THE COMPOSITION AND STRUCTURE OF BACTERIAL SPORES

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ABSTRACT

The composition of the insoluble "integuments" and soluble "contents" fractions of spores of four *Bacillus* species of widely differing heat resistance were compared. Electron microscopy of thin sections was also used to determine and compare the morphological structures in the integument preparations. The soluble fractions of the thermophiles, *B. coagulans* and *B. stearothermophilus*, had a higher content of hexose and dipicolinic acid. The hexose content of both fractions of the four species was related to heat resistance. Integument fractions consisted chiefly of protein together with variable amounts of the mucopeptide constituents, α, ϵ-diaminopimelic acid (DAP) and hexosamine. In the thermophiles the DAP and hexosamine were found chiefly in the insoluble integuments fractions, while in *B. cereus* and *B. subtilis* most of this material was soluble. Integument preparations, containing mainly protein with little mucopeptide, consisted chiefly of outer and inner spore coats, while preparations having more mucopeptide contained also residual cortical material and a cortical membrane (possibly the germ cell wall). The results suggest that spore integuments consist of mainly proteinaceous outer and inner coats together with variable amounts of residual cortex and cortical membrane which contain the mucopeptide material.

INTRODUCTION

Studies on the composition of spores have been concerned with spore coat preparations (30, 32, 40), germination exudates (25, 35), or specific constituents such as dipicolinic acid (DPA) (3, 18, 37), phosphorus fractions (7), or inorganic cations (4, 37). The chemical composition of individual morphological components of the spore other than coat preparations has not been defined. In view of the possible role of the spore integuments, in stabilizing the protoplasm by excluding water by mechanisms such as water impermeability (10, 27) or the postulated contractile cortex of Lewis *et al.* (20), a comparison of the composition and morphological structure of the integuments of several species was desirable.

Recent evidence suggests that the spore "core" may contain the dipicolinic acid (11), and that most of the mineral matter may be located in the cortical region (17). Chemical analyses of preparations of spore integuments, variously referred to as "coats" (13, 32), "membranes" (40), or "walls" (30), show these to consist largely of protein. Most of these preparations also contained diaminopimelic acid (DAP) or hexosamine, indicating the presence of some "spore peptide" material. The spore peptide (35) is released from germinating spores and from the insoluble fraction of disintegrated spores, probably by the action of lytic enzymes present in the spore (33, 34). Mayall and Robinow (22) observed that the cortex breaks down during germination and suggested that the spore peptides may come from the cortex.

Lewis *et al.* (20) have assumed it to be of "unusual biochemical composition" in support of their hypothesis of a contractile cortex.

In the studies on the composition of spore coats it is not clear which of the several spore integuments (22, 24) were present in most of the coat preparations, as sections were not examined in the electron microscope. The presence of DAP and hexosamine in the coat preparations may have been due to adhering cortical material if the cortex is the site of the spore peptide.

In this study, comparative analyses of the composition of the soluble "contents" and insoluble "integuments" from disrupted spores of four species with a wide range of heat resistance have been made. Electron microscopy of sectioned material was used to determine the morphological components present in the integument preparations analyzed, and to compare the structure of integuments in each species.

MATERIALS AND METHODS

Organisms

Spores of the following organisms were used: *Bacillus cereus* (Knaysi strain C3), *Bacillus subtilis*, *Bacillus coagulans* (strain 320), and *Bacillus stearothermophilus* (ATCC 7953; NCA strain 1518).

Preparation of Spore Crops

The spores were grown in 14 liter lots of medium, vigorously aerated in 20 liter bottles, with silicone antifoam A added to prevent foaming. *B. coagulans* and *B. stearothermophilus* were grown at 50°C and the other species at 30°C. *B. coagulans* was grown in a medium containing 0.5 per cent casamino acids (Difco), 0.5 per cent casitone (Difco), 0.1 per cent yeast extract (Difco), and a salt mixture (24). The other organisms were grown on potato infusion medium (26). For *B. stearothermophilus* the potato infusion was used at half strength and supplemented with the salt mixture. Residual vegetative cells and sporangia in the crops of *B. coagulans* and *B. stearothermophilus* were removed by treatment with papain (1 mg/ml) at 1°C for 2 to 3 weeks in the presence of 0.1 per cent 1:1 chloroform-toluene mixture. Each spore crop was washed 8 times with water to give a clean preparation (>99 per cent free spores).

Heat resistance was determined by survival counts on dextrose tryptone agar after heating spore suspensions (1.5 ml of a 10^6/ml suspension in 75 × 9 mm ampoules) in 0.05 M phosphate buffer, pH 7, at 100° or 110°C ± 0.05° for various periods. For comparison of decimal reduction times (D) at 110°C, $D_{110°}$ values were computed from $D_{100°}$ values assuming a temperature coefficient (Q_{10}) of 10 (z = 18°F).

