J. gen. Microbiol. (1970), **60**, 347–355
With 2 plates
Printed in Great Britain

Structure and Composition of Resistant Layers in Bacterial Spore Coats

By G. W. GOULD, J. M. STUBBS AND W. L. KING
Unilever Research Colworth/Welwyn Laboratory, Coleworth House, Sharnbrook, Bedford

(*Accepted for publication 12 November 1969*)

SUMMARY

Treatment with mercaptoethanol caused spores of various bacteria to become sensitive to lysis by lysozyme, a spore enzyme and hydrogen peroxide; further treatment with alkali caused greater sensitization to these lytic agents. Alkali removed a protein from the outer coats of mercaptoethanol-treated spores. The protein from *Bacillus cereus*, *B. coagulans* and *Clostridium sporogenes* contained high levels of acidic and basic amino acids; that from *B. coagulans* contained a high proportion (over 7 % of its residues) of tyrosine. Electron microscopy of thin section and freeze-etch samples showed that the alkali-soluble protein contributed to a striking banding pattern containing 57 Å spaced fibres on the outer surface of *B. coagulans* spores. This layer may function as a protection against the enzymes of potential predators.

INTRODUCTION

Spore coats of various bacteria consist mainly of protein (Salton & Marshall, 1959; Warth, Ohye & Murrell, 1963*a*) which contains a disulphide-rich component (Vinter, 1961) with a structure resembling that of keratins (Kadota, Iijima & Uchida, 1965). Also there is a tyrosine-rich component, which can be extracted from isolated coats of *Bacillus megaterium* spores by alkali, and a resistant residue protein associated with a muramyl phosphate polymer (Kondo & Foster, 1967). Although, as yet, the roles of the various components have not been elucidated, they are presumably important in maintaining the survival of spores in adverse environments. We noticed earlier (Gould & Hitchins, 1963) that chemical reduction or oxidation of disulphide bonds, which were probably mostly in the disulphide-rich coat component, could sensitize spores to the enzyme lysozyme. More recently we noticed that exposure of spores to alkali, following the reduction of coat disulphide bonds, caused further changes in sensitivity to lysozyme and to an autolytic enzyme from spores of *B. cereus* (Gould & King, 1969). The observation that the alkali treatment removed one of the coat components prompted the present investigation, which deals with the chemical and cytological nature of the alkali-soluble component and its role in resistance of spores.

METHODS

Organisms and production of spores. Bacteria used included *Bacillus cereus* strains T, PX and NCTC 945, *B. subtilis* strains A and NCTC 3610 (Marburg), *B. coagulans* NCTC 3991, *B. brevis* NCTC 7577, *B. megaterium* ATCC 9885, *Clostridium sporogenes*. These

were from the National Collection of Type Cultures or the American Type Culture Collection, or were described previously (Jones & Gould, 1968).

Spores of the aerobes were grown at 30° on the surface of agar containing Oxoid potato extract (0·4 %, w/v), yeast extract (0·4 %, w/v) and glucose (0·25 %, w/v). Sporulation and lysis of sporangia occurred in 2 to 6 days, after which spores were collected and washed four times by centrifuging suspensions in cold distilled water. Spores of *Clostridium sporogenes* were grown at 37° in Reinforced Clostridial Medium (Gibbs & Hirsch, 1956), then washed as above. Spores were stored at a concentration of about 20 mg. equiv. dry wt/ml., at −20°.

Sensitization of spores to enzymes. The sensitization procedure was based on that described by Gould & King (1969). Conditions were chosen which caused sensitization of spores to lysozyme, and also to hydrogen peroxide, but were insufficiently severe to cause inactivation of spores. Spores (10 mg. equiv. dry wt/ml.) were incubated in 7 M urea (adjusted to pH 2·8 with HCl) containing β-mercaptoethanol (10 % v/v) for 1 hr at 37°, then cooled, centrifuged and washed four times with cold water. Alkali treatment (see Results) consisted of resuspending the pellets in 0·1 N-NaOH and incubating for 15 min. at 4° before washing four times in cold water.

Electron microscopy. Samples for sectioning were fixed by resuspending pellets in freshly filtered $KMnO_4$ solution (2 %, w/v) for 2 hr at 20° (Mollenhauer, 1959). The fixed organisms were then washed ten times by suspending in cold distilled water and separating by centrifugation, dehydrated in a graded ethanol series, embedded in Epon 812 and sectioned on an LKB Ultratome 3. Sections were examined in a JEM 7A electron microscope. Pellets for freeze-etching were resuspended in 20 % (v/v) glycerine immediately before freezing in liquid Freon at −150°. Addition of glycerol immediately prior to freezing was found to minimize formation of ice crystals around the organisms, and thus best preserved their shape. Replicas were prepared in a Polaron/MRC freeze-etching machine, and examined as above.

