

ELECTRON MICROSCOPY OF SPORES OF *BACILLUS MEGATERIUM* WITH SPECIAL REFERENCE TO THE EFFECTS OF FIXATION AND THIN SECTIONING

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ABSTRACT

Resting spores of *Bacillus megaterium* appear uniformly opaque and undifferentiated under the electron microscope. Germinated spores and spores which have lost their dipicolinic acid underwent characteristic changes in structure. Spores fixed with KMnO_4 lose their dipicolinic acid. Spores fixed with OsO_4 under certain conditions retain their dipicolinic acid. When conventional sectioning procedures are used with either method of fixation, abnormal spore structure is produced as a result of the solution of cellular constituents. Dry sections of unfixed spores embedded in methacrylate reveal the spore structure in a more normal state. Indirect evidence has been obtained for the existence of a penetration barrier at or near the outer edge of the cortex.

INTRODUCTION

Of the many electron microscope studies of thin sections of bacterial spores (Robinow, 1953; Chapman, 1956; Mayall and Robinow, 1957; Tokuyasu and Yamada, 1959; Hashimoto and Naylor, 1958; Holbert, 1960; Fitz-James and Young, 1959; Fitz-James, 1959), a few mention difficulties arising from the fixation step (Robinow, 1953; Tokuyasu and Yamada, 1959; Fitz-James, 1959). The other procedures in electron microscopy, namely dehydration, embedding, and sectioning, may also influence conclusions as to spore morphology.

For example, one could visualize a loss or distortion of structures composed of water-soluble materials taking place during preparation of sections for electron microscopy. Hashimoto and Gerhardt (1960) allude to the loss of dipicolinic

acid from spores during sectioning, and Robinow (1960a) likewise points out that spore components may dissolve in the water used in the preparation of sections. For the most part, however, little experimental work on these potential complications has been published. The present study was designed to investigate some of these problems, with special reference to the fate of dipicolinic acid, and their implications for spore morphology as judged by electron microscopy.

MATERIALS AND METHODS

Suspensions of *Bacillus megaterium*, Texas strain, which consisted entirely of refractile spores resistant to heat (Rode and Foster, 1960a), were stored in deionized water at 4°C. Aliquots were centrifuged as the spores were needed.

One per cent osmium tetroxide in veronal-acetate buffer, pH 7.2 to 7.4, and 2 per cent aqueous potassium permanganate (Mollenhauer, 1959) were employed as fixatives. Following fixation, the spores were washed several times with distilled water and dehydrated by serial passage through 30, 50, 70, 95, 100 and 100 per cent aqueous ethanol. The spores were then distributed in a solution of 1 part mixed methacrylate (8 parts butyl methacrylate, 1 part methyl methacrylate) and 2 parts absolute ethanol, and allowed to stand for 1 hour at approximately 26°C. They were centrifuged and suspended in a solution of 2 parts mixed methacrylate containing 1 per cent benzoyl peroxide catalyst, and 1 part absolute ethanol. After 2 or 3 hours of standing at 26°C, the spores were centrifuged, resuspended in pure methacrylate-catalyst mixture, and allowed to stand for another hour at 26°C. Then they were embedded in capsules containing pure methacrylate-catalyst mixture. Polymerization took place during storage at 37°C.

Thin sections were cut with a Porter-Blum microtome fitted with a freshly broken glass knife. Most of the sections were cut wet, and were floated on distilled water or lanthanum nitrate solution (Mayall and Robinow, 1957), or they were collected in octane. Other sections were cut dry, in which case they were usually somewhat thicker, thereby facilitating manual transfer of sections from the knife edge to the grids without flotation.

All of the specimens were mounted on 200-mesh copper grids surfaced with collodion on which a thin layer of carbon had been evaporated. A model EMU 3-D RCA electron microscope was employed. Initial magnifications were approximately 8,000 to 22,000. Dipicolinic acid was estimated spectrophotometrically (Perry and Foster, 1955; Martin and Foster, 1958) and hexosamine colorimetrically (Immers and Vasseur, 1950).

RESULTS

Microscopy of Whole Spores

RESTING SPORES

Spores of *B. megaterium*, Texas strain, appear as undifferentiated, oval bodies uniformly opaque to electrons (Fig. 1). They vary considerably in size. The polar knob and equatorial ridge seen in other strains (Fitz-James and Young, 1959; Levinson and Wrigley, 1960) are not evident.

