhexyl)cysteine, 123330-48-3.

REFERENCES

Aminuddin, M., & Nicholas, D. J. D. (1973) *Biochim. Biophys. Acta 325*, 81–93.

Baxter, R. L., Scott, A. I., & Fukumura, M. (1982) *J. Chem. Soc., Chem. Commun. 1982*, 66–68.

Belkin, S., Wirsen, C. O., & Jannasch, H. W. (1985) *Appl. Environ. Microbiol. 49*, 1057–1061.

Bonomi, F., Pagani, S., & Cerletti, P. (1977a) *FEBS Lett. 84*, 149–152.

Bonomi, F., Pagani, S., Cerletti, P., & Cannella, C. (1977b) *Eur. J. Biochem. 72*, 17–24.

Burgstahler, A. W., Weigel, L. O., & Shaefer, C. G. (1976) *Synthesis 26*, 767–768.

Cairns, T. L., Evans, G. L., Larchar, A. W., & McKusick, B. C. (1952) *J. Am. Chem. Soc. 74*, 3982–3983.

Cerletti, P. (1986) *Trends Biochem. Sci. (Pers. Ed.) 129*, 369–372.

Cohen, V. I. (1976) *Helv. Chim. Acta 59*, 840–843.

Cook, P. F., & Wedding, R. T. (1976) *J. Biol. Chem. 251*, 2023–2029.

Daniels, L., Belay, N., & Rajagopal, B. S. (1986) *Appl. Environ. Microbiol. 51*, 703–709.

Delavier-Klutchko, C., & Flavin, M. (1965) *Biochim. Biophys. Acta 99*, 375–377.

DeMoll, E., & Shive, W. (1985) *Biochem. Biophys. Res. Commun. 132*, 217–222.

Fischer, F., Zillig, W., Stetter, K. O., & Schreiber, G. (1983) *Nature 301*, 511–513.

Frappier, F., Jouany, M., Marquet, A., Olesker, A., & Tabet, J.-C. (1982) *J. Org. Chem. 47*, 2257–2261.

Greenberg, D. M., Mastalerz, P., & Nagabhushnam, A. (1964) *J. Biol. Chem. 253*, 8854–8859.

Grekov, A. P., & Veselov, V. Y. (1978) *Russ. Chem. Rev. 47*, 631–648.

Hartzell, P., Zvilius, G., Escalante-Semerena, J., & Donnelly, M. I. (1985) *Biochem. Biophys. Res. Commun. 133*, 884–890.

Hong, J.-S., & Rabinowitz, J. C. (1970) *J. Biol. Chem. 245*, 6582–6587.

Hylin, J. W., & Wood, J. L. (1959) *J. Biol. Chem. 234*, 2141–2144.

Jarabak, R., & Westley, J. (1980) *Biochemistry 19*, 900–904.

Lalancette, J. M., & Freche, A. (1969) *Can. J. Chem. 47*, 739–742.

Lovenberg, W., Buchanan, B. B., & Rabinowitz, J. C. (1963) *J. Biol. Chem. 238*, 3899–3913.

Lovley, D. R., Greening, R. C., & Ferry, J. G. (1984) *Appl. Environ. Microbiol. 48*, 81–87.

Malkin, R., & Rabinowitz, J. C. (1967) *Biochemistry 6*, 3880–3891.

Mazumder, T. K., Nishio, N., Fukuzaki, S., & Nagai, S. (1986) *Appl. Environ. Microbiol. 52*, 617–622.

Moriarty, D. J. W., & Nicholas, D. J. D. (1970) *Biochim. Biophys. Acta 197*, 143–151.

Muth, E., Morschel, E., & Klein, A. (1987) *Eur. J. Biochem. 169*, 571–577.

Nagasawa, H. T., Goon, D. J. W., & Shirota, F. N. (1981) *J. Heterocycl. Chem. 18*, 1047–1051.

Ohkishi, H., Nishikawa, D., Kumagai, H., & Yamada, H. (1981) *Agric. Biol. Chem. 41*, 253–257.

