JOURNAL OF BACTERIOLOGY, Aug. 1972, p. 510-515

Vol. 111, No. 2
Printed in U.S.A.

Effect of Cyclopentaneglycine on Metabolism in *Salmonella typhimurium*

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Received for publication 1 March 1972

Cyclopentaneglycine (CPG) inhibited the growth of wild-type *Salmonella typhimurium*. The inhibition was overcome by isoleucine or any isoleucine precursor formed after threonine. CPG appeared to mimic isoleucine as a strong inhibitor of the activity of L-threonine deaminase. The analogue was a poor inhibitor of isoleucyl-transfer ribonucleic acid synthetase. CPG did not appear to be incorporated into protein nor did it replace isoleucine in repression. Cells that had recovered from growth inhibition by CPG had derepressed levels of the isoleucine-valine biosynthetic enzymes.

Amino acid analogues have proven extremely useful in studying the regulation of bacterial metabolism (12, 14, 23). In particular, utilization of these compounds to select for mutants has been invaluable in elucidating the mechanism of repression in amino acid biosynthesis (1, 3, 13, 15, 17). An early study on the effect of DL-cyclopentaneglycine (CPG) on the growth of *Escherichia coli* indicated that this compound was an isoleucine antagonist (9). The present paper presents evidence that CPG is a strong inhibitor of the growth of *Salmonella typhimurium*. The analogue appeared to exert its effect primarily as a false feedback inhibitor of L-threonine deaminase, the initial enzyme in isoleucine biosynthesis.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The organisms used in this report were *S. typhimurium* strain LT-2 *ara-9* and strain 3 *ilvA*, a mutant derived from strain LT-2 *ara-9*, which lacks L-threonine deaminase. Except where otherwise noted, the bacteria were grown in 0.4% glucose-minimal medium (5) in 50-ml Erlenmeyer flasks at 37 C in a New Brunswick water-bath gyratory shaker. Growth was measured in a Klett-Summerson colorimeter with a blue no. 42 filter.

Enzyme methods. The preparation of the crude extracts used to measure the isoleucine-valine biosynthetic enzymes was the same as described previously (22) except that a Branson 20-k cycle magnetostrictive ultrasonic oscillator was employed. Dihydroxy acid dehydrase (7), L-threonine deaminase (8), and acetohydroxy acid synthetase (16) were measured according to the methods cited. The reaction mixture for transaminase B contained in 1 ml (micromoles): potassium phosphate (*p*H 8.0), 100; pyridoxal phosphate, 0.1; α-ketoglutarate, 10; L-isoleucine, 40. The α-keto-β-methylvalerate formed was determined as described previously (18), except that toluene was used in place of ethyl acetate and 3 ml of potassium carbonate replaced the carbonate-sulfate solution. After extraction, 2 ml of the carbonate phase was transferred to a test tube, 0.5 ml of 20% KOH was added, and the brown color was read at 540 nm (green filter) in a Klett-Summerson colorimeter. Protein was measured by the method of Lowry et al. (11). Enzyme specific activity is expressed as the number of micromoles of product formed per milligram of protein per hour.

The assay used to measure the attachment of L-^{14}C-isoleucine to *S. typhimurium* transfer ribonucleic acid (tRNA) was that of Böck et al. (2), except that the washing of the samples and the radioactivity determinations were done as described by Freundlich (6). Crude isoleucyl-tRNA synthetase was initially prepared from *S. typhimurium* by procedures previously described (6). The crude extract was heated at 55 C for 45 min in 0.2 M potassium phosphate buffer (*p*H 7.5) containing 0.01 M sodium adenosine triphosphate, 0.04 M potassium chloride, 0.04 M magnesium chloride, and 0.02 M L-isoleucine. Denatured protein was removed by centrifugation at 20,000 × *g* for 30 min. Partially purified isoleucyl-tRNA synthetase free of ribonuclease was then prepared as described by Yegian and Stent (24). Protein and RNA were measured by the uptake of ^{14}C-phenylalanine and ^{14}C-uracil as described previously (19) except that isoleucine replaced leucine in the growth medium.

