LOCATION AND COMPOSITION OF SPORE MUCOPEPTIDE IN *BACILLUS* SPECIES

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ABSTRACT

Spore integuments of *Bacillus coagulans* were prepared containing nearly all the hexosamine and α,ϵ-diaminopimelic acid (DAP) present in intact spores. Subsequent autolytic action resulted in the destruction and removal of the residual cortical structure and "cortical membrane" leaving the appearance of the inner and outer spore coats unchanged in electron micrographs. Concurrently, all the hexosamine and DAP in the preparation was released mainly as non-diffusible mucopeptide containing alanine, glutamic acid, DAP, and all the glucosamine and muramic acid. Some diffusible peptides containing alanine, glutamic acid, and DAP were also present but there was little protein or carbohydrate. Lysozyme digestion of integument preparations from heated spores of *Bacillus* 636, *B. subtilis*, *B. coagulans*, and *B. stearothermophilus* specifically removed the residual cortex and cortical membrane with the release of the mucopeptide. In *B. cereus* T, only the residual cortex and part of the mucopeptide were solubilized by lysozyme. The effect of several reagents and enzymes upon the appearance and removal of hexosamine from *B. coagulans* spore integuments is reported. The results show that spore mucopeptide is mainly located in the residual cortex and cortical membrane and suggest that these structures consist essentially of mucopeptide. The implications of these results in relation to the "contractile cortex" theory of heat resistance in spores are discussed.

INTRODUCTION

In the preceding paper (36) on the composition of spore fractions of several *Bacillus* species it was shown that, in the integument fractions, the presence of increased amounts of cortical material was associated with a greater content of the mucopeptide constituents, α,ϵ-diaminopimelic acid (DAP) and hexosamine. It was suggested that mucopeptide was located in the spore cortex. Since spores of the more heat-resistant species were found to have a greater content of hexosamine and DAP (35), the mucopeptide component may have an important function in relation to the heat-protective mechanism of spores. The cortex constitutes a major part of the spore volume, and, although it has been implicated in several important theories on the mechanism of heat resistance (16, 26), little is known about its composition or properties. It was considered important, therefore, to confirm the morphological location of the spore mucopeptide and to determine more precisely the composition of the residual cortical structure.

The mucopeptide component in spores has been studied previously only as the "spore peptide" which was isolated from germination exudates (24) or spore extracts (33), or slowly released from a spore coat preparation (32). The spore peptide, like bacterial cell wall mucopep-

tides (29), contains alanine, glutamic acid, DAP, glucosamine, and muramic acid (33). In some species, most of the mucopeptide in spores remains in the insoluble fraction (integuments) of disrupted spores (32, 36), from which it is slowly released.

Preliminary studies of the conditions affecting the rate of solubilization of hexosamine from the insoluble fraction of disrupted *B. coagulans* spores enabled the preparation of washed *B. coagulans* spore integuments, retaining nearly all the spore hexosamine and DAP, which could then be solubilized during a suitable incubation. The changes in the morphological composition of *B. coagulans* integument preparations which accompanied removal of the mucopeptide component, either by autolysis or by lysozyme, were studied by electron microscopy of thin sections. The results with this species confirm our previous suggestion (36) that the mucopeptide is located in the cortical structure and cortical membrane. The composition of the soluble products from the breakdown of the cortical structure and cortical membrane in *B. coagulans* has been investigated and the effect of some enzymes and reagents upon the mucopeptide components reported. Studies of the effect of lysozyme upon spore integuments of four other *Bacillus* species, heated to inactivate the spore lytic systems, confirm the findings with *B. coagulans*.

MATERIALS AND METHODS

Organisms

Spore crops of *Bacillus coagulans* strain 320, *B. stearothermophilus* ATCC 7953 (NCA strain 1518), and *B. subtilis* were grown as described previously (36). Spores of *B. cereus* strain T were grown at 30°C on G medium (30) and spores of *Bacillus* strain 636 (an unidentified strain isolated from canned cabbage) were grown at 50°C in nutrient broth.

Preparation of Spore Integuments

B. coagulans spores were disrupted as described previously (36) in 1 per cent EDTA (Na) solution, pH 8.5, for 12 minutes at 1°C. After removal of the glass beads, the disrupted spore suspension was centrifuged at 12,000 *g* for 15 minutes. The sediment was washed by resuspension and centrifugation with 0.05 M tris buffer, pH 8.4 (twice), 0.1 M citrate buffer, pH 5.0 (twice), and water (twice). The integuments were kept between 0° and 4°C for the entire procedure, which was completed within 4 hours. This disruption and washing procedure, designed to minimize autolytic solubilization of the spore mucopeptide, also resulted in a partial separation of the inner and outer coats, which was not observed previously in integuments prepared in water (36). The rate of solubilization of hexosamine-containing material from *B. coagulans* spore integuments was stimulated by the presence of divalent cations such as Ca^{2+} (0.001 M) and sulfhydryl compounds (cysteine) and was at a maximum at pH 6.1. During disruption of the spores in EDTA and washing at pH 8.5 the rate of autolysis was reduced by over 90 per cent. *B. coagulans* integument preparations after the complete procedure retained essentially all the glucosamine and DAP from the intact spores (Table I).

TABLE I

Retention of Material Containing Glucosamine and α,ε-Diaminopimelic Acid during Disintegration and Washing of Bacillus coagulans Spore Integuments

(Content as per cent dry weight intact spores)

	Glucosamime	Diamino-pimelic acid
B. coagulans spores	4.4	2.1
Sediment, after disintegration in EDTA solution, pH 8.5	4.2	1.9
Sediment after washing*	4.0	1.9

* Sediment washed at 0–4°C, twice at pH 8.4, twice at pH 5.0, and twice in water.

Spores of *B. subtilis*, *B. stearothermophilus*, *B. cereus* T, and 636 were heated for ½ hour at 120°C before disruption to inactivate the lytic system in the spores. The disrupted spore suspensions, freed from beads, were centrifuged at 10,000 *g* for 15 minutes and the sediment was washed twice by resuspension and centrifugation in water. Heat-coagulated cytoplasmic material was removed from the preparations by incubation (15 hours, 30°C) with tryspin (0.5 mg/ml) and ribonuclease (0.15 mg/ml) in 0.05 M phosphate, pH 7.4. The integuments were sedimented at 10,000 *g* and washed with phosphate buffer (twice) and water (twice). From 8 to 20 per cent of the spore glucosamine and DAP was released in a soluble form after the disintegration, enzyme treatment, and washing procedures (Table V).

Autolysis of Bacillus coagulans Integuments

After a final washing at pH 6.1 in 0.05 M ammonium acetate buffer, the integuments were sus-

pended in 0.001 M $CaCl_2$ and the pH was readjusted to 6.1 with dilute ammonia. Although under these conditions more than 95 per cent of the hexosamine was normally released in 3 hours at 30°C, complete autolysis was ensured by incubation for 16 hours. The sediment was washed twice with water. After incubation, the calcium in the combined supernatant and washings was precipitated with ammonium oxalate, and ammonium acetate was removed by vacuum sublimation at 60°C for 24 hours.

Lysozyme Treatment of Spore Integuments

The autolytic system present in the *B. coagulans* integument preparation was inactivated by heating in water at 120°C for ½ hour after the washing at pH 6.1. The heat treatment released 22 per cent of the hexosamine present in this preparation, but much less hexosamine (5 per cent) was released when similar preparations were heated at pH 7–8. Heated integuments were finally washed with water before incubation.

