Action of Protoplast-bursting Factor upon Microorganism

Part I. The Effects on the Permeability Barrier*

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Protoplast-bursting factor (P. B. factor) has a little antibacterial activity and is capable of inhibiting the growth of Bacillus megaterium.

The cell suspensions required P. B. factor and Mg++ for the oxidation of glucose-6-phosphate but did not require them for that of glucose.

Leakage of various cellular components into the surrounding menstruum occurred when the cell suspension was subjected to treatment with P. B. factor. These materials were identified as protein, deoxyribonucleic acid, ribonucleic acid, and amino acids.

Under an electron microscope, the cytoplasm of the cells treated with P. B. factor was apparently less dense than the control, which seemed to suggest that the cytoplasm had leaked out of the inside of the cell through the membrane by the treatment of P. B. factor.

INTRODUCTION

Protoplasmic membrane of bacterial cells has been assumed to be a very important site which controls the permeability of many substances, but its structures and mechanisms of permeability still have scarcely been elucidated.

In the preceding papers,1,2) we have described the discovery of protoplast-bursting factor from pig pancreas which has a strong bursting activity on the protoplasts of Bacillus megaterium. This substance was purified and crystallized with acetone-water. And various properties of the purified materials have been studied.

The fact that this substance acts on the protoplasmic membrane suggests us that it seems to affect upon the permeability of the membrane.

The present paper deals with the changes of permeability barrier in the cells of B. megaterium caused by the addition of P. B. factor.

MATERIALS AND METHODS

Preparation of protoplast-bursting factor. Puri-

fication of the protoplast-bursting factor (P. B. factor) was carried out as described in the previous paper.1) The crystalline preparation was used in all experiments.

Organism and culture medium. Bacillus megate-
erium strain KM was used in this study. Cultures were grown at 30°C with continuous aeration for 20 hr. in nutrient medium composed of polypeptide (Difco), 5.0 g; yeast extract (Difco), 3.0 g; sodium chloride, 3.0 g; glucose, 1.0 g; and 1,000 ml of distilled water. The cells were harvested and washed with cold 0.85% sodium chloride at 0°C. The packed cells were then stored at −18°C.

Cell free extract. To disrupt the cells, suspensions were prepared in 0.01 M phosphate buffer (pH 7.2) at a concentration of 15 mg of cells (dry weight) per ml.

The cell suspensions were disrupted in a Sonic
oscillator under a 10 KC for 20 min.
The colored viscous supernatant, obtained after centrifugation of the disrupted mass at 13,000 × g for 20 min., was used as the crude enzyme preparation.

Assay of substrate oxidation. Conventional manometric techniques were used to measure oxidation by whole and disrupted cells.

The final concentration of whole cells in the Warburg vessels was 1.5 mg (dry weight) per ml. The buffer concentrations in the vessels with both whole and disrupted cells were 0.04 M phosphate buffer of pH 7.2.

Characterization of materials leaked from cells. Leakage studies were performed on washed cells suspended in 0.05 M phosphate buffer (pH 7.2) containing 0.05 M MgSO4 such that an optical density (O.D.) 0.4 at 600 μm (1 cm diameter tube) was obtained at a 1:10 dilution. The O.D. was determined by a Hitachi Model EPU-2 spectrophotometer. Tubes containing 10.0 ml of the undiluted cell suspensions with or without P. B. factor were incubated at 37°C for varying periods of time. The cells were then removed by centrifugation at 27,000 × g for 10 min. at 0°C. The supernatant fluids were analyzed for protein,3) ribonucleic acid (RNA),4) deoxyribonucleic acid (DNA),5) and free amino acids.6)

Yeast RNA, DNA (salmon sperm, type III), and L-alanine (Sigma Chemical Co.) were used as the comparative standards.

Electron microscopy. Samples were fixed in an aqueous solution of 2% osmium tetroxide for 10 min., and dehydrated in aqueous ethyl alcohol (v/v) according to the following schedule: 20%, 50%, 75% ethyl alcohol for 30 min. each, 95% ethyl alcohol for 60 min., and two successive dehydrations in absolute ethyl alcohol for 60 min. Alcohol was removed with propylene oxide, and the samples were embedded in a mixture of 35% Araldite 6005 and 12% Epon 812. Sections were cut on a Porter-Blum ultramicrotome with use of glass knives, were mounted on 150 mesh copper grids coated with a carbon film, and were stained with lead hydroxide according to the procedure of Millonig.7)

All samples for electron microscopy were examined in Hitachi Model Hu-11B electron microscope.

RESULTS

Inhibition of Growth by P. B. factor

The growth inhibitory activity of P. B. factor was investigated by inoculating Bacillus megaterium to nutrient broth containing sterile (Seiz filtered) preparation of P. B. factor (10–500 μg/ml), shaking at 37°C and following changes in turbidity were determined at 60 min. intervals.

Control consisted of the similar inocula not added with P. B. factor.

As shown in Fig. 1, P. B. factor showed a little growth inhibitory activity against Bacillus megaterium at a high concentration.

Leakage of Various Components by P. B. factor

Fig. 2a through 2d show the kinetics of

The Leakage of Cellular Components from Cells of *Bacillus megaterium* in the Presence of Various Concentrations of P. B. factor.

Organisms were grown in defined medium, collected by centrifugation and washed twice with saline. The washed organisms were resuspended to equiv. 50 mg dry wt./ml. P. B. factor was added to the suspensions preheated to 37°C and kept at this temperature. Samples were taken at intervals, cooled and centrifuged. And (a) amino acids; (b) RNA; (c) DNA; (d) protein in the supernatant fluids were measured as described in the Methods.

