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Inhibitory Effect of Penicillin on Proline Synthesis in *Escherichia coli*

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The production of glutamic γ-semialdehyde, an intermediate in the synthesis of proline, was inhibited in *Escherichia coli* by physiological concentrations of penicillin. Sucrose (0.6 M) and sodium chloride (0.1 M) prevented penicillin inhibition of glutamic γ-semialdehyde synthesis. Cells which were in the stationary phase, or which had been permitted to metabolize without growth, were insensitive to the effects of penicillin on glutamic γ-semialdehyde synthesis.

Penicillin exerts its growth inhibitory action by preventing a transpeptidation which is required for the production of cross-linkages in cell walls of bacteria (7, 12, 14, 15, 17), and thus causes the formation of osmotically fragile cells. Penicillin is active as an antibiotic in growing organisms (8), and sensitivity of *Escherichia coli* to penicillin is maximal during cell division (10). Effects of penicillin which are not explainable in terms of its interference with cell wall biosynthesis have also been described. Hancock (6) found a decrease in the amino acid pool of cells growing in penicillin, and Prestidge and Pardee (13) found that exposure of *E. coli* to penicillin produced a change in the cells such that a membrane fraction from treated cells stimulated lysis of protoplasts. Both of these effects of penicillin were found in cells which were growing, or had been grown, in penicillin. This paper reports a physiological effect of penicillin which can be expressed in nonproliferating cells: inhibition of proline synthesis by inhibition of the formation of glutamic γ-semialdehyde from glutamic acid.

MATERIALS AND METHODS

Bacterial cultures and media. The strains of *E. coli* used are described in Table 1. Bacteria were grown in an inorganic salts medium, with 0.5% glucose as the carbon source (1). Cultures grown to a turbidity of 50, as measured in a Klett colorimeter, were considered to be young cultures.

Analytical methods. Protein was determined by the method of Lowry et al. (9), with bovine serum albumin as a standard. Glutamic γ-semialdehyde was measured by the system of Albrecht and Vogel (Fed. Proc. **19:** 2, 1960), and assays for the rate of synthesis of this compound were performed as described previously (1). Pyrroline-5-carboxylate reductase (L-proline:nicotinamide adenine dinucleotide phosphate 5-oxido-reductase, EC 1.5.1.2) assays were performed by the method of Yura and Vogel (16)

Chemicals. Penicillin G was obtained from Sigma Chemical Co., St. Louis, Mo., and contained 1,597 units of penicillin per mg. Radioactive penicillin [potassium 6-phenyl(acet-*1*-^{14}*C*)amidopenicillanate], 20.7 c/mole, was obtained from Nuclear-Chicago Corp., Des Plaines, Ill. Radioactivity measurements were made in a Packard scintillation counter. Samples were prepared for counting by mixing 0.1 ml of a water suspension of bacteria with 20 ml of Bray's (4) scintillation mixture.

Design of experiment. Cultures were centrifuged, and the cells were washed twice with distilled water. The cells were dispersed in distilled water and added to flasks containing phosphate buffer (*p*H 7.0, 0.02 M), glucose (0.5%), glutamic acid (1 mg/ml, final concentration), and the indicated amount of penicillin. The concentration of bacterial protein in the flasks varied between 0.01 and 0.03 mg/ml from experiment to experiment, but was constant for any one experiment.

Samples (2 ml) were withdrawn from the flasks at the indicated times for glutamic semialdehyde assay.

Since *E. coli* strains 55-1 and WP1-30 require proline for growth, no overall growth would be expected to occur under the conditions of the experiment.

RESULTS

Incubation of cells of *E. coli* strain 55-1 or strain WP1-30 with penicillin caused inhibition of the production of glutamic γ-semialdehyde by these cells; there was little difference between the responses of the two strains. Maximal inhibition of synthesis occurred at 2,000 units of penicillin per ml; concentrations of penicillin greater than 2,000 units/ml produced no further effect on the synthetic process (Fig. 1). This concentration is double that used by Lederberg (8) for the formation of spheroplasts in growing cells. The cells behave as though they have become saturated with

TABLE 1. *Strains of Escherichia coli used*

Name	Description	Nutritional requirement
W	Wild type	None
55-1	W, lacks pyrroline-5-carboxylate reductase, and excretes glutamic γ-semialdehyde	Proline
WP1	W, lacks control of proline synthesis and excretes proline	None
WP1-30	WP1, lacks pyrroline-5-carboxylate reductase, and excretes glutamic γ-semialdehyde	Proline