The integument preparations (~6 mg/ml) of the five species were incubated with lysozyme (0.1 mg/ml) (obtained from Nutritional Biochemical Corp., Cleveland) in 0.05 M ammonium acetate buffer at pH 7.2 for 22 hours at 37°C. Toluene + chloroform (2:1) was added as preservative during the autolytic, trypsin, and lysozyme digestions.

Treatment of Bacillus coagulans Integuments with Other Enzymes and Reagents

The integuments for these treatments were prepared from *B. coagulans* spores, disrupted, and washed (5 times) in water at 0–4°C. Heat inactivation (120°C, ½ hour) of the preparation used in Table VII released 3 per cent of the hexosamine present. After a final washing, the integument preparations were incubated with the reagent for the appropriate time and then centrifuged (12,000 *g*, 15 minutes), and washed once with reagent and twice with water. The reagents and conditions are recorded with the results obtained in Tables VI and VII.

Enzymes and reagents were obtained from the following sources: trypsin, British Drug Houses, Laboratory Reagent; ribonuclease, L. Light and Co., Colnbrook, England; papain, B.P.C., Zimmerman and Co., Perivale England; polysept 103S (quaternary ammonium-glycine compound), Polymer Corp., Sydney, Australia.

Analytical Methods

Material for amino acid and amino sugar analysis was hydrolyzed with 6 N HCl at 100°C for 5 hours. Amino acids and amino sugars were identified by two-dimensional paper chromatography using butanol + acetic acid + water (BAW) (4:1:1) and phenol + ammonia, and by paper electrophoresis at pH 2.4 followed by paper chromatography (BAW) in the second direction. Muramic acid was identified also from its elution with 0.33 N HCl from a Zeocarb 225 column ($R_{glucosamine}$ 1.05) (5) and from the absorption spectrum produced in the Elson and Morgan reaction (31). Taurine was identified by comparison of the compound isolated from acid hydrolyzates of strain 636 spores with an authentic sample by the following criteria: two-dimensional paper electrophoresis and chromatography (as above); elution with water from Amberlite IR-120 (H^+); m.p. 210–215°C (dec); mixed melting point; and their infrared spectra (KBr disc). Amino acids were separated both by paper chromatography (BAW, 40 hours) and by paper electrophoresis (7 per cent acetic acid, pH 2.4, 1 hour, 60 v/cm). After separation of three samples by each method, each amino acid spot was cut out and estimated as described previously (36). DAP was estimated by the method of Work (37) after paper electrophoresis at pH 2.4. Glucosamine was determined by the method of Cessi and Piliego (4), and hexosamine as described previously (36).

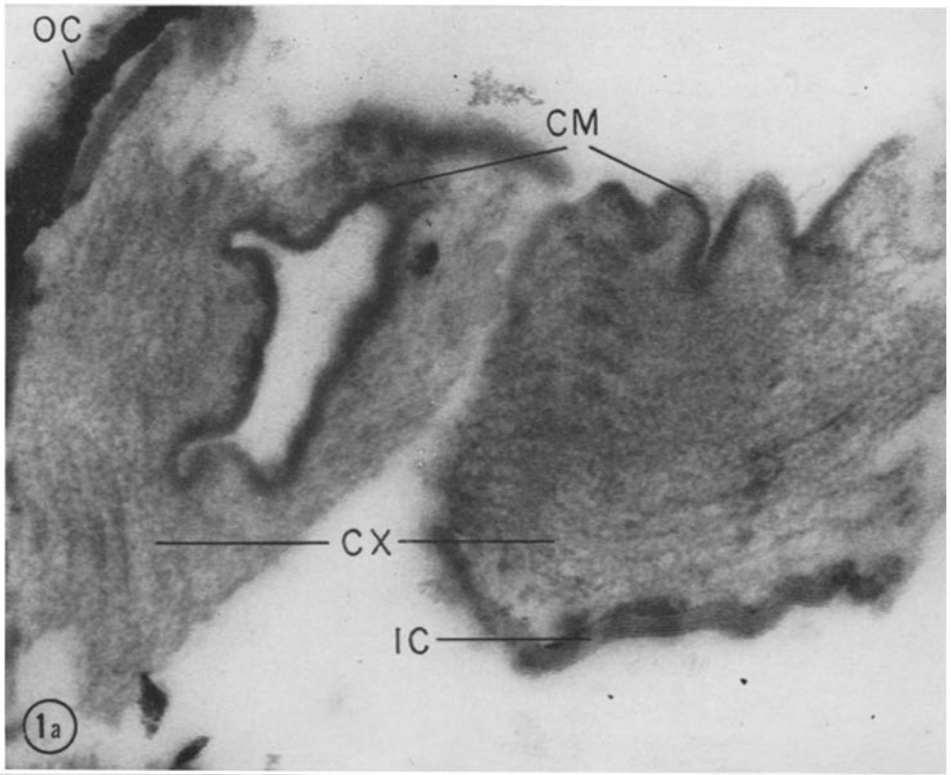
Electron Microscopy

The material was fixed with osmium tetroxide and embedded in Araldite as described previously (23).

RESULTS

Location of Mucopeptide in Bacillus coagulans Spores

EFFECT OF AUTOLYSIS ON *BACILLUS COAGULANS* SPORE INTEGUMENTS: The washed *B. coagulans* integument preparation consisted of electron-opaque outer coats, laminated inner coats, cortical material, and a layer, possibly the germ cell wall (36), attached to the inner surface of the cortex (Figs. 1 *a* and 1 *b*). We have referred to this layer tentatively as the "cortical membrane" (36). The residual cortex appears prominently as an open network of granular material, arranged in concentric bands. This preparation contained 60 per cent of the spore hexosamine, some having been lost during the final wash at pH 6.1. Following incubation of the preparation at pH 6.1 with 0.001 M $CaCl_2$ for 16 hours, electron micrographs showed complete breakdown and loss of the residual cortex and cortical membrane from the sedimented integuments, while the inner and outer coats remained unchanged in appearance (Fig. 2). Hydrolyzates of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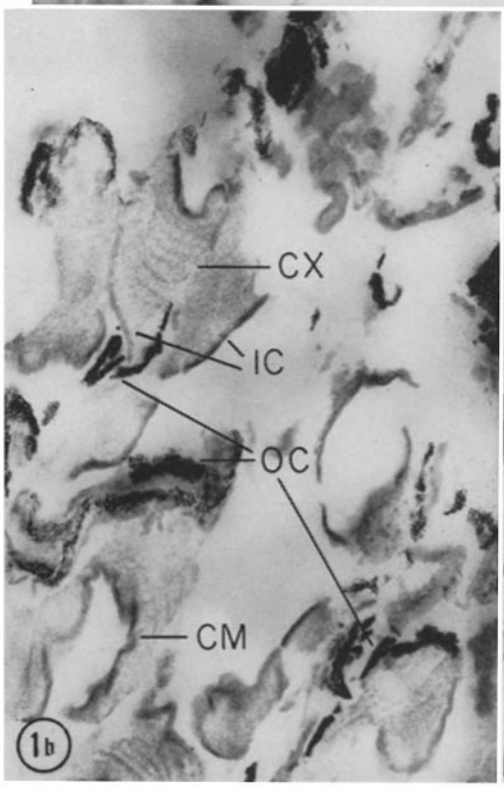
CX
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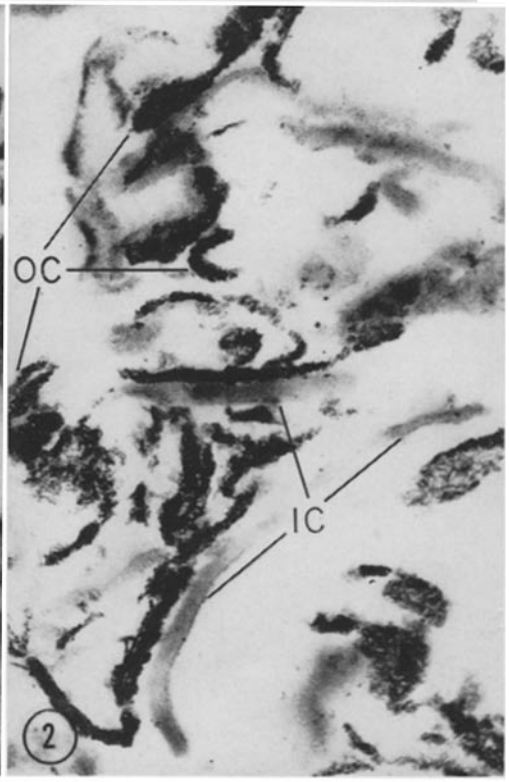

