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A Bacteriolytic Principle Associated with Cultures of *Bacillus cereus*

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SUMMARY: A lytic principle associated with cultures of *Bacillus cereus* is described and some of its properties discussed. The principle is relatively non-specific, acting on several species of aerobic spore-formers. It has properties in common with similar agents reported recently and comparisons with these are made. Lysis results in the loss of cell contents whilst at least part of the cell-wall structure remains intact.

Delpy & Chamsy (1949) found that in suspensions of old cultures of *Bacillus anthracis*, incubated in the presence of 1/20,000 thiomersalate, the vegetative rods underwent lysis which was complete in 4 days, the spores retaining their viability and serological properties. It is an extension of this observation that has led to the demonstration of a bacteriolytic principle associated with cultures of *B. cereus*.

METHODS

Organisms. The strain which produces the lytic principle used in this work (*Bacillus cereus* M. 8) was originally isolated from Egyptian soil (Mahmoud, 1955). It satisfied the main diagnostic criteria of Smith, Gordon & Clark (1952) and was strongly lecithinase-positive. It has been maintained by subculture on nutrient agar at 30°. The other organisms used for testing the principle were *B. subtilis* NCTC 3610 and the following strains from the Wellcome Research Laboratories: *B. circulans* 2925, *B. sphaericus* 1652, *B. polymyxa* 2002, *B. alvei* 2198 and *B. brevis* 2934 (Knight & Proom, 1950).

Thiomersalate. The preparation used was a 1/1000 solution of sodium mercurithiosalicylate obtained from Eli Lilly and Co. Ltd., Basingstoke.

Filter membranes. Courtauld's cellulose acetate membranes were used.

Culture media. Cultures were grown on Oxoid nutrient agar in 20 oz. Roux bottles which were each inoculated with 1 ml. of an overnight broth culture and incubated at 30°. For fluid cultures Oxoid nutrient broth was inoculated with a loopful of an overnight broth culture and incubated at 30°, with gentle shaking, in a water bath.

The production of the lytic principle. A Roux bottle containing nutrient agar was inoculated with *Bacillus cereus* M 8 and incubated for 12 hr. at 30°. The growth was then washed off into 10 ml. of sterile distilled water and the organisms centrifuged down to give an actively lytic supernatant fluid which could then be treated with thiomersalate or filtered through a sterilizing membrane as required.

The production of a bacterial suspension for use as substrate in the assay of lytic activity. For assaying lytic activity it was essential to use an organism

which showed no tendency to autolyse when suspended in distilled water (with or without thiomersalate) or in a preparation of the lytic principle which had been inactivated by heat. Most of the species investigated did in fact autolyse to some extent under these conditions, but *Bacillus sphaericus* 1652, whilst fully susceptible to the lytic principle, was stable in its absence and this organism was used throughout.

Bacillus sphaericus 1652 was grown in nutrient broth for 12–16 hr., centrifuged down, washed twice in sterile distilled water and resuspended in sterile distilled water to give a concentration of approximately 4×10^8 organisms/ml. This concentration, when diluted 10 times, gave a reading of about 100 units on the EEL nephelometer with the instrument set at two-thirds maximum sensitivity.

Method of assay of lytic activity. Samples (8.5 ml.) of the preparations to be tested were placed in sterile rubber-stoppered test tubes; 1 ml. of the *Bacillus sphaericus* 1652 suspension and 0.5 ml. of a 1/1000 solution of thiomersalate were then added to give a final thiomersalate concentration of 1/20,000. Each assay was performed in duplicate, the tubes being incubated in a water bath at 37° and nephelometer readings taken at intervals.

During the lysis of initially viable organisms there was a preliminary increase in nephelometer reading. The extent of this increase tended to differ from test to test and interfered with the comparison of the influence of variables such as temperature on the rate of lysis. When the *B. sphaericus* suspension was allowed to incubate in the presence of 1/20,000 thiomersalate for 3 hr. before addition of the lytic agent, no initial increase of optical density took place and a suspension pretreated in this manner was used whenever direct comparisons were to be made. It should be emphasized that such organisms were non-viable, since when washed *B. sphaericus* 1652 was suspended in distilled water there was a rapid decrease in viable count, the rate of which was not affected by the addition of thiomersalate (Fig. 1).