Chemicals. All of the chemicals used were obtained from Sigma Chemical Co., St. Louis, Mo., except the following: CPG was a gift from W. Shive; α,β-dihydroxyisovaleric acid was a gift from H. E. Umbarger; ^{14}C-amino acids were obtained from New England Nuclear Corp., Boston, Mass.

RESULTS AND DISCUSSION

Effect of CPG on growth. Strong inhibition

of growth of an exponentially growing culture of *S. typhimurium* LT-2 *ara-9* was found with concentrations of CPG above 1 mM (Fig. 1). At lower concentrations of the analogue, inhibition was progressively weaker. The effect of CPG on growth was more severe if the inoculum was previously grown under conditions where the isoleucine-valine enzymes were repressed (data not shown).

Reversal by isoleucine of growth inhibition caused by CPG. As has been previously reported (9), small amounts of isoleucine prevented growth inhibition by CPG (Fig. 2). Inhibition was reversed if isoleucine was added together with the analogue or 1.5 hr after the cells had grown with CPG. All isoleucine precursors formed after threonine prevented growth inhibition effectively. Leucine and valine slightly reduced the growth lag caused by the analogue.

Inhibition of L-threonine deaminase by CPG. Strong inhibition of the activity of L-threonine deaminase was found with CPG (Table 1). CPG was only from 5- to 10-fold less inhibitory than isoleucine, the normal feedback inhibitor of the enzyme (20). In *S. typhimurium*, L-threonine deaminase has a number of distinct binding sites for small molecules (8). Inhibition of L-threonine deaminase activity by CPG could be due to its action at any one of these sites. However, when crude enzyme extracts were treated with mercuric chloride,

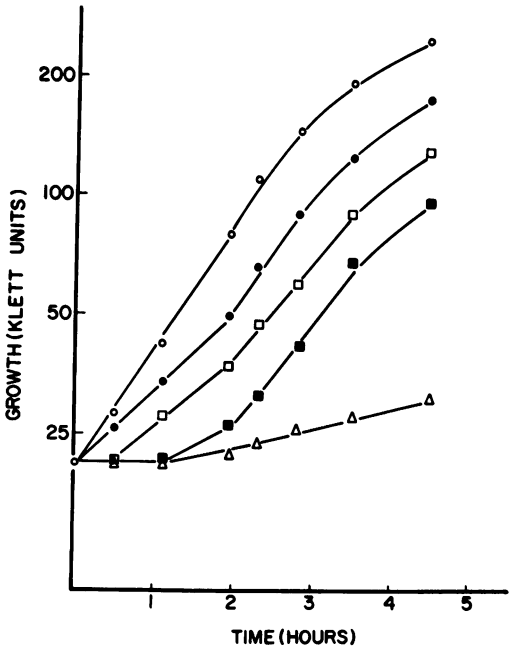

FIG. 1. *Inhibition of growth of S. typhimurium by cyclopentaneglycine (CPG). Exponentially growing cells in minimal medium were centrifuged, washed once in minimal medium, and suspended in minimal medium containing no supplement* (○), *and CPG: 0.1 mM* (●); *0.15 mM* (□); *0.3 mM* (■); *1.2 mM* (△).