Preparation of Spore Fractions

Spore suspensions (10 ml, 30 mg/ml) were shaken in bottles 3 × 1¼ inches in diameter with 20 gm of 0.1 mm glass beads (Chance Brothers, Smethwick, England, grade 12–13), and 2 drops tri-*n*-butyl citrate as an antifoam, on a reciprocating shaker (940 × 1 inch oscillations per minute) at 1°C. Shaking was continued until practically no refractile or stainable intact spores remained, but was stopped before the spores were disintegrated into unidentifiable fragments. Spores of *B. cereus* and *B. subtilis* tended to remain in typical laterally split forms after 20 to 30 minutes shaking, while spores of *B. coagulans* and *B. stearothermophilus* disintegrated more readily into small fragments. Disrupted suspensions, from 4 to 6 gm spores, were separated from the beads by decanting and passage through a no. 2 sintered glass filter. The insoluble material was sedimented (10,000 *g*, 15 minutes at 0°C) to give integument preparations (I fractions) which were washed 6 to 8 times with water and freeze-dried. The combined supernatants, containing all the water-soluble spore material, were freeze-dried to give the soluble or spore "contents" fraction (S). Before analysis, fractions were stored at room temperature *in vacuo* over P_2O_5. Centrifuging disrupted spores of *B. coagulans* gave a sediment with two distinct layers. The upper and lower layers were separated by several fractional resuspensions until homogeneous sediments were obtained. The upper layer will be referred to as fraction L (light) and the lower as H (heavy).

Electron Microscopy

Disrupted spore fractions were fixed with osmium tetroxide by the method of Kellenberger *et al.* (16) and embedded in Araldite (9). The blocks were sectioned onto 20 per cent acetone and observed in a Siemens Elmiskop I electron microscope.

Analytical Methods

Except for carbohydrate estimations, the analytical figures reported are the average of determinations on two samples.

Total nitrogen was estimated by the Kjeldahl method of McKenzie and Wallace (23). A digestion time of 45 minutes was used since other digestion procedures gave low recoveries of N from dipicolinic acid.

α-Carboxylic amino N was estimated on 6 N HCl (16 hours, 107°C) hydrolyzates by the manometric method of Van Slyke *et al.* (36).

Phosphorus was determined by Allen's (1) method.

Hexosamine was estimated after hydrolysis, by

the Rondle and Morgan (28) method using a glucosamine standard. Muramic acid gives a lower color yield than glucosamine (31). Hydrolysis was carried out for 5 hours at 100°C in 6 N HCl, which was found to be optimum for *B. coagulans* spores.

Dipicolinic acid (DPA) was estimated from the absorbance at 273 mμ of an aqueous solution of ether extracts of spore fractions acidified with 0.1 N HCl.

Sugars were identified by paper chromatography of deionized 2 N HCl hydrolyzates, using the solvents ethyl acetate + pyridine + water (2:1:2 *v/v*), *n*-butanol + acetic acid + water (18:2:5 *v/v*), and 80 per cent aqueous phenol. Descending chromatograms were run on Whatman no. 1 paper for 24 to 40 hours. Spots were detected with *p*-anisidine, aniline hydrogen phthalate, and alkaline silver nitrate. Total carbohydrate was estimated by the anthrone method (12) and rhamnose by the method of Dische and Shettles (6). A correction, due to rhamnose present, was applied to the anthrone results to give the hexose content, expressed as glucose, the principal sugar. Ribose, which was generally present, enhanced the extinction given by glucose in the anthrone reaction (14), and a glucose-ribose standard was used. Carbohydrate found by this reaction would be overestimated when greater quantities of ribose were present, but underestimated when galactose or mannose was present.

Amino acids were estimated by the method of Kay *et al.* (15) after separation of neutral and acidic amino acids by two-dimensional paper chromatography (19), and of basic amino acids and glucosamine by paper electrophoresis in collidine–acetic acid buffer (pH 6.6) for 100 minutes at 50 v/cm.

RESULTS

Electron Microscopy of Insoluble Fractions

Electron micrographs of thin sections of spores have revealed a number of integuments surrounding the spore protoplasm (Fig. 1), (24). It was therefore necessary to define which of these were present in each of the fractions analyzed and to compare the morphological structures in each species. Electron micrographs of thin sections of the insoluble fractions (Figs. 2 to 5) showed the presence of four morphological components. These were (*a*) the electron-opaque outer coat, (*b*) an underlying laminated inner spore coat, (*c*) cortical material, and (*d*) a thin cortical membrane. The appearance and structure of these components are shown more clearly in electron micrographs of partially disrupted or disrupted spores (Figs. 6 to 9).

Outer and inner coats, which usually remained attached to each other, were major components in each preparation used for analysis (Figs. 2 to 5). The *B. cereus* and *B. subtilis* I fractions (Figs. 2 and 3) consisted chiefly of the two spore coats. The outer coat was much thinner in *B. stearothermophilus* (Figs. 5 and 12) than in the other species. In *B. cereus* and *B. subtilis* the outer coat showed a network type of structure (Figs. 2, 3, 6, and 7) not seen in *B. coagulans* (Figs. 4 and 8) and *B. stearothermophilus* (Figs. 5 and 9), where the structure had a uniform fine-grained appearance. The inner coat was seen to be laminated and similar in appearance in all the species. Cortical material, often attached to the inner spore coat, was present in considerable amount in *B. coagulans* and *B. stearothermophilus* I fractions (Figs. 4 and 5). Much smaller quantities were found in the *B. cereus* and *B. subtilis* preparations (Figs. 2 and 3). The cortical material in micrographs of disrupted spores had the appearance of a structural residue from which soluble constituents had been leached (Figs. 4, 5, 7, and 8). Partial autolytic degradation of the cortex may have occurred during preparation of the fractions. A membrane component, clearly distinct from either of the coat layers, was present in *B. coagulans*, *B. subtilis*, and *B. stearothermophilus*. In many electron micrographs (Figs. 3 to 5, 7, and 8), this membrane remained attached to the inner surface of the cortex. In a partly disrupted spore (Figs. 1 and 9) it is located between the cortex and the spore protoplasmic membrane and may be the germ cell wall. Until further evidence is obtained on the nature of this membrane, it will be referred to as the "cortical membrane." In *B. coagulans* most of these cortical membranes were concentrated in the L fraction.

