

Reversal by Citrate of the Iodoacetate and Fluoride Inhibition of Glutamic Acid Production by *Corynebacterium glutamicum*

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Citrate reversal of iodoacetate inhibition of glutamate synthesis is nonmetabolic. Reversal of fluoride inhibition is metabolic, occurring only at low Mg concentrations.

The addition of penicillin to growing cells of *Corynebacterium glutamicum* triggers the excretion of high levels of glutamic acid (2, 4). This production of glutamic acid from sugar is thought to proceed predominantly through the Embden-Meyerhof-Parnas (EMP) pathway and the early steps of the tricarboxylic acid cycle, oxygen acting as terminal electron acceptor (A. L. Demain and J. Birnbaum, *Curr. Top. Microbiol. Immunol.*, *in press*). The effects of inhibitors on the conversion of glucose to glutamate by penicillin-treated resting cells were studied to confirm the importance of such a pathway. Surprisingly, it was observed that the inhibition of glutamate synthesis by iodoacetate and fluoride could be prevented or overcome by the addition of citrate to the resting cell medium.

C. glutamicum MB-1645 was grown at 28 C in the following medium: MgSO₄·7H₂O, 0.5 g; MnSO₄·H₂O, 0.03 g; FeSO₄·7H₂O, 0.01 g; K₂SO₄, 1.5 g; (NH₄)H₂PO₄, 1.0 g; (NH₄)₂HPO₄, 1.0 g; (NH₄)₂SO₄, 4.0 g; urea, 1.0 g; *d*-biotin, 30 μg; *p*-aminobenzoic acid, 0.05 g; glucose, 10 g; Difco Yeast Extract, 5 g; Difco Vitamin Free Casamino Acids, 5 g; and distilled water, 1,000 ml. The glucose was added separately from a sterile concentrated solution after autoclaving of the medium. After 16 hr, penicillin G was added (10 units/ml). The cells were rapidly chilled in an ice bath, centrifuged in the cold, and suspended at 5 g/liter (dry weight) in the following resting cell medium: KH₂PO₄, 1 g; MgSO₄·7H₂O, 0.25 g; urea 6.1 g; (NH₄)₂SO₄, 13.8 g; glucose, 60 or 80 g (added aseptically from a sterile concentrated solution); CaCO₃, 30 g; and distilled water, 1,000 ml. The pH was 7.0 to 7.1. Penicillin G was added (10 units/ml) to maintain the excretion of glutamate at a high level for several hours and to insure that the cells were maintained in a non-

proliferating state. Incubation was at 32 C on a gyrotary shaker. At intervals, samples were removed and were centrifuged to sediment the cells. The supernatant fluid was used with no further treatment for the assay of residual glucose by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.). For assay of glutamic acid, the clarified broth was acidified, heated for 20 min at 60 C, and neutralized to pH 5.0 to 5.5. Glutamic acid was then assayed manometrically by using glutamic acid decarboxylase from *Escherichia coli*. The glutamate assay was automated with a Technicon autoanalyzer.

In preliminary experiments it was found that 10⁻⁴ to 10⁻³ M concentration of methylene blue, phenazine methosulfate, and sodium azide, inhibitors of terminal electron transfer, was highly inhibitory to the glutamate excretion. Dinitrophenol, an inhibitor of oxidative phosphorylation, was less active, requiring 10⁻² M for complete inhibition. Iodoacetate and NaF, which affect glycolysis, caused, respectively, a 90 and 65% inhibition of glutamate production (Fig. 1A and 1B). The addition of 1% citrate to the resting cell medium completely prevented the effect of either inhibitor. Although some of the citrate was converted directly to glutamate, assays of residual glucose revealed that the major effect of citrate was to reverse the inhibition of sugar utilization. Inhibition of glutamate production caused by azide or dinitrophenol was not prevented by 1% citrate. This failure suggested that the citrate effect was specific for the glycolysis inhibitors, iodoacetate and fluoride. When citrate was added to the medium as late as 6 hr after sodium fluoride, reversal of inhibition was still observed. However, the addition of citrate as early as 45 min after the addition of iodoacetate did not reverse the inhibition. In fact, the only time that citrate pre-

