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

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SUMMARY

The production of bacitracin by *Bacillus licheniformis* closely paralleled growth in a synthetic medium without glucose. Glucose inhibited bacitracin production during the first hours of growth, whereas growth was not affected. Bacitracin was produced mainly during the later stages of growth. Formation of bacitracin was apparently not under catabolite repression control by glucose since the inhibitory effect of glucose upon the early bacitracin production was prevented by neutralizing the culture fluid with CaCO_3 . The inhibitory effect of glucose may be due to the low pH created by its metabolism.

Addition of 0.5 % glucose markedly increased the maximum titre of bacitracin. This stimulation could also be due to the effect of glucose metabolism upon the pH of the medium. The observation that peptide antibiotics are produced mainly after growth is not always true; in appropriate media they might also be produced during the phase of rapid growth.

INTRODUCTION

Bacitracin is a peptide antibiotic produced by some strains of *Bacillus licheniformis* and *B. subtilis* (Anker, Johnson, Goldberg & Meleney, 1948; Hickey, 1964; Weinberg, 1967). Bernlohr & Novelli (1960; 1963) have shown that it is produced after growth and before sporulation. They proposed that bacitracin is produced by the bacteria at this stage of their life cycle only.

Peptide antibiotics are frequently reported to be produced mainly after growth, e.g. penicillin, gramicidin S, cephalosporin C, tyrocidin, actinomycin, mycobacillin, edeine, polymyxins, triostin (Jarvis & Johnson, 1947; Winnick, Lis & Winnick, 1961; Demain, 1963; Mach, Reich & Tatum, 1963; Katz, Wise & Weissbach, 1965; Banerjee, Majumdar & Bose, 1967; Kurylo-Borowska, 1967; Paulus, 1967; Yoshida & Katagiri, 1969).

It is generally believed that the producing organisms initiate the synthesis of peptide antibiotics towards the end of the exponential phase of growth. Consequently there has been much speculation about what function peptide antibiotics may play in the producer organisms at this stage of their life cycle (Katz, 1968; 1971; Hurst, 1969; Sermoniti, 1969; Hanson, Peterson & Yousten, 1970; Hodgson, 1970; Weinberg, 1970; Jayaraman & Kennan, 1972; Sadoff, 1972; Sarkar & Paulus, 1972).

It has also been suggested that peptide antibiotics, like other secondary metabolites, are only produced by organisms that have recently stopped dividing (Bu'lock, 1961; Woodruff, 1966; Weinberg, 1970; 1971).

Snoke (1960; 1961) reported that glucose markedly stimulated the synthesis of bacitracin by *Bacillus licheniformis* in defined medium. Glucose is a common constituent of media for

antibiotic production and has also been reported to stimulate the production of mycobactin, polymyxin A and actinomycin (Banerjee, Majumdar & Bose, 1967; Paulus, 1967; Gallo & Katz, 1972). No explanation of why antibiotic production is stimulated by glucose has been offered.

During a study of bacitracin production by *Bacillus licheniformis* in different synthetic media, both the kinetics of bacitracin formation and the maximum titre of bacitracin were greatly affected by the composition of the medium. The results obtained are described and discussed in this paper.

METHODS

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

Media and growth conditions. The chemically defined medium (M2) for growth and bacitracin production had the following composition (g/l doubly distilled water): L-glutamic acid, 20.0; L-alanine, 0.2; citric acid, 1.0; $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 2.0; KCl, 0.5; Na_2SO_4 , 0.5; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.01.

The pH was adjusted to 7.0 with 1 N-NaOH before autoclaving at 121 °C for 20 min. The buffer medium (M20) had the same composition as M2 except for the sodium phosphate content which was 20.0 g/l. Additions to the media are noted in the text. Glucose was sterilized separately before addition. The inoculum consisted of spores. Growth and bacitracin production occurred in 500 ml Erlenmeyer flasks incubated at 37 °C at about 360 rev./min on a New Brunswick rotatory shaker (Model G-53).

Microbiological assay and detection of bacitracin. Bacitracin was determined by an agar diffusion method and detected by growth of *Micrococcus flavus* after thin-layer chromatography (t.l.c.) as described previously (Haavik & Thomassen, 1973).

Growth. Bacterial growth was measured as extinction at 650 nm (E_{650}) in a Spectronic 20 spectrophotometer.

