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Cell-Wall Lytic Enzymes at Sporulation and Spore Germination in *Bacillus* Species

BY R. E. STRANGE AND F. A. DARK

Microbiological Research Establishment, Porton, Wiltshire

SUMMARY: When washed sporulating cells of *Bacillus cereus* were incubated in buffer at 37° in the presence of toluene, a partial autolysis occurred resulting in the freeing of mature and immature spores. The autolysate contained lytic enzymes which attacked vegetative cells and cell-wall preparations, releasing hexosamine-containing peptides of characteristic constitution. The most active enzyme preparations were obtained from sporulating cells incubated for 1–2 hr. in buffer at pH 5·0–6·0. Two water-soluble lytic systems, enzyme V and enzyme S with pH optima near 4·5 and 8·0 respectively, were separated from the autolysate. Enzyme S is probably identical with the lytic system present in spores of *B. cereus* and other *Bacillus* species and further observations on this system are described. When non-sporulating cells of *B. cereus* were incubated under similar conditions no obvious lysis or sporulation occurred and no cell-wall lytic activity could be demonstrated. In growing cultures of *Bacillus cereus*, considerable amounts of hexosamine-containing peptides were released into the medium during the period between the appearance of intracellular spores and free spores. It is suggested that enzyme V may be mainly concerned with the release of free spores from sporangia and enzyme S with the lytic processes which accompany spore germination.

Spore release from sporulating cells is a process which can be observed under the microscope and it occurs by dissolution of the vegetative cell. The process might be due to the independent or combined activity of lytic systems present in the cell and the culture medium. Intracellular and extracellular lytic systems derived from *Bacillus* species have been reported by Greenberg & Halvorson (1955), Nomura & Hosoda (1956) and Norris (1957). The significance of the development of these lytic enzymes in growing cultures is not evident from the reports, although the suggestion is made that the autolytic substance described by Greenberg & Halvorson might be responsible for vegetative cell lysis at sporulation (Nomura & Hosoda, 1956). Powell & Strange (1956) found that an intracellular cell-wall lytic system developed during sporulation of *Bacillus cereus* and, in a further investigation (Strange & Dark, 1957), the partial purification and some properties of a lytic enzyme present in extracts of disintegrated resting-spores were described. It was considered that this enzyme released peptide from the spore coat during spore germination but it was not clear whether it or another enzyme dissolved the vegetative cell wall at sporulation.

In this report evidence is presented that sporulating cells of *Bacillus cereus* contain two cell-wall lytic enzymes, 'enzyme V' and 'enzyme S'. A partial separation of these enzymes is described and their properties compared. Further results are given which support the view that spore peptide (Strange &

Powell, 1954) is associated with the coat of the resting spore (Strange & Dark, 1956) and that it is released during germination or disintegration by enzymic activity of enzyme S.

METHODS

Preparation of cell-free solutions of lytic enzymes from sporulating cells and spores of Bacillus cereus

Cultures of a laboratory strain of *Bacillus cereus* were grown at 37° in 1 l. flasks, each containing 200 ml. of potato extract medium (Robinow, 1951) enriched with $\frac{1}{10}$th vol. of casein hydrolysate and yeast extract (Gladstone & Fildes, 1940), on a rotary shaker as previously described (Powell & Strange, 1956). Incubation was continued until intracellular spore formation was at an advanced stage and spore release had begun. The time of incubation varied from 17 to 19 hr. depending on the appearance of the cells in heat-fixed films stained with carbol fuchsin-nigrosin-methylene blue (Powell, 1950) which were examined at intervals. The sporulating cells were harvested by centrifugation at 0° and washed twice with 0·9 % (w/v) saline and once with distilled water. Washed cells (*c.* 0·9–1·0 g. dry weight from 1 l. medium) were suspended in distilled water (20 ml.). A measured volume of suspension was mixed with an equal volume of McIlvaine's buffer and after the addition of toluene (0·025 ml./ml. suspension) the mixture was incubated at 37°. The effect of pH value and time of incubation on the recovery of enzymic activity from autolysates is described in Results. After incubation, the suspension was centrifuged at 6000 ***g*** for 20 min. and the supernatant fluid filtered through sintered glass (porosity 5/3). The filtrate contained cell-wall lytic enzymes and was stored at −10°.

Partially purified cell-wall lytic enzyme was prepared from disintegrated resting spores of *Bacillus cereus* as described by Strange & Dark (1957).