PHYSIOLOGICALLY GERMINATED SPORES

Spores germinated with L-alanine and inosine (Hills, 1949; Powell, 1957) show a characteristic change (Fig. 2). Two prominent, collapsed and folded coats which appear to be non-rigid are the most conspicuous features of such spores. A large fragment of the outer coat is clearly visible, whereas the entire inner coat appears intact. These coats surround an opaque, featureless core.

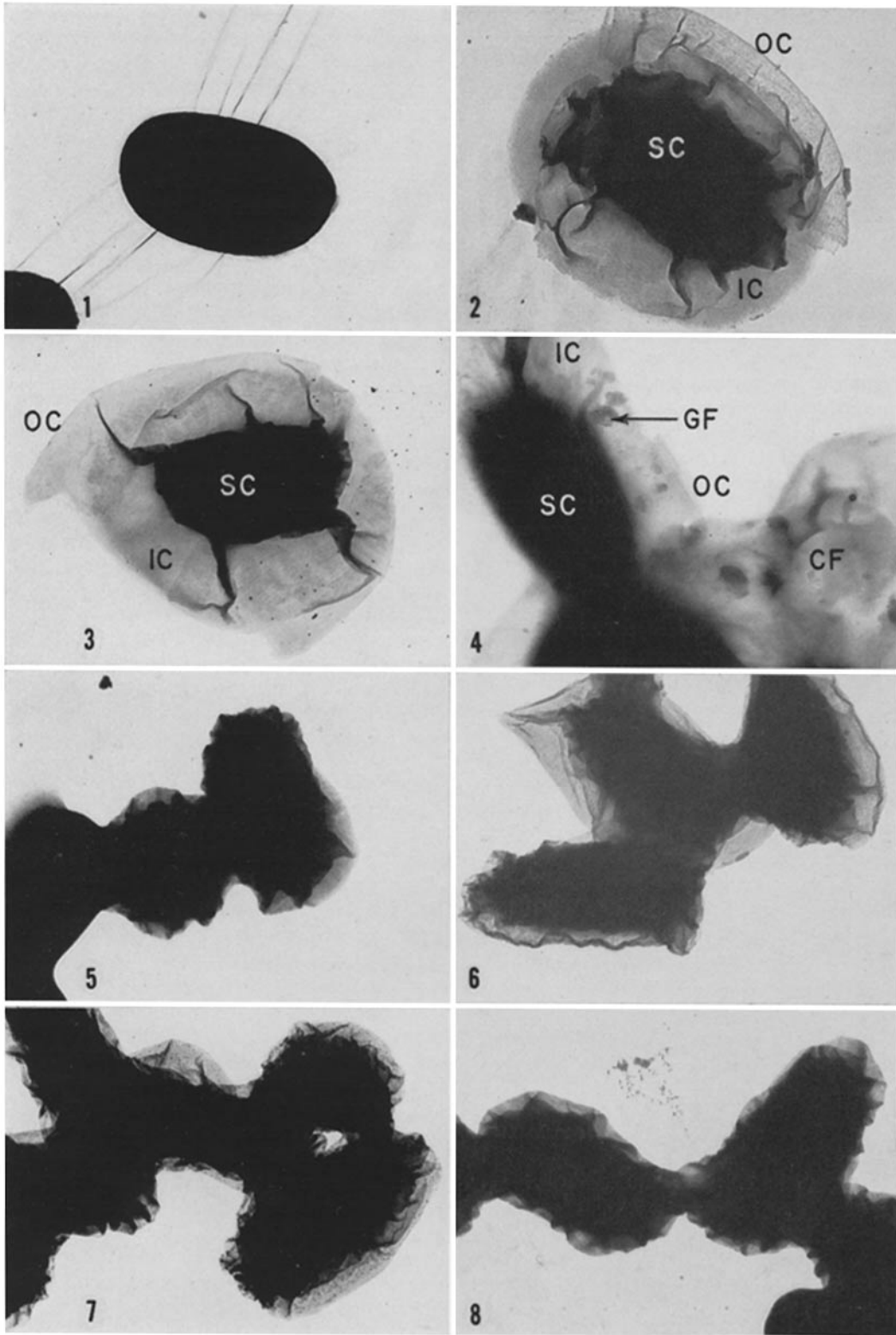
CHEMICALLY GERMINATED SPORES

A change similar to that brought about by physiological germination occurs in spores germinated with *n*-dodecylamine (Rode and Foster, 1961*a, b*). The expanded double coats and the opaque core are visible in Fig. 3.

