JOURNAL OF BACTERIOLOGY, Apr., 1966
Vol. 91, No. 4

Printed in U.S.A.

Metabolism of a Proline Analogue, L-Thiazolidine-4-Carboxylic Acid, by *Escherichia coli*[1]

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Received for publication 17 January 1966

ABSTRACT

UNGER, LEON (University of Illinois, Urbana), AND R. D. DEMOSS. Metabolism of a proline analogue, L-thiazolidine-4-carboxylic acid, by *Escherichia coli*. J. Bacteriol. **91**:1564–1569. 1966.—Resting cells of *Escherichia coli* K-12, pregrown in a proline- and thioproline-free medium, oxidize the proline analogue, L-thiazolidine-4-carboxylic acid (L-thioproline), without a lag with the consumption of 1 atom of oxygen per mole of thioproline. The organism also oxidizes cysteine and formaldehyde, the chemical precursors of thioproline. The total oxygen consumed is the same whether the substrate is thioproline, cysteine, formaldehyde, or an equimolar mixture of cysteine and formaldehyde. The results suggest that neither cysteine nor formaldehyde are free intermediates in the oxidative pathway. Thioproline is available as a metabolic carbon source for the synthesis of the ribonucleic acid bases, guanine and uracil.

Enzymatic oxidation of L-thiazolidine-4-carboxylic acid (L-thioproline) by mammalian preparations has been demonstrated in a number of laboratories. Oxidation by purified rat liver mitochondria proceeds without an induction lag with the consumption of 1 atom of oxygen per mole of thioproline and the formation of *N*-formylcysteine as a major end product (2, 10). The total oxygen uptake is identical whether the substrate is thioproline, cysteine, or equimolar amounts of cysteine and formaldehyde. Additionally, thioproline serves as a substrate for a purified proline-oxidizing system (8) and a proline-degrading D-amino acid oxidase (18) prepared from mammalian sources.

It has been reported (17) that thioproline is a specific analogue of proline which inhibits the growth of *Escherichia coli* by mimicking proline in certain of its essential metabolic functions. In the course of these studies, several observations were consistent with the view that thioproline is a metabolically labile compound.

The purpose of the present study was to elucidate the metabolic fate of thioproline in *E. coli* K-12. The results indicate that thioproline serves as a substrate for an active oxidizing system and is available as a carbon source for the synthesis of ribonucleic acid bases.

[1] This paper was taken from a thesis submitted by L. Unger to the faculty of the University of Illinois Graduate College in partial fulfillment of the requirements for the Ph.D. degree.

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MATERIALS AND METHODS

Organism. E. coli strain K-12 was used in these experiments. Growth at 37 C on a rotary shaker was measured turbidimetrically in an Evelyn colorimeter at 490 mμ and was related to dry weight by a previously prepared standard curve.

Oxidation of L-thiazolidine-4-carboxylic acid (thioproline) by resting cells of E. coli. To obtain resting cells for studying the ability of *E. coli* to oxidize thioproline, cultures were grown aerobically on Friedlein's medium (5) containing 1% sodium lactate (Fisher Scientific Co., Pittsburgh, Pa.). The cells were harvested after 14 to 16 hr of growth and were washed twice with 0.85% NaCl. The washed culture was resuspended in a volume of saline such that 0.3 ml of the cell suspension would have an absorbancy (*A*) of approximately 0.43 when diluted in a total of 10 ml of Friedlein's medium.

The conventional Warburg technique (16) was used to measure oxygen consumption. The center well contained 4% lead acetate in 20% KOH to absorb H_2S and CO_2, respectively. Substrates were added to the main compartment, and cells were placed in the side arm and tipped into the main cup at zero time. Incubation was at 30 C in an air atmosphere.

Preparation of cells for isotope studies and for the isolation of soluble ribonucleic acid (sRNA). Cultures were grown in modified C medium (1 × C; 17), with

or without 200 μg of L-proline per ml, to 0.2 *A* (dry weight, 310 μg/ml). The exponentially multiplying cells were harvested by centrifugation at 4 C, washed once with 40 ml of double-strength C medium (2 × C) from which NH_4Cl had been omitted (2 × C without N), and resuspended in 2 × C without N to 0.4 *A*. The cells were starved for nitrogen for a total period of 2 hr. When desired, chloramphenicol was added at the end of the 1st hr of nitrogen-deprivation at a final concentration of 100 or 300 μg/ml, and the incubation was continued for 1 additional hour. Nitrogen was reintroduced as NH_4Cl, L-thiazolidine-2-C^{14}-4-carboxylic acid (L-thioproline-2-C^{14}) was supplied as indicated in each case, and an appropriate volume of distilled water was added to redilute the cell suspension to 0.2 *A* and the 2 × C to single-strength C medium.