Determination of enzymic activity. To determine the cell-wall lytic activity of enzyme preparations, a substrate of cell walls from non-sporulating cells of *Bacillus cereus* was used. The method for the preparation of cell walls was described in a previous paper and spontaneous lysis was prevented by heating for 15 min. at 100° (Strange & Dark, 1957). Buffer systems in the pH range 3·0–9·6 were: McIlvaine's (0·1 M-citric acid + 0·2 M-Na_2HPO_4), pH 3·0–8·0; 0·1 M-tris-(hydroxymethyl)aminomethane/HCl (Gomori, 1946), pH 8·0–9·0; 0·5 M-Na_2CO_3/$NaHCO_3$, pH 9·6. The effect of the lytic enzymes on the substrate was determined by estimation of the soluble combined amino-sugars released (Strange & Dark, 1957) or by turbidity measurements in a Coleman Junior spectrophotometer at 450 mμ. For turbidity measurements a suspension (2·5 ml.) containing buffer (1 ml.) enzyme solution, cobalt sulphate, (50 μg. Co^{++}) and sufficient substrate to give a $-\log T$ reading of *c.* 0·35 (*c.* 1·5 mg.) was prepared in an optical cuvette (12 × 75 mm.). A control solution containing enzyme but no substrate was prepared to determine turbidity changes caused by precipitation which occurred mostly at acid pH values and usually increased during incubation. A second control tube contained substrate without enzyme. The optical densities of the three tubes

were measured and they were incubated at 56°. Readings were taken at intervals after the tubes had been rapidly cooled to 15° and lysis was expressed as percentage decrease of turbidity, allowance being made for changes due to precipitation of the enzyme preparation. The maximum decrease in turbidity varied from 55 to 75% according to the particular preparation of cell walls. When a number of enzyme solutions were compared they were tested under identical conditions and their activities expressed in terms of the time (min.) taken to produce 50% of the maximum possible lysis.

Analytical methods. These were as described in a previous paper (Strange & Dark, 1957), but the method for the determination of $\alpha\epsilon$-diaminopimelic acid (DAP) was modified. An acid-hydrolysed sample containing 10–60 μg. DAP was chromatographed on Whatman no. 1 paper using the technique of Rhuland, Work, Denham & Hoare (1955). After reaction with ninhydrin in acetone, the olive-green spot corresponding in position to that of a standard of DAP was cut out and dropped into a test tube. Distilled water (1 ml.), glacial acetic acid (1 ml.) and acid ninhydrin solution (1 ml.; Chinard, 1952) were added and the tube heated at 100° for 5 min. (Work, 1957). After cooling, glacial acetic acid (3 ml.) was added and the yellow solution freed from paper by centrifugation. The optical density of the solution was determined at 430 mμ. against a blank prepared from a non-coloured area of the chromatogram and compared with those of solutions prepared from known amounts of DAP treated in the same way.

RESULTS

The release of soluble components from vegetative cell walls of Bacillus cereus *during sporulation*

Vegetative cell walls of *Bacillus cereus* contained 30–35% amino sugars (as glucosamine) and 3–4% DAP. During vegetative growth, small amounts of these substances were present in the culture medium, but during sporulation the amounts increased rapidly. Shaken cultures of *B. cereus* incubated for 10·5, 17·75, 18·75 and 22 hr. at 37° were centrifuged and the supernatant fluids filtered through sintered glass (porosity 5/3). Samples of each filtrate were hydrolysed with 6N-HCl and analysed for hexosamine and DAP (Table 1). After growth for 22 hr. free spores predominated and the concentration of hexosamine and DAP in the culture filtrate was four times that found in the 10 hr. filtrate from vegetative cells. When samples of the culture filtrates were dialysed, it was found that the bulk of these constituents were non-diffusible indicating that they were present in components of relatively high molecular weight. To obtain these components free from other high molecular weight substances present in the culture medium, washed sporulating cells were allowed to partially autolyse in buffer, pH 6·0, containing toluene for 20 hr. at 37°. After removal of the cells by centrifugation and filtration through sintered glass, protein was precipitated by saturation of the solution with ammonium sulphate. The protein-free filtrate was dialysed exhaustively against distilled water at 2° and freeze-dried. A white solid was obtained (97 mg. from

1·74 g. dry wt./sporulating cells) which contained 45% amino sugars (as glucosamine), 12·7% carbohydrate excluding hexosamine (as glucose) and 4·2% DAP. After acid hydrolysis followed by paper chromatography, DAP, glutamic acid, alanine, glucosamine and a substituted hexosamine (Strange, 1956) were found to predominate with small amounts of other amino acids. The composition of this material was very similar to that of the peptides released from the isolated cell walls of *Bacillus cereus* by the enzyme present in spores (Strange & Dark, 1957).

