Studies on the Formation of Bacitracin by *Bacillus licheniformis*: Effect of Inorganic Phosphate

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(Received 14 March 1974; revised 1 May 1974)

INTRODUCTION

Many inorganic constituents of fermentation media can be added in excess without inhibiting antibiotic formation. Inorganic phosphate, however, is usually very critical. Phosphate concentrations in excess of that necessary for growth have been reported to inhibit the production of streptomycin, bacitracin, chlortetracycline, actinomycin, novobiocin, oxytetracycline, neomycin, nystatin, monamycin and polymyxin (Dulaney & Perlman, 1947; Hills, Belton & Blatchley, 1949; Biffi, Boretti, Di Marco & Pennella, 1954; Katz, Pienta & Sivak, 1958; Hoeksema & Smith, 1961; Zygmunt, 1964; Donovan & Brown, 1965; Hall & Hassall, 1970; Chigaleichik, Makhfud & Nefelova, 1973).

Excess inorganic phosphate may generally distort the formation of secondary metabolites in a wide variety of micro-organisms (Weinberg, 1970; Gentry *et al.* 1971).

We report the effect of inorganic phosphate upon the production of the secondary metabolite bacitracin by *Bacillus licheniformis*.

METHODS

Organism. The bacitracin-producing strain *Bacillus licheniformis* ATCC10716 was kept as a spore suspension at 4 °C throughout the investigation.

Preparation of inoculum. The inoculum consisted of spores. Bacteria were spread on the surface of agar plates (Difco, Antibiotic Medium No. 1) and incubated at 37 °C for 7 days. The spores produced were washed off with sterile 0.9 % (w/v) NaCl and treated with 0.1 % lysozyme to destroy whole cells and cell debris. The spores were washed three times with sterile 0.9 % NaCl and finally diluted in 0.9 % NaCl to give an extinction of 24 at 650 nm. The inoculum was 0.05 ml of the spore suspension.

Media and growth conditions. The chemically defined medium for growth and bacitracin production had the following composition (g/l glass-distilled water): L-glutamic acid, 20.0; L-alanine, 0.2; citric acid, 1.0; NaH₂PO₄·2H₂O, 0.2; KCl, 0.5; Na₂SO₄, 0.5; MgCl₂·6H₂O, 0.2; CaCl₂·2H₂O, 0.01; FeSO₄·7H₂O, 0.01; MnSO₄·H₂O, 0.01. The pH was adjusted to 6.0 with 1 M-NaOH before autoclaving at 121 °C for 20 min. Additions to the medium are noted in the text.

Growth and bacitracin production occurred in 500 ml Erlenmeyer flasks at 37 °C at about 360 rev./min on a New Brunswick rotatory shaker (Model G-53). In some of the experiments a shaking waterbath (120 cycles/min) was used (Model HETO TB SH02).

Microbiological assay of bacitracin. Bacitracin was determined by the agar diffusion method of Haavik & Thomassen (1973).

Fig. 1. (a) Growth, (b) bacitracin production and (c) pH of culture fluid as a function of incubation time in media containing: ○, 0.2 g phosphate/l; ●, 1.0 g phosphate/l; △, 10.0 g phosphate/l; ▲, 20.0 g phosphate/l.

Thin-layer chromatography (t.l.c.). Bacitracin was detected by t.l.c. and identified by overlaying with *Micrococcus flavus* as described by Haavik & Thomassen (1973).

Growth. Bacterial growth was measured as extinction at 650 nm using a Spectronic 20 spectrophotometer. The cultures were appropriately diluted with distilled water before measurement and growth was expressed as the E_{650} recorded multiplied by the dilution factor. The relationships between this value and bacterial density was linear over the range 0.1 to 8.5, corresponding to viable counts of 3.0×10^7 and 4.0×10^8 bacteria/ml respectively.

RESULTS AND DISCUSSION

Bacillus licheniformis ATCC10716 produced about 22 i.u. bacitracin/ml after 28 h growth in the medium with 0.2 g inorganic phosphate/l. Further incubation decreased the titre. Bacitracin was produced throughout the period of active growth.

