JOURNAL OF BACTERIOLOGY
Vol. 87, No. 6, pp. 1397-1401 June, 1964
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BIOSYNTHESIS OF MYCOBACILLIN, A NEW ANTIFUNGAL PEPTIDE

I. ROLE OF NUCLEIC ACID

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Received for publication 22 January 1964

ABSTRACT

BANERJEE, ARUN B. (University of Calcutta, Calcutta, India), AND S. K. BOSE. Biosynthesis of mycobacillin, a new antifungal peptide. I. Role of nucleic acid. J. Bacteriol. 87:1397-1401. 1964.—The biosynthesis of mycobacillin, a cyclic polypeptide antifungal antibiotic, was studied in relation to the effect of chloramphenicol, 6-azathymine, and 5-bromouracil on the process. It was found that chloramphenicol inhibits both mycobacillin synthesis and growth, whereas nucleic acid base analogues inhibit only growth and nucleic acid synthesis but not mycobacillin formation. A change in the concentration of labeled aspartic acid in the general metabolic pool led to a corresponding change in the specific activity of aspartic acid isolated from different peptide fragments of the mycobacillin molecule, suggesting that mycobacillin synthesis occurs by way of linear addition of amino acid to the peptide chain.

Studies on the biosynthesis of proteins and peptides indicate some interesting differences. That ribonucleic acid (RNA) of large molecular weight is not involved in biosynthesis of peptides seems fairly well established. Mycobacillin, a new antifungal antibiotic isolated in this laboratory, is a cyclic peptide consisting of 13 amino acids of seven different residues whose sequence has been worked out (Majumdar and Bose, 1960). The molecular weight of this peptide is 1,527. The biosynthesis of a peptide of this size poses an interesting question as to the role of nucleic acid in its formation. In this paper, we report on a study of biosynthesis of mycobacillin in relation to the role of nucleic acid. Attempts were also made to determine whether simultaneous or stepwise biosynthesis is involved in this process.

MATERIALS AND METHODS

Organisms. Bacillus subtilis B_3 was used to study biosynthesis of mycobacillin, and for its assay a sensitive strain of *Aspergillus niger* G_3Br was used.

Media and fermentation experiments. The chemically defined medium for production of mycobacillin contained (per liter): glucose, 10.0 g; L(+)glutamate, 5.0 g; potassium dihydrogen phosphate, 1.0 g; magnesium sulfate, 200 mg; manganous sulfate, 10 mg; and ferrous sulfate, 10 mg; the pH was adjusted to 7.0. The medium used for the maintenance of *B. subtilis* B_3 was the same as above, except that it contained 3.0% agar. The test organism, *A. niger*, was maintained on Czapek agar. Usually the bacteria were grown at 30 C in stationary culture in a 250-ml conical flask containing 100 ml of medium adjusted to pH 7.0. As inoculum, 1 ml of a 24-hr culture of an absorbancy value of 0.3 was used.

Isolation, purification, and assay of mycobacillin. Mycobacillin was isolated and purified from the culture fluid as described by Majumdar and Bose (1958*a*). The mycobacillin content of fermented broth was assayed by cup-plate method with 5×10^4 spores of *A. niger* per plate (Majumdar and Bose, 1958*b*) and 50 μliters of Millipore-filtered broth per cup.

Partial hydrolysis of mycobacillin and fractionation of constituent peptides. Purified mycobacillin was subjected to partial hydrolysis in a sealed tube with 11.4 N HCl at 37 C for 50 hr. After total removal of HCl, the peptides were fractionated by two-dimensional paper chromatography with water-saturated phenol and *n*-butanol–acetic acid–water (4:1:1, v/v) as developing solvents (Majumdar and Bose, 1960). The locations of radioactive peptides were marked by radioautography. Amino acids were also chromatographed in two dimensions with the same solvent systems.