Preparation of inoculum. The 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 saline and treated with 0.1 % lysozyme in order to destroy whole bacteria and debris. The spores were washed three times with sterile saline and finally diluted in saline to give an E_{650} of 24.0. The size of inoculum was 0.05 ml of the spore suspension.

RESULTS

Growth and bacitracin production in M2 medium

The growth of *Bacillus licheniformis* ATCC10716 in medium M2 gave bacitracin titres of approx. 13 i.u./ml after 24 h incubation (Table 1). Further incubation reduced the titre. The release of bacitracin from the bacteria closely paralleled growth, maximum titre of bacitracin being found in the culture fluid at about the same time as the culture entered the stationary phase. During all stages of growth significant amounts of bacitracin were detected by t.l.c. and subsequent overlaying with *Micrococcus flavus*. It appears that the bacitracin is formed as the organisms are actively dividing.

Effect of glucose upon bacitracin formation

By adding glucose to the M2 medium, bacitracin formation was retarded (Table 2). The more glucose added the later bacitracin was detected in the culture fluid, whereas growth was not influenced.

Table 1. *Bacitracin production during growth in M2 medium*

Time of incubation (h)	Growth (E_{650})	pH of the culture fluid	Bacitracin in culture fluid (i.u./ml)
7	0.14	7.37	< 0.1*
8	0.20	7.48	< 0.1*
9	0.34	7.58	0.1
10	0.64	7.77	0.4
11	1.10	7.90	0.5
12	1.50	8.05	0.6
14	3.70	8.28	4.0
16	5.90	8.35	8.4
18	7.40	8.47	9.8
20	8.60	8.65	10.4
24	9.45	8.83	12.5
26	9.50	8.92	12.0
28	8.10	9.25	7.7

* Less than 0.1 i.u. bacitracin/ml detected.

The maximum titre of bacitracin was also affected by glucose. When 0.5 % glucose was added to the medium a maximum titre of about 20 i.u./ml was found (Table 2). This is a marked stimulation as compared with the 13 i.u./ml of bacitracin produced when glucose is absent (Table 1). With higher concentrations of glucose in the medium bacitracin production fell.

Effect of CaCO₃

Addition of glucose to the M2 medium led to a lowered pH of about 5.5 during the first hours of growth (Table 2). The more glucose added, the longer was the duration of the low pH. This decrease in pH may reflect the accumulation of pyruvic acid and acetic acid formed from glucose (Halvorson, 1962).

CaCO₃ (0.5 %) in the M2 medium with 1.0 % glucose prevented both the fall in pH and the inhibition of the early bacitracin production by glucose (Table 3).

Effect of phosphate buffer

Growth of *Bacillus licheniformis* in the medium with increased phosphate (M20) gave bacitracin titres of about 19 i.u./ml after 28 h incubation. When 0.5 % glucose was added to this medium a maximum titre of about 40 i.u. bacitracin/ml was found (Table 4).

Since glucose causes an early fall in pH, the pH is lower during later stages of growth than in media without glucose (Tables 1 and 2). The pH of the M20 medium was reduced with HCl to see whether this lower pH could stimulate bacitracin production. When the initial pH was lowered to 6, the pH of the culture during later stages of growth was found to be practically identical to that recorded when glucose was a constituent of the medium (Table 4). The lower pH values during later stages of growth gave approx. 40 i.u. bacitracin/ml, the same high bacitracin production as when glucose was added.

Effect of NaOH

The pH of the M20 medium with initial pH 6 reached about 7.0 after 16 h. Adding NaOH to pH 8.5 produced only 13.6 i.u. bacitracin/ml in the culture fluid after an additional 4 h, and about 16 i.u./ml after 10 h. The final pH was 8.7. The control culture (M20 medium with initial pH 6) produced 21 i.u. bacitracin/ml and a pH of 7.3 after an additional 4 h, and about 40 i.u./ml and a final pH of 8.1 after 10 h (a total of 26 h of incubation).