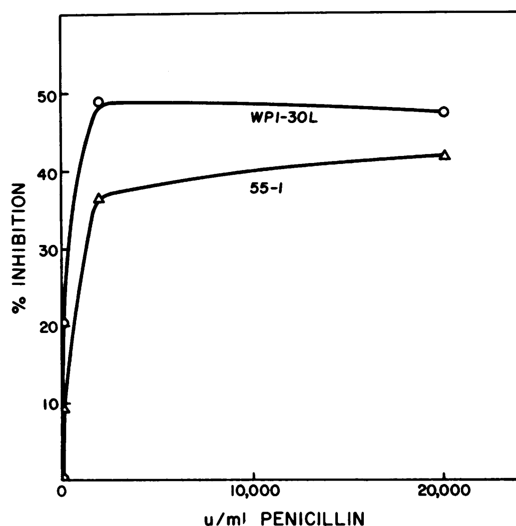

FIG. 1. *Effects of penicillin on the synthesis of glutamic γ-semialdehyde in cells of E. coli.*

penicillin, and one may infer a finite number of binding sites for this compound.

Cells of strain 55-1 were incubated for 120 min with glucose and glutamic acid medium (1) supplemented with 0.25 μc of radioactive penicillin per ml. The cells were washed twice. Penicillin was bound by cells from either old or young cultures, and binding was not prevented by 0.6 M sucrose or 0.2 M NaCl (Table 2). Cells incubated in the presence of sucrose showed consistently higher binding than cells incubated in a medium of low osmotic pressure. This observation suggests that sucrose exposes additional binding sites for the antibiotic.

Binding of penicillin to cells appears to be irreversible, since, after incubation of the cells in the presence of penicillin for 90 min, washing did not lead to a reversal of the inhibitory effect (Fig. 2) nor to a loss of bound radioactive penicillin. Preincubation for as little as 30 min was effective in producing cells in which the synthesis of glutamic γ-semialdehyde was inhibited. Preincubation in the absence of glucose or glutamic acid decreased binding of penicillin to the cells.

Sucrose (0.6 M) prevented inhibition of glutamic γ-semialdehyde synthesis by penicillin almost completely, although sucrose itself diminished the production of glutamic γ-semialdehyde (Fig. 3). Sodium chloride (0.1 M) also protected bacterial cells from penicillin inhibition of glutamic γ-semialdehyde synthesis (Fig. 4). The actions of sucrose and sodium chloride were additive, both in protection against penicillin and in inhibition of glutamic γ-semialdehyde production (Table 3).

Although sucrose prevented penicillin from inhibiting the formation of glutamic γ-semialdehyde, the addition of sucrose to a preparation previously incubated in the presence of penicillin

TABLE 2. *Binding of ^{14}C-penicillin to cells of E. coli*

Age of culture	Incubation medium	Per cent binding
Young	Glucose-glutamic acid (1)	100
Young	Glucose-glutamic acid + 0.6 M sucrose	257
Young	Glucose-glutamic acid + 0.2 M NaCl	97
Old (overnight culture)	Glucose-glutamic acid (1)	253

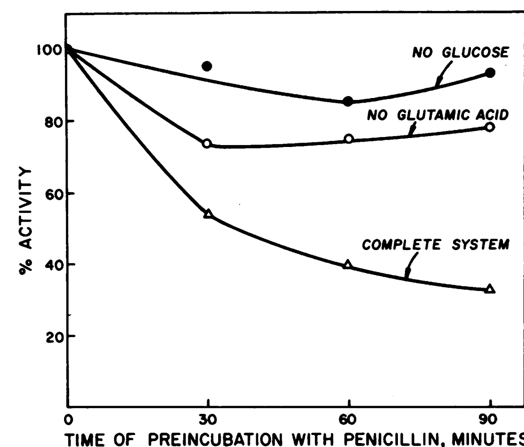

FIG. 2. *Effect of glucose (0.5%) and glutamic acid (1 mg/ml) on the binding of penicillin (2,000 units/ml) to suspensions of cells of strain 55-1.*

did not reverse the effect. Both sucrose and sodium chloride cause plasmolysis of the cell (3, 11), and may, by this effect prevent penicillin from acting on the sensitive cellular component.

The age of the culture had a profound effect on the sensitivity of cells to inhibition by penicillin. Cells from young cultures were very sensitive, but cells from cultures which had been in the stationary phase for several hours were unresponsive to penicillin (Fig. 5). It appears that, although cells from older cultures bind penicillin, this binding of penicillin does not lead to inhibition of glutamic γ-semialdehyde synthesis.