Estimation of deoxyribonucleic acid (DNA), RNA, and amino acids. For estimation of RNA and DNA, cells were harvested by centrifugation

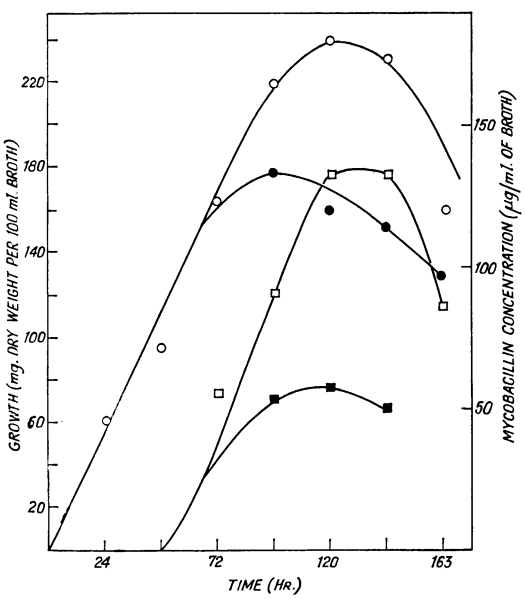

FIG. 1. *Effect of chloramphenicol on growth and mycobacillin production. Chloramphenicol (20 μg/ml) was added after 66 hr. Symbols:* ○, *growth in control;* ●, *growth in the presence of chloramphenicol;* □, *mycobacillin production in control;* ■, *mycobacillin production in the presence of chloramphenicol.*

TABLE 1. *Distribution of mycobacillin in cell pool and fermentation broth**

Cell age	Distribution (%) of mycobacillin			
	Without chloramphenicol		With chloramphenicol†	
	Cell pool	Broth	Cell pool	Broth
hr				
24	—	—	—	—
48	—	—	—	—
72	5.1	94.9	—	—
96	3.9	96.1	7.2	92.8
120	6.8	93.2	2.0	98.0
144	5.2	94.8	4.2	95.8

* Cells were grown in the presence of C^{14} aspartic acid (20 μc per liter), separated from the medium, and washed by centrifugation at 3,000 × *g* at 0 C. Mycobacillin was isolated from cells and also from broth as described in the text, with the help of carrier mycobacillin where necessary.

† Chloramphenicol was added at the 66th hr of growth (60 μg/ml).

at 3,000 × *g* for 20 min at 0 C. Nucleic acids were extracted by the method of Schneider (1945). RNA was estimated by the Orcinol method of Mejbaum (1939) and DNA by the method of Burton (1956) after hydrolysis of nucleic acids with 5% trichloroacetic acid at 95 C. Amino acids were measured by the photometric ninhydrin method of Troll and Cannan (1953).

Radioactivity measurements. Samples were uniformly plated on glass planchets, and dried in a thin layer with an infrared lamp. Radioactivity was measured in a windowless gas-flow counter with a M5 semiautomatic sample changer (model 182X scaler, Nuclear-Chicago Corp., Des Plaines, Ill.).

Chemicals and radio chemicals. 6-Azathymine and 5-bromouracil were obtained from Calbiochem. Chloramphenicol was recrystallized (mp, 150 to 151 C) from ethyl acetate. All these chemicals were dissolved in water and sterilized by Millipore filtration. Uniformly labeled C^{14}-aspartic acid was the product of Radiochemical Centre, Amersham, England.

RESULTS

Growth and mycobacillin production. Growth and mycobacillin production with time were studied in chemically defined medium (control, Fig. 1).

Distribution of mycobacillin in cell pool and fermentation broth. Distribution of radioactive mycobacillin in cell and culture fluid was followed throughout the fermentation process (Table 1). It appears from Table 1 that almost all of the mycobacillin formed is released in the broth. The ratio of concentration of mycobacillin in cell pool and that in fermentation broth is fairly constant at any stage of the growth process. It is of interest to note that cells cannot accumulate more than 7% of formed mycobacillin at any stage of growth process.