Table 1. *DAP and amino sugar contents of acid-hydrolysis culture medium—filtrates from vegetative and sporulating cells of* Bacillus cereus

Incubation period of culture (hr.)	State of cells	Dry wt. cells/ml. (mg.)	μg./ml. culture medium filtrate	
			DAP	Amino sugars (as glucosamine)
10·5	Vegetative forms	1·37	1·8	20·0
17·75	Intracellular spores	1·40	3·0	24·0
18·75	Intracellular spores	1·40	4·4	32·0
22·0	Free spores	1·30	9·0	85·0

Activity of the cell-free preparations of lytic enzymes from sporulating cells

It was established that the soluble products released during partial autolysis of washed sporulating cells of *Bacillus cereus* included hexosamine-containing peptides and cell-wall lytic enzymes. Preliminary experiments with cell-free preparations indicated that activity was very much greater at 56–58° than at 37° and that cobalt ions were stimulatory (see below). The conditions required to give maximum recovery of lytic activity were defined by investigation of the effect of pH value, time of incubation and the presence of toluene on the autolysis of sporulating cells. A series of suspensions (6 ml.) containing 20 mg. dry wt. of sporulating cells/ml. in buffer pH 5·4–7·5 with toluene were incubated in parallel with similar suspensions without toluene. Samples (1 ml.) were removed at intervals, freed from cells and tested for lytic activity by turbidity measurements at pH values of pH 4·5 and 7·0. The results expressed in terms of the time (min.) taken by 0·1 ml. autolysate to produce 50% maximum lysis (Table 2), indicated that optimum conditions for recovery of lytic enzymes were present when a suspension of sporulating cells buffered at pH 5·0–6·0 was incubated for 1–4 hr. at 37°. Lytic activity decreased during prolonged incubation but this decrease was lowest at pH values near 5·0. At acid pH values toluene had little effect, but above pH 6·0 its presence substantially increased the yield of lytic activity. Examination of films stained for spores (Powell, 1950) and cell walls (Webb, 1954) indicated that after incubation for 1 hr. at 37°, complete or substantial spore release occurred under all the conditions used and that vegetative cell walls were dissolved. Of the free spores, a variable number did not stain red in outline which is typical of resting spores but appeared as weakly staining blue rings. It is

possible that these were immature spores released from the sporangia because of the relatively high concentration of lytic enzymes present in the thick suspensions, before the spore coats had properly developed. They appeared to be as refractile as normal spores when examined in wet preparations. Germinated spores, i.e. forms permeable to stains (Powell, 1950) were most abundant in autolysates incubated in the presence of toluene and vegetative cell structure persisted at pH values of 6·9 and 7·5 in the absence of toluene. Investigation of autolysis at pH values of 3·0 and 4·5 were also made. At pH 3·0 little lysis occurred and only a small amount of lytic enzyme was recovered. Substantial autolysis occurred at pH 4·5 but the activity recovered was less than at pH 5–6.

Table 2. *Concentration of lysins in cell-free preparations from partial autolysates of sporulating cells of* Bacillus cereus

Cell-free autolysates (0·1 ml. derived from 2 mg. dry wt. sporulating cells) tested for lytic activity. Lytic test suspensions (2·5 ml.) contained McIlvaine's buffer, pH 4·5 or 7·0 (1 ml.), *B. cereus* cell walls (1·5 mg.) Co^{++} (50 μg.). Incubation at 56°.

		Time (min.) to produce 50 % max. lysis by 0·1 ml. autolysate					
		Lytic tests at pH 4·5			Lytic tests at pH 7·0		
pH during autolysis	Toluene	1*	4	20	1*	4	20
5·4	+	22·5	26·5	31·5	37·5	66·0	132
5·4	–	17·0	26·5	39·0	22·5	57·0	375
6·1	+	24·0	26·5	375	34·5	79·5	375
6·1	–	19·5	22·5	†	27·0	64·5	375
6·9	+	25·5	84·0	†	45·0	124·5	†
6·9	–	48·0	†	†	†	†	†
7·5	+	180	375	†	63·0	375	†
7·5	–	375	375	†	†	†	†

* Incubation time (hr.) of autolysate at 37°.
† No detectable activity.