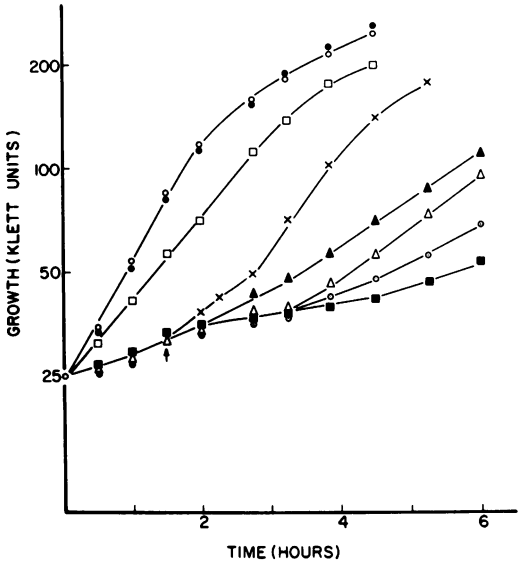

FIG. 2. *Reversal of growth inhibition by cyclopentaneglycine (CPG) by isoleucine. Cells were prepared as described in Fig. 1 and placed in minimal medium with the following supplements: none* (○); *1.4 mM CPG* (■); *1.4 mM CPG and 0.1 mM isoleucine* (●); *1.4 mM CPG and 0.15 mM α-ketobutyrate or 0.15 mM α-ketomethylvalerate* (□); *1.4 mM CPG and 0.1 mM of either leucine* (▲), *valine* (△), *or threonine* (○). *At the time indicated by the arrow, 0.1 mM isoleucine was added to a sample of the culture containing 1.4 mM CPG* (×).

TABLE 1. *Inhibition of L-threonine deaminase by isoleucine and cyclopentaneglycine (CPG)*[a]

Additions (M)		L-Threonine (4×10^{-2} M)		L-Threonine (10^{-2} M)	
		Specific activity[b]	% Inhibition	Specific activity[b]	% Inhibition
None		15.0		12.6	
L-Isoleucine	5×10^{-5}	15.6	0	12.8	0
	10^{-4}	15.0	0	7.2	35
	2×10^{-4}	8.1	46	4.7	64
	3×10^{-4}	4.5	70	2.8	78
	4×10^{-4}	2.7	82		
CPG	6×10^{-4}	15.6	0	13.0	0
	1.2×10^{-3}	15.2	0	6.0	54
	2×10^{-3}	8.4	44	3.9	70
	2.5×10^{-3}	6.7	55	3.2	78

[a] Wild-type cells grown in minimal medium.

[b] Expressed as micromoles of product per milligram of protein per hour.

the ability to inhibit the enzyme by isoleucine or CPG was abolished (Table 2). Treatment of L-threonine deaminase from *S. typhimurium* and *E. coli* with mercuric ions has been shown to desensitize the isoleucine binding site while having little effect on other binding sites on the enzyme (4, 8). An additional indication that CPG is mimicking the effect of isoleucine on L-threonine deaminase is shown in Fig. 3. CPG was almost as effective as isoleucine in stabilizing L-threonine deaminase at 0 C. It has been shown previously that threonine and valine, which are thought to bind at sites other than the isoleucine site, do not stabilize L-threonine deaminase (8).

Effect of CPG on isoleucyl-tRNA synthetase. In contrast to the strong inhibition by CPG on L-threonine deaminase, the analogue was a poor inhibitor of isoleucyl-tRNA synthetase (Table 3). Isoleucyl-tRNA formation was inhibited only 13% by a concentration of CPG 4,000 times greater than that of the substrate, isoleucine. At a ratio of analogue to isoleucine of 18,000 to 1, the reaction was inhibited 50%. A plot of data obtained with partially purified isoleucyl-tRNA synthetase indicated that inhibition by CPG was competitive with respect to isoleucine (Fig. 4).

Effect of CPG on RNA and protein synthesis. The incorporation of radioactive phenylalanine and uracil into cells of an isoleucine auxotroph was used as a measure of protein and RNA synthesis. The addition of 100 μg of CPG per ml to strain *ile-3* starved for isoleucine did not allow protein or RNA synthesis.