OC
IC
2

the soluble material released from the integument suspension consisted chiefly of alanine, glutamic acid, DAP, glucosamine, and muramic acid (Table II). This included more than 96 per cent of the glucosamine and DAP present initially in the integument preparation. The insoluble residue on hydrolysis gave amino acids of the type normally encountered in protein hydrolyzates.

TABLE II

Composition of Material Released from Bacillus coagulans Integument Preparation by Autolytic and Lysozyme Digestion*

	Autolysis‡	Lysozyme
Alanine	2.00	2.00
Glutamic acid	0.98	1.05
α,ϵ-Diaminopimelic acid	1.01	1.00
Glucosamine	1.87	1.96
Muramic acid	Not determined	

* Analyzed after hydrolysis (6 N HCl, 5 hours, 100°C).

‡ Integument preparation incubated 16 hours, 30°C, with 0.001 M $CaCl_2$, pH 6.1.

EFFECT OF LYSOZYME ON *BACILLUS COAGULANS* SPORE INTEGUMENTS: Spore integument preparations, in which the autolytic system had been inactivated by heating, were incubated with lysozyme. Heating did not alter the appearance of the integuments in the electron microscope, although some hexosamine (22 per cent) was released. Digestion with lysozyme specifically removed the cortical and membrane components from the *B. coagulans* preparation (Fig. 3). Soluble mucopeptide was released which contained more than 96 per cent of the hexosamine and DAP initially present in the preparation. After incubation of the heated integuments in the absence of lysozyme, 4 per cent of the hexosamine and 2 per cent of the DAP became soluble. Since lysozyme increased four-fold the rate of release of hexosamine from *B. coagulans* spores disrupted in water, the sensitivity of the residual cortex and cortical membrane to lysozyme was not induced by heating or treatment with EDTA or buffers.

Composition of the Material Released from Bacillus coagulans Integuments by Autolysis and Lysozyme

Lysozyme and autolysis both released material of similar composition from the *B. coagulans* integument preparation (Table II). The principal constituents in the hydrolyzates were five amino acids and amino sugars, characteristic of spore peptide (33) or cell wall mucopeptide of *Bacillus* species (29). In addition, some glycine (Table III) and traces of most amino acids common to protein hydrolyzates were present, mainly in the autolyzate. After dialysis of the autolyzate for 36 hours, most of the alanine, glutamic acid, and DAP, and all the hexosamines were retained in the non-diffusible fraction (Table III). The diffusible material contained small amounts of alanine, glutamic acid, DAP, and glycine, but no amino sugars. Lesser amounts of various amino acids common to protein hydrolyzates together with a small amount of neutral carbohydrate were also present in this fraction.

The diffusible fraction was separated after paper electrophoresis at pH 2.7, 3.9, or 5.5 into four main bands reacting with ninhydrin or a chlorine–potassium iodide–tolidine reagent (25).

FIGURES 1 *a* AND 1 *b*

Thin sections of *B. coagulans* spore integument preparation. The cortical structure (*CX*) appears as a sponge-like network of fibrils showing a number of distinct bands. The cortex is bounded at its inner surface by the cortical membrane (*CM*) and at its outer surface by the multilaminated inner spore coat (*IC*). Fragments of the outer coat (*OC*) are seen detached from the inner coat. *a*, × 110,000; *b*, × 37,500.

FIGURE 2

The *B. coagulans* spore integument preparation shown in Fig. 1, but after autolytic digestion. The cortical structure and cortical membrane have been almost completely degraded, leaving only fragments of inner (*IC*) and outer (*OC*) spore coats unchanged in appearance. × 88,000.

Paper chromatography (BAW) for 5 days separated two slowly moving spots from those moving more rapidly. The procedure is shown schematically in Fig. 4, and the qualitative composition of material eluted from the principal bands separated by paper chromatography and by paper electrophoresis in pyridine + acetic acid buffer, pH 3.9, is shown in Table IV.

Location and Composition of Mucopeptide in Other Species

The location of the mucopeptide in spores of *B. subtilis*, *B. stearothermophilus*, *B. cereus* T, and 636 was investigated by lysozyme treatment of integuments prepared from heat-killed spores. As in *B. coagulans*, the integument preparations of each

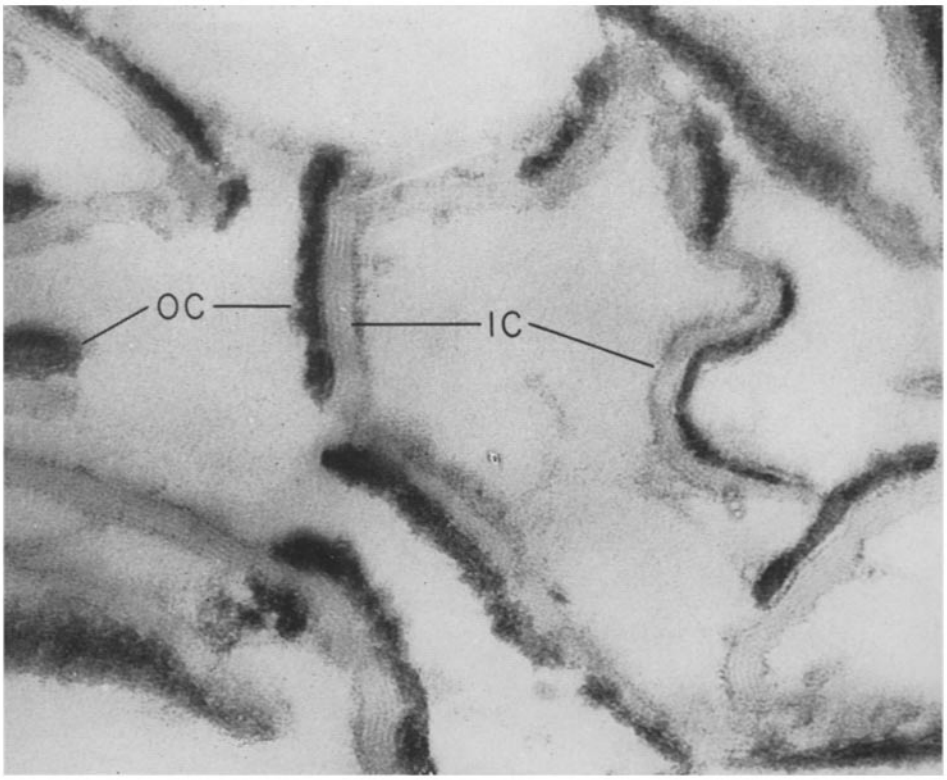


FIGURE 3

This figure shows the effect of lysozyme on heat-inactivated *B. coagulans* integuments. As after autolysis (Fig. 2), the cortical components but not the spore coats (*IC*, *OC*) were destroyed. × 180,000.

