Involvement of Autolysis of Cytoplasmic Membranes in the Process of Autolysis of *Bacillus cereus*

By Y. KOGA AND I. KUSAKA

Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan

(Accepted for publication 8 April 1968)

SUMMARY

When exponentially growing *Bacillus cereus* organisms were suspended in buffer or buffered hypertonic sucrose solution and incubated at 37°, a rapid decrease in turbidity of the suspension was observed (autolysis). During autolysis ultraviolet-absorbing substances leaked from the bacteria and over 50 % of the total phospholipids was released. Of the amino sugars, main components of the cell walls of this organism, less than 20 % was released. Phase-contrast microscopy showed the empty rod-shaped ghosts which increased in number, while the total counts were constant during autolysis.

Mg²⁺ and Mn²⁺ inhibited the autolysis; these cations either prevented leakage of ultraviolet absorbing substances or release of phospholipids. Isolated cytoplasmic membranes autolysed when the membranes were incubated at 37° in buffer solution (pH 7·0 to 7·5). It could be assumed, therefore, that the cytoplasmic membranes of *Bacillus cereus* were lysed faster than the cell walls during autolysis and that the autolysis of the membranes was inhibited by Mg²⁺ and Mn²⁺.

INTRODUCTION

Bacterial cell-wall lytic enzymes (mureinases) are known to be associated closely with their own cell walls and work from several laboratories has suggested that bacterial autolysis was a lytic event caused by the action of these enzymes (Stolp & Starr, 1965). Autolysis is, however, a very complex event and decomposition of cellular components other than cell walls might be involved in the autolytic process. Norris (1957) showed that the lysis of *Bacillus cereus* resulted in loss of cell contents whilst at least part of the cell-wall structures remained intact. This would suggest that the cell membranes were lysed faster than the cell walls. The present paper gives some cytological and biochemical evidence for the involvement of lysis of cytoplasmic membranes in the autolysis of *B. cereus*.

METHODS

Organism. *Bacillus cereus* IAM 1656 was used throughout.

Medium. GYC medium (pH 7·0) contained the following nutrients: glucose, 10 g.; yeast extract (Difco), 5 g.; casein enzymic hydrolysate (Nutritional Biochemicals Corporation), 5 g.; 1000 ml. of water in which were dissolved: MgSO₄·7H₂O, 200 mg.; MnSO₄·4H₂O, 10 mg.; FeSO₄·7H₂O, 6 mg.; K₂HPO₄, 500 mg.; KH₂PO₄, 150 mg.; CaCl₂, 100 mg.; NaCl, 100 mg.

Culture conditions and handling of organisms. Fifty ml. of GYC medium in a 2 l. Erlenmeyer flask were inoculated with 0·25 ml. of *Bacillus cereus* culture grown in

brain heart infusion (Difco) at 37° for 15 hr on a rotary shaker. At exponential phase of growth, the bacteria were harvested by centrifugation and washed with 0.85 % (w/v) NaCl solution. Centrifugation was done (at 4°), the packed bacteria suspended in 3 ml. of tris HCl buffer (pH 7.5) and stored in an ice water bath. This suspension had to be used for autolysis experiments within about 30 min.

Measurement of autolysis. To 5 ml. of 0.05 M-tris-HCl buffer (pH 7.5) was added a small amount of the concentrated bacterial suspension to give an extinction of 0.3 at 650 m μ , autolysis being followed by the optical change in extinction at 650 m μ .

Preparation of protoplasts and cytoplasmic membranes of Bacillus cereus. For the preparation of protoplasts, the bacteria were suspended in 0.4 M-sucrose solution buffered with 0.1 M-phosphate (pH 6.8) containing 500 μ g. lysozyme/ml. and incubated at 30°. Over 99 % of the bacteria were converted to protoplasts within 60 to 70 min. Protoplasts were centrifuged down and suspended in aqueous 5 mM-MgCl₂ (Mg water). Crude membrane fraction was obtained by centrifugation of burst protoplast suspension at about 30,000 g for 15 min. The pellets were washed twice with Mg water and then dialysed against distilled water at 4° for 15 hr.