The rate of cell-wall lysis in the presence of 0·025–0·1 ml. of a typical cell-free autolysate (pH 6·0, 1 hr.) as determined by turbidity measurements is shown in Fig. 1. In the presence of 0·1 ml. of the preparation (derived from 2·6 mg. dry wt. of sporulating cells), the optical density of a suspension of cell walls (1·46 mg./2·5 ml.) buffered at pH 4·6, was decreased from 0·365 to 0·154 (58 % decrease) in 1 hr. at 56°. Films prepared after incubation and stained for cell walls (Webb, 1954) showed only amorphous debris. The optical density of a control suspension incubated without enzyme remained stationary. The effect of increasing concentrations of Co^{++} (0–200 μg./ml.) on lytic activity was determined at pH values of 5·0 and 7·0. In both cases stimulation occurred which reached a maximum at a concentration of 100 μg. Co^{++}/ml. (Fig. 2). When acetate buffer (0·5M) replaced McIlvaine's buffer at pH 5·0, a similar stimulation occurred in the presence of Co^{++} which showed that citric acid was not concerned with this effect.

Some inhibition of activity occurred when the salt concentration of the system was increased. In McIlvaine's buffer (pH 4·5) containing KCl in

concentrations of 0·05, 0·1, 0·2 and 0·4M, the time taken to produce 50% maximum lysis of 1·3 mg. cell walls by 0·1 ml. autolysate was 13·5, 16·5, 20 and 24 min. respectively, compared with 13·0 min. in the absence of KCl. The effect of the added salt on the pH value of the system was not sufficient to account for the inhibition. Addition of NaCl or increasing the concentration of the buffer salts also reduced activity. Enzymic activity was completely destroyed by heating preparations at 100° for 15 min.

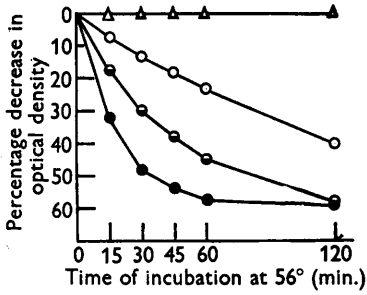

Fig. 1

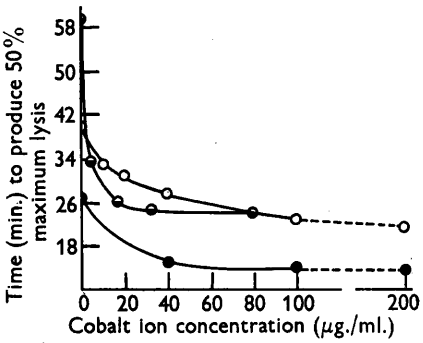

Fig. 2

Fig. 1. The rate of lysis at 56° of *Bacillus cereus* cell walls in the presence of various amounts of cell-free autolysate from *B. cereus* sporulating cells: the decrease in turbidity of a cell-wall suspension (1·46 mg./2·5 ml.) buffered at pH 4·6 and incubated with 0·025 ml. (○), 0·5 ml. (◒), 0·1 ml. (●) of the preparation; cell-wall control, △.

Fig. 2. Effect of increasing amounts of cobalt ions (0–200 μg./ml.) on the activity of lytic enzymes from *Bacillus cereus* sporulating cells: results expressed in terms of the time taken to produce 50% maximum lysis of cell walls (1·46 mg./2·5 ml.) by a cell-free autolysate (0·1 ml.) in McIlvaine's buffer, pH 5·1 (○), in acetate buffer, pH 5·0 (●), in McIlvaine's buffer, pH 7·0 (◒). Incubation at 56°.

Effect of pH *value on the activity of lytic enzymes from sporulating cells*

The effect of pH value on lytic activity was determined turbidimetrically and also by estimation of the combined hesoxamine released from the cell-wall substrate. The enzyme preparations contained combined hexosamine and this interfered with subsequent analyses; active material was freed from it by precipitation with $(NH_4)_2SO_4$ (1·0 saturation) at 0°. The precipitate was separated by filtration through sintered glass (porosity 5/3) and dissolved in 0·01M-$NaHCO_3$. When this solution was diluted to the same volume, it was as active as the original preparation but contained an insignificant amount of hexosamine. A series of suspensions (2·5 ml.) buffered in the pH range 3·0–9·6, containing substrate (3·65 mg.), enzyme (0·05 ml. from 1·3 mg. sporulating cells) and Co^{++} (50 μg.) were prepared and optical density readings taken. After incubation at 56° for 2 hr. in parallel with controls, further optical density readings were taken, pH determinations were made and the suspensions were centrifuged. A measured volume (0·2 ml.) of each supernatant fluid was dried at 100°, hydrolysed with 6N-HCl for 2 hr. at 100° and analysed for hexosamine. Decrease in turbidity and release of hexosamine

followed a similar course and were greatest at pH values near 4·5 and 8·0 (Fig. 3). It thus appeared that two distinct lytic systems were released from sporulating cells and attempts were made to separate them.