TABLE 2. *Effect of isoleucine and cyclopentaneglycine (CPG) on densensitized L-threonine deaminase*[a]

Additions (M)	Specific activity[b]	
	Native	Desensitized
None	13.6	18.8
L-Isoleucine 1.5×10^{-4}	8.0	19.7
2×10^{-4}	4.3	19.4
3×10^{-4}	2.2	19.7
CPG 1.2×10^{-3}	5.8	19.7
2×10^{-3}	3.6	19.2

[a] Mercuric chloride (1.7×10^{-5} M) was added to a portion of an extract prepared from wild-type cells grown on minimal medium. The extract was incubated at 26 C for 5 min and then added immediately to the assay mixture supplemented as indicated. The control (native enzyme) was a portion of the same extract without added mercuric chloride.

[b] Expressed as micromoles of product per milligram of protein per hour.

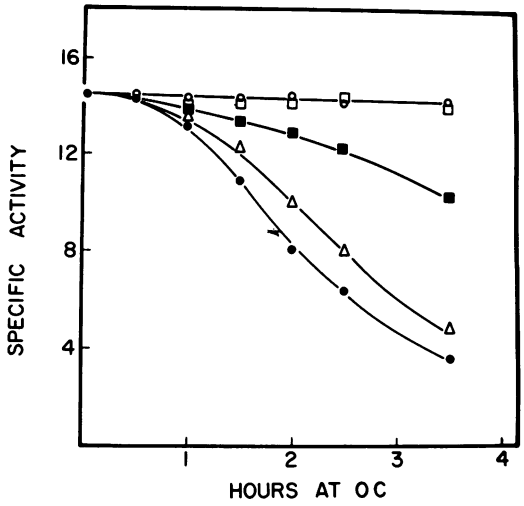

FIG. 3. *Effect of isoleucine and cyclopentaneglycine (CPG) on stabilization of L-threonine deaminase at 0 C. The extract was prepared from wild-type cells and divided into five portions. L-Isoleucine and CPG were added immediately and the extracts were stored at 0 C. Samples were removed at the indicated times, and the specific activity expressed as micromoles of product per mg of protein per hour was determined. Symbols: ●, no amino acid added to extracts; ○, 0.2 mM L-isoleucine; □, 0.7 mM CPG; ■, 0.2 mM CPG; △, 0.04 mM L-isoleucine.*

TABLE 3. *Effect of cyclopentaneglycine (CPG) on the attachment of isoleucine to tRNA*[a]

CPG (M)	Counts/min (V_i)	% Inhibition
None	2,500	
3.6×10^{-3}	2,180	13
7.2×10^{-3}	2,030	20
1×10^{-2}	1,693	32
1.8×10^{-2}	1,304	52

[a] Crude isoleucyl-tRNA synthetase was prepared as described in Materials and Methods. The concentration of L-^{14}C-isoleucine was 5×10^{-7} M.

In addition, CPG did not appear to replace isoleucine in repression. No reduction in the derepressed levels of the isoleucine-valine enzymes were found when 50 to 250 μg of CPG per ml was added to strain *ile-3* growing on growth-limiting amounts of isoleucine (data not shown).

Recovery of cells from growth inhibition by CPG. Wild-type cultures containing 1×10^{-3} to 5×10^{-3} M CPG usually resumed growth 5 to 15 hr after addition of the analogue (Fig. 5A). When these cells were washed and again placed in contact with CPG, no growth lag was observed (Fig. 5B). However, strong

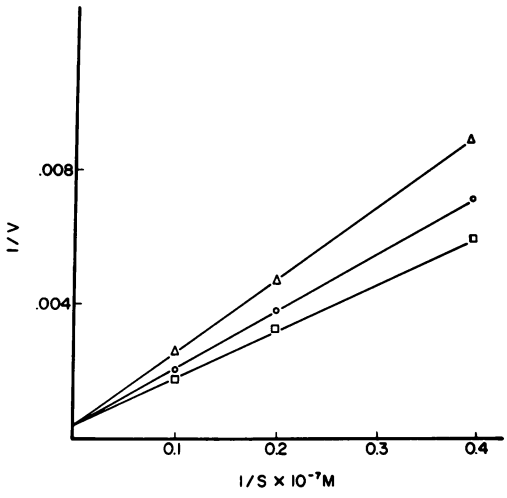

FIG. 4. *Nature of cyclopentaneglycine (CPG) inhibition of isoleucyl-tRNA synthetase. The enzyme extract was prepared from wild-type cells. Isoleucyl-tRNA synthetase was partially purified as described in Materials and Methods. Symbols: □, no CPG; △, 5 mM CPG; ○, 2.5 mM CPG. The K_i calculated from these data (other CPG concentrations not shown) is 1×10^{-2} M CPG.*