Analyses of Insoluble Fractions

The results for four species together with the ratios of the heat resistance of the spores from which the integuments were prepared are given in Table I. Generally a higher proportion of insoluble material was obtained from the more heat-resistant species, *B. coagulans* and *B. stearothermophilus*, but this would be affected slightly by losses during preparation. Greater amounts of hexosamine occurred in the I fractions of the more heat-resistant spores. Small amounts of

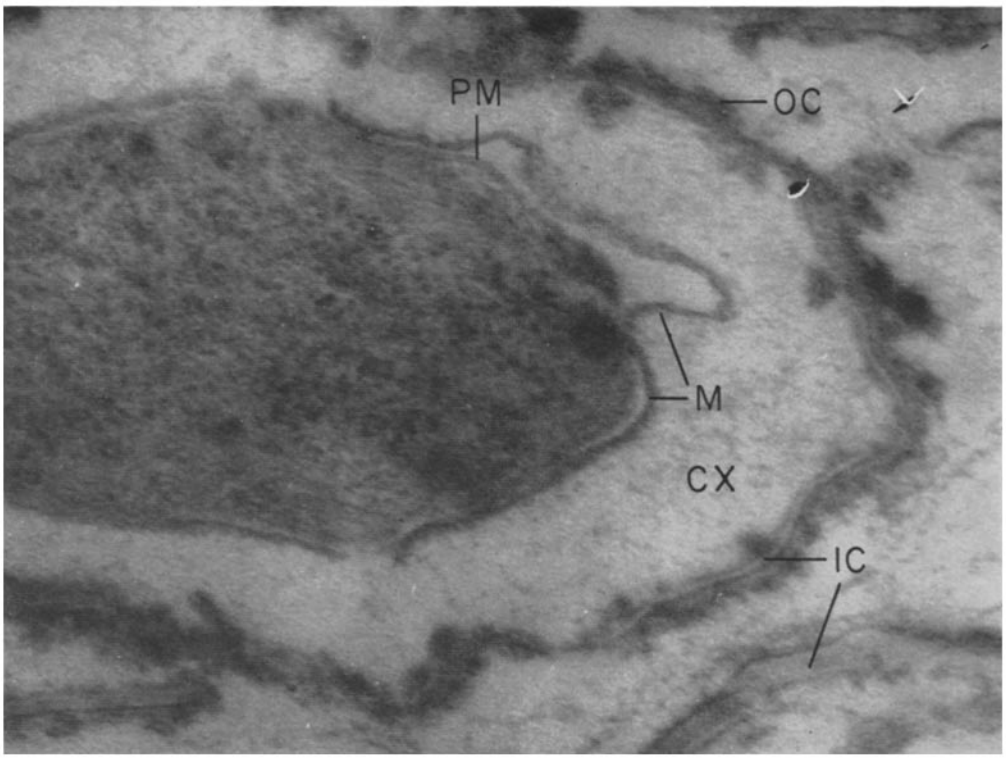


FIGURE 1

Sections of partly disrupted spores of *B. stearothermophilus* showing the relative location and structure of the outer (*OC*) and inner (*IC*) coats, cortex (*CX*), a membrane (*M*) at the inner surface of the cortex, probably the germ cell wall, and the spore protoplasmic membrane (*PM*). The protoplasmic membrane has the typical appearance of a "unit" membrane and is distinct from the cortical membrane (*M*); it was not observed in integument preparations. × 130,000.

FIGURE 2

B. cereus integument preparation. This consists almost entirely of coat fragments (*OC*, *IC*). Very little residual cortex and debris and no cortical membranes can be seen. × 60,000.