Isolation of sRNA. Chloramphenicol-treated cells, pregrown in proline-containing C medium, were incubated for 2 hr at 37 C in the presence of thioproline-2-C^{14} (final concentration, 2.25 μmoles/ml, 1.12×10^5 counts/min per μmole. The culture was harvested, washed twice with 40 ml of cold 0.01 M tris(hydroxymethyl)aminomethane (Tris)—0.05 M $MgCl_2$ buffer (*p*H 7.4; TM buffer), and resuspended in TM buffer containing 50 μg/ml of deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.). The cell suspension was disrupted by extrusion twice through a small orifice in a French pressure cell (4). Cell debris was removed by centrifugation at 4 C for 20 min at 22,000 × *g*.

The clear supernatant fluid was centrifuged in a Spinco model L ultracentrifuge at 100,000 × *g* for 4 hr. The sRNA was purified from the resulting supernatant fluid by the phenol procedure of Gierer and Schramm (7).

Localization of thioproline-C^{14} in purified sRNA. The following samples were treated with 0.5 ml of 1 N HCl at 100 C for 1 hr in sealed tubes according to the method of Smith and Markham (15). This hydrolysis procedure yields the free purine bases and the pyrimidine nucleotides of RNA. (I) Purified radioactive sRNA (187.5 μg, 2.80×10^4 count/min per mg) isolated from *E. coli* which had been incubated in the presence of thioproline-2-C^{14}. (II) A mixture of 500 μg of each of the four nucleotides (guanylic, adenylic, cytidylic, and uridylic acids) and 1.5 μmoles of thioproline-2-C^{14} (1.12×10^5 counts/min per μmole). (III) Thioproline-2-C^{14} (1.5 μmoles, 1.12×10^5 counts/min per μmole). (IV) A mixture of 500 μg of each of the four nucleotides. (V) Yeast RNA, 5,000 μg (Schwartz Laboratories, Inc., Mount Vernon, N.Y.). After hydrolysis, a sample from mixture IV equivalent to approximately 34 μg of each nucleotide was added as carrier to the sRNA hydrolysate. Appropriate samples of each hydrolysate were chromatogrammed on Whatman no. 1 filter paper and developed for 2 days in a descending 70% *t*-butanol-0.8 N HCl solvent system (15). The bases were located by screening with UV light in a dark room. The position of thioproline-2-C^{14} was determined by scanning with a Nuclear-Chicago model C 100B strip counter. The spots corresponding to the purine bases, the pyrimidine nucleotides, and thioproline were cut out and measured for radioactivity.

Analytical methods. Cells were fractionated by the procedure of Roberts et al. (13). Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer in solutions of organic phosphors in toluene.

Protein was determined according to the method of Lowry et al. (9). RNA was measured by the orcinol procedure (1) or by absorption at 260 mμ in a Zeiss spectrophotometer.

Thioproline was assayed quantitatively with the chromotropic acid (Eastman, 1,8-dihydroxy-naphthalene-3,6-disulfonate) reagent as described by Frisell, Meech, and Mackenzie (6).

Chemicals. L-Thiazolidine-4-carboxylic acid (L-thioproline) was synthesized according to the procedure of Ratner and Clarke (12) and Schubert (14), as modified by Mackenzie and Harris (10).

Radioactive compounds. L-Thiazolidine-2-C^{14}-4-carboxylic acid (L-thioproline-2-C^{14}) was synthesized from formaldehyde-C^{14} (Research Specialties Co., Richmond, Calif.) and L-cysteine (Mann Research Laboratories, Inc., New York, N. Y.) by the method indicated above, and was recrystallized to constant specific activity.

RESULTS

Oxidation of thioproline by resting cells of E. coli K-12. Since a structurally related, metabolic derivative of thioproline appears to be responsible for inhibiting the growth of *E. coli* (17), and in view of reports that the analogue is oxidized by rat liver preparations (2, 10), it was of interest to establish and examine the ability of *E. coli* to oxidize thioproline.