The non-diffusible fraction on paper electrophoresis at pH 5.5 showed an anionic spot (distance migrated relative to glutamic acid 0.53) together with a leading and tailing streak. After paper chromatography for 2 days, only a stationary spot was detected. Material eluted from both spots had a composition similar to that of the material applied. The spots were detected with chlorine–potassium iodide–tolidine reagent, ninhydrin, and light green SF, but did not react with $AgNO_3$-NaOH, TCA-diphenylamine, or bromophenol blue.

species contained inner and outer coats, residual cortex, and cortical membranes (Figs. 5, 7, and 10). (The observations on *B. stearothermophilus* integuments were similar to those obtained for *B. subtilis* and *B. coagulans* and are not included.) The *B. cereus* T preparation (Fig. 7) contained in addition exosporia, loosely enveloping the outer coat. These had the appearance of an electron-opaque membrane *ca.* 45 A thick enclosed between two faint layers. A number of small granules were embedded in these layers and in the space between the exosporium and the outer coat. Sur-

rounding the outer coat in 636 spores (Fig. 9) was a thick structure (140 to 1100 A) consisting probably of two layers, the outer having a number of folds similar to that shown in *B. polymyxa* by Holbert (11). This structure was also present in the 636 integument preparation (Fig. 10).

TABLE III

Composition of Autolyzate from Bacillus coagulans Integument Preparation after Dialysis*

	Non-diffusible	Diffusible
Alanine	2.00	0.23
Glutamic acid	0.82	0.17
α,ϵ-Diaminopimelic acid	1.01	0.12
Glucosamine	1.90	0.00
Muramic acid	—	0.0
Glycine	0.0	0.15

Results are expressed in moles relative to alanine, which is taken as 2.00 in the non-diffusible fraction. *Analyzed after hydrolysis (6 N HCl, 100°C, 5 hours).

Incubation with lysozyme specifically removed the residual cortex in each species (Figs. 6, 8, and 11). The cortical membrane was removed from preparations of *B. subtilis*, *B. stearothermophilus*, and 636 (Figs. 6 and 11) but not from that of *B. cereus* T (Fig. 8). The inner and outer coats, the exosporium in *B. cereus* T, or the ridge structure of 636 did not appear to be affected by lysozyme.

Alanine, glutamic acid, DAP, glucosamine, and muramic acid were the major compounds present in hydrolyzates of the soluble material released by lysozyme from each species; however, amino acids derived from protein were present in greater amounts than in the *B. coagulans* lysozyme digest. In *B. subtilis* and *B. stearothermophilus* essentially all the hexosamine and DAP present in the integument preparations was released by lysozyme (Table V).

In strain 636, however, nearly all the DAP present was released but only half the glucosamine. This species has much more glucosamine in proportion to DAP than the species without

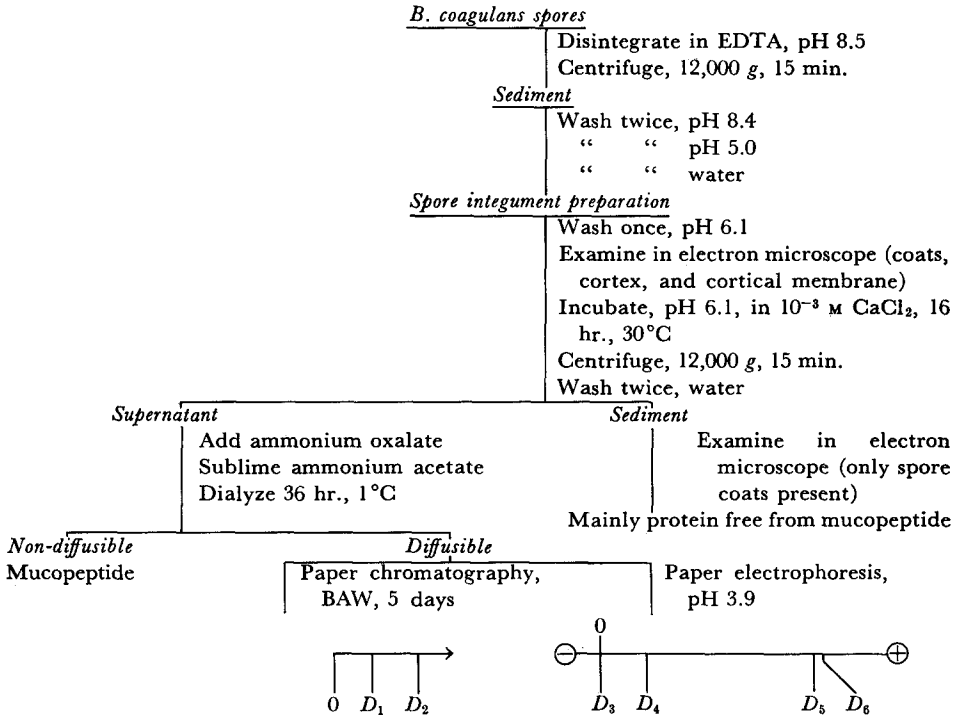


FIGURE 4

Preparation of *B. coagulans* spore integuments and analysis of the soluble material released after autolytic destruction of the cortex and cortical membrane.

structures external to the outer coat. If the inner and outer coats in this species are free of glucosamine as in other species, then the glucosamine in the residue after lysozyme treatment would be located in the ridge structure. It was not possible to demonstrate the presence or absence of muramic acid in the hydrolyzed residue. The residue after lysozyme digestion of 636 integuments also differed from that of other species in containing much greater amounts of glutamic acid and an unusual amino acid, identified as taurine (see Methods). These amino acids may also be derived from the ridge structure. Taurine was not present in the water-, trypsin-, or lysozyme-soluble fractions of 636 or in whole spore hydrolyzates of fourteen other *Bacillus* species (35).

TABLE IV

Composition of Dialyzable Peptides from Autolyzed Bacillus coagulans Integument Preparations

		Alanine	Glutamic acid	DAP	Glycine
D_1	Very slow spot*	4	4	3	1
D_2	Slow spot*	4	1	3	2
D_3	Neutral spot‡	4	1	3	2
D_4	Slow anionic‡	4	4	0	0
$D_5 + D_6$	Fast anionic‡	4	4	3	1

Relative strengths of spots: strong, 4; medium, 3; weak, 2; very weak or doubtful, 1; absent, 0.
* Separated by paper chromatography in butanol + acetic acid + water, 5:1:2, 5 days.
‡ Separated by paper electrophoresis at pH 3.9 (pyridine + acetic acid + water, 30:100:870).