FIGURES 1 THROUGH 8

Electron micrographs of whole spores. Structures identified are outer coat (*OC*), inner coat (*IC*), spore core (*SC*), coat fraction (*CF*), and glass fragments (*GF*). Magnification, 30,000.

- FIGURE 1 Resting spore.
- FIGURE 2 Spore germinated 20 minutes at 40°C with a mixture of L-alanine (10^{-4} M) and inosine (1.4×10^{-4} M) in 0.067 M phosphate buffer, pH 8.
- FIGURE 3 Spore germinated 2 minutes at 40°C with 6×10^{-5} M *n*-dodecylamine in 0.067 M phosphate buffer, pH 8.
- FIGURE 4 Spore germinated mechanically with fine glass particles in the Mickle tissue disintegrator.
- FIGURE 5 Spores boiled 15 minutes in water.
- FIGURE 6 Spores treated 2 hours with 10 per cent H_2O_2 at 56°C.
- FIGURE 7 Spores treated 2 hours with 80 per cent ethanol at 56°C.
- FIGURE 8 Spores treated 1 hour with 0.01 per cent cetyl trimethyl ammonium bromide at 56°C.



MECHANICALLY GERMINATED SPORES

Spores of *B. megaterium* germinated by mechanical abrasion with fine glass particles (Rode and Foster, 1960a) contain cells (Fig. 4) whose appearance corresponds to physiologically and chemically germinated spores (Figs. 2 and 3). Some spore coats from which the opaque core has been lost are also visible in Fig. 4.

SPORES WITHOUT DIPICOLINIC ACID (OTHER TREATMENTS)

Certain non-germinative treatments simulate germination by inducing the release of dipicolinic acid from spores (Rode and Foster, 1960b). Electron micrographs have been prepared of spores which have been thus affected by boiling (Fig. 5), by hydrogen peroxide (Fig. 6), by ethanol (Fig. 7), and by cetyl trimethyl ammonium bromide (Rode and Foster, 1960c) (Fig. 8). The spores from each of these treatments exhibit a morphologically intact, opaque core surrounded by wrinkled coats. The appearance is similar to that of germinated spores (Figs. 2, 3, and 4).

The enlargement of the spores during germination results from enlargement of the core and subsequent flattening of the flaccid coats on the grids during drying. Spores in which non-germinative loss of dipicolinic acid has been induced do not appear enlarged (Figs. 5 through 8), possibly because denaturation of the cytoplasmic proteins prevents hydration. The over-all dimensions of these spores are comparable to those of ungerminated spores, but the opaque central portion appears smaller than the uniformly opaque body of the resting spore. If the dipicolinic acid occupies a site peripheral to the core (Mayall and Robinow, 1957), its loss by induction might account for the diminished central area of opacity. The fact that the core of germinated spores is apparently not smaller (Figs. 2, 3, and 4) could be due to the hydration and swelling mentioned previously.

Microscopy of Thin Sections

A study was undertaken of fixation, embedding, and sectioning techniques for preparation of thin sections of bacterial spores, with special reference to the fate of dipicolinic acid.

KMnO₄ AND OsO₄ AS FIXATIVES

Spores fixed with KMnO₄ and OsO₄ were compared. Fixation in each case took place for 2

hours at 26°C followed by routine dehydration, embedding in methacrylate, and wet sectioning with flotation on water. The 2-hour fixation period was selected on the basis of preliminary tests wherein both the time and the temperature of fixation with KMnO₄ and OsO₄ were varied widely.

The KMnO₄ fixation yielded spores with a uniform and characteristic appearance (Figs. 9 and 10). They had a thin outer coat, a thick inner coat, a wide zone of electron transparency, and a core wall and plasma membrane (Tokuyasu and Yamada, 1959; Robinow, 1960b) surrounding an opaque, compact core. Membrane structures were well preserved (Bradbury and Meek, 1960). Structures corresponding to the cortex (Mayall and Robinow, 1957) were not found in these preparations, even with lanthanum staining.

Spores fixed with OsO₄ exhibited a variable, poorly differentiated appearance (Figs. 11 and 12). The outer coat was seldom observed, and the inner coat was prominent, though ill defined. A structure corresponding to the cortex was occasionally seen (Figs. 11 and 12), though poorly differentiated even with lanthanum staining. The core wall and plasma membrane were rarely observed. The core was homogeneous and amorphous, as though partially solubilized (Robinow, 1953). It appeared that the OsO₄ had not penetrated the spores adequately.