As shown in Fig. 1, oxidation of thioproline by *E. coli* K-12 proceeds immediately, whereas that of cysteine begins only after a lag of approximately 25 min. Formaldehyde is oxidized after a very slight delay. Although thioproline and cysteine (and an equimolar mixture of cysteine and formaldehyde) are oxidized at approximately the same rates, formaldehyde is degraded at an appreciably greater velocity. However, the total oxygen consumption is the same with each of the substrates, provided appropriate corrections are applied for endogenous oxygen uptake, and for auto-oxidation, in the cases of cysteine and formaldehyde.

Table 1 represents a summary of the enzymatic oxidations of thioproline, cysteine, formaldehyde, and an equimolar mixture of cysteine and formaldehyde. It is clear that resting cells of *E. coli* oxidize thioproline and each of its chemical precursors separately, with the consumption of 1 μatom of oxygen per μmole of substrate.

Incorporation of thioproline-C^{14} into nucleic acids. Isotope experiments designed to determine the distribution of thioproline-C^{14} within the cell revealed the presence of a high concentration of

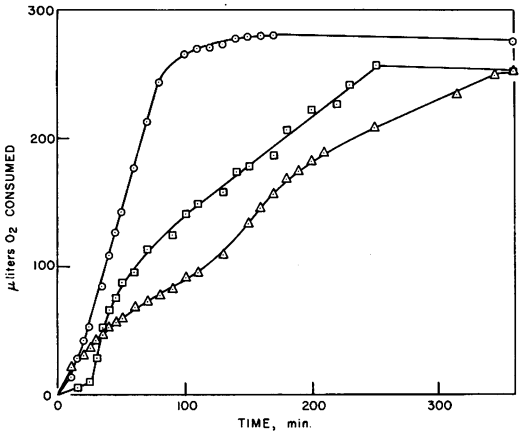

FIG. 1. *Oxidation of thioproline and precursors by resting cells of Escherichia coli K-12. Per Warburg vessel: cells, 0.5 ml; phosphate buffer, 0.066 M (pH 7.0); total volume, 3.0 ml; 30 C; atmosphere, air; center well contained 20% KOH-4% lead acetate. Symbols: △, L-thioproline, 22.8 μmoles; □, L-cysteine, 22.4 μmoles; ○, formaldehyde, 21.6 μmoles. The curves have been corrected for endogenous values and for auto-oxidation of cysteine and formaldehyde.*

TABLE 1. *Oxidation of L-thioproline, L-cysteine, and formaldehyde by resting cells of Escherichia coli*

Substrate (μmoles)			O_2 consumption*	Oxygen (μatoms) Substrate (μmoles)
L-Thioproline	L-Cysteine	Formaldehyde		
			μatoms	
22.8	0	0	22.4	0.98
0	22.4	0	24.6	1.1
0	0	21.6	24.6	1.1
0	22.4	21.6	23.8	1.1

* Corrected for endogenous values and for auto-oxidation in the absence of added cells in the cases of cysteine and formaldehyde. Incubation time, 6 hr.

radioactivity in the hot trichloroacetic acid-soluble fraction. Table 2 shows that when protein synthesis is inhibited by chloramphenicol, all of the cold trichloroacetic acid-insoluble thioproline-C^{14} incorporated in 1 hr, either in the presence or absence of proline, is extractable with hot trichloroacetic acid. Similar results were obtained when the cultures were incubated for 9 hr. Since the free amino acid pool was previously removed with cold trichloroacetic acid, it is concluded that measurements of cold trichloroacetic acid-insoluble radioactivities from chloramphenicol-treated cells primarily reflect thioproline-C^{14}

TABLE 2. *Hot trichloroacetic acid-extractability of L-thioproline-2-C^{14} incorporated in the presence of chloramphenicol**

Additions	Counts/min per ml of cells		Thioproline-C^{14} extracted
	Cold trichloroacetic acid-insoluble	Hot trichloroacetic acid-soluble	
			%
Thioproline-C^{14}	939	933	99
Thioproline-C^{14}; proline	848	840	99

* L-Thioproline-2-C^{14}, 2.25 μmoles/ml, 1.12 × 10^5 counts/min per μmole; L-proline, 0.2 μmole/ml; chloramphenicol, 300 μg/ml. Thioproline-2-C^{14} and proline were introduced simultaneously. Incubation time, 60 min.

incorporation into nucleic acids. Additionally, Table 2 demonstrates that proline inhibits thioproline incorporation by approximately 10% in this experiment. It is likely that this small but consistent antagonism reflects proline interference with the formation of thioprolyl-RNA.