FIGURE 3

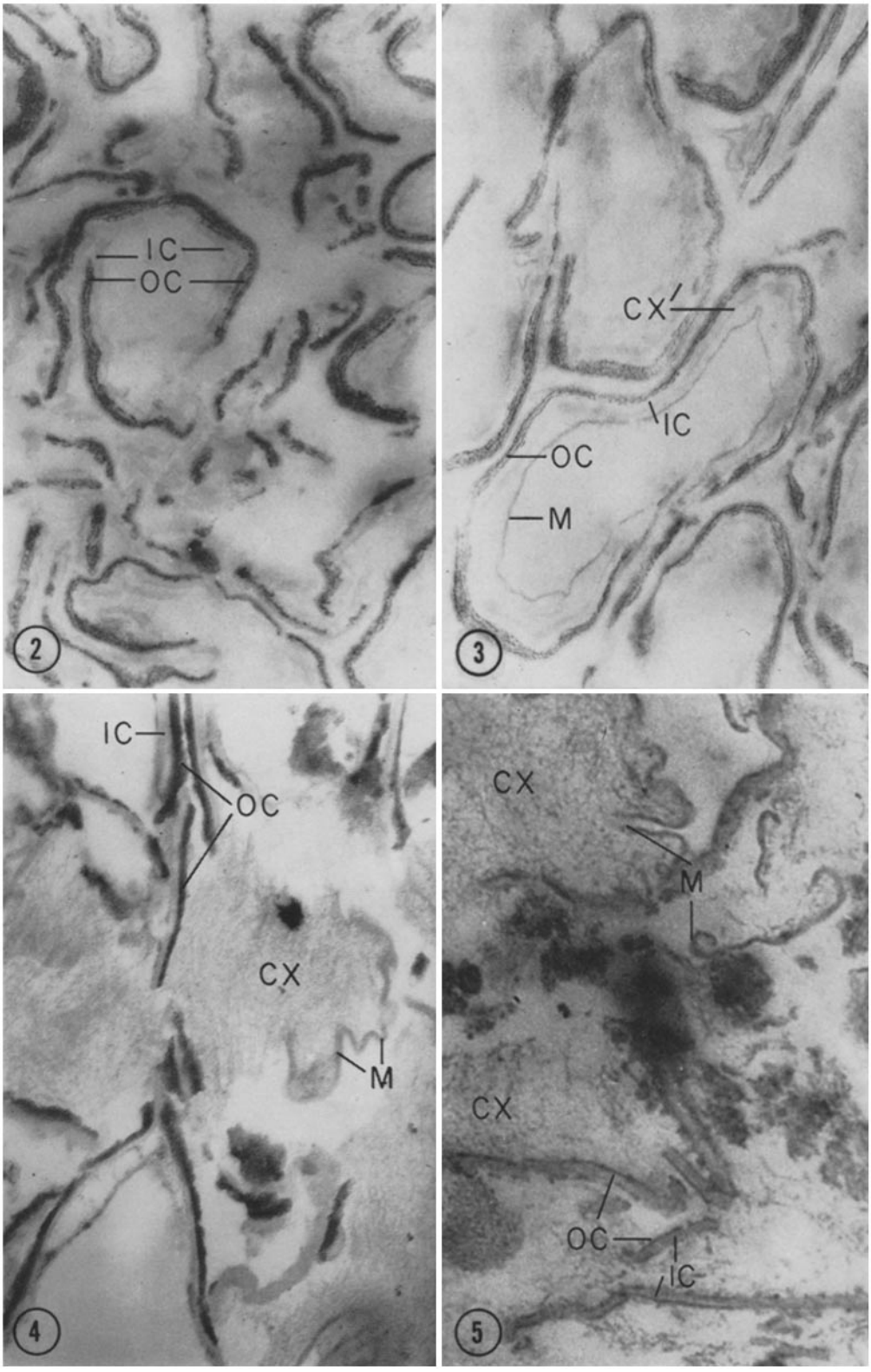
B. subtilis integument preparation. This consists largely of spore coats (*IC*, *OC*), but with some residual cortex (*CX*) and cortical membranes (*M*) present within the coats. × 60,000.

FIGURE 4

Section of the heavy (H) fraction of the insoluble material from disrupted *B. coagulans* spores. This preparation contains outer coats (*OC*), inner coats (*IC*), and cortical material (*CX*), together with a few cortical membranes (*M*). The *B. coagulans* light (L) fraction contained the same components as the H fraction; however, there were many more cortical membranes but fewer coats present. × 60,000.

FIGURE 5

Thin section of the *B. stearothermophilus* integument preparation (I fraction). The preparation consists of spore coats (*IC*, *OC*), cortical residue (*CX*), and cortical membranes (*M*). The cortex has a fibrous structure similar to that seen in the *B. coagulans* integument preparations. × 60,000.


IC
OC
2
CX
IC
OC
M
3
IC
OC
CX
M
4
CX
M
CX
OC
IC
5

several sugars, including traces of ribose, were present in all spore fractions (Tables I, II). Hexose estimation was subject to errors due to the complex mixture of component sugars, interference from proteins and amino acids, and the effect of ribose present (see Methods). The hexose content of both I and S fractions was related to heat resistance (Tables I, IV). No uronic acids were detected by the Dische carbazole method (5) and no polyols were detected on paper chromatograms of the 2 N HCl hydrolyzates with alkaline silver nitrate. Significant amounts of phosphorus were present in each I fraction (Table I).

The amino acid constituents of the I fractions were estimated semiquantitatively after paper chromatographic separation (Table III). Other amino acids common to protein hydrolyzates, including cystine, proline, and methionine, were present, but were not estimated. No hydroxyproline or β-alanine was detected. The typical spore peptide constituents (DAP, glucosamine, and muramic acid) were also present, although a considerable loss of amino sugars had occurred during hydrolysis. The relative content of the spore peptide constituents increased with an increase in heat resistance of the spores (Tables I, III). Amino acids derived from the protein component, in particular glycine and tyrosine, tended to be present in inverse proportion to DAP, hexosamine, and heat resistance.

Ultraviolet Absorption Spectra of Spore Coat Extracts

The occurrence of both ribose and phosphorus in spore integument preparations suggested the presence of nucleic acid. As Barkulis and Jones (2) have shown that the presence of ultraviolet-absorbing material cannot be properly demonstrated by spectrophotometry of cell wall suspensions, the ultraviolet spectra of hot acid extracts (2) were examined. Extracts from each of the I fractions showed significant ultraviolet absorption (Fig. 10). A peak at 255 mμ was present in the *B. stearothermophilus* extract, which corresponded, according to spectrophotometric data (21), to a maximum RNA content of 4 to 5 per cent. The RNA may be present, adsorbed upon the spore coats, as is found with the streptococcal cell walls (2). The other extracts showed much

FIGURE 6

Section of a *B. cereus* spore showing more clearly the layered appearance of the outer coat (*OC*) and the regular laminar structure of the inner coat (*IC*). Cortex, *CX*; plasma membrane, *PM*. × 190,000.