inhibition by the analogue was found when cells that had recovered from inhibition by CPG were allowed to grow for six to eight generations in minimal medium and then were placed in medium containing CPG (Fig. 5C). Cells that had recovered from inhibition by CPG (Fig. 5B) were derepressed for the isoleucine-valine enzymes (Table 4). These data suggest that these high enzyme levels are responsible for eventual growth of wild-type cells in the presence of CPG.

Inhibition by CPG of strain ile-3 grown on D-threonine. The previous data indicate that the primary cause of growth inhibition by CPG is as a false feedback inhibitor of L-threonine deaminase. If this interpretation is correct, the analogue should be unable to block the growth of an L-threonine deaminase-requiring mutant growing on D-threonine. Mutants lacking L-threonine deaminase are able to synthesize α-ketobutyrate from D-threonine using degradative D-threonine deaminase (21). This enzyme is apparently not subject to feedback inhibition by isoleucine (10) or by CPG (O'Neill and Freundlich, *unpublished data*). The concentration of D-threonine used was

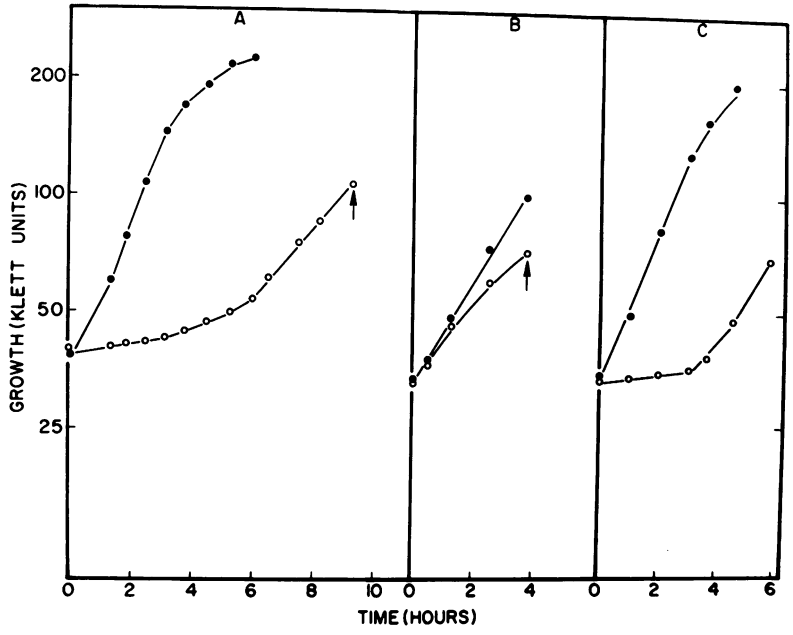

FIG. 5. *Effect of cyclopentaneglycine (CPG) on cells previously grown with CPG. A, Wild-type cells were prepared as described in Fig. 1 and placed in minimal medium (●), and minimal medium plus 1 mM CPG (○). At the time indicated by the arrow, cells that had grown with CPG were centrifuged, washed, and resuspended in minimal medium. B, These cells were inoculated into minimal medium (●) and minimal medium plus 1 mM CPG (○). At the time indicated by the arrow, cells that had grown with CPG were centrifuged, washed, and resuspended in minimal medium and allowed to grow for six generations. C, These cells were incoulated into minimal medium (●) and minimal medium plus 1 mM CPG (○).*