Effect of chloramphenicol on growth, mycobacillin production, and retention. The effect of chloramphenicol on mycobacillin production and distribution was studied in view of its strong inhibitory effect on protein biosynthesis (Gale and Folkes, 1953). The results (Fig. 1) indicate that both growth and mycobacillin production are inhibited by chloramphenicol. It is of interest to note that addition of chloramphenicol did not change the original pattern of distribution of mycobacillin in cell pool and fermentation broth, showing that chloramphenicol does not impair the release of

mycobacillin from the cell into the medium. The apparent inhibitory effect may be due to inhibition of cellular growth. The result is inconclusive unless the effect of chloramphenicol on the growth process is isolated from antibiotic production.

Effect of azathymine and bromouracil on growth, mycobacillin production, and nucleic acid synthesis. A study was made using the inhibitory effect of base antagonists on nucleic acid synthesis to assess the role of these acids, if there be any, on the biosynthesis of mycobacillin. The results of the experiment with 5-bromouracil are given in Fig. 2 and 3. It is interesting to note that, though this compound adversely affects growth and nucleic acid synthesis (both RNA and DNA, particularly the former), it has no effect on mycobacillin production. The results were essentially the same when 6-azathymine was used (not shown in the figures) in place of 5-bromouracil.

Simultaneous or stepwise biosynthesis of mycobacillin. Protein biosynthesis has been shown to occur by simultaneous or by stepwise methods. It is, therefore, of interest to determine which occurs in the case of mycobacillin. In experimental technique, the concentration of a given labeled amino acid in the metabolic pool was altered, and its effect on the distribution of specific activity of the same amino acid in different peptide fragments of the mycobacillin molecule so formed was determined. C^{14}-labeled aspartic acid was selected for the purpose, because five aspartic acid residues occur in the mycobacillin molecule (Majumdar and Bose, 1960). This acid was added aseptically in a dose of 50 μc per liter at the 66th hr of growth. Thereafter, at 2-hr intervals C^{12} aspartic acid (2 mg/100 ml) was added to bring about a rapid change in the specific activity of labeled aspartic acid in the medium. At 10 hr after the addition of the radioactive amino acid, an equal amount of *n*-butanol was added to fermentation broth. Mycobacillin was isolated, purified (Majumdar and Bose, 1958*a*), and then partially hydrolyzed to obtain small peptides. Four peptides which are well separated and distinctly marked on the radioautogram were taken, and the rest was discarded. These peptides were completely hydrolyzed, and the amino acids were chromatographed to obtain aspartic acid

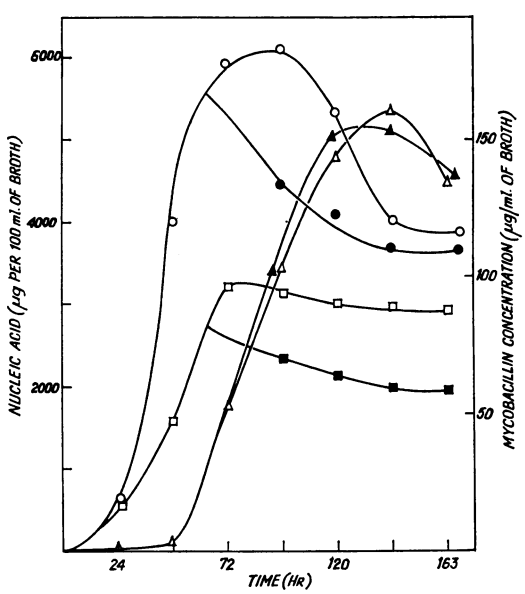

FIG. 2. *Effect of 5-bromouracil on RNA, DNA, and mycobacillin production. 5-Bromouracil (20 μg/ml) was added after 66 hr. Symbols:* ○, *RNA;* □, *DNA;* △, *mycobacillin production. Open symbols represent the control system and solid symbols represent the 5-bromouracil-treated system.*