Isolation of alkali-soluble material. The alkaline extracts of mercaptoethanol-treated spores were dialysed against distilled water to remove NaOH, then dialysed against the appropriate buffer for Sephadex experiments (below) or precipitated by titration to pH 3 with HCl. Samples for amino acid analysis were freeze-dried from aqueous solutions.

Gel filtration. Alkali-soluble protein solutions (1 ml.) from *Bacillus coagulans* spores were eluted from Sephadex G200 columns (about 1 × 25 cm.) in 0·05 M-sodium phosphate (pH 8·0)+0·15 M-NaCl and in 0·05 M tris (hydroxymethylaminomethane)-HCl (pH 9·0)+0·15 M-NaCl. Eluate was monitored by ultraviolet absorption at 254 nm.

Amino acid analysis. Freeze-dried samples of alkali-soluble protein were hydrolysed by refluxing under N_2 in 6 N-HCl for 21 hr. Amino acids were determined by Moore and Stein analysis using a Technicon method based on that described by Thomas (1966). Diaminopimelic acid was determined separately using an autoanalyser method (F. J. Bailey, unpublished data) based on the procedure of El Shazly & Hungate (1966).

Heat and radiation. Aqueous suspensions of variously treated *Bacillus cereus* T spores (about 10^8 in 1 ml.) were sealed into thin walled glass ampoules which were completely immersed in a water bath at 90°. Duplicate ampoules were removed at intervals, cooled, and the survivors counted using pour plates of nutrient agar. Incubation time was 2 days at 30°. Samples of variously treated spores of *B. coagulans* (about

10^8 in 1 ml.) were sealed into glass freeze-drying ampoules and irradiated at room temperature with Co 60 γ-radiation at a dose rate of about 0·7 Mrad./hr. Survivors were enumerated as above except that the incubation temperature was 37°.

RESULTS

Sensitivity of mercaptoethanol and alkali-treated spores to enzymes and hydrogen peroxide

When spores of *Bacillus coagulans* were treated with mercaptoethanol, they became lysozyme-sensitive as expected, but when, subsequent to mercaptoethanol treatment, they were treated with alkali, their lysozyme-sensitivity increased markedly (Fig. 1).

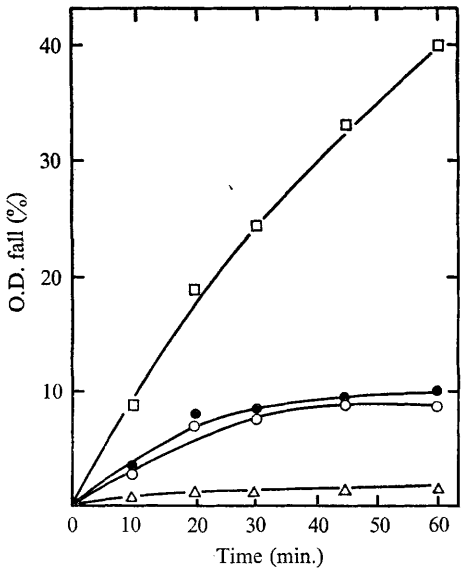


Fig. 1

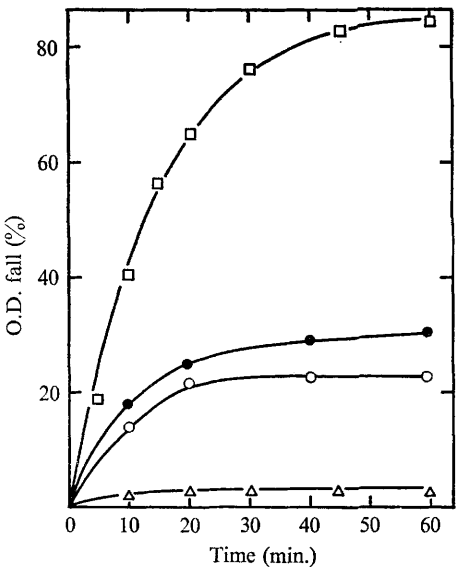


Fig. 2

Fig. 1. Lysis of spores by lysozyme. The figure shows the decrease in optical density (at 580 nm.) that occurred when variously treated spores of *Bacillus coagulans* were incubated at 37° in lysozyme (20 μg./ml. in 100 mM sodium phosphate, pH 8.0). The spores had been previously incubated with mercaptoethanol, as described in Methods, then extracted for 15 min. at 4° with: 0·1 M-sodium phosphate (pH 8·0), ○; 0·01M-NaOH (pH 12), ●; 0·1 M-NaOH, □; 0·1 M-NaOH-treated, lysozyme-free, control, △.