The *B. cereus* T integuments retained 20 per cent of the spore DAP and 36 per cent of the spore glucosamine after lysozyme treatment (Table V). This was consistent with the observation that in this species the cortical membrane was not removed by lysozyme, and supports the view that both the residual cortex and the cortical membrane contain mucopeptide. As in strain 636, *B. cereus* T spores contained more glucosamine in proportion to DAP than other species (Tables I, V). This ratio was even greater in lysozyme-treated integuments, suggesting that some of the glucosamine retained in the integuments was associated with the DAP in the cortical membrane, while the excess was located in the exosporium.

Incubation of the integument preparations in the absence of lysozyme caused no change in their appearance in the electron microscope, and released insignificant quantities of glucosamine or DAP. Small quantities of neutral carbohydrate, when present, were largely solubilized after lysozyme treatment.

Effect of Enzymes and Chemical Reagents upon the Spore Mucopeptide and the Spore Integument Structures in Bacillus coagulans

The spore mucopeptide was very resistant to treatment with formic and performic acids, NaOH, phenol, surfactants, and trypsin (Tables VI, VII). Papain released material containing most of the hexosamine, but much more slowly than lysozyme. The papain preparation used also lysed *B. megaterium* strain KM vegetative cells and possibly contains lysozyme as an impurity. Papain also accelerated the release of hexosamine from *B. megaterium* spore integuments (32) and removed some glucosamine from the mucopeptide preparation from *E. coli* cell walls (18). Hexosamine was probably released by acid hydrolysis of the mucopeptide during the trichloroacetic acid (TCA) treatment. Heating with NaOH at 100°C would have partly destroyed the hexosamine in the mucopeptide. The surfactants cetyltrimethyl-

FIGURES 5 *a* AND 5 *b*

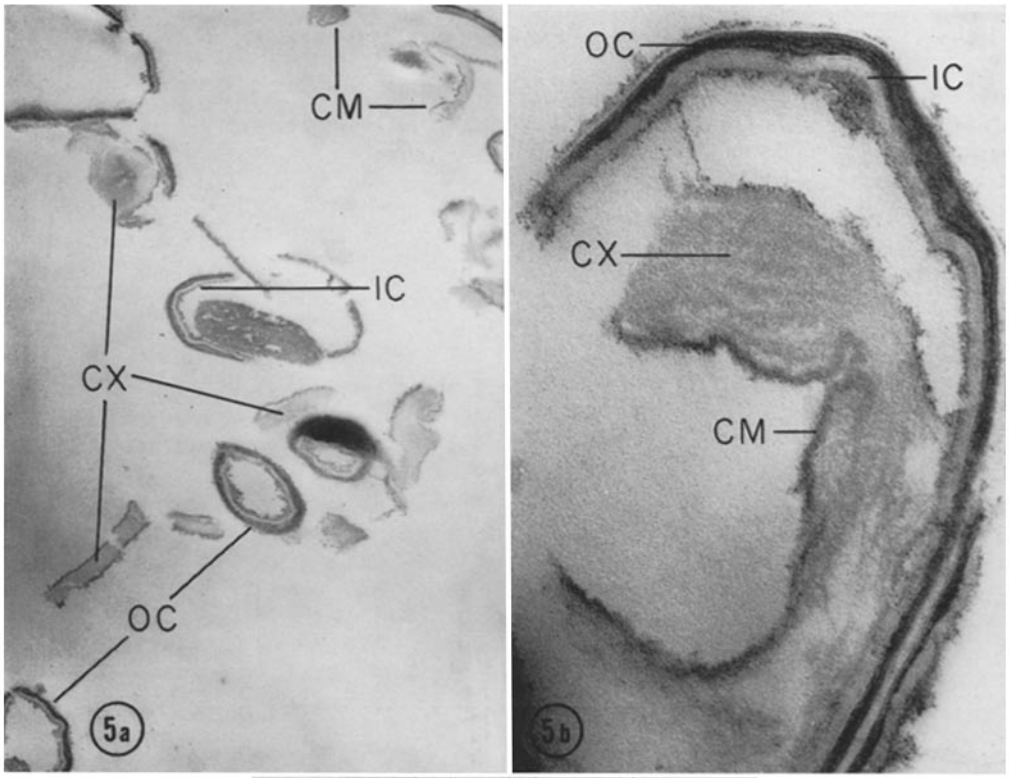
Integument preparation from heat-killed *B. subtilis* spores.

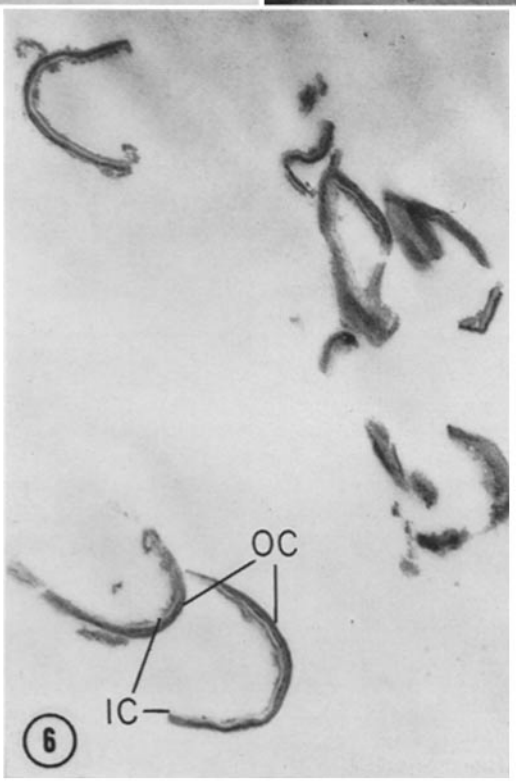
a. The preparation contains fragments of the two spore coats (*IC*, *OC*), the residual cortex (*CX*), and cortical membrane (*CM*). × 24,000.

b. The structure and location of each of the spore integuments is shown more clearly at greater magnification in an unfragmented disrupted spore. × 108,000.

FIGURE 6

The *B. subtilis* spore integument preparation after incubation with lysozyme. No cortical material or cortical membranes can be seen. The preparation contains only the two spore coats (*IC*, *OC*). × 27,000.


CM
IC
CX
OC
5a
OC
IC
CX
CM
5b


OC
IC
6

ammonium bromide (CTAB) and Polysept 103S were tested because of a report (27) that these substances stimulated germination. CTAB had no stimulating effect upon the rate of autolysis in unheated integuments. Intact *B. coagulans* spores lost 93 per cent of their dipicolinic acid after heating with Polysept 103S (70°C, ¼ hour). Except for lysozyme and papain digestion, none of the treatments in Tables VI and VII resulted in a noticeable change in the appearance or structure of either the cortex or the cortical membrane. Treatment with other reagents such as 8 M LiBr, 8 M urea, and 2 M $CaCl_2$ (24 hours, 37°C, then ½ hour, 90°C) was also without apparent effect upon the appearance of the cortical membrane, residual cortex, or spore coats (Fig. 12). These properties are all consistent with those of the mucopeptide from Gram-positive bacterial cell walls. The resistance of the mucopeptide components and the two spore coats, residual cortex, and cortical membrane to concentrated salt solutions, performic acid, surfactants, and alkali suggests that these structures are covalently cross-linked and do not entirely depend upon disulfide cross-linking or hydrogen, ionic, or hydrophobic bonding. The resistance of the spore integuments to alkali and surfactants indicates that normal lipid or lipoprotein was not an important structural element in the residual cortex, cortical membrane, or spore coats.