Staining techniques. The Gram method was carried out in the usual way with an acetone/ethanol mixture as decolorizing agent (Kopeloff & Cohen, 1928). Robinow's tannic acid-crystal violet method (Robinow, 1945) was used to stain cell walls.

Viable counts. In the absence of thiomersalate viable counts were performed in the usual way, using quarter-strength Ringer's solution as diluent and nutrient agar as the medium. When thiomersalate was present it was inactivated by the incorporation of glutathione to 3×10^{-3} M into the medium.

RESULTS

Lytic activity and age of culture

Preliminary experiments showed that the lytic activity associated with fluid cultures of *Bacillus cereus* M 8 was of a low order compared with that of cultures grown on solid media. A number of nutrient agar cultures was set up in Roux bottles and incubated at 30°. The total growth from a bottle was harvested into 10 ml. sterile distilled water at 8, 12 and 16 hr., the organisms

centrifuged down and the supernatant fluid treated with thiomersalate and assayed for lytic activity against *B. sphaericus* 1652. Comparison of the initial rates of decrease in nephelometer reading indicated that there was little difference in the activities of the three preparations (Fig. 2). A 12 hr. incubation period was adopted for the preparation of the lytic principle.

Fig. 1

Fig. 1. The loss of viability of washed vegetative organisms from a 16 hr. broth culture of *Bacillus sphaericus* when suspended in distilled water. In the presence ○—○ and absence ●—● of 1/20,000 thiomersalate.

Fig. 2

Fig. 2. A comparison of the rates of lysis of vegetative organisms of *Bacillus sphaericus* suspended in supernatant fluids derived from cultures of *B. cereus* in the presence of thiomersalate. *B. cereus* cultures harvested at — 8 hr., ●—● 12 hr. and ○—○ 16 hr.

Since Ivanovics & Alföldi (1955) found that their agent, Megacine, was released during the lysis of the organism which produced it, the deposit from the 12 hr. culture of *Bacillus cereus* M8 in the above experiment was washed and resuspended in sterile distilled water to which thiomersalate was added. Autolysis, as judged by nephelometer reading and microscopically, occurred at 37° over a period of 24 hr. and samples of supernatant fluid taken at intervals were assayed for lytic activity against *B. sphaericus* 1652. There was no significant increase in lytic activity accompanying autolysis.

Properties of the lytic principle

Filterability. There was only slight loss of activity on passage through a membrane filter.

Optimum temperature for activity. Replicate assays were set up in water baths at different temperatures and the rates of lysis compared (Fig. 3); activity was optimal at 37°. Controls consisting of suspensions of *Bacillus sphaericus* 1652 in heat-inactivated preparations of the lytic principle, and similar suspensions in distilled water, both containing 1/20,000 thiomersalate, were set up at all the different temperatures and showed no significant change in optical density during the course of the experiment.

Dialysability. A preparation was dialysed in a cellophan bag against running water at 4°. There was no significant loss of activity at 20 hr. and the agent appeared to be non-dialysable.

Thermal stability. Samples of an active preparation were heated for 30 min. at different temperatures in water baths and their activities compared (Fig. 4). Loss of activity was almost complete following exposure to 70° for 30 min., and complete at 80° for the same length of time.

Fig. 3

Fig. 4

Fig. 3. The effect of temperature on the rate of lysis of vegetative *Bacillus sphaericus* suspended in a preparation of the *B. cereus* lytic principle + 1/20,000 thiomersalate.

Fig. 4. The thermal stability of the *Bacillus cereus* lytic principle. Vegetative *B. sphaericus* was suspended in preparations of the principle in the presence of thiomersalate. Preparation unheated $\square$ — $\square$, and heated for 30 min. at $\times$ — $\times$ 55°, $\bullet$ — $\bullet$ 63°, $\circ$ — $\circ$ 70° and $\bullet$ — $\bullet$ 80°.

Optimum pH value. The normal pH value of the preparation was pH 8.0–8.2 and adjustment of reaction to the acid side resulted in considerable precipitation of material which interfered with the use of the nephelometer so that it was not possible to survey the activity at a range of pH values.

Specificity. The agent was tested against strains of the following members of the genus *Bacillus* and lysed all of them: *B. cereus*, *B. circulans*, *B. polymyxa*, *B. subtilis*, *B. alvei*, *B. brevis*, *B. sphaericus*. There was no lysis of *Staphylococcus aureus* (Oxford strain) or a strain of *Escherichia coli*.