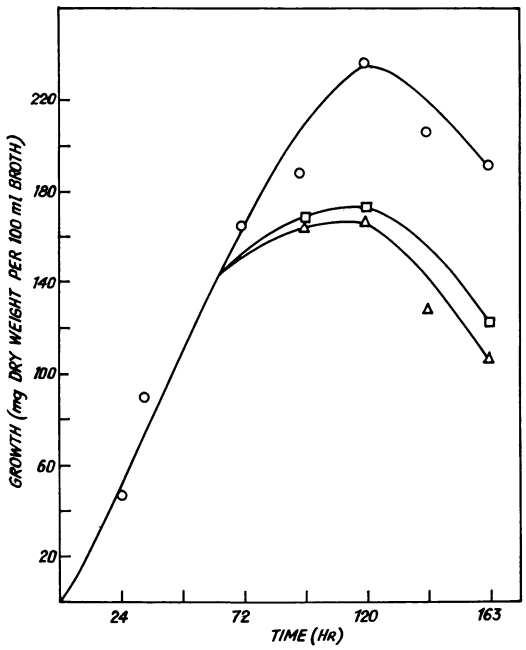

FIG. 3. *Effect of 5-bromouracil and 6-azathymine on growth of Bacillus subtilis. Symbols:* ○, *control;* □ *and* △, *experimental, in the presence of 5-bromouracil and 6-azathymine, respectively (20 μg/ml). Analogues were added at the 66th hr of growth.*

TABLE 2. *Distribution of radioactive aspartic acid in mycobacillin molecule**

Peptide no.	Aspartic acid content	Specific activity of C^{14} aspartic acid†
	μmoles	
1	0.31	2,088
2	0.24	2,817
3	0.46	1,361
4	0.29	3,172
Mycobacillin molecule	4.01	2,060

* Initial count for added aspartic acid per 100 ml of broth was 9.36×10^6 per min.

† Expressed as counts per min per μmole.

whose specific activity was determined as usual (Table 2). These results show the uneven distribution of radioactivity throughout the mycobacillin molecule. This may be taken to mean that the pathway of mycobacillin formation may involve multiple intermediary steps. Nonuniform incorporation of amino acid in α-amylase of *B. subtilis* was demonstrated in a similar way by Yoshida and Tobita (1960).

DISCUSSION

The present work was concerned with the biosynthesis of mycobacillin with special reference to the role of nucleic acid template in the process. It is now fairly well established that nucleic acid template is not involved in the biosynthesis of a small peptidelike glutathione (Snoke and Bloch, 1955), opthalmic acid (Lane and Lipmann, 1961), etc. The situation may, however, be a little different with mycobacillin whose molecular weight is 1,527.

In the first place, the action of chloramphenicol, a well-known inhibitor of protein biosynthesis, was studied. It inhibited both growth and mycobacillin production. It was not, however, possible to isolate the growth process from mycobacillin formation. Hence, the result is inconclusive, unless a cell-free system is developed to decide the issue.

Experiments relating to the effect of base antagonists on antibiotic production yielded interesting results. Base antagonists were added after 66 hr of growth. Immediately after its addition, growth and RNA and DNA synthesis stop, whereas mycobacillin production continues as usual. This may be an indication that RNA of high molecular weight is not involved as a template for the formation of the molecule. It is of interest to note that almost the same conclusion was reached by Winnick, Lis, and Winnick (1961) for gramicidin S.

In cases of protein synthesis where template is involved, the process may occur either simultaneously or in a stepwise fashion (Askonas et al., 1955; Bishop, Leahy, and Schweet, 1960; Dintzis, 1961; Steinberg, Vaughan, and Anfinsen, 1956), whereas in case of peptides in whose synthesis template is not involved the stepwise fashion may be the only alternative left. This hypothesis was tested by studying how the changes in the concentration of labeled aspartic acid (which has been repeated five times in the mycobacillin molecule) in the pool affect the distribution of specific activity of aspartic acid in different peptide fragments of mycobacillin. It has been observed that the specific activities of aspartic acid in different peptide fragments are not the same. This may be an indication that the synthesis may occur in many steps, which agrees with the stepwise hypothesis for biosynthesis. A similar pathway was reported by Strominger (1962) to be involved in the uridine peptide formation in the cell wall of gram-positive organisms.

ACKNOWLEDGMENTS

The authors wish to express their thanks to S. K. Majumdar and H. K. Das for valuable discussions. This work was aided by grants from the Council of Scientific and Industrial Research. One of us (A. B. B.) is grateful to the same authority for a scholarship.

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