Table 3. *Effect of 0.5% CaCO₃ on bacitracin production in M₂ medium with 1.0% glucose*

Time of incubation (h)	Calcium carbonate added:				
	None		0.5%		
	pH of culture fluid	Bacitracin in culture fluid (i.u./ml)	pH of culture fluid	Bacitracin in culture fluid (i.u./ml)	Bacitracin in culture fluid (i.u./ml)
12	5.72	n.d.	7.54	1.5	1.5
14	5.54	n.d.	6.97	6.8	6.8
16	6.45	0.3	6.88	15.0	15.0
17	7.47	1.3	7.30	21.9	21.9
20	7.50	6.3	7.78	27.0	27.0
24	8.42	8.0	7.89	30.8	30.8
28	8.50	10.9	8.50	27.0	27.0

Table 4. *Effect of glucose and pH on bacitracin production in M₂₀ medium*

Time of incubation (h)	Initial pH		7		6	
	Glucose added		0		0	
	Growth (<i>E</i> ₆₅₀)	pH	Growth (<i>E</i> ₆₅₀)	pH	Growth (<i>E</i> ₆₅₀)	pH
20	5.10	8.37	4.70	7.40	5.10	7.48
24	7.50	8.58	5.90	7.95	7.20	7.93
26	8.10	8.60	6.80	8.22	8.70	8.08
28	7.50	8.78	8.90	8.43	7.80	8.45
32	6.90	8.98	8.70	8.75	6.90	8.65
			Growth (<i>E</i> ₆₅₀)	Bacitracin (i.u./ml)	Growth (<i>E</i> ₆₅₀)	Bacitracin (i.u./ml)
			14.7	13.0	5.10	18.1
			17.4	25.0	7.20	32.5
			17.9	32.5	8.70	41.5
			19.0	40.5	7.80	42.0
			16.4	34.4	6.90	41.0

DISCUSSION

Bacitracin production by *Bacillus licheniformis* is greatly affected by the external pH of the bacteria. Simply by manipulating the buffer capacity of the medium the maximum titre increases from 13 i.u. bacitracin/ml in the M2 medium (Table 1) to about 40 i.u. bacitracin/ml in the M20 medium with an initial pH of 6 (Table 4). Both high and low pH values inhibit bacitracin synthesis. Our findings are in agreement with Snoke (1961) who reported a pH optimum of 8.0 for bacitracin formation by protoplasts of *B. licheniformis*.

Glucose metabolism stimulates bacitracin production, probably by reducing the pH during later stages of growth (Table 4). If too much glucose is added, a reduction of bacitracin titres is found (Table 2). Perhaps the long duration of the low pH period inhibits bacitracin production for most of the period of active growth, particularly as the postulated pH value of 8.0 for optimal production (Snoke, 1961) is not reached before growth is completed. The poor bacitracin titres obtained with too much glucose added (1 % or more) can be avoided by adding CaCO_3 which prevents the fall in pH (Table 3).

The inhibitory effect of glucose upon early bacitracin synthesis may explain the results of Bernlohr & Novelli (1960; 1963) who reported that bacitracin production occurred after growth. A main constituent of their medium was glucose, and they also noted a fall in pH during the first period of incubation.

Snoke & Cornell (1965) had already shown that the production of bacitracin by *Bacillus licheniformis* paralleled growth. However, their observation was described as 'puzzling and unusual' (Weinberg, 1967) and has not influenced the general view that peptide antibiotics are produced after growth. Their medium contained neither glucose nor other carbohydrates. According to our observations the early bacitracin production would not therefore be inhibited by unfavorable conditions.

Demain (1968) points out that many fermentations require carbon sources other than, or in addition to, glucose for optimal antibiotic production. Excellent carbon sources for antibiotic production are as a rule very slowly oxidized by the bacteria. However, slow feeding of glucose has in many cases eliminated the need for a slowly utilizable carbon source and Demain (1968) suggested that the production of many antibiotics, among them bacitracin, may be controlled by catabolite repression by glucose. We propose that this is not true for bacitracin since inhibition of its production by glucose can be prevented by neutralizing the culture fluid with CaCO_3 (Table 3). It is more likely that the rapidly usable glucose inhibits antibiotic production because of its drastic influence upon the pH of the medium.

An initial fall in pH has also been reported in the production of penicillin, cephalosporin C, mycobacillin, polymyxins and nisin (Jarvis & Johnson, 1947; Demain, 1963; Banerjee *et al.* 1967; Paulus, 1967; Hurst & Dring, 1968). It is therefore tempting to propose that the frequently observed production of peptide antibiotics after growth may be pH dependent. Several peptide antibiotics may, like bacitracin, be produced during the whole period of active growth. It would therefore be of great interest to perform experiments which could explain the function of these peptide antibiotics in the dividing organism.

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