Parry, R. J., & Trainor, D. A. (1978) *J. Am. Chem. Soc. 100*, 5243–5244.
Parthasarathy, R., Paul, B., & Korytnyk, W. (1976) *J. Am. Chem. Soc. 98*, 6634–6643.
Ratner, S., & Clarke, H. T. (1937) *J. Am. Chem. Soc. 59*, 200–206.
Stetter, K. O., & Gaag, G. (1983) *Nature 305*, 309–311.
Szilagyi, L., & Gyorgydeak, Z. (1979) *J. Am. Chem. Soc. 101*, 427–432.
Takahashi, Y., Mitsui, A., Hase, T., & Matusbara, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 2434–2437.
Tanaka, H., Esaki, N., & Soda, K. (1977) *Biochemistry 16*, 100–106.
Taniguchi, T., & Kimura, T. (1974) *Biochim. Biophys. Acta 364*, 284–295.
Trainor, D., Parry, R. J., & Gitterman, A. (1980) *J. Am. Chem. Soc. 102*, 1467–1468.
White, R. H. (1978) *Biochemistry 17*, 3833–3840.
White, R. H. (1980a) *J. Am. Chem. Soc. 102*, 6605–6607.
White, R. H. (1980b) *Biochemistry 19*, 15–19.
White, R. H. (1981) *Anal. Biochem. 114*, 349–354.
White, R. H. (1983) *Biochem. Biophys. Res. Commun. 112*, 66–72.
White, R. H. (1986) *Biochemistry 25*, 5304–5308.
White, R. H. (1988) *Biochemistry 27*, 7458–7462.
White, R. H. (1989) *Arch. Biochem. Biophys. 270*, 691–697.
Whitman, W. B., Ankwanda, E., & Wolfe, R. S. (1982) *J. Bacteriol. 149*, 852–863.
Wood, J. L. (1982) in *Structure and Function Relationships in Biochemical Systems* (Bossa, F., Chiancone, E., Finazzi-Agro, A., & Strom, R., Eds.) pp 327–342, Plenum, New York.
Wood, J. L. (1987) *Methods Enzymol. 143*, 25–29.
Yukawa, Y., & Kiski, Y. (1951) *J. Chem. Soc. Jpn., Pure Chem. Sect. 72*, 371–373.
Zophel, A., Kennedy, M. C., Beinert, H., & Kroneck, P. M. H. (1988) *Arch. Microbiol. 150*, 72–77.

Binding of Pyrimidin-2-one Ribonucleoside by Cytidine Deaminase as the Transition-State Analogue 3,4-Dihydrouridine and the Contribution of the 4-Hydroxyl Group to Its Binding Affinity†

L. Frick, C. Yang, V. E. Marquez, and R. Wolfenden*

Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27599, and Laboratory of Medicinal Chemistry, National Cancer Institute, Developmental Therapeutics Program, Division of Cancer Treatment, National Institutes of Health, Bethesda, Maryland 20892

Received May 23, 1989; Revised Manuscript Received July 26, 1989

ABSTRACT: Cytidine deaminase, purified to homogeneity from constitutive mutants of *Escherichia coli*, was found to bind the competitive inhibitors pyrimidin-2-one ribonucleoside (apparent $K_i = 3.6 \times 10^{-7}$ M) and 5-fluoropyrimidin-2-one ribonucleoside (apparent $K_i = 3.5 \times 10^{-8}$ M). Enzyme binding resulted in a change of the λ_{max} of pyrimidin-2-one ribonucleoside from 303 nm for the free species to 239 nm for the bound species. The value for the bound species was identical with that of an oxygen adduct formed by combination of hydroxide ion with 1,3-dimethyl-2-oxopyrimidinium (239 nm), but lower than that of a sulfur adduct formed by combination of the thiolate anion of *N*-acetylcysteamine with 1,3-dimethyl-2-oxopyrimidinium (259 nm). The results suggest that pyrimidin-2-one ribonucleoside is bound by cytidine deaminase as an oxygen adduct, probably the covalent hydrate 3,4-dihydrouridine, rather than intact or as an adduct involving a thiol group of the enzyme. In dilute solution at 25 °C, the equilibrium constant for formation of a single diastereomer of 3,4-dihydrouridine from pyrimidin-2-one ribonucleoside was estimated as approximately 4.7×10^{-6}, from equilibria of dissociation of water, protonation of 1-methylpyrimidin-2-one, and combination of the 1,3-dimethylpyrimidinium cation with the hydroxide ion. On the basis of this equilibrium constant and the apparent K_i value observed for pyrimidin-2-one ribonucleoside, the equilibrium constant for dissociation of a single inhibitory isomer of 3,4-dihydrouridine from the enzyme was estimated as 1.2×10^{-12} M, more than 8 orders of magnitude lower than the K_m value of the substrate cytidine. Replacement of the 4-hydroxyl group of 3,4-dihydrouridine, in 3,4-dihydropyrimidin-2-one ribonucleoside (apparent $K_i = 3.0 \times 10^{-5}$ M), reduced its negative free energy of binding by 10.1 kcal/mol.

Cytidine deaminase catalyzes a thermodynamically favorable reaction whose product, uridine, can undergo phosphorolysis to yield pentose derivatives. Thus, the presence of this enzyme in bacteria allows cytidine to serve as a sole carbon source (Hammer-Jespersson & Nygaard, 1976). The bacterial enzyme enhances the rate of hydrolytic deamination of cytidine by a factor of approximately 4×10^{11} (Frick et al., 1987). The activity of this enzyme is not affected by the presence of EDTA but is inhibited reversibly by mersalyl ($K_i = 3 \times 10^{-5}$ M) (Wolfenden et al., 1967) and irreversibly by 5-mercuriocytidine (Ashley & Bartlett, 1984a), suggesting the presence of an essential sulfhydryl group.

† Supported by Research Grants GM18325 from the National Institutes of Health and PCM7823016 from the National Science Foundation.

0006-2960/89/0428-9423$01.50/0