TABLE 3. *Effect of sucrose and sodium chloride on penicillin inhibition of glutamic γ-semialdehyde production*

Addition	Glutamic γ-semialdehyde produced (%)	
	Without penicillin	With penicillin
None	100	30
Sucrose (0.6 M)	91	76
NaCl (0.2 M)	104	66
Sucrose (0.6 M) + NaCl (0.2 M)	39	33

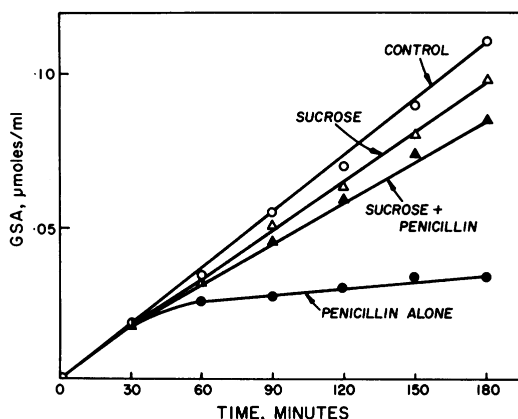

FIG. 3. *Effect of sucrose on the synthesis of glutamic γ-semialdehyde (GSA). Sucrose (0.6 M) was added to the incubation mixture in the absence (△) and presence (O) of penicillin (2,000 units/ml).*

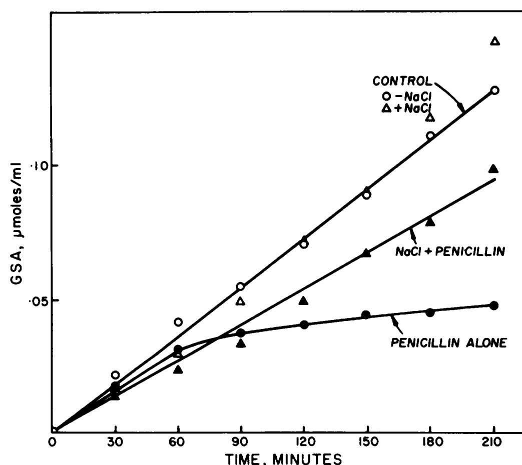

FIG. 4. *Effect of sodium chloride on the synthesis of glutamic γ-semialdehyde (GSA). NaCl (0.2 M) was added to the incubation mixture in the absence (△) and presence (▲) of penicillin (2,000 units/ml).*

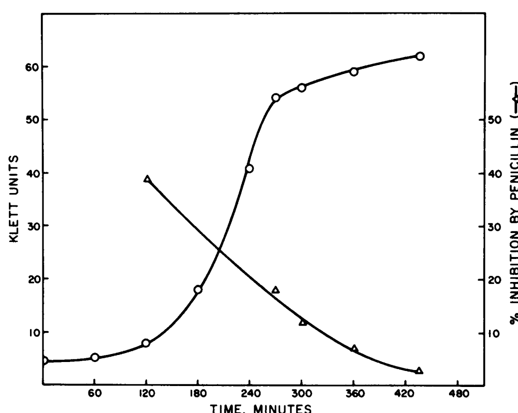

FIG. 5. *Effect of age of the culture on the inhibition of glutamic γ-semialdehyde synthesis by penicillin. Cells of strain 55-1 were grown in limiting proline (5 μg/ml) and assayed for their ability to produce glutamic γ-semialdehyde in the presence and absence of penicillin (2,000 units/ml).*

Loss of sensitivity to penicillin occurred when cells were incubated under nongrowing conditions in the presence of glucose and glutamic acid. Inhibition of glutamic γ-semialdehyde synthesis by penicillin decreased in a linear fashion with time of preincubation in the absence of penicillin (Fig. 6). This loss of sensitivity to penicillin could be achieved with several carbon sources and several amino acids (Table 4), whereas preincubation with neither glutamic acid nor glucose alone bestowed protection against penicillin inhibition. Sensitivity of the cells to penicillin was not decreased by an increase in the glutamic acid concentration. As is shown in Table 5, a variation of 200-fold in the concentration of glutamic acid had little effect on the extent of inhibition of the reaction by penicillin. Pyrroline-5-carboxylate reductase, the second reductive step in the pathway, was not inhibited by penicillin.