Kinetics of incorporation of thioproline-C^{14} into nucleic acids in the presence of chloramphenicol. The time course of incorporation of thioproline-C^{14} into the nucleic acids of cells incubated in the presence of chloramphenicol is presented in Fig. 2. Uptake appears to proceed linearly without a detectable lag at an initial rate of approximately 20 μμmoles per hr per μg (dry weight) of cells for 50 to 60 min prior to the onset of a slower, second phase of incorporation.

Utilization of thioproline as a source of carbon for the synthesis of sRNA bases. Only a small fraction of the extensive analogue radioactivity incorporated into nucleic acids can be reasonably associated with sRNA-bound thioproline molecules (Table 2). Since thioproline is oxidized by *E. coli*, it became of interest to examine the availability of thioproline carbon for the synthesis of purine and pyrimidine bases.

sRNA, with a specific radioactivity of 2.80 × 10^4 counts per min per mg was isolated and purified from chloramphenicol-treated cells incubated in the presence of thioproline-2-C^{14}. Since all of the radioactivity associated with the purified product is rendered acid-soluble by treatment with ribonuclease (Worthington Biochemical Co., Freehold, N.J.), it is concluded that the sRNA preparation is free from significant contamination with radioactive DNA and protein.

A sample of the purified C^{14}-sRNA (I), together with appropriate parallel controls (II, III, IV, V), was treated and analyzed as described in

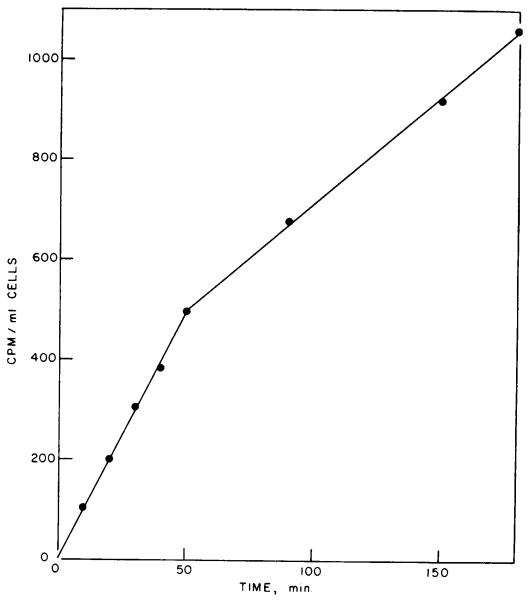

FIG. 2. *Incorporation of thioproline-C^{14} into nucleic acids in the presence of chloramphenicol. An overnight culture was inoculated into fresh C medium, grown to approximately 0.2 A, washed, and starved for N. After 60 min of starvation, the suspension received chloramphenicol (final concentration, 100 μg/ml) and incubation was continued for an additional 60 min. Thereupon, NH_4Cl and L-thioproline-2-C^{14} (final concentration, 2.25 μmoles/ml, 9.85 × 10^4 counts/min per μmole) were introduced.*

Materials and Methods. The data are summarized in Table 3. Although the results for the control samples IV and V have been omitted from the table for the sake of clarity in the presentation and discussion of the findings, it should be noted that the chromatographic patterns of the hydrolyzed C^{14}-RNA from *E. coli* were identical to those of a mixture of the authentic nucleotides (II, IV) and to hydrolyzed yeast RNA (V). The significant findings of these experiments are as follows.

(i) Four major components, corresponding in R_F values to guanine, adenine, cytidylic acid, and uridylic acid, were detected in the C^{14}-sRNA hydrolysate (I) subjected to one-dimensional chromatography in the *t*-butanol-HCl solvent system.

(ii) The sum of the radioactivities recovered in these four bases represents approximately 94% of the original radioactivity of the sRNA sample prior to hydrolysis and chromatography (I). It is concluded that the methods employed allow essentially quantitative recovery of the major components of the purified sRNA.

(iii) Thioproline is stable under the conditions of hydrolysis and chromatography as evidenced by the retention of 94 to 95% of the original thioproline radioactivity in a single spot (III) with the same R_F (0.56 to 0.58) as that of the analogue not treated with 1 N HCl.

(iv) Thioproline and cytidylic acid have identical R_F values (0.56 to 0.58) whether chromatographed separately (III, IV) or together (II) and cannot be resolved by one-dimensional chromatography in the solvent system used.