Fig. 2. Lysis of spores by hydrogen peroxide. Details as for Fig. 1 except that spores were incubated in 1·7 M-hydrogen peroxide in place of lysozyme.

Qualitatively similar results were obtained with spores of *B. cereus* T and *B. subtilis* Marburg. Furthermore, the alkali treatment increased the sensitivity of *B. cereus* spores to the *B. cereus* spore autolytic enzyme and also made sensitive to this enzyme the otherwise resistant mercaptoethanol-treated spores of *B. coagulans*.

In addition to causing an increase in sensitivity to enzymes, the alkali treatment also made mercaptoethanol-treated spores more sensitive to lysis by hydrogen peroxide (Fig. 2).

Resistance of treated spores to heat and γ-radiation

Spores were treated with the mercaptoethanol and alkali or left untreated as controls, then washed and subjected to heat or γ-radiation. The survivor curves obtained (Fig. 3 and 4) showed that, although reducing resistance of spores to the lytic enzymes and hydrogen peroxide, the mercaptoethanol and alkali treatments had negligible effects on resistance of spores to heat and ionizing radiation.

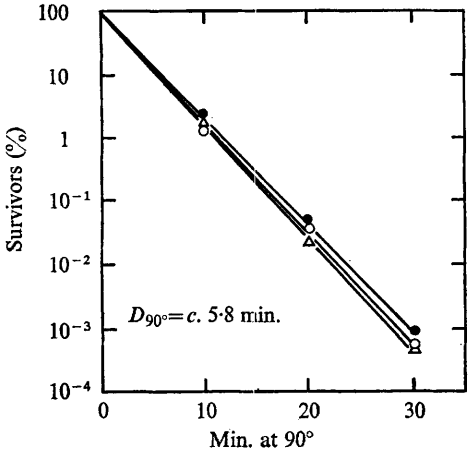


Fig. 3

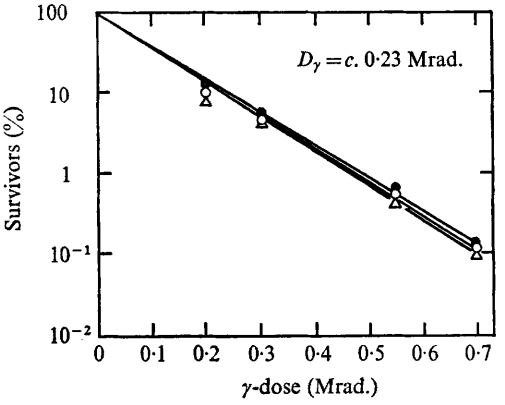


Fig. 4

Fig. 3. Heat resistance of mercaptoethanol- and alkali-treated spores. Variously treated spores of *Bacillus cereus* T were heated at 90°, and survivors enumerated as described in Methods. The figure shows the survivor curves obtained when spores were: untreated, △; treated with mercaptoethanol, ●; treated with mercaptoethanol and alkali, ○.

Fig. 4. γ-Radiation resistance of mercaptoethanol- and alkali-treated spores. Symbols as in Fig. 3.

Alkali-soluble spore protein

The alkali treatment removed some component(s) from mercaptoethanol-treated spores. Acidification of the alkaline extracts from spores of various species caused precipitation as shown in Fig. 5. The pH versus precipitation curves were similar for the eight Bacillus species tested, but precipitation from alkali extracts of mercaptoethanol-treated spores of *Clostridium sporogenes* occurred at lower pH values (Fig. 5 and Table 1). The precipitable material was dialysed to remove low molecular weight solutes, dried at 105° and weighed. It represented between about 1 and 5 % of the dry matter of the different spores.

The dialysed material eluted in about the void volume from a Sephadex G200 column, suggesting it was high molecular weight or aggregated material. The elution peak was symmetrical at pH 9·0 but tailed at pH 8·0, suggesting heterogeneity.

Composition of alkali-soluble protein

Moore and Stein analysis of hydrolysed samples suggested that the alkali-soluble material was predominantly protein with an unusual amino acid constitution (Table 2). In particular, the levels of acidic amino acids (aspartic and glutamic) and basic amino acids (lysine and arginine) were high. The low pH for precipitation of alkali-soluble

protein from *Clostridium sporogenes* (Table 1) may be reflected in its high content of acidic amino acids (Table 2). The level of tyrosine, accounting for over 7 % of the residues in the material from *Bacillus coagulans* spores, was high for proteins, approaching the high level of 11 % previously found in an alkali-soluble protein component isolated from spore coats of *B. megaterium* (Kondo & Foster, 1967). The samples contained no diaminopimelic acid (i.e. less than 0·2 %, w/w) and were therefore probably free of peptidoglycan components.