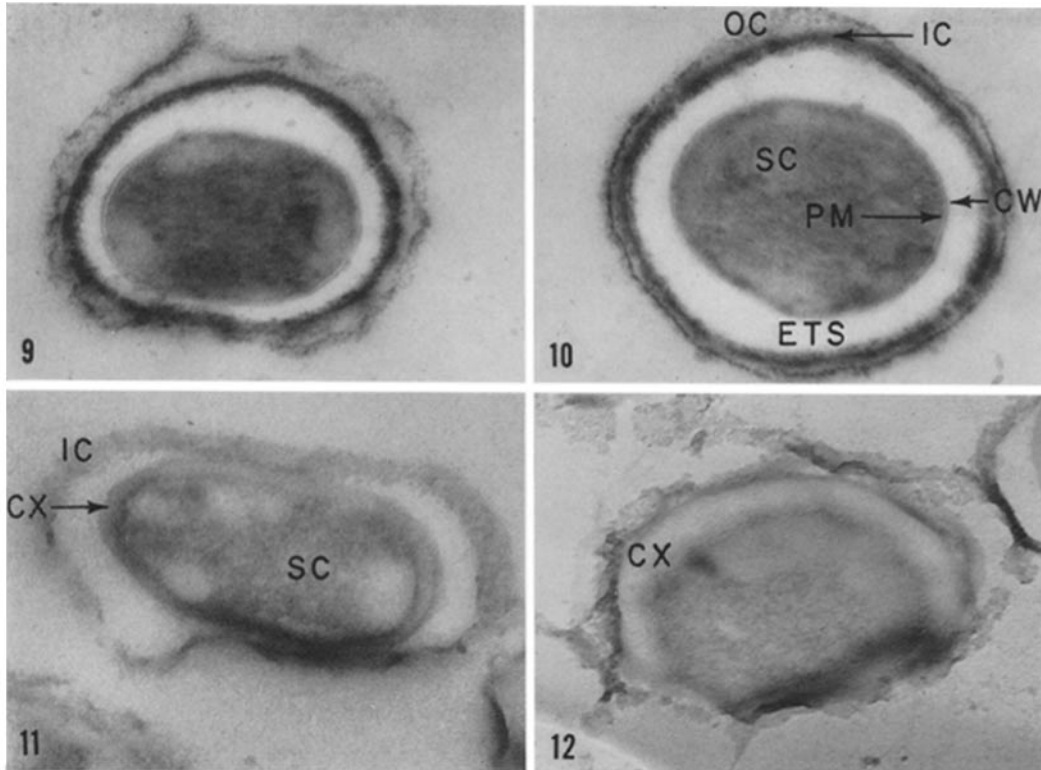
SPORES DEVOID OF DIPICOLINIC ACID

Spores which had been induced to lose their dipicolinic acid by various methods were fixed by KMnO₄. Whether induced mechanically (Fig. 13) (Rode and Foster, 1960a), or chemically with H₂O₂ (Fig. 16), with ethanol (Fig. 17), or with cetyl trimethyl ammonium bromide (Fig. 18), the spores showed an inner coat that was characteristically fragmented, but usually no outer coat. The intact core and its limiting membranes were in evidence though not well preserved, and a pronounced zone of electron transparency was seen between the core and the inner coat. Spores which had been boiled also had this appearance (Figs. 14 and 15). They contained, in addition, vestiges of the cortex (Hashimoto, Black and Gerhardt, 1959). The supernatant liquid from boiled spores contained the dipicolinic acid of the spore (Rode and Foster, 1960b), but not the hexosamine, a characteristic component of the polypeptide excreted by *Bacillus* spores upon germination (Powell and Strange, 1953).

EXTRACTION OF SPORE DIPICOLINIC ACID
DURING KMnO_4 FIXATION

Spores of *B. megaterium* were suspended in 2 per cent aqueous KMnO_4 for 2 hours at room temperature. After thorough washing with water,

germination" (Rode and Foster, 1961a). The appearance of whole spores fixed with KMnO_4 (Fig. 19) is similar to that of germinated spores (Figs. 2, 3, and 4) and, like germinated spores, they lack dipicolinic acid and possibly also other soluble components.