DISCUSSION

From the electron microscopy studies, the effect of lysozyme on spore integuments of several species appeared to be limited to the specific removal of the residual cortex and cortical membrane. During autolysis of *B. coagulans* integuments these structures were also specifically removed. Analysis of the soluble material released in *B. coagulans* by these enzymes indicated that these components consisted mainly of mucopeptide material, similar in composition to the "spore peptide" of Strange and Powell (33) and the mucopeptide component of cell walls of *Bacillus* species (29). Relatively little protein, amino acids, or carbohydrate was found in the enzyme digests of *B. coagulans* integuments. The material released after lysozyme digestion of the spore integuments of the four other *Bacillus* species also consisted mainly of mucopeptide but contained more protein than *B. coagulans* digests. Protein would be less readily removed from the cortex after the heat treatment given these spores. The disintegration of the residual cortex and cortical membrane by lysozyme showed that the basic structure maintaining their morphological integrity was dependent upon lysozyme-susceptible linkages (probably β-(1–4)-*N*-acetyl hexosamine bonds) (3, 28) and demonstrated a structural similarity to cell wall mucopeptide. The resistance shown by the cortical structure and cortical membrane to trypsin, performic acid, surfactants, and alkali suggests that protein, lipoprotein, or lipids were not important in the structural integrity of these components, and probably were present only as minor constituents.

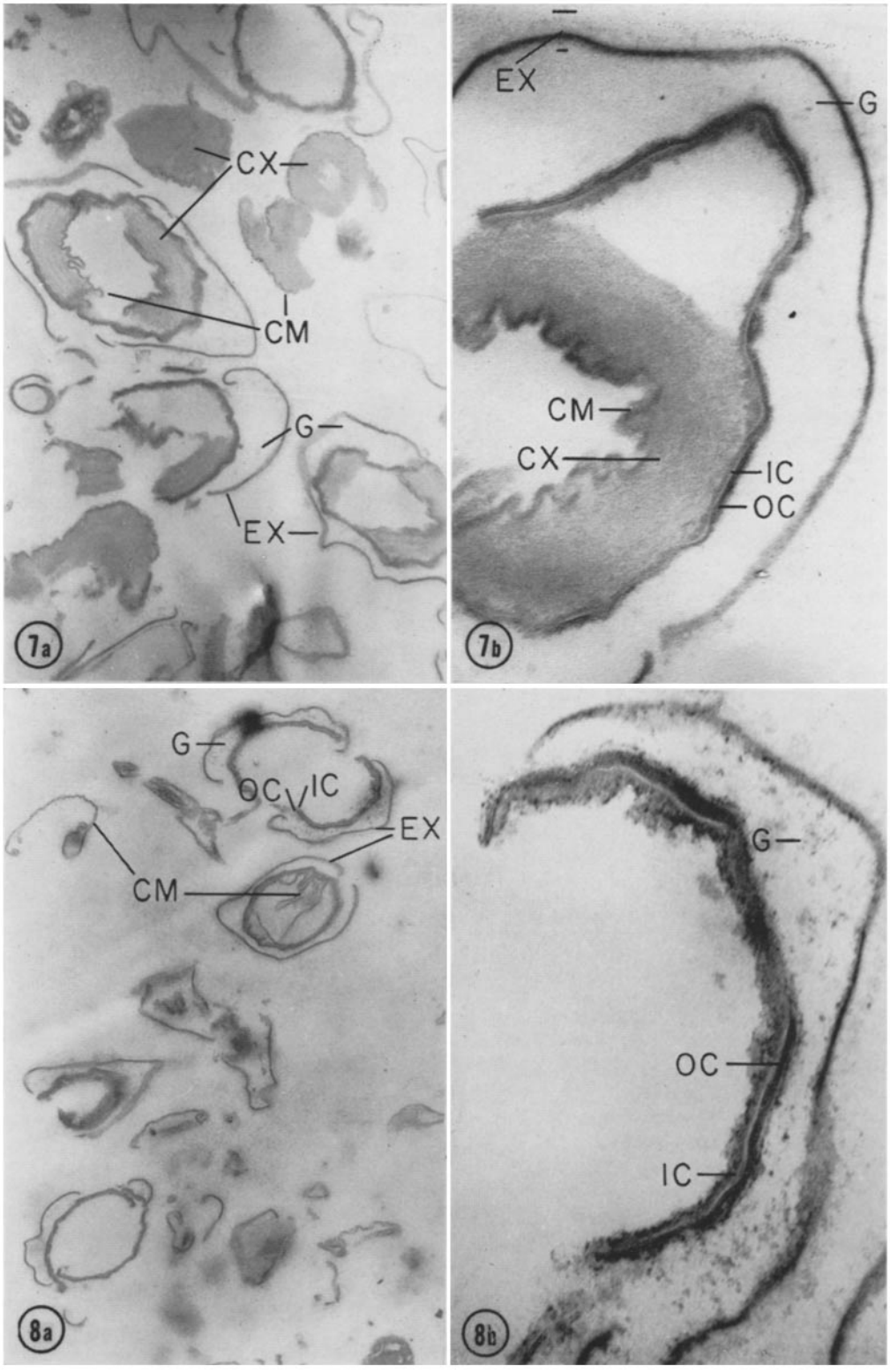
The analytical data gave the composition of only those structures which survived the disintegration and washing procedure and were subsequently removed by lysozyme or autolysis. During preparation of the *B. coagulans* integuments, the lytic system was not completely inactive and some

FIGURES 7 *a* AND 7 *b*

Spore integument preparation of heat-killed *B. cereus* T. In this organism the exosporium (*EX*), a loosely fitting membrane enveloping the spore coats, is present in addition to the two spore coats (*IC*, *OC*), cortex (*CX*), and cortical membrane (*CM*). The exosporium consists of a relatively dense membrane approximately 45 A thick between two much less dense layers each approximately 150 A thick. Many grains (*G*) are embedded in these layers and also in the space between the coats and the exosporium. *a*, × 27,000; *b*, × 110,000.

FIGURES 8 *a* AND 8 *b*

B. cereus T integument preparation after digestion for 16 hours with lysozyme. The spore coats (*OC*, *IC*), exosporium (*EX*), and granules (*G*) remain in the preparation, unaffected in appearance. The cortical structure is absent, but the cortical membrane (*CM*) is present and readily identified within some disrupted spores. *a*, × 25,000; *b*, × 120,000.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IC
8b

hexosamine and DAP were released before the chemical and morphological study. Analyses showed that nearly all the spore hexosamine and DAP in *B. coagulans* were present in the insoluble integument fraction immediately after disintegration of the spores and throughout most of the washing procedure (Table I). Also, by heat inactivation of the lytic system before disruption, integument preparations were obtained containing most of the spore glucosamine and DAP (Table V). These results show that most of the spore mucopeptide is located in the cortex and cortical membrane. In two species, *B. cereus* T and 636, additional glucosamine, apparently not associated with DAP, was present in integuments after lysozyme digestion and could be located in the exosporia or ridge structures present in these species. Berger and Marr (1) have associated the release of hexosamine during sonic treatment of *B. cereus* spores with the removal of the exosporium. The above experiments do not indicate the nature of soluble substances such as calcium, dipicolinic acid (DPA), or enzymes which may be held in the cortex of the intact spore. The micro ashing studies of Knaysi suggest the cortex as the site of most of the inorganic constituents (15).