Chemical estimations. Hexosamine was determined by the method of Elson & Morgan modified by Svennerholm (1956). For the determination of lipid-phosphorus from material extractable by chloroform + methanol (2 + 1 by vol.), lyophilized samples of bacteria were digested with HClO₄ at 200° for 3 hr. Inorganic phosphorus was estimated by the method of Allen modified by Nakamura (1950).

Total bacterial counts were made by using a Petroff-Hausser counting chamber.

RESULTS

When exponentially growing *Bacillus cereus* organisms were suspended in tris HCl or phosphate buffer (pH 7.5) and incubated at 37°, a rapid decrease of the turbidity of the suspension was observed (Fig. 1). The optimum condition for the autolysis was 0.05 M-tris HCl buffer (pH 7.5) and 37°. The decrease in number of viable bacteria followed the decrease of turbidity (Fig. 1). On the other hand, total bacterial counts were constant during this time. Phase-contrast microscope observation showed that 'empty forms' appeared, in which the cell walls might be intact while the cytoplasmic contents were almost completely lost (Pl. 1, fig. 1-3). As shown in Fig. 2, the 'empty forms' increased very rapidly; almost all the bacteria were turned into the empty forms within 20 min.

Leakage of intracellular ultraviolet-absorbing substances during autolysis. During autolysis of *Bacillus cereus* in tris HCl buffer containing 0.4 M-sucrose, samples were taken at intervals, immediately chilled and filtered through Millipore Filter HA (Millipore Corporation, U.S.A.). The filtrates were measured for extinction at 260 m μ . As shown in Fig. 3 substances which absorbed at 260 m μ had leaked out of the bacteria. The decrease in turbidity of the suspension corresponded well with the leakage of the substances, which were composed mainly of partly degraded RNA and protein.

The effect of divalent cations on autolysis and leakage from the bacteria. Autolysis was markedly suppressed when Mn²⁺ was supplied to the suspension; Mg²⁺ was less effective than Mn²⁺; Ca²⁺ had no effect (Table 1). The leakage of ultraviolet-absorbing substances was markedly inhibited by Mn²⁺ or Mg²⁺ (Fig. 4).

Fig. 1

Fig. 2

Fig. 1. Changes in turbidity and viability during autolysis. ○, Turbidity; ●, viable counts.

Fig. 2. Changes in counts of total and empty forms during autolysis. ▲, Total cell counts; ●, empty form counts; ○, turbidity.

Fig. 3

Fig. 4

Fig. 3. Leakage of intracellular ultraviolet-absorbing substances. Bacteria were suspended in tris-HCl buffer (○) or tris-buffered sucrose (●) and incubated at 37°. —, Turbidity; ---, extinction at 260 mμ.

Fig. 4. Effect of divalent cations on the leakage. Bacteria were suspended in tris-buffered sucrose in the absence (○) or presence of 5 mM (▲) and 0.5 mM (■) of $MnCl_2$ or 5 mM of $MgCl_2$ (●). —, Turbidity; ---, extinction at 260 mμ.

Fig. 5. Release of lipid-phosphorus and hexosamines during autolysis. Bacteria were suspended in tris-HCl buffer only (●—●) or tris + 5 mM MnCl_2 (●---●). △—△, unhydrolysed supernatant fluid.

Table 1. *Effect of divalent cations on autolysis*

Additions	Autolysis*	% of control
None	43	100
MnCl_2 , 5 mM	19	44
MgCl_2 , 5 mM	29	68
CaCl_2 , 5 mM	40	93

$$\frac{*E_{650} (0 \text{ min.}) - E_{650} (10 \text{ min.})}{E_{650} (0 \text{ min.})} \times 100.$$

Release of phospholipids and hexosamines during autolysis. Supernatant fluid of autolysed suspensions after centrifugation at 10,000 g contained phosphorus in a form extractable with chloroform + methanol (2 + 1 by vol.; lipid-P). The release of lipid-P increased during autolysis and about 50% of the total lipid-P was released within 30 min. (Fig. 5).