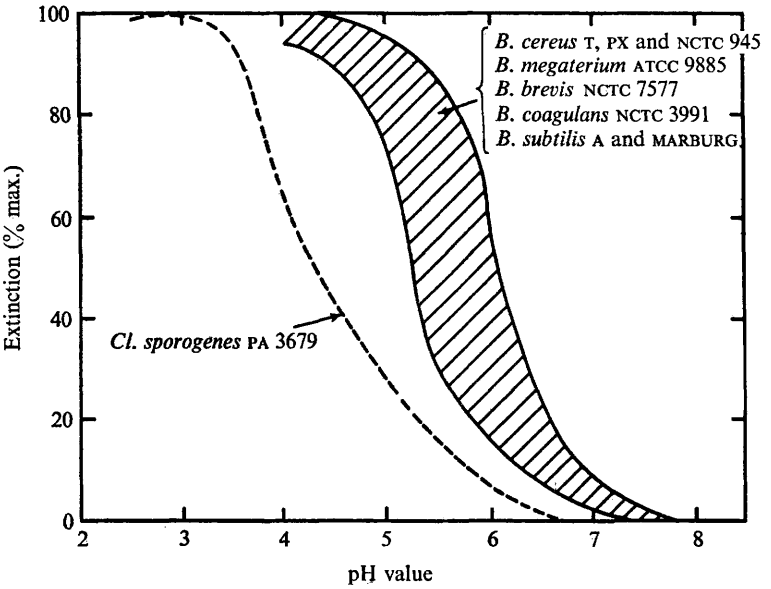


Fig. 5. Precipitation of alkali-soluble spore proteins. Preparations of alkali soluble proteins, in dilute NaOH at about pH 8, were titrated with HCl. The hatched area encompasses the precipitation curves of alkali-soluble protein from *Bacillus cereus* (three strains) and *B. subtilis* (two strains; see Methods), *B. megaterium*, *B. brevis* and *B. coagulans*. The dashed line is the precipitation curve for alkali-soluble protein from *Clostridium sporogenes*.

Table 1. *Yield and properties of alkali-soluble protein from different spores*

Organism	Yield of alkali-soluble protein (μg./mg. dry wt of spores)	pH value for precipitation	
		50 % precipitation	Maximal precipitation
Bacillus cereus T	13	5·4	4·5
B. cereus PX	21·5	5·9	4·5
B. cereus NCTC 945	15	6·1	4·6
B. subtilis A	17·5	6·1	4·4
B. subtilis MARBURG	54	6·1	4·2
B. brevis NCTC 7577	12	5·8	4·6
B. coagulans NCTC 3991	13	5·6	4·4
B. megaterium ATCC 9885	12·5	5·5	4·2
Clostridium sporogenes	47·5	4·3	2·8

Table 2. *Amino acid composition of alkali-soluble protein from spores of different species*

Amino acid	Residues amino acid per 100 residues		
	Bacillus cereus T	*B. coagulans*	*Clostridium sporogenes*
Half cystine	0·3	0	0
Aspartic acid	11·6	8·2	10·0
Threonine	7·7	6·3	7·1
Serine	3·7	5·6	5·3
Glutamic acid	12·7	12·6	11·1
Glycine	9·7	8·1	10·2
Alanine	9·3	6·1	8·7
Valine	7·0	7·1	8·1
Methionine	1·5	1·5	1·8
Isoleucine	5·5	5·3	4·5
Leucine	8·0	7·2	9·3
Tyrosine	3·5	7·1	4·0
Phenyl alanine	4·2	4·3	5·0
Lysine	6·3	8·8	7·6
Histidine	2·6	5·3	3·6
Arginine	5·4	4·2	3·7
Ornithine	0·9	2·1	0
Overall yield based on weight of sample	67·5%	60·8%	78%

Electron microscopy of treated spores

It seemed likely that the observed increase in sensitivity of mercaptoethanol-treated spores to enzymes and to hydrogen peroxide, following treatment with alkali, resulted from solubilization of the alkali-soluble protein from the spore. In order to detect the location of the alkali-soluble protein in spores, the variously treated samples were examined by electron microscopy.