Lecithinase and gelatinase activities. Complex preparations derived from a strongly proteolytic organism such as *Bacillus cereus*, may be expected to possess a variety of enzymic properties, and tests for lecithinase and gelatinase activities were both strongly positive. What part, if any, such activities play in the lytic process remains to be shown.

Action on heat-killed organisms. Vegetative *Bacillus sphaericus* 1652, killed by heating at 80° for 20 min., was rapidly lysed by preparations of the principle. Lysis of such organisms was not preceded by an increase in turbidity of the suspension as was noted earlier with unheated organisms (Fig. 5).

Cytological changes during lysis. Examined by the Gram method, lysis of *Bacillus sphaericus* 1652 appeared to consist of two distinct stages. The initial decrease in turbidity was associated with a loss of Gram-positive reaction. The rods, initially uniformly Gram-positive, rapidly became granular and the

Gram-positive granules then became fewer in number and stained less intensely. At the end of this phase the organisms stained well with the counterstain. During the second phase staining became fainter until the organisms appeared as barely visible ghosts; finally only debris was seen. When such a lysed preparation was stained with tannic acid-crystal violet, however, the cell walls were seen to be intact although almost devoid of contents, an observation confirmed by electron microscopy (Pl. 2, fig. 6). The details of lysis were essentially similar with all the seven organisms studied. Control suspensions of vegetative organisms in heat-inactivated lytic principle showed none of these changes during the course of the experiment (Pl. 2, fig. 5).

Bactericidal activity. The bactericidal activity of sterile (membrane filtered) thiomersalate-free preparations of the principle was investigated by adding 1 ml. of a 16 hr. broth culture of test organism to 9 ml. of the lytic preparation and following changes in viable count over a period of 15 hr. Controls consisted of similar inocula added to heat-inactivated material. The preparations showed a marked bactericidal activity against *Bacillus cereus*, *B. subtilis* and *Staphylococcus aureus* but did not affect *Bacillus sphaericus*, *B. circulans* or *Escherichia coli*. Death did not occur in the controls, and in most cases active growth took place (Table 1).

Table 1. *The bactericidal activity of the lytic principle from Bacillus cereus M 8*

1 ml. of an overnight broth culture of each test organism was added to 9 ml. of filtered lytic principle preparation, and the changes in viable count followed during incubation at 37°.

Test organisms	Organisms suspended in solution containing lytic principle			Organisms suspended in solution containing lytic principle inactivated by heating at 80° for 20 min.		
	Time of observation (hr.)			Time of observation (hr.)		
	0	5	16	0	5	16
	Viable counts (organisms/ml.)					
<i>Bacillus cereus</i>	235×10^3	24×10^2	162×10^1	219×10^3	51×10^4	30×10^5
<i>B. subtilis</i>	42×10^4	16×10^4	89×10^3	38×10^4	42×10^4	95×10^5
<i>Staphylococcus aureus</i>	71×10^4	9×10^1	18×10^0	77×10^4	23×10^4	99×10^4
<i>B. sphaericus</i>	140×10^4	26×10^5	162×10^6	131×10^4	22×10^5	22×10^6
<i>B. circulans</i>	52×10^4	127×10^3	187×10^4	46×10^4	59×10^4	48×10^5
<i>Escherichia coli</i>	57×10^5	65×10^5	46×10^6	52×10^5	74×10^5	57×10^6

The role of thiomersalate in the lytic process. Reference to Figs. 4–6 shows that in each of the control preparations vegetative *Bacillus sphaericus* 1652 showed no tendency to lyse in the absence of active lytic principle, and it may be concluded that thiomersalate had no lytic activity of its own under the conditions described. Thiomersalate proved to be essential for the initial increase in turbidity of the suspensions of *B. sphaericus* 1652 which were undergoing lysis, and its presence greatly facilitated the action of the lytic principle (Fig. 6).

DISCUSSION

Early reports of bacteriolytic activity associated with spore-forming aerobic bacteria are numerous (Waksman, 1945). Recently attention has been redirected to this subject by workers who have paid more attention to characterizing the agents concerned. Ivanovics & Alfoldi (1955) induced rapid lysis in cultures of certain strains of *Bacillus megaterium* by ultraviolet irradiation and found that the resulting lysate was very active in promoting the lysis of all

Fig. 5

Fig. 6

Fig. 5. The action of the *Bacillus cereus* lytic principle on heated and unheated vegetative *B. sphaericus*. Unheated organisms suspended in lytic principle, ●—● and in heat-inactivated principle ●---●. Organisms heated at 80° for 20 min. and suspended in lytic principle ○—○, and in heat-inactivated principle ○---○.