(v) The radioactivity of the sRNA component with an R_F corresponding to cytidylic acid-thioproline is negligible (I). This observation indicates the absence of thioproline molecules in the preparation of sRNA purified under the conditions described above. Moreover, thioproline carbon is not appreciably incorporated into cytidylic acid.

(vi) Uridylic acid and guanine are the only sRNA constituents detected which are significantly labeled (I); together they represent 98.5% of the total radioactivity recovered. The conclusion that the radioactivity of uridylic acid resides in the base moiety, rather than in the ribotide residue, would appear to be a reasonable extension of the finding that cytidylic acid is not

TABLE 3. *Incorporation of thioproline carbon into sRNA bases**

Component (after hydrolysis)	I			II		III	
	R_F	Total counts/min	Counts/min per mg of sRNA	R_F	Total counts min (× 10^5)	R_F	Total counts/min (× 10^5)
Guanine	0.25	1,864	9,950	0.25	—	—	—
Adenine	0.37	60	320	0.37	—	—	—
Cytidylic acid	0.57	24	128	0.58	1.58	—	—
Uridylic acid	0.78	3,015	16,100	0.79	—	—	—
Thioproline	—	—	—	0.57	1.58	0.58	1.59

* (I) Purified *Escherichia coli* C^{14}-sRNA, 187.5 μg, 2.80 × 10^4 counts/min per mg. (II) Mixture of guanylic, adenylic, cytidylic, and uridylic acids; L-thioproline-2-C^{14}, 1.5 μmoles, 1.12 × 10^5 counts/min per μmole. (III) L-Thioproline-2-C^{14}, 1.5 μmoles, 1.12 × 10^5 counts/min per μmole.

labeled. If the radioactivity were harbored in the ribotide portion of uridylic acid, then it could be expected that cytidylic acid also would have been extensively labeled.

It is concluded that thioproline carbon 2 is available for the synthesis of uracil and guanine. The experiments do not provide information as to whether other thioproline carbons are incorporated into sRNA bases.

DISCUSSION

Resting cells of *E. coli* K-12, pregrown in a proline-free medium, possess an active thioproline oxidizing system (Fig. 1). The consumption of 1 atom of oxygen per mole of substrate (Table 1) strongly suggests that thiazolidine-4-carboxylic acid (thioproline) is dehydrogenated to form 2,3-thiazoline-4-carboxylic acid. Although product formation was not determined, this desaturated intermediate would be expected to undergo hydrolytic cleavage at the double bond followed by opening of the ring to yield N-formylcysteine.

If it is presumed that desaturation to the thiazoline ring is the first step in the enzymatic oxidation of thioproline, it must be considered unlikely that the oxidative pathway in *E. coli* involves either free cysteine or formaldehyde as an intermediate. If such were the case, the predicted oxygen consumption per mole of thioproline would be two to three times greater than that observed with its individual chemical precursors. The data are not in accord with this prediction in that the total oxygen consumption is the same whether the substrate is thioproline, cysteine, formaldehyde, or an equimolar mixture of cysteine and formaldehyde. Moreover, whereas further metabolism of N-formylcysteine cannot be ruled out, thioproline does not appear to be converted to products more oxidized than this compound.

Thioproline has been shown to be oxidized to N-formylcysteine via this postulated pathway by rat-liver preparations (2, 10). *E. coli* differs from the mammalian system (10) and is similar to *Aerobacter aerogenes* and *Pseudomonas aeruginosa* (11) in its ability to oxidize exogenously supplied formaldehyde.

Of interest to this discussion is the careful demonstration by Frank and Rybicki (3) of a proline-oxidizing system in *E. coli* B which is inducible by proline in the wild-type strain. The thioproline-oxidizing activity of *E. coli* K-12 reported in the present work appears to differ from this proline-degrading system in that it does not have the characteristics of an inducible enzyme, since it is demonstrable in resting cells pregrown in a proline- and thioproline-free medium.

The utilization of thioproline carbon 2 for the synthesis of guanine and uracil is in apparent discord with its negligible incorporation into adenine and cytosine. An explanation for these observations is elusive and would have to be consistent with the following empirical and theoretical considerations.

(i) Rapid initial incorporation of thioproline-2-C^{14} into nucleic acids proceeds linearly without a lag in the presence of chloramphenicol at a rate of approximately 20 $\mu\mu$moles per hr per μg (dry weight) of cells for 50 to 60 min, followed by a slower phase of uptake (Fig. 2).