FIGURE 7

Section of a disrupted *B. subtilis* spore. The outer coat (*OC*) has a layered structure similar to that in *B. cereus* (Fig. 6), and the inner coat (*IC*) shows the laminar structure common to each species. The cortical residue (*CX*) consists of an open network of slightly granular fibrils similar in appearance to that seen in *B. coagulans* and *B. stearothermophilus* integument preparations (Figs. 4 and 5). Cortical membrane, *M*. × 150,000.

FIGURE 8

Section of *B. coagulans* integument preparation showing the structure and relative location of each morphological component. The cortical membrane (*M*), residual cortex (*CX*), and inner coat (*IC*) each have a structure similar to that seen in the other species. The electron-opaque outer coat (*OC*), however, does not show the layered structure seen in *B. cereus* and *B. subtilis*, but possibly has a slightly granular structure like that in *B. stearothermophilus* (Fig. 9). × 150,000.

FIGURE 9

Section through part of two disrupted *B. stearothermophilus* spores showing more clearly the lamination in the inner coats (*IC*). At the inner boundary of the cortex (*CX*) the cortical membrane (*M*) can be distinguished from the plasma membrane (*PM*). Outer coat, *OC*. × 130,000.

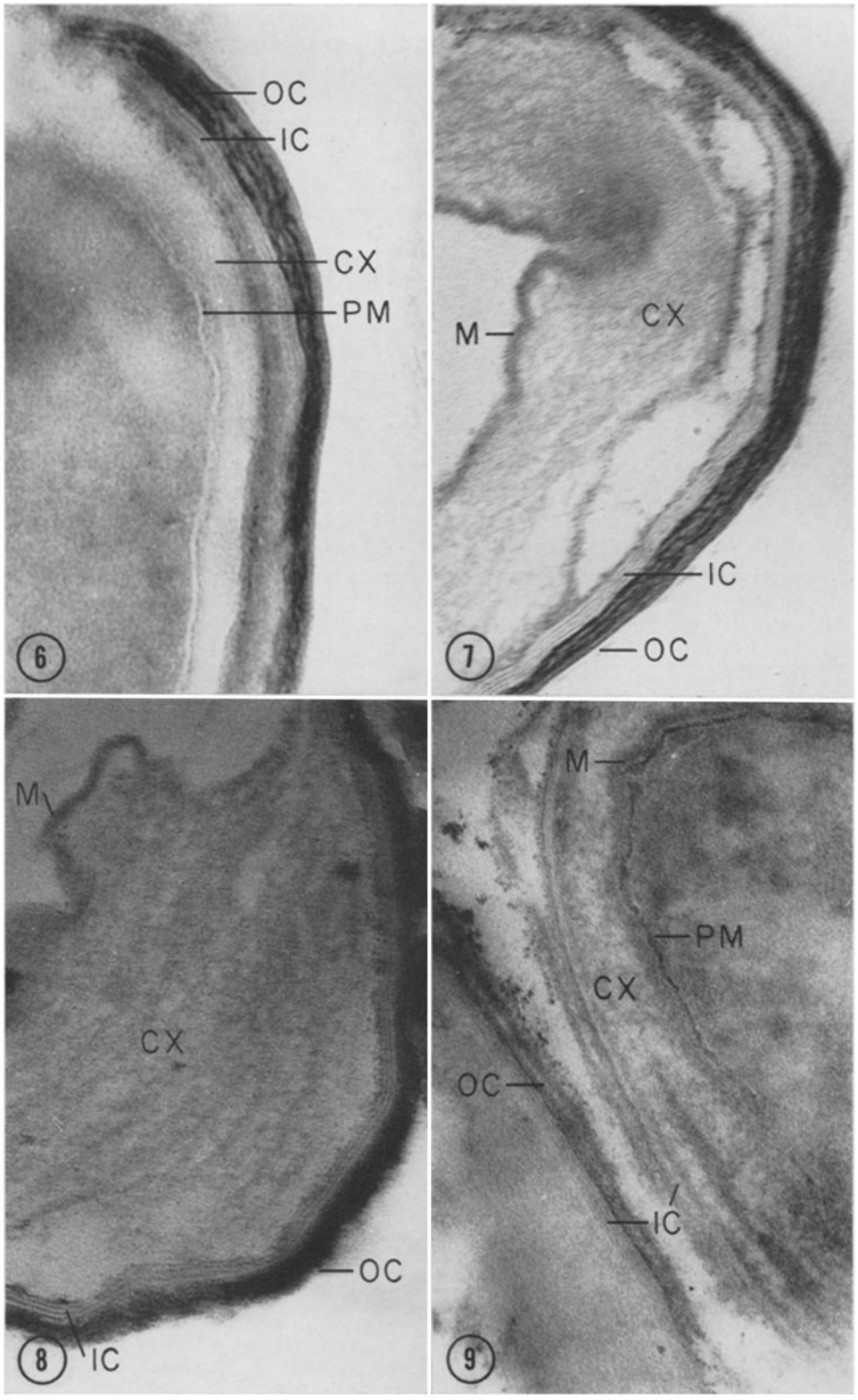

OC
IC
CX
PM
6
M
CX
IC
OC
7
M
CX
OC
8
IC
M
PM
CX
OC
IC
9

TABLE I

Chemical Analysis of Spore Integument Preparations

(Per cent dry weight)

	B. cereus	*B. subtilis*	*B. coagulans*		*B. stearothermophilus*
			Heavy	Light	
Heat resistance (ratio) of spores	1.0*	1.3	114	—	189
Total N	10.6	11.5	9.7	9.7	11.1
α-Carboxylic amino N	6.9	7.5	6.2	4.5	7.0
Total P	1.32	1.26	2.80	1.60	0.70
Hexosamine (as glucosamine)	2.27	2.40	4.81	11.9	7.06
Hexose (as glucose)	0.4	0.5	0.7	2.5	4.3
Methyl pentose (as rhamnose)	0.25	0.24	0.0	0.0	0.0
Yield of preparation (% whole spores)	48	52	26	40	60

* Decimal reduction time (D) of 0.36 minutes at 110°C.