Fig. 6. Vegetative *Bacillus sphaericus* suspended in lytic principle ●—● with 1/20,000 thiomersalate, and ○—○ without thiomersalate. ●---●. Organisms suspended in heat-inactivated lytic principle + thiomersalate.

strains of this organism tested. The agent was released during the lysis of the organism which produced it, was bactericidal and had properties indicative of a protein-like nature; it was largely species-specific. Greenberg & Halvorson (1955) demonstrated a lytic agent in old fluid cultures of *B. terminalis* (*B. cereus*). This agent was also largely species-specific, attacking only *B. cereus* strains, and had protein-like properties. Similar agents were demonstrated in two strains of *B. cereus* and *B. polymyxa*. Neither of these papers described the cytological changes which accompanied lysis. Boroff (1955) with the anaerobe *Clostridium botulinum* demonstrated in old cultures a lytic agent which was active against young actively growing cultures of the same organism. Lysis was followed microscopically with Gram-stained organisms; the series of changes seen closely resembled those described in the present paper. Cell-wall stains were not used, however, and the presence of cell-wall material at the end of lysis in these experiments must remain problematical. Boroff's agent was not characterized but was destroyed by boiling for 5 min.

The lytic principle described in the present paper resembles those investigated by the above workers in its main properties, which are consistent with its being protein in nature. The conditions under which it is formed and its marked non-specificity, however, serve to distinguish it from them. One cannot put forward more than tentative suggestions as to the mode of action of the agent. The nature of the lysis, the marked non-specificity and the ability to act on non-viable organisms speak against a phage mechanism, a conclusion supported by failure to demonstrate any increase in lytic activity during lysis of the substrate organism. Lysis might well be the result of the combined action of more than one constituent of the preparation, one possibly acting on the cell walls in such a way as to facilitate the entry of an actively lytic component into the cell. Concerning the role of thiomersalate, at the concentration used it causes little protein denaturation (McCulloch, 1945) and its activity in facilitating the action of the lytic principle may be attributable to interference with the normal metabolic processes of the organism, resulting in an enhanced susceptibility to the agent. Greenberg & Halvorson (1955) obtained a correlation between decrease in viable count and decrease in turbidity, attributing both effects to the action of their lytic agent. It should be borne in mind that preparations from organisms of this type may well possess additional antibiotic substances (Snell, Ijichi & Lewis, 1956) and a direct correlation between lysis and bactericidal activity need not necessarily exist. In the case of the agent here described the bactericidal action against *Staphylococcus aureus* was not associated with lysis; the two effects must be distinguished. In addition to their more general applications these observations serve to emphasize that decrease in optical density, which has often been used as an indicator of lysis, is a criterion which must be interpreted with caution since, although the contents of the cells are apparently going into solution, it does not necessarily follow that the cell walls are being disintegrated.

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EXPLANATION OF PLATES

PLATE 1

Changes in the appearance of vegetative *Bacillus sphaericus* 1652 suspended in solution containing the lytic principle and 1/20,000 thiomersalate; incubated at 37°. Light microscope; $\times 1250$ approximately.

Fig. 1. Organisms at the beginning of lysis. Gram stain.

Fig. 2. Organisms after 2 hr. of lysis. Gram stain.

Fig. 3. Debris left at the end of 16 hr. of lysis. Gram stain.

Fig. 4. Washed deposit from the preparation in fig. 3. Cell-wall stain.

PLATE 2

Electron micrographs of vegetative *Bacillus sphaericus* 1652.

Fig. 5. Organisms suspended in heat-inactivated lytic principle + 1/20,000 thiomersalate and incubated for 16 hr. $\times 4300$.

Fig. 6. Organism suspended in active lytic principle + 1/20,000 thiomersalate and incubated for 16 hr. $\times 21,500$.

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J. R. NORRIS—A LYTIC PRINCIPLE FROM *B. CEREUS*. PLATE 1

(Facing p. 8)

J. R. NORRIS—A LYTIC PRINCIPLE FROM *B. CEREUS*. PLATE 2