During sporulation, the cortex forms between the two cytoplasmic membranes of the forespore (8, 23). The surface of each membrane adjacent to the cortex is the "outer" surface, which would normally be adjacent to the vegetative cell wall (23). Hence, the morphogenesis of the cortex and cortical membrane is similar to that of the cell wall. The similarity in the composition of the residual cortex and cell wall mucopeptide of *Bacillus* species confirms that the cortex in part is analogous to an endogenous cell wall.

The cortical membrane in spore integument preparations is possibly the germ cell wall (36). If this is so, it is surprising that in *B. coagulans* it should be degraded by an autolytic system along with the cortex, unless treatment with EDTA or buffers has made it susceptible to attack. In *B. cereus* T the cortical membrane was resistant to lysozyme. Differences in the relative susceptibility of the residual cortex and cortical membrane to lysozyme and spore lytic enzymes may depend upon very small differences in their composition and structure and on slight differences in enzyme specificity.

The mucopeptide composition of the cortical structure supports the view that the spore peptide in germination exudates arises from the cortical region of the spore, which disintegrates during germination (19). Electrophoretic evidence (6), which has been interpreted as demonstrating that the outer surface of spores consists of mucopeptide, may have been influenced by small amounts of adsorbed mucopeptide, released during lysis of the sporangium. Lysozyme affected the mobility (7) but not the viability (34) of intact spores.

It has long been considered probable that labile constituents in resting spores are protected from heat by their location in a dry region of the spore (17). Until recently, it was postulated that a dry core could be maintained by a water-impermeable barrier (26) formed by the coats or possibly the cortex (9). Lewis *et al.* (16) pointed

FIGURE 9

Section of intact spore of *Bacillus* sp. strain 636. The outermost layer of the spore consists of a ridge structure (*R*) with seven or eight points. Except for a slightly less dense layer 80 A thick adjacent to the outer coat (*OC*), it has a uniform electron opacity and varies in thickness from 140 A to 1100 A at the points. Underlying the ridge structure is the typical opaque outer coat (*OC*) and laminated inner coat (*IC*). Cortex, *CX*. × 140,000.

FIGURE 10

Section of integument preparation from heat-killed 636 spores. Fragments of ridge structure (*R*), outer and inner coats (*C*), and residual cortex (*CX*) are present in this field. × 34,500.

FIGURE 11

Spore integument preparation of 636, after lysozyme treatment. Residual cortex and cortical membranes are absent. The preparation consists of fragmented spore coats (*C*) and ridge structure (*R*). × 31,500.

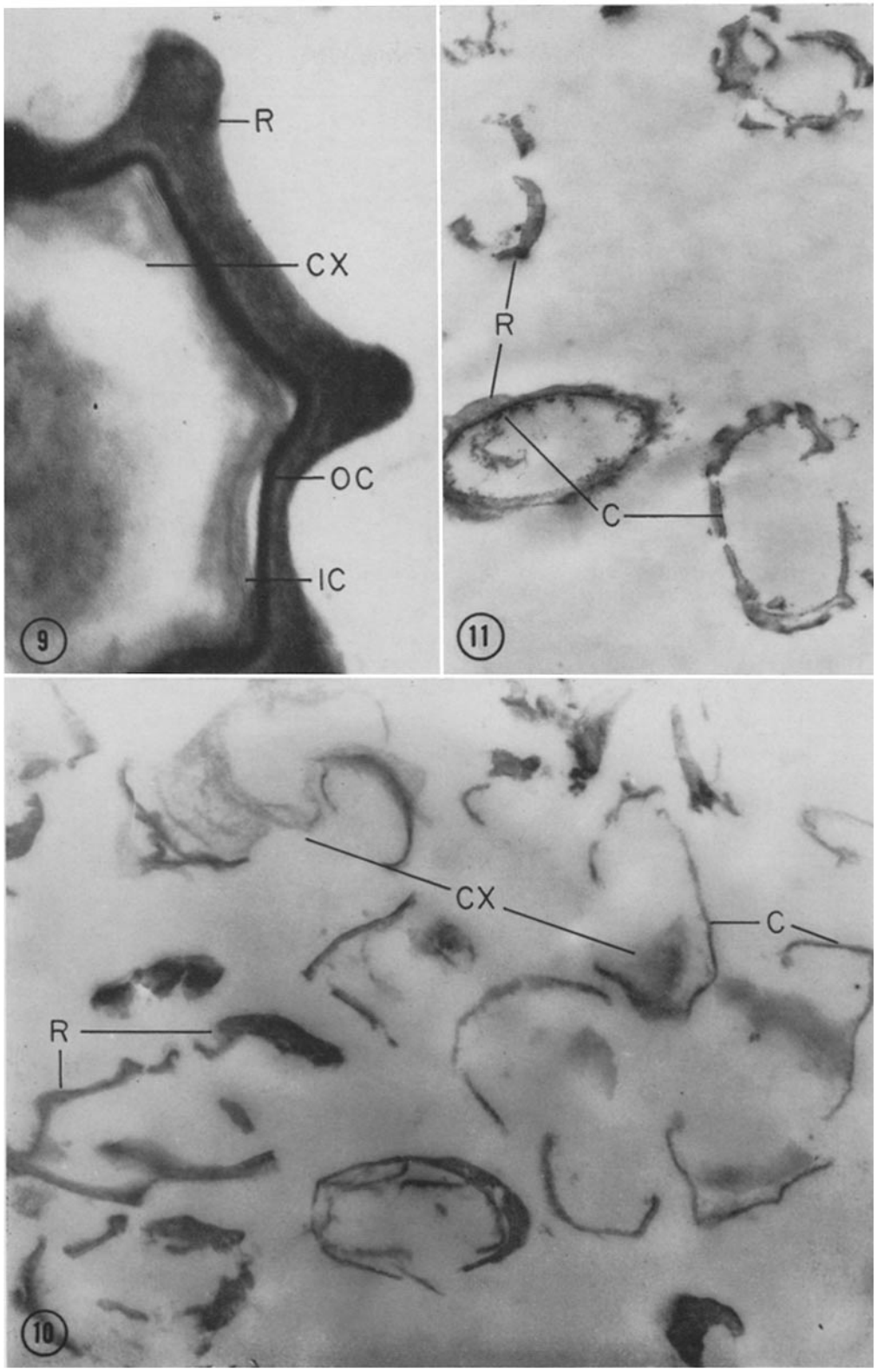
R
CX
OC
IC
9
R
C
11
CX
C
R
10

TABLE V

Release by Lysozyme of Material Containing Glucosamine and DAP from Disrupted Heat-Killed Spores

	B. stearothermophilus		*B. subtilis*		636		*B. cereus* T	
	GM*	DAP	GM	DAP	GM	DAP	GM	DAP
Soluble spore material‡§	9	8	15	11	20	17	15	11
Material solubilized by lysozyme§	90	91	81	87	39	80	49	69
Material solubilized after incubation in absence of lysozyme§	1.5	—	3	—	1.7	—	2	—
Residue after incubation with lysozyme§	0.8	1	4	2	41	3	36	20
Glucosamine and DAP content of intact spores (% dry wt.)	4.5	2.21	3.2	1.28	6.2	1.00	3.8	0.75

* Glucosamine.