TABLE II

Sugars Present in Acid Hydrolyzates of Spore Fractions

	B. cereus		*B. subtilis*		*B. coagulans*			*B. stearothermophilus*	
Sugar	I*	S*	I	S	H‡	L‡	S	I	S
Glucose	+	++	+	++	+	+++	+++	+++	+++
Galactose	±	+	±	+	±	++	+++	−	+
Mannose	−	−	−	−	−	−	−	±	++++
Rhamnose	+	+++	+	++	−	−	−	−	−
Ribose	±	++	±	++	±	+	+++	+++	++

* I, insoluble fraction—spore integuments; S, soluble fraction—spore contents.

‡ H, heavy fraction; L, light fraction.

less absorption than that of *B. stearothermophilus* at 255 mμ and had a maximum at 270 mμ, suggesting the presence of DPA. In the *B. coagulans* H and L extracts, both of which showed a more intense and distinct maximum than *B. cereus* or *B. subtilis*, no more than 1 per cent DPA would be present according to calculations.

Analyses of Soluble Fractions

Analyses of the soluble component of spores are given in Table IV. The concentration of DPA in these fractions increased strikingly with heat resistance mainly because the DPA, on disruption of the spore, goes into the soluble fraction, and less soluble material was obtained from the more heat-resistant species. It was only with *B. stearothermophilus* that whole spores showed a greater DPA content (38). Most of the hexosamine in *B. cereus* and *B. subtilis* spores was fractionated into the soluble fraction, whereas in *B. coagulans* and *B. stearothermophilus* the hexosamine was associated chiefly with the insoluble integuments. The difference in the proportion of soluble to insoluble material obtained from the different species appears to be due largely to the degree of solubilization of the hexosamine-containing component. The amino acid patterns of hydrolyzed S fractions were similar to those of hydrolyzed I fractions (Table III) except that no cystine was detected in the former. The spore hexose and phosphorus contents (Tables I, II, IV, V) were not associated specifically with either the I or the S fractions, except that in *B. stearothermophilus* more glucose occurred in the I and mannose mainly in the S fraction. Rhamnose, when present, was predominantly in the S fraction (Tables I, II, IV).

Fractionation of the Insoluble Integuments

In view of the complex morphological composition of the spore integument preparations, the possibility of mechanical fractionation was explored. On centrifugation of disrupted *B. coagulans* and *B. stearothermophilus* spores, several distinct layers appeared in the sediment. It proved possible to separate these layers by resuspending each layer in turn, and purifying each separated layer by further centrifugations and resuspensions (fractional resuspension).

The effect of the fractionation was studied by chemical analysis and electron microscopy. The two insoluble fractions obtained from *B. coagulans* (H and L) were analyzed with the other integument preparations (Tables I, II, III). The *B. stearothermophilus* preparation gave three insoluble fractions. Analytical data are given in Tables III and V. In both species the uppermost layer in the sediment contained more DAP, glucosamine, and alanine, and correspondingly less of the other amino acids, particularly tyrosine and glycine. The bottom layer of *B. stearothermophilus* (fraction 1) was almost free of DAP and glucosamine, but the differences between the other fractions and the two *B. coagulans* fractions were not so sharp.

In both species the lowest layer (fractions H and 1) contained most of the inner and outer coats. In *B. stearothermophilus* fraction 1 these were the only components seen (Fig. 12), but *B. coagulans* H fraction also contained cortical matter and occasional cortical membranes (Fig. 4). The lighter fractions consisted mainly of cortex and cortical membranes (Fig. 11), but coat fragments were also present. Protein matter, not removed by washing with water, may have been present in integument preparations, and silicone antifoam was probably adsorbed on the coat layers.

TABLE III

Amino Acid Composition of Spore Integument Preparations and Insoluble Fractions

(Mole ratios)

Amino acid*	*B. cereus*	*B. subtilis*	*B. coagulans*		*B. stearothermophilus*	*B. stearothermophilus*		
			Heavy	Light		1§	2§	3§
Alanine	9.7	9.9	13.2	13.5	15.6	13.6	13.6	16.0
Glutamic acid	10	10	10	10	10	10	10	10
Glucosamine	3.6	2.6	4.4	7.3	5.1	1.4	5.3	—
Hexosamine‡	4.8	4.4	11.3	26	9.4	1.3	7	18
DAP	1.3	1.5	2.1	3.4	2.3	0.7	2.7	3.5
Glycine	21.7	23.5	13.6	9.6	12.5	25.0	16.1	9.4
Serine	9.8	10.1	5.8	4.6	3.1	4.7	3.6	2.3
Threonine	6.7	6.9	5.1	3.9	4.1	5.8	4.5	3.2
Valine	6.8	5.9	4.9	3.6	5.3	6.9	5.1	6.2
Leucine/isoleucine	11.3	9.6	8.8	6.5	8.4	10.9	9.0	9.8
Phenylalanine	5.7	6.4	3.5	3.8	2.9	5.7	3.5	2.8
Tyrosine	10.0	10.2	6.0	3.9	5.3	11.6	5.7	2.8
Aspartic acid	9.6	9.8	6.8	5.6	6.4	10.4	7.5	6.6
Arginine	7.2	6.1	6.3	5.1	4.8	5.6	4.0	—
Histidine	7.4	7.1	4.6	2.4	3.3	3.8	3.9	—
Lysine	16.0	14.2	7.4	6.2	6.4	5.8	4.3	—

* Amino acids and glucosamine determined on 6 N HCl hydrolyzates by paper chromatography (see Methods).