Examination of freeze etched preparations suggested that the alkali treatment removed a surface layer from spores of *Bacillus coagulans*. The surface layer in untreated spores had a well defined 'bandage' pattern (Pl. 1, fig. 1) which contained a regular substructure consisting of parallel fibrils at about 57 Å spacing (Pl. 1, fig. 2). Treatment of spores with mercaptoethanol in urea at pH 2·8, as described in Methods, hardly influenced this pattern (Pl. 1, fig. 3) but subsequent treatment with alkali caused the pattern to disappear (Pl. 1, fig. 4).

Loss of material from the surface of alkali-treated spores was less apparent in thin-sectioned than in freeze-etched preparations. Nevertheless, some loss of electron density in the outer coat region could be seen (compare Pl. 2, fig. 5, 6).

The alkali-soluble protein was dialysed, negatively stained with phosphotungstic acid and examined by electron microscopy. Plate 2, fig. 7, shows the appearance of the protein sedimenting onto the grid from a solution at pH 8·0, when small particles and also fibres could be seen. When the pH value was lowered to 6·0, the fibres coalesced and precipitated in long strands (Pl. 2, fig. 8).

DISCUSSION

The resistance of bacterial spores to heat, radiation and chemical agents has been intensively studied in the past. In contrast, the resistance of spores to enzymes has

received little attention, even though resistance to enzymes must be an important attribute contributing to the survival of spores in natural environments.

The results in this paper suggest that at least two reasonably well-defined spore components are important in determining their resistance to enzymes. Both are thought to be located in the outer coat regions of spores. The first component is the disulphide-rich protein, originally described by Vinter (1961), and shown by Kadota *et al.* (1965) to have a physical structure resembling keratins in spores of *Bacillus subtilis*. The evidence suggesting involvement of this protein in resistance of spores to enzymes is based on the observations that reagents which break disulphide bonds, by oxidation or reduction, alter the coat sufficiently to allow lysozyme or lysozyme-like enzymes to pass through the altered coat and reach their peptidoglycan substrates, which probably constitute the underlying cortex layer (Warth, Ohye & Murrell, 1963*b*). The second component is the alkali-soluble protein and the evidence for its implication in resistance of spores to enzymes is presented in this paper. Alkali treatment of spores, which had been treated with mercaptoethanol, increased their sensitivity to enzymes and to hydrogen peroxide at the same time as the protein was extracted from the spores. The weight of protein certainly accounted for the bulk of the material extracted by alkali. The alkali may have caused further (undetected) changes affecting enzyme sensitivity, but the important spore properties of resistance to heat and to γ-radiation were unaffected by the various treatments used. The relationship of lysozyme resistance to hydrogen peroxide resistance is not clear. However, hydrogen peroxide is known to primarily attack spore coats by metal-catalysed reactions involving free radicals (King & Gould, 1969).

The striking patterns on the outer surface of *Bacillus coagulans* spores resemble most closely the patterns described on some Bacillus spores by Leadbetter & Holt (1969), on spores of *B. subtilis* and *B. licheniformis* by Holt & Leadbetter (1969) and on *Aspergillus conidia* by Hess & Stocks (1969). The same patterns were not visible on freeze-etch preparations of sporulating *B. cereus* (Remsen, 1966) or *Clostridium perfringens* (Hoeniger, Stuart & Holt, 1968), but Holt & Leadbetter (1969) described a rope-like substructure overlaying a pitted layer in *B. cereus* spore coats, and showed that in other Bacillus spores various similar layers could be recognized. The patterns on spores of *B. coagulans* resemble closely those on vacuoles of *Halobacterium halobium* cells (Stoeckenius & Kunau, 1968). The regularity of the substructure suggests crystallinity. This is also suggested by examination of the areas where 'bandage' patterns meet (see Pl. 1, fig. 2). The 'bandages' do not appear to overlap each other at these points, but rather to meet at an angle, as in a crystal dislocation line.

The patterned alkali-soluble layer is only readily removed from spores following mechanical rupture (Kondo & Foster, 1967) or treatment with a reagent like mercapto-ethanol, which will rupture disulphide bonds. Presumably the disulphide-rich layer holds the alkali-soluble layer onto the spore in some manner; for instance, by physical admixture, or through disulphide bridge from the one component to the other. The latter possibility seems less likely, because the cyste(i)ne contents of the alkali-soluble components were low. Preparations of the alkali-soluble material *in vitro* tended to form fibrils in some respects resembling the fibrils on spores. Presumably the amino acid composition of the material resulted in a tertiary structure which favoured fibril formation. It would be interesting to find whether fibrils were formed and spontaneously coalesced onto the coats of spores during the process of sporulation.