- ●●●, Control; ×××, 50 µg/ml P. B. factor; △△△, 100 µg/ml; ○○○, 500 µg/ml

release of amino acids, DNA, RNA and protein in the cells with or without P. B. factor.

In the control suspension (without P. B. factor) the values of these four materials remained permanently very low during the whole period of the experiment. This facts illustrates that in these conditions neither autolysis nor plasmolysis of the cell is arisen.

On the other hand, if the cells were incubated with P. B. factor, a great deal of these four materials appeared in supernatant fluids after some lag period.

In addition of 50 µg/ml of P. B. factor, this leakage opened after the cells had been treated for at least 45 min. But when added 50 µg/ml of P. B. factor, this leakage appeared much earlier, after 15 min. of incubation.

The order of leaked amounts were as follows:

Protein > Amino acid > RNA > DNA

The total amount of RNA leaked by P. B. factor (500 µg/ml) was about 20% of the whole amount of RNA in the cells.

**Effect of P. B. Factor on Substrate Oxidation.**

Oxidation of various substrates by intact

**FIG. 3. Oxidation of Glucose by Intact and Disrupted Cells of *Bacillus megaterium* with and without P. B. Factor or Mg++.**

Each vessel contained 2 ml of intact or disrupted cells and 0.1 ml of water containing 20 µmoles of glucose, and 0.2 ml of 500 µM of P. B. factor or 0.01 M of MgSO₄. Control was added with none of P. B. factor and Mg++. Whole cells were present in a concentration of 1.5 mg (dry weight) of cells per ml, and disrupted cells in a concentration equivalent to 9.5 mg (dry weight) whole cells per ml. And 0.2 ml of 10% KOH was present in the center well to absorb CO₂. The gas phase was air, and the incubation temperature was 30°C.
The conditions were identical to those described for the oxidation of glucose (Fig. 3).

As shown in Fig. 3 whole cells and disrupted cells oxidize glucose and their rates are little influenced by neither P. B. factor nor Mg++. On the other hand, apparently different results were obtained for the oxidation of glucose-6-phosphate (Fig. 4).

In this figure, whole cells required both P. B. factor and Mg++ for the oxidation of glucose-6-phosphate and there was little oxidation in the absence of P. B. factor of Mg++. Whereas disrupted cells oxidize glucose-6-phosphate for themselves, and the rate was uninfluenced by the addition of P. B. factor or by Mg++.

The possibility that glucose-6-phosphate may be utilized after hydrolyzed to glucose was omitted by the following facts:

P. B. factor itself was devoid of any glucose-6-phosphatase activity. And the activity of this enzyme was not detected in the supernatant fluid of the reaction mixture in which the cells were incubated with P. B. factor.

These results suggested that glucose-6-phosphate which was known to be difficult to enter the bacterial cells did enter by the addition of P. B. factor.

As reported in a previous paper, P. B. factor required Mg++ to act on protoplasts. From this fact and these results, Mg++ seems to have a function as a cofactor of P. B. factor.

Electron Microscopic Studies

The cells of B. megaterium incubated with or without P. B. factor were studied with an electron microscopy.

Plate 1 shows the cells incubated in 0.05 M phosphate buffer (pH 7) containing 0.01 M MgSO₄ without P. B. factor at 37°C for 30 min.

The cytoplasm was tightly packed with...
dense granules, about 30\,\textmu\text{m} in size, and these granules are now generally identified with the ribonucleoprotein particles.\footnote{8}

Plate 2 shows the cells incubated in the same buffer under the same conditions with P. B. factor. In this plate, the cytoplasm of the cells was much less dense than that of Plate 1, and some of the cells apparently looked empty. And the cell wall was left uninjured or only slightly injured, indicating that the primary action of P. B. factor was exerted not on the cell wall itself but on deeper layers of the organisms. This fact suggests that P. B. factor increased the permeability of the osmotic barrier.

The data in the leakage studies above mentioned make sure this suggestion.

\textbf{DISCUSSION}

Wiley and Stokes\footnote{9,10} has shown that an alkaline pH and ammonium salts are essential for the oxidative metabolism of \textit{B. pasteurii}. These requirements are reported to be indispensable to oxidation of various substrates.

Whereas our data indicate that P. B. factor and Mg\textsuperscript{++} are required for the oxidation only of glucose-6-phosphate. In other words P. B. factor and Mg\textsuperscript{++} specifically affect on cell permeability barrier.

P. B. factor appeared to destroy the permeability barrier of sensitive organism and protoplasts. Concentrations producing these changes were similar to those inhibiting the growth of such cells, which suggests that the disruption of the permeability barrier and protoplast membrane is a primary cause of the lethal action of P. B. factor.

It was interesting that P.B. factor did not alter the turbidity of protoplast membrane fraction of sensitive bacteria. P. B. factor seems to bring about a subtle change within the permeability barrier and whilst such changes may be caused by a highly specific enzyme, there is as yet no direct evidence for this.

In the previous reports,\footnote{11} the structure of the protoplasmic membrane of \textit{B. megaterium} was described. The relationship between the membrane structure and action of P. B. factor on it will be presented in a later paper.

\footnote{11} T. Yamaguchi, G. Tamura and K. Arima, \textit{J. Bact.}, \textbf{93}, 403 (1967).
\footnote{12} T. Yamaguchi, N. Tsukakoshi, G. Tamura and K. Arima, This Journal, 475 (1967).