

Product Inhibition of the Fermentative Formation of Glutamic Acid

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The addition of penicillin to cells of *Corynebacterium glutamicum* growing in 5-liter fermentors initiated the excretion of glutamic acid. The rate of glutamate production in fermentors declined continuously with time and reached 75% of the initial rate in 24 hr after penicillin had been added. The addition of glutamate to resting cell suspensions had only a slight effect on sugar utilization but caused a marked decrease in glutamate excretion. It is suggested that the high level of glutamate accumulating in the fermentation broth is responsible for inhibiting its own production.

When penicillin is added to exponentially growing cells of *Corynebacterium glutamicum*, permeability is altered and glutamate excretion is triggered (3). Although production of the amino acid continues for 50 hr or more, the rate of production decreases continuously. The reason for the declining production rate has yet to be ascertained. The present study shows that inhibition of the conversion of glucose to glutamate by glutamate is an important factor leading to a decline in the production rate.

MATERIALS AND METHODS

Culture. *C. glutamicum* MB-1645 was used in all experiments. The organism was maintained by serial passage on a medium containing, per liter: peptone (Difco), 10 g; beef extract (Difco), 5 g; yeast extract (Difco), 5 g; NaCl, 2.5 g; and agar (Difco), 25 g; pH 7.1. Incubation was as a slant culture at 28 C for 24 hr.

Glutamic acid production in 5-liter fermentors. Cells from the maintenance medium were used to inoculate 200 ml of medium which contained, per liter: Casamino Acids (Difco), 5 g; yeast extract (Difco), 5 g; K₂SO₄, 1.5 g; (NH₄)₂HPO₄, 1.0 g; (NH₄)H₂PO₄, 1.0 g; urea, 1.0 g; MgSO₄·7H₂O, 0.5 g; MnSO₄·1H₂O, 0.01 g; FeSO₄·7H₂O, 0.007 g; and glucose, 10 g. After incubation for 12 to 16 hr at 27 C on a rotary shaker (220 rev/min, 2-inch thrust), the entire contents were used to inoculate a single 5-liter fermentor (glass jar type, New Brunswick Scientific Co.). The fermentor was charged with 2,700 ml of medium which contained, per liter: MgSO₄·7H₂O, 0.25 g; MnSO₄·1H₂O, 0.01 g; (NH₄)₂SO₄, 5.2 g; FeSO₄·7H₂O, 0.01 g; KH₂PO₄, 0.52 g; K₂HPO₄, 0.52 g; yeast extract (Difco), 0.5 g; Casamino Acids (Difco), 0.5 g; and *d*-biotin, 10 μg. Blackstrap molasses (Pacific Molasses

Co.) was added before inoculation to 25 g/liter and was also added during the fermentation until an amount equivalent to 17% sugar had been added. When the cell population reached 6 g/liter (dry weight), 6 units of potassium benzyl penicillin per ml was added to initiate glutamic acid excretion. The medium was maintained at pH 7.0 during the growth phase (before penicillin addition) and raised to pH 7.6 at the beginning of the production phase. The pH was controlled by automatic NH₄OH addition. The whole process was carried out at 33 C, an agitation rate of 700 rev/min, and an aeration rate of 1.75 liters/min.

Glutamate production by resting cells. To prepare nonproliferating suspensions, cells either were removed from the 5-liter fermentors at intervals or were grown in 2-liter baffled flasks containing 250 ml of the following medium (per liter): glucose, 10 g; yeast extract (Difco), 5.0 g; vitamin-free acid-hydrolyzed casein (Nutritional Biochemicals Corp.), 5.0 g; K₂SO₄, 1.5 g; urea, 1.0 g; (NH₄)₂HPO₄, 1.0 g; (NH₄)H₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.5 g; MnSO₄·H₂O, 0.1 g; FeSO₄·7H₂O, 0.0033 g (pH 6.8). The cells were then incubated for 16 hr at 27 C on a rotary shaker (220 rev/min, 2-inch thrust). Potassium benzyl penicillin (filter-sterilized) was added at 10 to 20 units/ml, and the cells were harvested by centrifugation in the cold. To the cells was added 130 ml of the sterile resting cell medium containing, per liter: blackstrap molasses, 80 g (on a sugar basis); (NH₄)₂SO₄, 13.8 g; urea, 6.4 g; KH₂PO₄, 1.0 g; MgSO₄·7H₂O, 0.25 g; and penicillin, 10 to 20 units/ml; adjusted to pH 7.2 with KOH. The sugar sources were added aseptically from sterile concentrated stock solutions. After rapid mixing, the cell suspension was subdivided into 35-ml portions in sterile 250-ml Hinton flasks (Pyrex) each containing 1.0 g of CaCO₃. Resting cell incubation was on the rotary shaker at 32 C. Glutamic acid and residual sugar were assayed as described previously (1, 2). Growth was measured as dry weight of cells per milliliter of culture.