The amino acid composition of alkali-soluble proteins from different spores differed in detail and yet certain similarities were evident. For instance, these samples, and the alkali-soluble protein isolated from *Bacillus megaterium* spore coats by Kondo & Foster (1967), characteristically contained very high levels of acidic and basic amino acids. Unlike that from *B. megaterium* and *B. coagulans*, the alkali-soluble materials from *B. cereus* and *Clostridium sporogenes* were not exceptionally rich in tyrosine. Similarities in chemical composition of the fractions from different spores would suggest a relationship of structure to function of the alkali-soluble proteins, for example, resistance to enzymic degradation or some physical property like hydrophobicity which may be an important property of the outer layers of spores. In this respect, however, it is worth bearing in mind that although the alkali-soluble protein appears to constitute the outer layer of *B. coagulans* spore coats, Kawasaki, Nishihara and Kondo (1969) interpreted their electron microscope study of sectioned spore coats of *B. megaterium* differently. They suggested that the alkali-soluble component was mixed with the keratin-like component in the inner coats, and that the outer coat consisted of a resistant residue containing a phosphomuramic acid polymer. Aronson & Fitz-James (1968) showed that extraction of coats of spores of *B. cereus* T with alkaline thioglycollate resulted in rapid loss of detail from the dense layers and partial removal of the inner coat layers as seen by electron microscopy of thin sections. The extraction procedure caused loss of a dotted fine structure visible on the outer coat surface in negative-stained preparations. In the present study, the freeze-etch technique greatly aided location of the alkali-soluble layer, and in fact revealed details not apparent in thin sections.

There is ample evidence that the keratin-like and alkali-soluble components of spore coats protect spores from lysis by lysozyme-like enzymes: one can speculate that the specialized structures containing these components have evolved as protective layers which guard the spore protoplast against the hydrolytic enzymes of predators.

We would like to thank Mr F. J. Bailey for the amino acid analyses and Mr F. J. Judge for technical assistance.

REFERENCES

ARONSON, A. I. & FITZ-JAMES, P. C. (1968). Biosynthesis of bacterial spore coats. *Journal of Molecular Biology* **33**, 199.

EL SHAZLY, K. & HUNGATE, R. E. (1966). Method for measuring diaminopimelic acid in total rumen contents and its application to the estimation of bacterial growth. *Applied Microbiology* **14**, 27.

GIBBS, B. M. & HIRSCH, A. (1956). Spore formation by Clostridium species in an artificial medium. *Journal of Applied Bacteriology* **19**, 129.

GOULD, G. W. & HITCHINS, A. D. (1963). Sensitization of bacterial spores to lysozyme and hydrogen peroxide with reagents which rupture disulphide bonds. *Journal of General Microbiology* **33**, 413.

GOULD, G. W. & KING, W. L. (1969). Action and properties of spore germination enzymes. In *Spores IV*. Ed. by L. L. Campbell, p. 276. Washington D.C.: American Society of Microbiology.

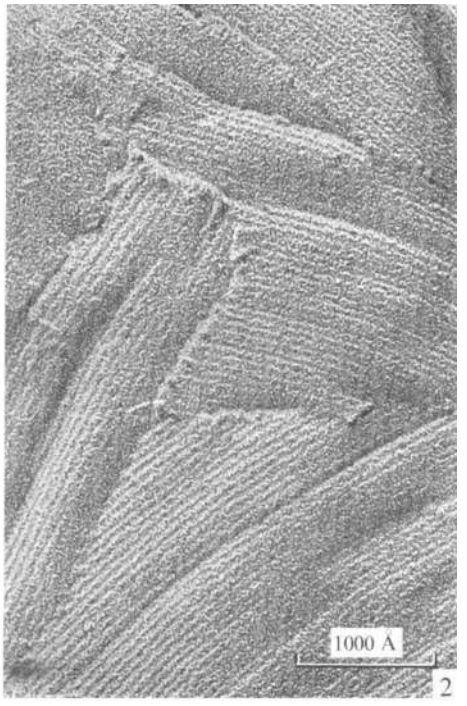
HESS, W. M. & STOCKS, D. L. (1969). Surface characteristics of *Aspergillus conidia*. *Mycologia* **61**, 560.

HOENIGER, J. F. M., STUART, P. F. & HOLT, S. C. (1968). Cytology of spore formation in *Clostridium perfringens*. *Journal of Bacteriology* **96**, 1818.