(ii) Incorporation of thioproline carbon 2 into sRNA does not appear to affect the coding for specific amino acids, since the transfer RNA present in analogue-treated cells accepts amino acids essentially as well (with the exception of proline) as nontreated cells (17). Additionally, it follows that thioproline does not significantly reduce the size of the acceptor RNA pool by inhibiting sRNA synthesis.

(iii) Negligible incorporation of thioproline carbon into adenine and cytosine is consistent with the view that the analogue does not contribute C^{14} to the 1-carbon pool of purine and pyrimidine precursors.

Thioproline must be regarded as a metabolically labile analogue of proline which can undergo enzymatic oxidation as well as provide carbon for the synthesis of guanine and uracil in *E. coli*.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant E-2971 from the National Institute of Allergy and Infectious Diseases and by grant G-9849 from the National Science Foundation. L. U. was the recipient of Public Health Service training grant GM-510 from the Division of General Medical Sciences.

LITERATURE CITED

1. ASHWELL, G. 1957. Colorimetric analysis of sugars, p. 73–105. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 3. Academic Press, Inc., New York.
2. CAVALLINI, D., C. DEMARCO, B. MONDOVI, AND F. TRASARTI. 1956. Studies of the metabolism of thiazolidine carboxylic acid by rat liver homogenate. Biochim. Biophys. Acta **22**:558–564.
3. FRANK, L., AND P. RYBICKI. 1961. Studies of proline metabolism in *Escherichia coli*. I. The degradation of proline during growth of a proline-requiring auxotroph. Arch. Biochem. Biophys. **95**:441–449.
4. FRENCH, C. S., AND H. W. MILNER. 1955. Disintegration of bacteria and small particles by high-pressure extrusion, p. 64–67. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in

enzymology, vol. 1. Academic Press, Inc., New York.

5. FRIEDLEIN, F. 1928. Der quantitative verwendungsstoffwechsel des *Paratyphus-B-Bazillus*, des *Bacterium coli* und des *Bacillus pyocyaneus*. Biochem. Z. **194**:273–291.
6. FRISELL, W. B., L. A. MEECH, AND C. G. MACKENZIE. 1954. A simplified photometric analysis for serine and formaldehyde. J. Biol. Chem. **207**:709–716.
7. GIERER, A., AND G. SCHRAMM. 1956. Infectivity of ribonucleic acid from tobacco mosaic virus. Nature **177**:702–703.
8. JOHNSON, A. B., AND H. J. STRECKER. 1962. The interconversion of glutamic acid and proline. IV. The oxidation of proline by rat liver mitochondria. J. Biol. Chem. **237**:1876–1882.
9. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. MACKENZIE, C. G., AND J. HARRIS. 1957. N-formylcysteine synthesis in mitochondria from formaldehyde and L-cysteine via thiazolidine carboxylic acid. J. Biol. Chem. **227**:393–406.
11. NEELY, W. B. 1963. Action of formaldehyde on microorganisms. I. Correlation of activity with formaldehyde metabolism. J. Bacteriol. **85**: 1028–1031.
12. RATNER, S., AND H. T. CLARKE. 1937. The action of formaldehyde upon cysteine. J. Am. Chem. Soc. **59**:200–206.
13. ROBERTS, R. B., P. H. ABELSON, D. B. COWIE, E. T. BOLTON, AND R. J. BRITTON. 1957. Studies of biosynthesis in *Escherichia coli*. Carnegie Inst. Wash. Publ. 607.
14. SCHUBERT, M. P. 1936. Compounds of thiol acids with aldehydes. J. Biol. Chem. **114**:341–350.
15. SMITH, J. D., AND R. MARKHAM. 1950. Chromatographic studies on nucleic acids. 2. The quantitative analysis of ribonucleic acids. Biochem. J. **46**:509–513.
16. UMBREIT, W. W., R. H. BURRIS, AND J. F. STAUFFER. 1957. Manometric techniques. Burgess Publishing Co., Minneapolis.
17. UNGER, L., AND R. D. DEMOSS. 1966. Action of a proline analogue, L-thiazolidine-4-carboxylic acid, in *Escherichia coli*. J. Bacteriol. **91**: 1556–1563.
18. WELLNER, D., AND H. SCANNONE. 1964. Oxidation of L-proline and L-3,4-dehydroproline by D-amino acid oxidase. Biochemistry **3**:1746–1749.