FIGURES 9 THROUGH 12

Electron micrographs of sections of spores embedded in methacrylate and floated on water. Structures identified are outer coat (OC), inner coat (IC), core wall (CW), plasma membrane (PM), spore core (SC), cortex (CX), and electron-transparent space (ETS). Magnification, 50,000.

FIGURES 9 and 10 Spores fixed 2 hours in 2 per cent aqueous KMnO_4 at 26°C.

FIGURES 11 and 12 Spores fixed 2 hours in 1 per cent OsO_4 in veronal-acetate buffer, pH 7.2 to 7.4, at 26°C.

the spores contained only 6 per cent of the initial level of dipicolinic acid. Also, they stained readily with gentian violet. The dipicolinic acid lost from the spores was recoverable from the KMnO_4 solution. It was found that 30 minutes of exposure to KMnO_4 at 4°C converted 50 per cent of the population to stainable cells. After 1 hour at 4°C all of the spores were stainable. This effect of KMnO_4 may be akin to the first stages of chemical germination, and may be an example of "lethal

EXTRACTION OF DIPICOLINIC ACID DURING
 OsO_4 FIXATION

Resting spores were fixed for varying times in 1 per cent OsO_4 at 26°C, 37°C, and 56°C, then thoroughly washed with water and analyzed for dipicolinic acid. Routine fixation (2 hours at 26°C) did not induce loss of dipicolinic acid. However, in 5 hours a 10 per cent loss occurred, and in 24 hours a 20 per cent loss. At 37°C the losses were

35 and 55 per cent in 5 and in 24 hours, respectively. At 56°C the loss amounted to 70 per cent in less than 2 hours, whereas at 100°C the loss was complete in less than 15 minutes.

Suitable analytical tests for dipicolinic acid, performed during and after dehydration and embedding of spores which had been fixed for 2 hours at 26°C in 1 per cent OsO₄, showed that loss of dipicolinic acid did not take place during these stages.

Large numbers of sections of OsO₄-fixed, methacrylate-embedded spores were cut on water in the usual way. An ultraviolet-absorbing material with the characteristic absorption spectrum of calcium dipicolinate was present in the water in relatively high concentrations.

These experiments indicate that spores of *B. megaterium* fixed for 2 hours at 26°C with 1 per cent OsO₄ retain their dipicolinic acid, which is lost when thin sections of the embedded specimens are floated on water. Hence, electron micrographs of thin sections of such spores in reality are images lacking a portion if not all of their dipicolinic acid which, amounting to 15 per cent of the dry spore weight in this instance (Rode and Foster, 1961a), represents a major component of the spore. Its loss may, therefore, be expected to result in altered morphology.

APPEARANCE OF THIN SECTIONS OF OsO₄-FIXED SPORES CUT "WET" AND CUT "DRY"

Spores fixed for 2 hours with OsO₄ and embedded in methacrylate were sectioned dry in order to obtain sections retaining their dipicolinic acid and other constituents which would be lost during wet-sectioning. Electron micrographs of these "dry" sections are shown in Figs. 20 and 21. These sections are, of necessity, quite thick and

should be viewed with this in mind. Notable features include the textured cytoplasm of the core, limiting membranes surrounding the core, a structure corresponding to the cortex, and poorly differentiated coats. Other noteworthy features are the absence of conspicuous electron-transparent zones (compare with Figs. 9 and 10), the absence of an amorphous appearance (compare with Figs. 11 and 12), and the intimate association of adjacent structures.

Thick sections cut wet from this same block of OsO₄-fixed spores and washed with water on the grids had a totally different appearance (Fig. 22). This difference is due to the effects of water. The diffuse, amorphous appearance of the spore cytoplasm, bounded by an undifferentiated area of low electron opacity, is characteristic. The appearance suggests that internal spore components had dissolved. There is an over-all enlargement of the wet spore sections (Fig. 22) compared to those cut dry (Figs. 20 and 21). A sharply defined boundary may be noted in the wet sections. This presumably is the limit of penetration of the methacrylate during the embedding process.

UNFIXED SPORES CUT "WET" AND CUT "DRY"

The feasibility of dry-sectioning having been demonstrated, sectioning of unfixed spores was next undertaken. Spores were dehydrated and embedded in methacrylate in the usual manner, without prior fixation. Spores thus treated retained their dipicolinic acid.

Dry sections of unfixed spores (Fig. 23) were again quite thick. It is possible to discern, however, the textured cytoplasm of the large core, the limiting membrane(s) surrounding the core, a compact cortex, the wrinkled coat(s), and the absence of conspicuous electron-transparent zones.