From whole organisms only 60% was extractable with extraction of chloroform + methanol; however, when the bacteria were autolysed for about 60 min. or treated ultrasonically at 10 kc for 10 min., lipid-P was easily extractable with this solvent mixture. The lipid-P gave the same value when extracted from bacteria disruption ultrasonically or autolysed; total lipid-P was taken as the value from ultrasonically treated suspensions.

The direct estimation of free hexosamines released from autolysed bacteria showed less than 0.6% of total hexosamines even after autolysis for 30 min. When such extracts were hydrolysed with 2 N-HCl at 105° for 16 hr, reactivity to the Elson-Morgan reagent was markedly increased. This meant that the hexosamines were released from the bacteria in the unhydrolysed state; calculation from the reactivity before and after acid hydrolysis showed that the hexosamines released by autolysis were polymers of at least 40 hexosamine units.

Estimation of hexosamines in the autolysis supernatant fluid after acid hydrolysis showed that the hexosamines released during autolysis were about 20% of the total. Mn^{2+} or Mg^{2+} inhibited the release of lipid-P but did not affect the release of hexosamine (Fig. 5).

Evidence for the autolysis of isolated membranes. When washed membranes, isolated by bursting protoplasts and centrifugations down, were suspended in tris-HCl or phosphate buffer (pH 7.5) and incubated at 37°, a rapid decrease of turbidity at 650 m μ was observed. The decrease of turbidity was completely inhibited by 1 mM- Mn^{2+} or Mg^{2+} (Fig. 6).

Fig. 6. Autolysis of isolated membranes of *Bacillus cereus* at 20° (pH 7.5). Washed and dialysed membranes obtained by bursting protoplasts of *B. cereus* were suspended in tris-HCl buffer in the absence (○) or presence of 5 mM- $MnCl_2$ (●) or 5 mM $MgCl_2$ (▲).

DISCUSSION

The involvement of changes of membraneous structures in autolysis of bacteria was suggested by Norris (1957) who isolated a lytic principle associated with culture of *Bacillus cereus*. He found loss of cell contents when the principle was added to bacterial suspensions. The results presented here show that the cytoplasmic membranes of *B. cereus* are digested in the process of autolysis. The leakage of ultraviolet-absorbing substances in hypertonic medium and the release of phospholipids (which are known to be one of the main constituents of bacterial membranes) from the bacteria were concomitant with the decrease of turbidity of the suspension. These observations show that decrease in turbidity paralleled the rate of loss of cell contents or the rate of damage of cell membranes. The cell walls after autolysis were comparatively intact.

Stolp & Starr (1965) proposed that bacterial autolysis might be defined as a lytic event caused by the action of the cell's own mureinases. However, the results described in this paper show that bacterial autolysis is a complex event and should not be defined as proposed by Stolp & Starr.

The isolated protoplast membranes of *Bacillus cereus* were autolysed when the membranes were incubated with buffer (pH 7.5) at 37°. The protoplast lytic activity is associated with the membranes of *B. cereus* (Kusaka, Tamaki & Koga, unpublished) and phospholipids are released from the membranes when they are incubated with buffer (pH 7.0 to 7.5) at 37° (Kusaka & Koga, unpublished). These facts suggest that certain factors which digest the membranes may be associated with the membranes themselves.

The authors are grateful to Dr S. Fukui for his interest and helpful suggestions.

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EXPLANATION OF PLATE

Magnification, $\times 2500$

Fig. 1. Intact cells.

Fig. 2. A photograph of a partially lysed suspension which is a mixture of intact and completely lysed cells (autolysed for 10 min.)

Fig. 3. Completely lysed cells (autolysed for 25 min.) All photographs are taken through phase contrast microscope.