By increasing the concentration of phosphate to 1.0 g/l bacitracin production was decreased by 50 %, even though total growth was markedly increased (Fig. 1 *a, b*), in agreement with the general assumption that excess phosphate is inhibitory to antibiotic production. Demain (1968) suggested that the inhibition of antibiotic formation by phosphate is generally caused by a feed-back effect of phosphate upon phosphatases necessary for the synthesis of the antibiotic from phosphorylated intermediates. Another suggestion was that excess inorganic phosphate suppresses antibiotic production by depriving the cells of an essential metal (Weinberg, 1970). When the concentration of phosphate in the medium was increased, bacitracin production gradually increased again and reached normal levels at 10 g phosphate/l (Fig. 1 *b*). At 20 g phosphate/l, a marked stimulation of bacitracin production was observed.

The antibiotic produced at the different phosphate concentrations was identified as bacitracin by t.l.c.

These results indicate that there is no feed-back effect of phosphate upon bacitracin production as suggested by Demain (1968). Nor is it likely that phosphate is depriving the cells of an essential metal (Weinberg, 1970), since bacitracin production was restored by further increasing the concentration of phosphate.

The pH of the culture fluids increased by different amounts at the various concentrations of phosphate used (Fig. 1 *c*). Cultures containing 1.0 g phosphate/l showed the most rapid increase in pH and the lowest antibiotic yield, whereas cultures containing 20.0 g phosphate/l showed the slowest increase in pH and the highest bacitracin titres. Intermediate effects on pH and antibiotic production titre were observed for cultures containing 0.2 and 10.0 g phosphate/l. (Fig. 1 *b, c*). This indicates that too rapid increase in pH may inhibit bacitracin production.

After 14 h incubation a pH of about 8.0 was produced in the medium containing 1.0 g phosphate/l. By maintaining the pH at about 8.0 by means of frequent additions of small amounts of 1 M-HCl during the succeeding period of incubation, a maximum bacitracin production of about 16 i.u./ml was obtained. The control culture (1.0 g phosphate/l) yielded 11.0 i.u. bacitracin/ml and had a pH of 8.9 after the same period of incubation. I have previously shown that bacitracin production was markedly inhibited when the pH was raised by the addition of NaOH about 16 h after the initiation of growth (Haavik 1974). pH-dependency of bacitracin production is in agreement with Snoke (1961) who reported a pH optimum of 8.0 for bacitracin production by *B. licheniformis*.

A widespread opinion is that growth and antibiotic production are competing or incompatible processes. Bu'Lock, (1961) and Weinberg (1970) have postulated that antibiotics are

produced by cells that have recently stopped dividing. Another view is that a rapid growth rate will result in a low antibiotic yield (Jarvis & Johnson, 1947; Demain, 1968). Since bacitracin is produced throughout the period of active growth, I find it unlikely that the antibiotic is produced by non-growing cells.

During the first 14 h of incubation the cultures showed the same growth rate at all concentrations of phosphate, the doubling time being about 60 min. In the succeeding period of incubation, cultures containing 0.2 g phosphate/l showed a slower growth rate than cultures containing 1.0 g phosphate/l (Fig. 1*a*), probably due to a rate-limiting effect of the low phosphate concentration. Cultures containing 0.2 g phosphate/l produced about 50 % more bacitracin than cultures containing 1.0 g phosphate/l, conforming with the belief that a rapid growth rate is adverse for antibiotic production. The same rapid growth rates as found with cultures containing 1.0 g phosphate/l were also observed with cultures containing 10.0 and 20.0 g phosphate/l, and in these cases high titres of bacitracin were produced (Fig. 1*b*). The lower bacitracin production by cultures grown at 1.0 g phosphate/l can therefore not be explained by the faster growth rate observed. Since both high and low pH inhibits bacitracin production (Haavik, 1974) I suggest that a rapid growth rate adversely affects antibiotic production only when it leads to a rapid drift in pH. If pH is controlled, rapid growth may be beneficial to antibiotic production.

My findings indicate that inorganic phosphate exerts an indirect effect upon bacitracin synthesis by affecting the pH development of the culture.

I thank Mr T. Höyland, Director of Research and Development, for his support of this work, Mr S. Thomassen, Head of the Microbiological Laboratories, for carefully reading the manuscript, and Mrs Eva Gustavsson for excellent technical assistance.

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