FIGURES 13 THROUGH 18

Electron micrographs of sections of spores fixed 2 hours in 2 per cent aqueous KMnO₄ at 26°C. Structures identified are outer coat (OC), inner coat (IC), core wall (CW), plasma membrane (PM), spore core (SC), and cortex (CX). Magnification, 50,000.

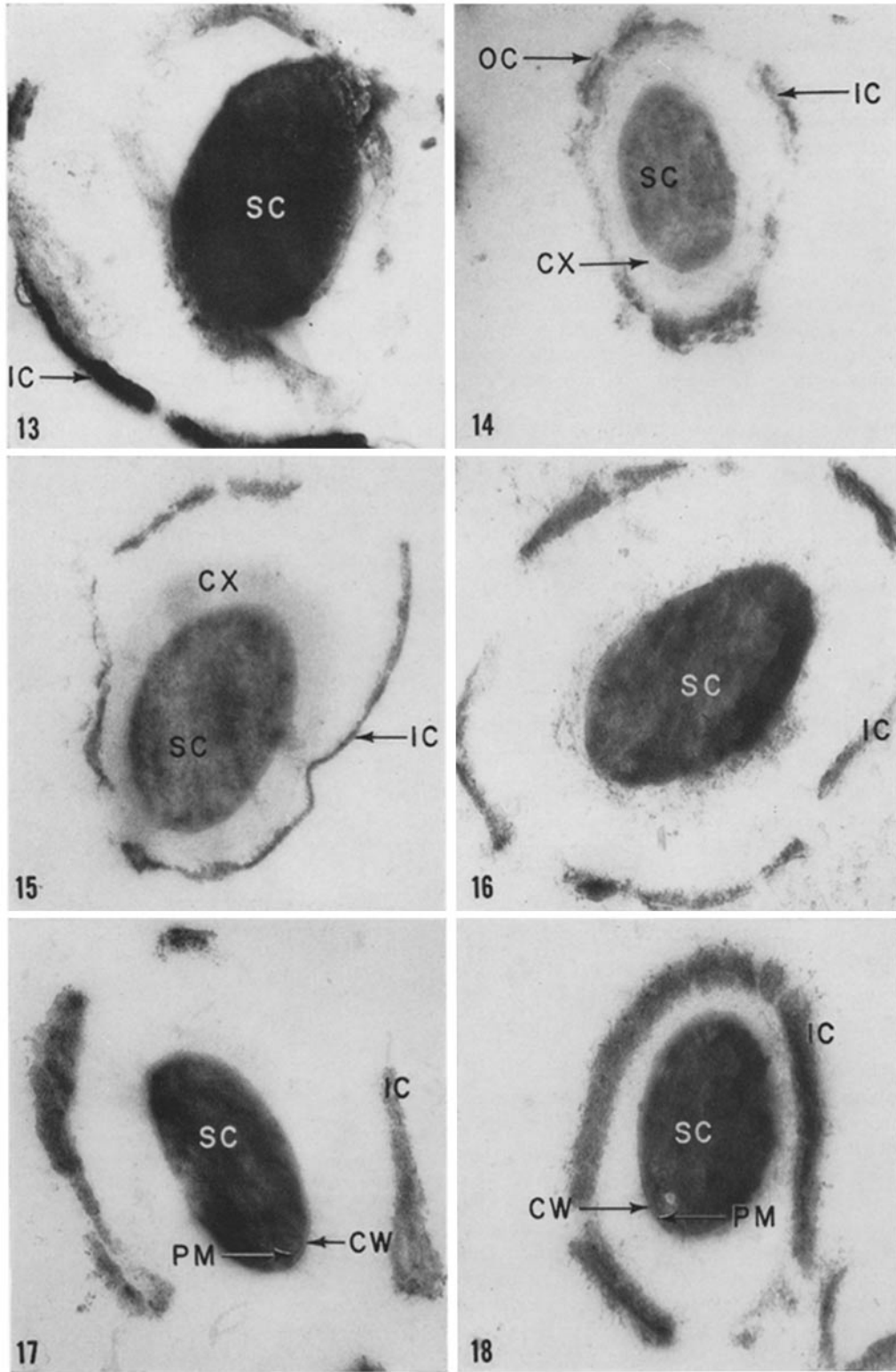
FIGURE 13 Spore germinated mechanically by abrasion with fine glass particles in the Mickle tissue disintegrator.