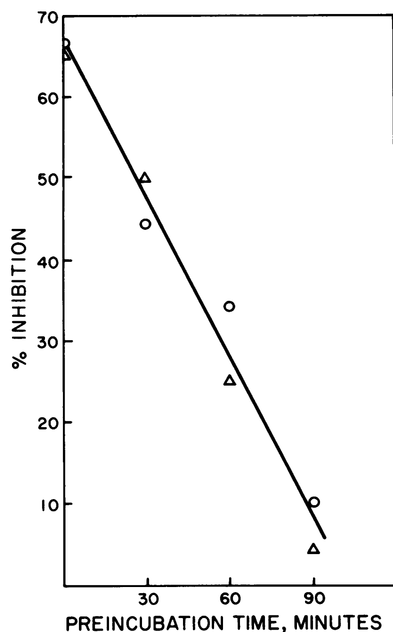

FIG. 6. *Effect of preincubation of cells of strain 55-1 (△) and WP1-30 (○) with glucose (0.5%) and glutamic acid (1 mg/ml) on their sensitivity to inhibition by penicillin (2,000 units/ml).*

TABLE 4. *Effect of preincubation with carbon sources and amino acids on sensitivity to penicillin*

Carbon source	Amino acid	Inhibition of glutamic γ-semialdehyde synthesis by penicillin (2,000 units/ml)
Glucose	Glutamate	5
	Aspartate	6
Pyruvate	Glutamate	7
	Aspartate	8
No preincubation	—	60-90

DISCUSSION

Inhibition of glutamic γ-semialdehyde synthesis by penicillin, and its prevention by 0.6 M sucrose, could be interpreted as an indirect response of the cells to loss of the amino acid pool reported by Hancock (6), since a lowered concentration of glutamic acid would explain the apparent inhibition of the reaction. Hancock suggested that loss of the amino acid pool can be attributed to disorganization of the cytoplasmic membrane, which results from blocking of cell wall synthesis. This theory predicts that an increase in the glutamic acid concentration would tend to reverse the apparent inhibition by penicillin. This was not found to occur. In the system described here, there was no growth, and the cells were not osmotically fragile after being treated with penicillin. Therefore, this interpretation may not be relevant to the present study.

An alternative interpretation is that the prevention of penicillin inhibition by sucrose and sodium chloride could be explained by a consideration that plasmolysis of the bacterial cell, which leads to a separation of the cell wall from the cell membrane, could physically separate the proline-synthesizing system in the membrane from the penicillin-binding site. Penicillin does not appear to penetrate the cell membrane (5, 7), and is bound at or near the surface of the cell. Inhibition of glutamic γ-semialdehyde synthesis would be a secondary response of the binding of penicillin, that is, an indirect effect on the sites of enzyme attachment, leading to inhibition of the enzyme, as is shown in Fig. 7.

Inhibition of glutamic γ-semialdehyde synthesis is strongly dependent upon the physiological state of the cells. The differential effect of penicillin on cells from young cultures (young cells), as opposed to cells from older cultures (old cells),

TABLE 5. *Effect of concentration of glutamic acid on sensitivity to penicillin inhibition*

Concn of glutamic acid	Inhibition by penicillin (20,000 units/ml)
mg/ml	%
0.10	45
0.50	54
1.0	53
10.0	60
20.0	59

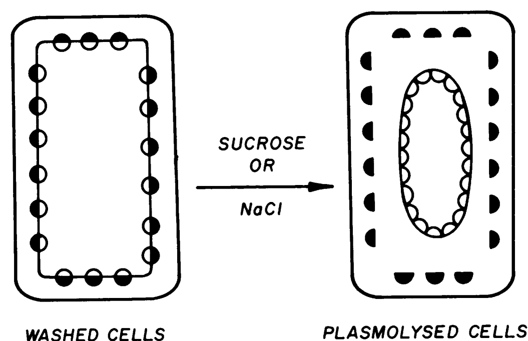

FIG. 7. *Proposed schematic representation of the effect of plasmolysis on glutamic γ-semialdehyde synthesis in the presence of penicillin.*

or on young cells incubated in the presence of an energy source and an amino acid in the absence of growth, could be explained on the basis that these cells are less responsive to penicillin because they have cell walls which are different from those in young cells. The difference in the cell walls is a subtle one, since older cells bind penicillin to a greater extent than younger cells but this binding does not lead to inhibition of glutamic γ-semialdehyde synthesis. This interpretation is supported by the observation of Bayer (2), who found that young cultures of bacteria are more sensitive to osmotic shock than are cells in stationary phase.

Inhibition of glutamic γ-semialdehyde synthesis in cells of *E. coli* may provide a sensitive tool for the determination of the average metabolic age of a culture.

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