Partial separation and properties of two lytic systems from sporulating cells

Attempts to separate the two lytic systems by fractionational precipitation with $(NH_4)_2SO_4$ at 0° were not very successful. A separation based on the relative solubilities of the enzymes at pH 3·0 was more satisfactory and was

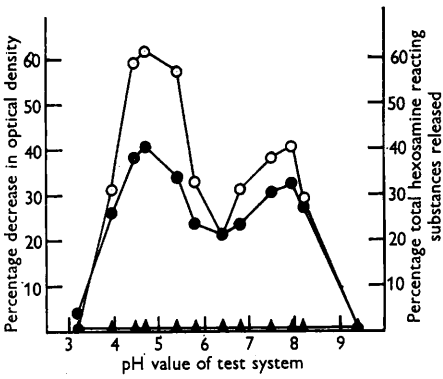

Fig. 3

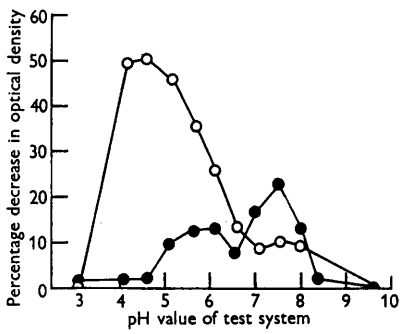

Fig. 4

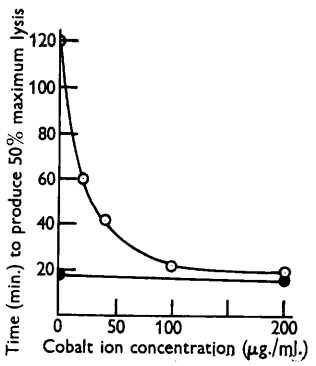

Fig. 5

Fig. 3. Effect of pH on the activity of lytic enzymes from *Bacillus cereus* sporulating cells in terms of the percentage total combined hexosamine released (○) and percentage decrease in turbidity (●) during incubation for 2 hr. at 56° with cell walls (3·65 mg./2·5 ml.), cell wall control (▲).

Fig. 4. Effect of pH value on the activity of two lytic enzymes separated from a 1 hr. autolysate (pH 6·0) of *Bacillus cereus* sporulating cells: percentage reduction in turbidity of cell-wall suspensions (1·4 mg./2·5 ml.) with 0·1 ml. (from 2·5 mg. cells) of enzyme V in 1 hr. at 56° (○); with 0·1 ml. (from 5 mg. cells) of enzyme S in 2 hr. at 56° (●).

Fig. 5. Effect of increasing amounts of cobalt ions (0–200 μg./ml.) on the activity of two lytic enzymes from *Bacillus cereus* sporulating cells: time taken to produce 50% maximum lysis of cell walls (1·5 mg./2·5 ml.) by 0·1 ml. (from 2·5 mg. cells) of enzyme V at pH 4·5 (●); by 0·1 ml. (from 5·0 mg. cells) of enzyme S at pH 6·2 (○). Incubation at 56°.

achieved as follows: cooled cell-free autolysate (1 vol.) was added to cold McIlvaine's buffer (2 vol.) pH 3·0, and the mixture allowed to stand for 30 min. at 0°. The precipitate which formed was separated by centrifugation and dissolved in 0·01 M-$NaHCO_3$ (0·5 vol.). This fraction was reprecipitated from the solution by the addition of an equal volume of buffer (pH 3·0), and redissolved in 0·01 M-$NaHCO_3$ (0·5 vol.). The supernatant fluid from the first precipitate at pH 3·0 was saturated with solid $(NH_4)_2SO_4$ and allowed to stand for 1 hr. at 0°. The precipitate which formed was separated by filtration through sintered glass, washed with 0·9 saturated $(NH_4)_2SO_4$ solution and dissolved in 0·01 M-$NaHCO_3$ (0·5 vol.). The effect of pH value on the activity of these two fractions, i.e. the pH 3·0 insoluble material and the $(NH_4)_2SO_4$ precipitated pH 3·0 soluble material, was determined turbidimetrically. It was found that maximum lysis occurred at pH values near 8·0 and 4·5 respectively (Fig. 4). The two enzymes are subsequently referred to as 'enzyme S' (pH 3·0 insoluble) and 'enzyme V' (pH 3·0 soluble). When the separation procedure was applied to a number of cell-free autolysates it was found that the relative amounts of the two enzymes varied considerably, depending on the time of incubation and pH value during autolysis. Another factor which could not be assessed but which probably affected the amounts of lytic enzyme recovered was the precise state of the sporulating cells before autolysis, e.g. the proportion of mature to immature intracellular spores. Enzyme V appeared to predominate in preparations from autolysates in buffer pH 5·0–6·0 incubated for 1–2 hr. and usually only small amounts of enzyme S were separated. When the suspensions of sporulating cells were incubated for longer periods, the ratio of enzyme S to enzyme V was greater. The two enzymes could also be distinguished by the effect of Co^{++} on their activities. Enzyme S was progressively more active in the presence of increasing amounts of Co^{++} (0–200 μg./ml.), whereas the activity of enzyme V was unaffected (Fig. 5).