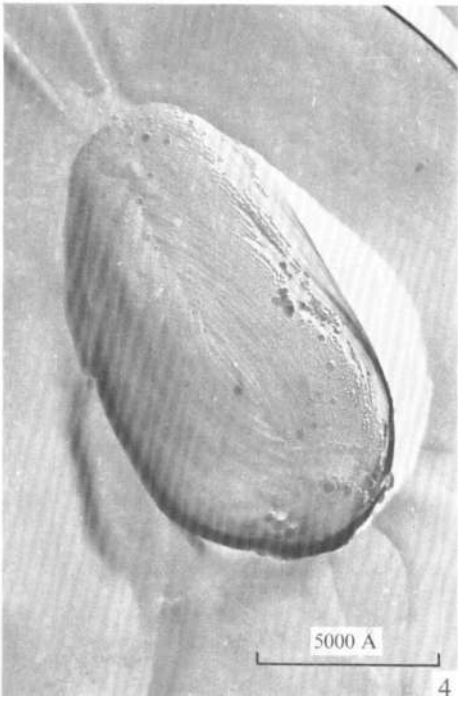
HOLT, S. C. & LEADBETTER, E. R. (1969). Comparative ultrastructure of selected aerobic spore-forming bacteria: a freeze-etching study. *Bacteriological Reviews* **33**, 356.

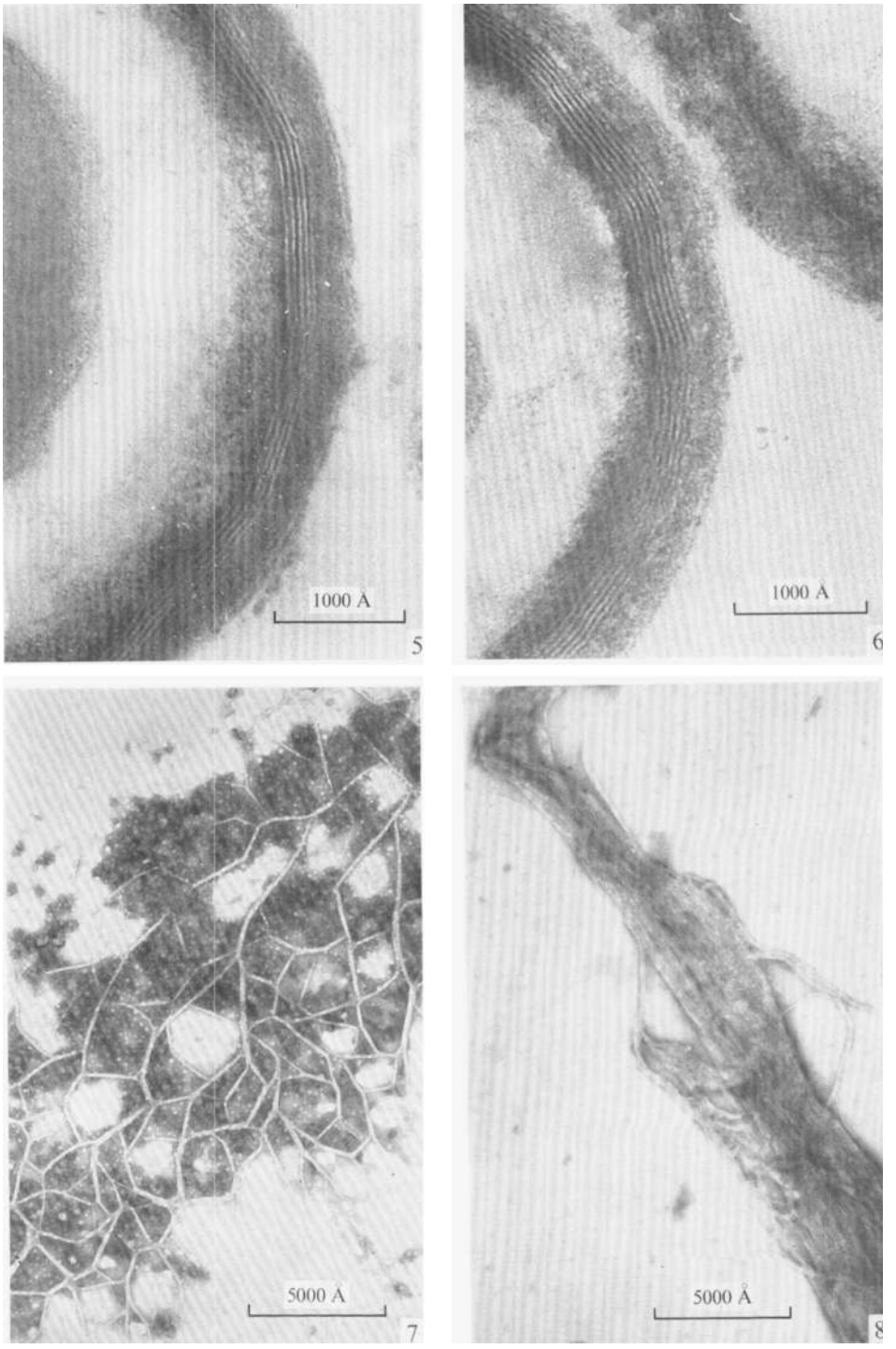
JONES, A. & GOULD, G. W. (1968). Stimulation of germination of bacterial spores by analogues of D-alanine. *Journal of General Microbiology* **53**, 383.