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RESULTS AND DISCUSSION

In a complete fermentation, the rate of glutamate production continually declined after it had been triggered by the penicillin addition. To test whether this was a function of the changing extracellular environment, cells were removed from the 5-liter fermentor at various times after penicillin addition, centrifuged, and added to the fresh resting cell medium in flasks. The amount of glutamate produced in 6 hr per gram (dry weight) of cells in the flasks was compared to the amount produced by the bulk of the cells left in the fermentor (Table 1). At early hours, the fermentor system supported double the specific rate of glutamate production when compared to the flask system. However, the loss of ability to produce glutamate in the complete fermentation was much more rapid than in the case of cells removed from the fermentation medium and placed in the fresh medium in flasks. Thus, by 24 hr, production of glutamate by cells in flasks was more rapid than in the complete fermentation. At 48 hr, production by cells in the fermentor was only one-third that of the identical cells that had been removed to the fresh resting cell medium.

Since changing of the extracellular environment seemed to allow a greater relative rate of glutamate production, we considered whether the high concentration of glutamate itself might cause inhibition. Cells were grown in flasks and subsequently resuspended in the resting cell medium containing increasing levels of glutamate, which resulted in an increasing inhibition of both the rate and extent of glutamate production. As little as 10 g/liter caused about 25% inhibition. Ammonium L-glutamate added at a concentration of 25 g/liter caused a 50% inhibition after 12 hr of incubation. The inhibition was not due to the ammonium ion since ammonium sulfate was non-inhibitory. Monosodium glutamate was also inhibitory to glutamic acid production in a resting cell medium, whereas the sodium ion had no significant effect.

Table 2 compares the sugar utilization with glutamate production when different levels of ammonium glutamate were added to the resting cell medium. The addition of 10 g of ammonium glutamate per liter had no effect on sugar consumption but decreased the glutamic acid synthesized by the organism by 25%. At 50 g of added ammonium glutamate per liter, there was a 40% decrease in sugar utilization with a much greater decrease (70%) in glutamate excretion. The result was a marked decrease in conversion efficiency. This suggests that the sugar was metabolized to a glutamate intermediate whose con-

TABLE 1. Decline in rate of glutamic acid production by *Corynebacterium glutamicum* in 5-liter fermentors

Age of cells removed after penicillin addition <i>hr</i>	Rate of glutamate production ^a		Ratio of cells in fermentor to cells removed from fermentor
	Cells remaining in fermentor	Cells removed from fermentor ^b	
1	2.9	1.5	1.9
6	1.6	1.3	1.2
24	0.7	1.8	0.39
48	0.4	1.3	0.31
55	0.3	1.0	0.30

^a Rate equals grams of glutamate produced per grams of dry cell per 6 hr. The rate in the fermentor was based on 6 hr of incubation subsequent to the removal of cells for resting cell suspensions.

^b At various times as indicated, cells were removed from the fermentor, washed, and resuspended at 5 mg/ml (dry weight) in the resting cell medium with blackstrap molasses as the carbon source.

TABLE 2. Effect of ammonium L-glutamate on sugar utilization and glutamic acid production by resting cells of *Corynebacterium glutamicum*^a

Ammonium-L-glutamate added at 0 hr	Sugar consumed ^b	Glutamate produced	Molar conversion efficiency ^c
<i>g/liter</i>	<i>g/liter</i>	<i>g/liter</i>	
0	35	20	0.69
10	35	16	0.55
50	21	6	0.35

^a Data are from 12-hr resting cell fermentations with blackstrap molasses as the carbon source.

^b Assayed as glucose equivalents.

^c Computed as the ratio of moles sugar (as glucose) consumed to moles of glutamate produced.

version to glutamic acid was inhibited by glutamate.

Under various conditions, *C. glutamicum* is able to produce and excrete alanine, valine, aspartate, glutamine, lactate, acetate, pyruvate, α -ketoglutarate, and other members of the tricarboxylic acid cycle as well as pyroglutamate (3). These acids were therefore added to the resting cell system to determine whether they contribute to the decline of the production rate. At the following levels (which were considerably higher than those found in fermentation broths), L-alanine (5 g/liter), L-glutamine (10 g/liter), L-valine (5

g/liter), L-aspartate (10 g/liter), and L-pyroglytamate caused some inhibition but were considerably less active than glutamate. Also tested at 10 to 30 g/liter were all the tricarboxylic acid cycle intermediates, acetate, pyruvate, glyoxylate, and α -ketoglutarate, but none had an inhibitory effect on glutamate synthesis. Besides L-glutamate, D-glutamate, which does not occur in significant concentrations in the spent fermentation broth, was the only material that prevented the excretion.

At present, we have no information as to the mechanism of this inhibition. Although glutamate exerts control of its own biosynthesis in *Escherichia coli*, *Bacillus subtilis*, and *B. licheniformis* by repression of one or more of the enzymes [citrate synthetase, aconitase, isocitrate dehydrogenase, nicotinamide adenine dinucleotide phosphate-specific glutamic acid dehydrogenase (4, 5, 6)], we are not aware of any reports of feedback inhibition of their activities by glutamate. The addition of L-glutamate to the growth medium of *C. glutamicum* has no effect on subsequent glutamate excretion by resting cells (*unpublished data*). This suggests that the amino acid regulates the tricarboxylic acid cycle enzymes in *C. glutamicum*

by a mechanism which is different from those operative in *E. coli* and *Bacillus* species.

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