‡ Hexosamine determined on 4 N HCl hydrolyzates by the Rondle and Morgan method (28).

§ See note to Table V.

Location of the Mucopeptide Material

The spore integument preparations of each species and the *B. coagulans* and *B. stearothermophilus* fractions evidently consisted largely of different proportions of two components. One of

these contained the typical spore peptide constituents DAP, hexosamine, and part of the alanine and glutamic acid, while the other component had typically protein constituents. The content of the mucopeptide component was closely related to the proportion of cortical material and cortical membranes seen in electron micrographs, suggesting that the mucopeptide is located specifically in one or both of these structures. This possibility has been confirmed by enzyme studies, which are described in the following paper (39).

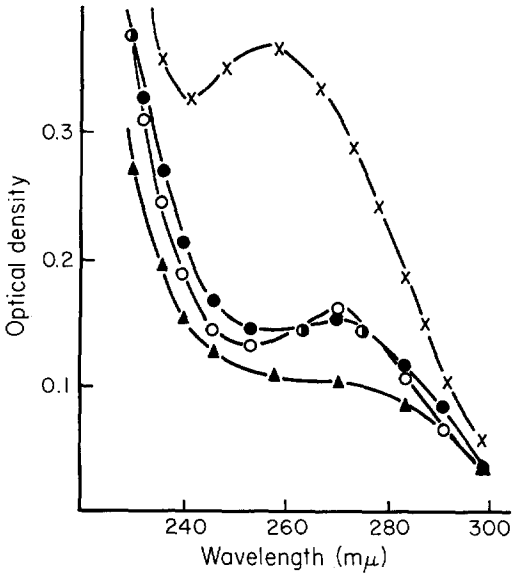


FIGURE 10

Ultraviolet absorption spectra of material extracted from spore coat preparations with HCl (pH 2, 5 minutes, 100°C). Extract from 0.25 mg coats per ml after neutralization. Crosses, *B. stearothermophilus*; solid circles, *B. coagulans* heavy fraction; open circles, *B. coagulans* light fraction; solid triangles, *B. subtilis* and *B. cereus*.

DISCUSSION

Electron microscopy of sectioned material has demonstrated that so-called spore coat preparations are variable mixtures of several of the spore integuments. The value of analyses of these preparations, in defining the chemical composition of particular spore integuments, therefore, is limited. Further progress in this respect will be greatly assisted by the development of techniques for separating the morphological components of the spore. Fractional resuspension by itself did not achieve a complete separation of components from disrupted spores. Methods are at present under study for parting the coat layers from each other, and for complete fractionation of the spore integuments. Homogeneous preparations of the inner and outer coats of *B. coagulans* have been obtained by treating disrupted spores with lysozyme to dissolve the residual cortex and cortical membrane, and with trypsin to digest cytoplasmic protein.

The structure of the spores of these species as revealed by thin sections of disrupted spores indicated that they have a similar morphological organization in that they are composed of two coats, cortex and cortical membrane, around the spore protoplasm (cf. 24). The structure of the outer coat, however, shows differences between the species, varying from the loose network type of structure in *B. cereus* and *B. subtilis* to the very electron-opaque structure in *B. coagulans* and the thin outer coat of *B. stearothermophilus*. These differences may be related to their resistant properties. The inner coat in each species appeared lami-

TABLE IV

Chemical Analyses of Spore Contents Fractions

(Per cent dry weight)

	B. cereus	*B. subtilis*	*B. coagulans*	*B. stearothermophilus*
Total N	9.1	9.7	—	9.2
α-Carboxylic amino N	—	—	5.6	4.3
Total P	2.3	0.7	2.2	0.8
Hexosamine (as glucosamine)	5.5	7.0	2.8	2.6
Hexose (as glucose)	2.3	0.5	2.1	3.9
Methyl pentose (as rhamnose)	1.09	0.75	0.0	0.0
Dipicolinic acid	9.4	12.3	17.5	26.4
Fraction (% whole spores)	52	48	34	40

nated, but the laminations were not so well resolved in *B. cereus* and *B. subtilis*. The cortical membrane appears to adhere more strongly to the cortex than to the spore protoplasmic membrane (Fig. 1). This may be expected since their morphogenesis (24) and composition appear similar.

The analytical results for the preparations containing most spore coat and least cortical material, *B. stearothermophilus* fraction 1 and the *B. cereus* and *B. subtilis* I fractions, suggest that the spore coats consist mainly of protein. The hexosamine-DAP component appeared to be associated specifically with the residual cortex and cortical membrane, phorus content (Table I). The absence of ribitol-type compounds (30) and, except in the *B. stearothermophilus* coat preparation, the absence of sufficient amounts of nucleic acid to account for it, suggest that the phosphorus may be present in a "residual P" fraction as reported in *B. megaterium* spores by Fitz-James (7). Fitz-James and Young (8) correlated a high residual P content of coat preparations from strains of *B. megaterium* with the presence of a dense outer coat. Our observation that *B. stearothermophilus* possessed a much thinner outer coat, and had much less phosphorus than the other species, supports their conclusion that