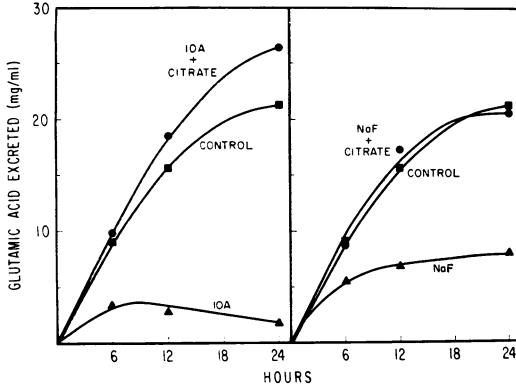


FIG. 1. Reversal of inhibition of glutamic acid production by citrate: (A) iodoacetate (5×10^{-3} M); (B) sodium fluoride (10^{-2} M). Citrate added at 10 mg/ml.

vented iodoacetate inhibition was when it was sterilized with the medium. These results show that the mechanism by which citrate prevents NaF and iodoacetate inhibition is different for each inhibitor. Subsequently, it was found that the only conditions which permitted glutamate production in the presence of iodoacetate were those in which citrate and the inhibitor were autoclaved together. Apparently, citrate directly inactivates iodoacetate during heating of the resting cell medium. Citrate reversal of fluoride inhibition, however, is a true metabolic effect. It is known that fluoride inhibition of enolase is dependent upon the formation of a magnesium-fluorophosphate complex, which inactivates the enzyme (1). A similar complex is responsible for inhibition of phosphoglucomutase (3). Fluoride inhibition of glutamate formation increases with increasing magnesium concentrations, and citrate reversal of fluoride inhibition is antagonized by increasing the magnesium concentration (Fig. 2). Apparently, citrate acts by chelating magnesium and preventing the formation of the inhibitory complex.

The inhibitory effects of methylene blue, phenazine methosulfate, azide, fluoride, and iodoacetate demonstrate the importance of terminal respiration, oxidative phosphorylation, and the EMP pathway for glutamate formation from glucose. The results also point out some dangers

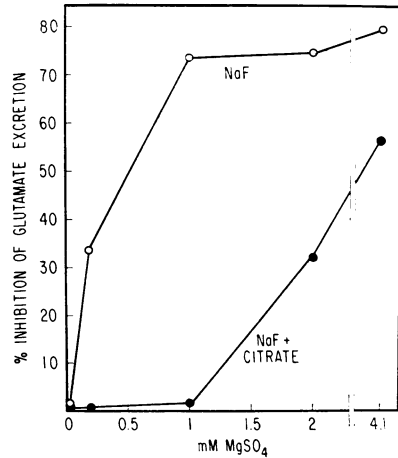


FIG. 2. Effect of magnesium on inhibition of glutamic acid production by fluoride and its reversal by citrate. Sodium fluoride concentration was 10^{-2} M. Citrate used at 10 mg/ml. At each level of $MgSO_4$, per cent inhibition was calculated based on glutamate excretion in flasks containing NaF compared with those lacking NaF (O). Similarly, glutamate excretion was compared in flasks containing NaF plus citrate with flasks lacking NaF and citrate (●). $MgSO_4$, at the levels tested, had no adverse effect on glutamate excretion.

involved in interpretation of experiments with inhibitors, i.e., the necessity of sterilizing iodoacetate separately if aseptic conditions are desired and the need for high magnesium concentrations when studying the effect of fluoride on glycolysis.

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LITERATURE CITED

- Hewitt, E. J., and D. J. D. Nicholas. 1963. Cations and anions: Inhibitions and interactions in metabolism and in enzyme activity, p. 401. In R. M. Hochster and J. H. Quastel (ed.), *Metabolic inhibitors*, vol. 2. Academic Press Inc., New York.
- Matsuo, T., Y. Oyama, H. Tanemoto, W. Hashida, and S. Terramoto. 1966. Fermentative production of glutamic acid and its application. X. Effects of antibiotics on the glutamic acid fermentation. Part III. Effect of penicillin and biotin. *Amino Acids and Nucleic Acids* 12:915-924.
- Najjar, V. A. 1948. The isolation and properties of phosphoglucomutase. *J. Biol. Chem.* 175:281-290.
- Somerson, N., and T. Phillips. 1961. Procédé d'obtention d'acid glutamique. Belgian Patent no. 593,807.