‡ Soluble material after heating (120°C, ½ hour) and disruption of spores and treatment of the insoluble integuments with trypsin and ribonuclease.

§ Result expressed as per cent of total glucosamine or DAP in intact spore. Determined after hydrolysis (6 N HCl, 5 hours, 100°C).

TABLE VI

Loss of Hexosamine from Bacillus coagulans Spore Integuments after Treatment with Chemical Agents

Reagent	Treatment		Hexosamine loss (% original content)
	Time (hr.)	Temperature (°C)	
Formic acid (98%)	2	1	10
Performic acid*	1	1	10
N NaOH	1	80	11
N NaOH	1	100	28
5% trichloroacetic acid	¼	90	43
80% phenol	1	18	14

* 0.3 per cent H_2O_2 in 98 per cent formic acid. Hexosamine determined in insoluble sediment after each treatment

out the unlikelihood that layers of these dimensions would be effectively impermeable to water. They proposed, instead, that water may be excluded from the core by pressure exerted by contraction of the cortex, possibly under the influence of calcium ions.

The water impermeability theory is not supported by recent studies on the water permeability of spores (2, 21). Furthermore, the hydrophilic mucopeptide composition of the cortical material makes this layer alone unlikely to be responsible for water impermeability. The high water content of spores (2, 21) suggests that any barrier impermeable to water is located beneath the coats of the spore.

On the other hand, the chemical nature and properties of the cortical structure studied fulfil the main requirements for a contractile cortex. Electron micrographs of the residual cortex (Figs. 1 *a*, 5 *b*, and 7) show a number of granules loosely cross-linked to form an insoluble matrix. The granules are of a size comparable to that expected for a non-diffusible "spore peptide" of mol. wt. ~10,000. The mucopeptide of which this structure is composed, if of a chemical structure similar to that proposed (3, 28, 29) for cell wall mucopeptide, consists of a hexosamine backbone with peptide chains attached to muramic acid. Our results suggest that in the *B. coagulans* cortex the peptide chains contain glutamic acid, DAP, and alanine. Each side chain in the structure then has the possibility of 3 free carboxyl groups (1 x glu, 1 x DAP, and 1 x COOH terminal) and 1 free amino group (DAP). This evidence suggests that the residual cortical structure could function as a weak acid-type ion exchanger with low cross-linking. Under normal physiological conditions of pH and ionic strength, analogous synthetic materials occur in a highly swollen form. The swelling depends upon pH and the cations present. For instance, CM-Sephadex C-25 at pH 7.0 contracts in 1 M $CaCl_2$ to 35 per cent of its volume in water. Heat-resistant spores have relatively high contents of calcium (35)

which may be concentrated in the cortex (15). The introduction into the cortex, during sporogenesis, of a high concentration of calcium or possibly calcium dipicolinate offers a likely mechanism for contracting an initially swollen cortical structure.

Unlike the lipid or lipoprotein structures which might constitute water-impermeable barriers, a mucopeptide structure would retain its strength at the higher temperatures where its protective effect is important. Sufficient strength may be available in a bacterial mucopeptide to maintain the con-

TABLE VII

Release of Hexosamine from Heat-Inactivated Bacillus coagulans Spore Integuments after Various Treatments

Treatment	Conc. (mg/ml)	Time (hr.)	Temperature (°C)	Hexosamine dissolved (% original content)
Lysozyme	0.1	24	37	96
Papain	0.1	24	37	73
Trypsin	0.5	40	37	11
CTAB	0.2	24	37	4.5
Polysept 103S	0.1	1	80	3
Phosphate buffer		24	37	3
EDTA, pH 7.0	10	24	37	2.2

Incubation of spore integument suspensions with each enzyme was in 0.05 M phosphate buffer, pH 7.2.

tractile pressure required, since it has been calculated that the vegetative cell wall of *Staphylococcus aureus* may withstand hydrostatic pressures of 30 atmospheres (20). The cortex in *B. coagulans* could possibly maintain much higher pressures since it is probably six times as thick and has one-half the inside diameter (23) of the cell wall of *Staphylococcus aureus*; it is also protected by two surrounding coats.

The contractile cortex theory does not necessarily predict a completely anhydrous "core." As dry proteins commonly show a contraction in volume of 5 to 8 per cent on solution (10), pressure would stabilize a state of low but not zero water content. This is in accord with the observation that spores (22), vegetative cells, and proteins

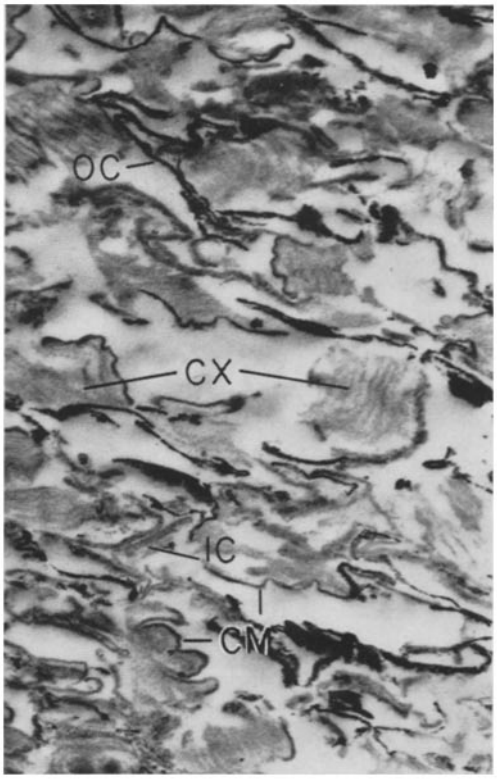

FIGURE 12

Section of *B. coagulans* spore integument preparation after treatment with 8 M urea. The treatment has not noticeably modified the structure or appearance of the residual cortex (*CX*), cortical membranes (*CM*), outer coats (*OC*), or inner coats (*IC*). The inner and outer coats are parted from each other and hence lose some rigidity. Preparations of similar appearance were obtained after treatment with a number of concentrated electrolytes. × 22,500.

(12) are less heat-stable under conditions of extreme dryness.

Pressure on the spore protoplasm, besides reducing the water content, would also have a direct stabilizing effect on the spore proteins. The heat stability of proteins (13) and spores (14) is increased by pressure (<1000 atmospheres).

These observations support the concept of a contractile cortex as advanced by Lewis *et al.* (16) and indicate some details of the contractile mechanism which may operate in the spore cortex. Thus the residual cortex may be visualized as a structural matrix carrying free carboxyl and amino groups, the contraction and swelling of which is controlled in spores by calcium or calcium

dipicolinate concentration. To evaluate this or alternative theories of heat resistance, more detailed information is necessary on the chemical structure of the cortex and the factors influencing the degree and force of its contraction. The location of calcium and DPA within the developing and mature spore is also of utmost importance.

Received for publication, November 15, 1962.

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