Effect of lytic enzymes from sporulating cells on intact vegetative cells

Cell-free autolysates from sporulating cells attacked suspensions of washed vegetative cells of *Bacillus cereus*. The decrease in turbidity during incubation at 58°, however, was small: values of *c.* 16% decrease were obtained after 1 hr. and no further decrease occurred when incubation was continued for several hours. It appeared likely that at 58° the vegetative cell contents were coagulated and that the decrease in turbidity represented only the dissolution of cell walls. Examination of stained films of the suspensions after incubation with enzyme showed that the walls were dissolved, leaving the cell contents as Gram-negative fragments. A similar result was obtained when cell suspensions were heated at 100° for 15 min. before incubation at 56° with lytic enzyme. At 37° in the presence of thiomersalate (1/5000) the lytic enzymes had no visible effect for several hours, but after 16 hr. a 60% decrease in turbidity occurred, whereas the turbidity of a control suspension incubated for the same period without enzyme increased by 10%. Stained films of the suspensions incubated with lysins at 37° showed no evidence of residual cell

structures. The lytic enzymes had no obvious effect on suspensions of viable or heat-killed vegetative cells of *B. megaterium* at either 37 or 56°.

Investigation of non-sporulating cells and culture medium filtrate for the presence of lytic enzymes

Washed *Bacillus cereus* harvested from potato-extract medium after growth for 9·5 hr. at 37° appeared as Gram-positive rods in stained films and showed no evidence of sporulation. After incubation of a suspension in buffer (pH 6·0) containing toluene for 4 hr. at 37° the organisms showed no evidence of lysis but were now Gram-negative. Examination of films stained by Webb's (1954) method showed that the cell walls had not been dissolved. A cell-free preparation from the incubated suspension had no detectable lytic activity against *B. cereus* cell walls. The culture medium filtrate from sporulating cells of *B. cereus* after growth for 18 hr. was saturated with ammonium sulphate and the resulting precipitate was tested for lytic activity. An insignificant amount of lytic enzyme was present.

Comparison of the lysins from sporulating cells with the lysin present in spores

It was previously found that a cell-wall lytic enzyme present in extracts of disintegrated resting spores of *Bacillus cereus* and other *Bacillus* spp. showed optimum activity at pH 7–8 in the presence of cobalt ions at 58° (Strange & Dark, 1957). Re-examination of the effects of pH value and increasing amounts of Co^{++} on the activity of this lysin indicated that its properties were very similar to those of enzyme S (Figs. 6, 7). Both the spore enzyme and enzyme S were insoluble at pH 3·0 and this property had been used for the

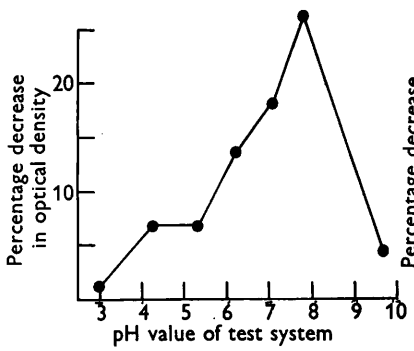

Fig. 6

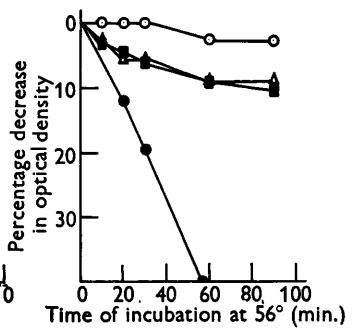

Fig. 7

Fig. 6. Effect of pH value on the activity of lytic enzyme in extracts of disintegrated resting spores of *Bacillus cereus*: percentage decrease in turbidity of cell-wall suspensions (1·3 mg./2·5 ml.) containing buffer (1 ml.) cobalt ions (20 μg.) and enzyme from 5 mg. spores, after incubation for 2 hr. at 56°.