TABLE V

Chemical Analysis of B. stearothermophilus Fractions

(Per cent dry weight)

	Fraction				
	1	2	3	4	5
Yield	14	10	5	20	50
Total N	10.8	10.8	10.4	9.9	8.4
α-Carboxylic amino N	7.0	9.4	3.6	4.7	3.8
Total P	0.5	0.9	0.8	1.2	0.4
Hexosamine (as glucosamine)	0.7	6.7	10.0	7.7	7.2
Hexose (as glucose)	2.7	2.6	3.5	—	2.3
Dipicolinic acid	—	—	—	11.1	20.8
Sugars:					
Glucose	+	+	++	±	−
Mannose	−	±	±	±	+
Ribose	++	++	++	++	+

Fractions obtained by fractional resuspension and centrifugation of disrupted *B. stearothermophilus* spores: 1, lowest layer of sediment; 2, intermediate layer; 3, top layer; 4, water washings from sediment; 5, soluble material.

since in the various fractions those containing the most hexosamine and DAP also contained the most cortex and cortical membrane. In the following paper (39) more detailed evidence will be presented to support this conclusion. Variation in the amount of mucopeptide or cortical material in the integument preparations of different organisms probably arises from differences in the rate of autolytic degradation of the cortex occurring during the disruption and washing procedures. The spore coat preparations of Strange and Dark (32), Salton and Marshall (30), and Hunnell and Ordall (13) all contained small amounts of hexosamine or DAP and so probably contained residual cortical material in addition to the spore coats.

As previously reported (30, 32), many spore coat preparations have a relatively high phos-

the residual P occurs in the dense outer spore coat. Higher amounts of sugars in both the soluble and insoluble fractions of *B. stearothermophilus* may suggest important differences in composition of the spores of this species, which forms the most heat-resistant spores known. Carbohydrate constituents have not been identified previously in spores or spore coats. The presence of rhamnose in *B. cereus* and *B. subtilis* spores (Table II) but not in their vegetative cells (29, 30, 40) suggests that the carbohydrate components are from spores and not residues of vegetative cells.

The correlation which appeared between heat resistance and the DPA concentration in the soluble fraction of spores does not necessarily mean that a higher concentration of DPA exists

in the protoplasm of the more heat-resistant species. It is not certain where the DPA is located in the spore, and varying amounts of soluble material including the spore peptide component may be derived from other regions of the spore such as the cortex. Possibly some protoplasmic material such as nucleic acid or protein, particularly in the more heat-resistant species, was fractionated with the coat material.

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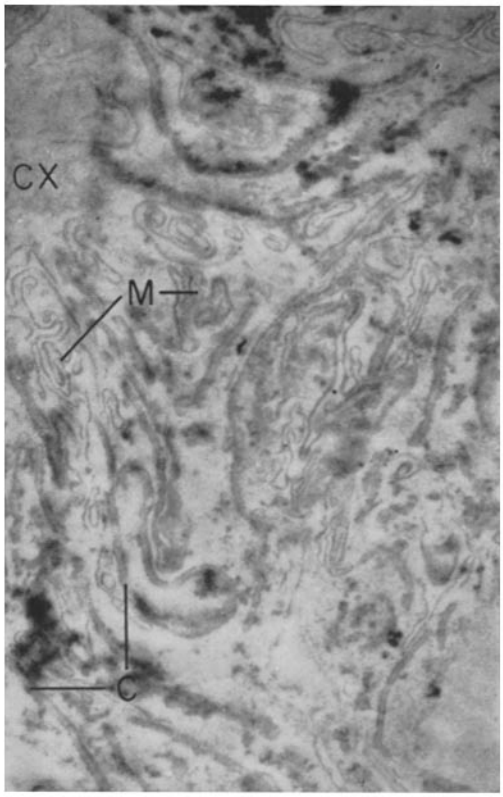

FIGURE 11

Section of *B. stearothermophilus* fraction 3 (top layer of sediment). This contains mostly cortical material (*CX*) and membranes (*M*) with some coat fragments (*C*). × 60,000.

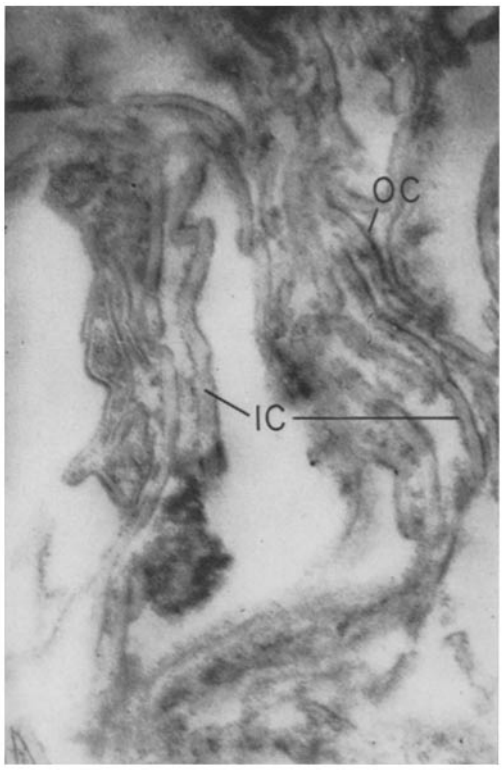

FIGURE 12

Section of *B. stearothermophilus* fraction 1 (bottom layer of sediment), consisting largely of coat fragments (*IC*, *OC*) practically free of cortex or membranes. × 60,000.

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