TABLE 4. *Levels of isoleucine-valine biosynthetic enzymes after growth in cyclopentaneglycine (CPG)*

Growth conditions[a]	Specific activity[b]			
	Threonine deaminase	Dihydroxy acid dehydrase	Aceto-hydroxy acid synthetase	Transaminase B
Minimal	16.0 (3.8)	6.4 (6.6)	2.8 (2.4)	9.2 (8.8)
CPG, 10^{-3} M	57.6 (14.2)	17.8 (17.8)	6.0 (5.2)	32.6 (28.8)

[a] Samples of cells from Fig. 5A that had grown to 140 Klett units were harvested, and enzymatic activity was assayed.

[b] Expressed as micromoles of product per milligram of protein per hour. The figures in parenthesis are the specific activities obtained when the assays were performed in the presence of 4×10^{-3} M CPG.

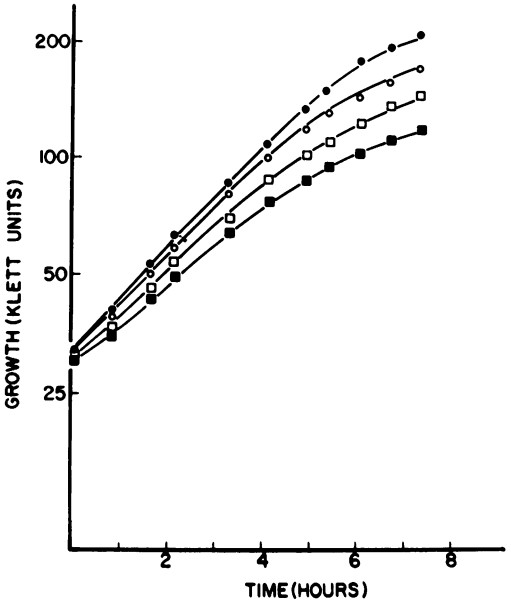

FIG. 6. *Effect of cyclopentaneglycine (CPG) on the growth of strain ile-3 grown with D-threonine. Exponentially growing cells in minimal medium and 200 μg of D-threonine/ml were washed once in minimal medium and suspended in minimal medium plus 30 μg of D-threonine per ml (●), and CPG: 1.5 mM (○); 3.5 mM (□); 7 mM (■).*

rate-limiting for growth of strain *ile-3*. Under these conditions, CPG was a much less effective growth inhibitor than in wild type (Fig. 6). However, some growth inhibition was seen with high levels of the analogue. These data indicate that CPG is able to affect growth by blocking some reaction other than that catalyzed by L-threonine deaminase. It does not appear that this reaction is one that is necessary for isoleucine biosynthesis, since other enzymes necessary for isoleucine synthesis are not inhibited by the analogue (Table 4). Although the in vitro effects of CPG on inhibition of isoleucine activation are slight, it is possible that when the intracellular concentration of isoleucine is very low, this inhibition becomes physiologically significant. Alternatively, although not seen in the present studies, CPG under certain conditions may be slowly incorporated into protein, causing a small inhibition of growth.

The strong growth inhibition of wild-type *S. typhimurium* by CPG has allowed the selection of numerous regulatory mutants in the isoleucine-valine pathway. These mutants fall into three general categories: (i) reduced feedback inhibition by isoleucine and CPG of L-threonine deaminase; (ii) partially derepressed isoleucine-valine enzymes; and (iii) partially derepressed isoleucine-valine enzymes and reduced feedback inhibition of acetohydroxy acid synthetase. The properties of these mutants will be presented elsewhere (O'Neill and Freundlich, *manuscript in preparation*).

ACKNOWLEDGMENTS

This work was supported by Public Health Service research grant GM 17152 from the National Institute of General Medical Sciences. M.F. is a Research Career Development Awardee of the Public Health Service (GM 14374). J.P.O. holds a predoctoral fellowship from the Public Health Service.

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