Fig. 7. Effect of cobalt on the activity of lytic enzyme from resting spores of *Bacillus cereus*: percentage decrease in turbidity of cell-wall suspensions (1·3 mg./2·5 ml.) containing enzyme from 2·5 mg. spores at pH 4·9 without cobalt (○) and with 20 μg. Co^{++}/ml. (△); at pH 7·1 without cobalt (■) and with 20 μg. Co^{++}/ml. (●).

separation of spore enzyme from spore peptide. The possibility that some enzyme V was also present in resting spores was checked by testing the activity of material precipitated from spore extracts with $(NH_4)_2SO_4$ (1·0 saturation). The results showed that resting spores contained little or no enzyme V.

Evidence that peptide is released from spore coats by enzymic activity during mechanical disintegration of spores

In previous reports (Powell & Strange, 1956; Strange & Dark, 1957) it was suggested that the function of enzyme S in spores is to release peptide from the spore coat during germination and that the presence of soluble peptide in aqueous extracts of disintegrated spores is due to the attack of enzyme S on the spore coat during mechanical disintegration. However, Powell & Strange (1957) were unable to demonstrate a cell-wall lytic enzyme similar to enzyme S in spores of *Bacillus sphaericus*. Spore extracts did not attack vegetative cell walls of this organism or those of *B. cereus*. Spore peptide was found in extracts from *B. sphaericus* spores, while the coats contained relatively small amounts of peptide as indicated by their low content of DAP and amino

Table 3. *Hexosamine content of acid-hydrolysed spore coats of* Bacillus cereus *and* B. sphaericus *NCTC* 9602 *isolated from spores disintegrated at different* pH *values*

Source of coats	pH during disintegration	Hexosamine (g./100 g. spore coats)
Bacillus cereus spores	5·0	3·0
B. cereus spores	7·4 (water)	2·3
B. cereus spores	9·6	6·2
B. sphaericus (NCTC 9602) spores	7·4 (water)	2·3
B. sphaericus (NCTC 9602) spores	9·6	6·5

sugars. It seemed possible, though unlikely, that in *B. sphaericus* the spore peptide was not attached to the spore coat and appeared in spore extracts independently of a lytic system. This possibility can now be excluded on the following grounds: enzyme S in spores of *B. cereus* was inactive at pH 9·6 (see above). If in fact the detachment of the peptide from the spore coat during disintegration depends on the activity of enzyme S, disintegration at pH 9·6 should yield spore coat preparations of relatively high DAP and hexosamine content. This was found to be the case. Thus spore suspensions of *B. cereus* and *B. sphaericus* (NCTC 9602) in distilled water (*c.* 10 mg. dry wt./ml.) were disintegrated in a Mickle (1948) tissue disintegrator and the spore coat fractions isolated by centrifugation. After washing several times in saline (0·9% NaCl, w/v) and distilled water, the coats were freeze-dried. Similar preparations were made from spores suspended in 0·5 M-Na_2CO_3/$NaHCO_3$ (pH 9·6) and also, in the case of *B. cereus*, from a suspension in 0·5 M-sodium acetate/acetic acid (pH 5·0). During the washing of the coats from buffered homogenates, possible enzymic activity was minimized by using dilute solutions (1/10) of the appropriate buffers and distilled water as the final wash. Analyses (Table 3)

of the acid-hydrolysed coats showed that considerably more hexosamine was present in those from spores disintegrated at pH 9·6 than at pH 7·4 or pH 5·0. When coats from the pH 9·6 homogenates were suspended in buffer (pH 7·1) containing Co^{++} (10 μg./ml.) and incubated for 2 hr. at 56° a considerable release of hexosamine-containing components occurred (Table 4). However, when the coats were heated at 100° for 15 min. before incubation in buffer, a much smaller release of material occurred. Addition of the homologous spore-extract or lysin S to heat-inactivated spore coats caused release of hexosamine-containing material in similar amounts to those released spontaneously from non-heated coats (Table 4).