5000 Å
1


1000 Å
2


5000 Å
3


5000 Å
4



G. W. GOULD, J. M. STUBBS AND W. L. KING

KADOTA, H., IIJIMA, K. & UCHIDA, A. (1965). The presence of keratin-like substance in spore coat of *Bacillus subtilis*. *Agricultural and Biological Chemistry* **29**, 870.

KAWASAKI, C., NISHIHARA, T. & KONDO, M. (1969). Ultrastructure and its relation to the fractions isolated from spore coat of *Bacillus megaterium*. *Journal of Bacteriology* **97**, 944.

KING, W. L. & GOULD, G. W. (1969). Lysis of bacterial spores with hydrogen peroxide. *Journal of Applied Bacteriology* **32**, 481.

KONDO, M. & FOSTER, J. W. (1967). Chemical and electron microscope studies on fractions prepared from coats of Bacillus spores. *Journal of General Microbiology* **47**, 257.

LEADBETTER, E. R. & HOLT, S. C. (1969). Electron microscopy of frozen-etched *Bacillus* species. In *Spores IV*. Ed. by L. L. Campbell, p. 39. Washington, D.C.: American Society of Microbiology.

MOLLENHAUER, H. M. (1959). Permanganate fixation of plant cells. *Journal of Biophysical and Biochemical Cytology* **6**, 431.

REMSEN, C. C. (1966). The fine structure of frozen etched *Bacillus cereus* spores. *Archiv für Mikrobiologie* **54**, 266.

SALTON, M. R. J. & MARSHALL, B. (1959). The composition of the spore wall and the wall of vegetative cells of *Bacillus subtilis*. *Journal of General Microbiology* **21**, 415.

STOECKENIUS, W. & KUNAU, W. H. (1968). Further characterization of particulate fractions from lysed cell envelopes of *Halobacterium halobium* and isolation of gas vacuole membranes. *Journal of Cell Biology* **38**, 337.

THOMAS, D. C. (1966). *Techniques in Amino Acid Analysis*. Monograph No. 1, p. 116. Technicon Instruments Co. Ltd.

VINTER, V. (1961). The formation of cystine-rich structure in sporulating cells and its possible role in the resistance of spores. In *Spores II*. Ed. by H. O. Halvorson, p. 127. Minneapolis, Minn.: Burgess Publishing Co.

WARTH, A. D., OHYE, D. F. & MURRELL, W. G. (1963*a*). The composition and structure of bacterial spores. *Journal of Cell Biology* **16**, 579.

WARTH, A. D., OHYE, D. F. & MURRELL, W. G. (1963*b*). Location and composition of spore mucopeptide in Bacillus species. *Journal of Cell Biology* **16**, 593.

EXPLANATION OF PLATES

PLATE 1

Electron micrographs of freeze-etched preparations

Fig. 1. Outer surface of a spore of *Bacillus coagulans* showing typical bandage pattern.

Fig. 2. Enlargement of a portion of the surface of *B. coagulans* showing parallel fibrillar structure within the 'bandage' pattern.

Fig. 3. Spore of *B. coagulans* treated with mercaptoethanol, showing little loss of surface detail.

Fig. 4. Spore of *B. coagulans* treated with mercaptoethanol and then with alkali, which strips off the outer 'bandage' coat layer.

PLATE 2

Electron micrographs of thin sections

Fig. 5. Multilayered spore coat of *B. coagulans* in spores treated with mercaptoethanol. (The picture is identical with that seen in untreated controls.)

Fig. 6. Multilayered spore coats in *B. coagulans* spores treated with mercaptoethanol and alkali. The main result of alkali treatment is loss of electron density in the outer coat region (compare Pl. 2, fig. 5).

Electron micrographs of phosphotungstic acid negatively stained preparations

Fig. 7. Alkali-soluble protein at pH 8·0 sedimented onto grid as a network of fibrils and particles.

Fig. 8. Alkali-soluble protein precipitated at pH 6·0 as irregular bundles of fibrils.