Table 4. *Release of combined hexosamine from spore coats of* Bacillus cereus *and* B. sphaericus *NCTC* 9602

Suspensions (0·5 ml.) contained McIlvaine's buffer (pH 7·1), 0·2 ml., cobalt, 5 μg. and spore coats. Incubation, 2 hr. at 58°. Hexosamine was determined in the supernatant fluids after hydrolysis with 6N-HCl for 2 hr. at 100°. The *Bacillus cereus* spore extract was freed from hexosamine, whereas that from *B. sphaericus* was not: this is allowed for in the results.

Source of spore coats	Wt. (mg.)	Source of extract	Substrate hexosamine (μg.)	Hexosamine released (μg.)	Percentage substrate hexosamine released
Bacillus cereus	5	—	310	108	35
B. cereus	5 (H)	—	310	12	4
B. cereus	5 (H)	6 mg. *B. cereus* spores	310	79	26
B. sphaericus	5	—	325	225	69
B. sphaericus	4·9 (H)	—	304	62	20
B. sphaericus	4·9 (H)	5 mg. *B. sphaericus* spores	304	290–56*	77

(H) = heated at 100° for 15 min.
* 56 μg. of hexosamine present in the added spore extract.

DISCUSSION

The results of the present work establish that there are two distinct lytic systems present in sporulating cells of *Bacillus cereus*. Neither of these was detected in non-sporulating vegetative cells and only small amounts were found in concentrates of the filtered culture medium from intact, i.e. non-autolysed sporulating cells. Lysis of the sporangia to allow spore release appears to be due to one or both of these intracellular lytic enzymes. One of these enzymes (S) is probably identical with that found in extracts of disintegrated *B. cereus* resting spores and might have been derived from immature spores which lysed under the experimental conditions used. The mode of attack of both enzyme S and enzyme V is readily distinguishable from that of the lytic principle associated with non-sporulating cells of a strain of *B. cereus* (Norris, 1957) which dissolved the protoplasts of vegetative cells of a number of *Bacillus* spp., leaving the cell walls. Dr J. R. Norris (private communication) has found that these residual cell walls are dissolved by a preparation of *B. cereus* enzyme S which we supplied. It is not possible to compare the activity of either enzyme S or V with that of the lytic substance from *B. cereus* var. *terminalis* described by Greenberg & Halvorson (1955), since these

authors did not define the lytic substrate. The lytic enzyme demonstrated in autolysates of *B. subtilis* vegetative cells (Nomura & Hosoda, 1956) resembled enzymes S and V in its mode of action, attacking vegetative cell-wall preparations of this organism; this system appeared in non-sporulating cultures. We have found that buffered suspensions of washed vegetative cells of a laboratory strain of *B. subtilis* showing no evidence of sporulation, autolysed on incubation at 37° or 56°, whereas suspensions of vegetative cells of *B. megaterium* and *B. cereus* were stable. Suspensions of cell walls isolated from vegetative *B. subtilis* also lysed spontaneously and this was not completely prevented by heating them at 100° for 15 min. (Strange & Dark, 1957).

The action of enzymes S and V on heat-killed vegetative cells and cell walls resembles that of lysozyme. Salton (1953) showed that the walls of heat-killed *Bacillus megaterium* were dissolved by lysozyme but the coagulated cell contents were left unchanged. However, whereas lysozyme was much more active against intact cells of *B. megaterium* and *B. subtilis* than those of *B. cereus* (Salton, 1956), enzymes S and V attacked intact cells of *B. cereus* but not those of *B. megaterium*. Enzyme S from *B. cereus* spores attacked the isolated cell walls from all three organisms (Strange & Dark, 1957).

The results of previous work with *Bacillus sphaericus* (Powell & Strange, 1957) showed that this organism differed biochemically in a number of ways from other members of the group. For example, the peptide component present in vegetative cell walls contained lysine instead of α,ϵ-diaminopimelic acid. *B. sphaericus* spore peptide did contain α,ϵ-diaminopimelic acid and resembled peptides present in vegetative cell walls and spores of other *Bacillus* spp. It has now been shown that *B. sphaericus* spores contain a lytic enzyme with similar properties to those of enzyme S. The inability of this enzyme to dissolve *B. sphaericus* vegetative cell walls might be due to the different composition of the peptide present.

The interpretation of lysis caused by substances of bacterial origin must take into account the possibility that the agent is a bacteriophage or bacteriocin. The enzymes described in the present work attacked substrates of heat-killed bacteria and their optimum temperature for lytic activity was near 56°; they appear to play a definite part in sporulation and spore germination. These properties are akin to those of an enzyme rather than a bacteriophage or bacteriocin.

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