FIGURES 14 and 15 Spores boiled 15 minutes in water.

FIGURE 16 Spore treated 2 hours with 10 per cent H₂O₂ at 56°C.

FIGURE 17 Spore treated 2 hours with 80 per cent ethanol at 56°C.

FIGURE 18 Spore treated 1 hour with 0.01 per cent cetyl trimethyl ammonium bromide at 56°C.



The textured appearance of the cytoplasm of unfixed spores sectioned dry (Fig. 23) is strongly suggestive of an anhydrous state.

Thick sections from the same methacrylate block of unfixed spores cut on water (Fig. 24) lost almost completely, through solution, the spore structures internal to a sharp boundary denoting the limits of penetration of the methacrylate. Dipicolinic acid appeared in the cutting liquid. Comparable sections were shadowed. It seems apparent (Fig. 25) that penetration of methacrylate into the spore had not extended beyond a sharply delineated region. Inside this point the spore components dissolved in the cutting water, leaving a crater in the methacrylate section. Fig.

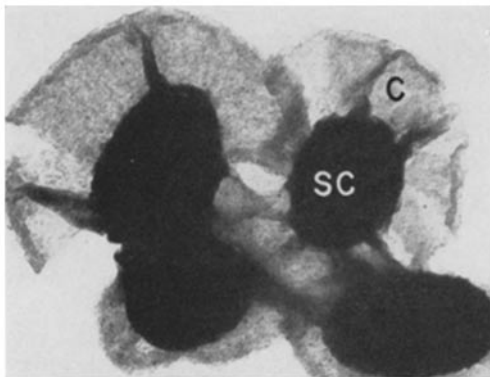


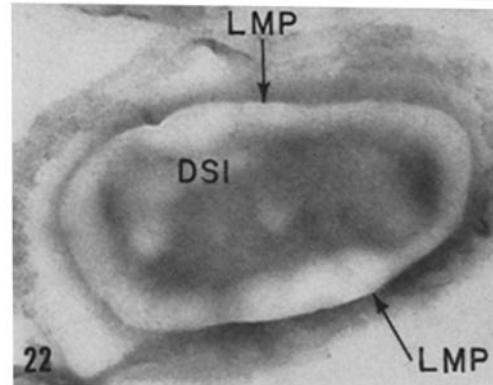
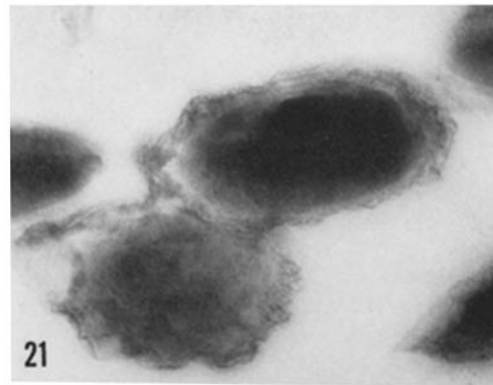
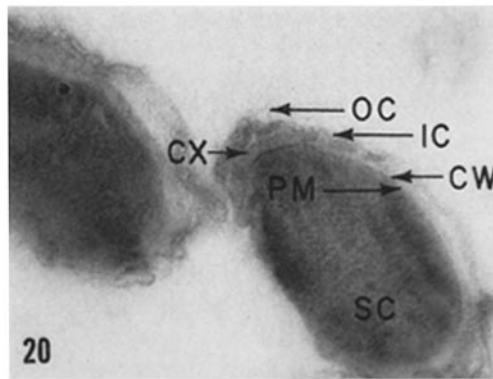
FIGURE 19

Electron micrograph of whole spores fixed 2 hours with 2 per cent aqueous KMnO_4 at 26°C . Structures identified are coats (C) and spore core (SC). Magnification, 30,000.

25 would correspond to the appearance of this boundary, visualized three-dimensionally by means of vapored palladium, viewed from inside the spore. The thickness of the section is evident from the shadow.

These experiments also suggest a method of locating the approximate site of the penetration barrier of the spore. Unfixed spores embedded in methacrylate were sectioned quite thin and floated on water in the usual way. Their appearance (Fig. 26) suggests that the methacrylate did not penetrate beyond a region corresponding to the juncture of the inner coat and the cortex. The barrier to methacrylate penetration appears to coincide with the outer surface of the cortex.

The appearance of thin, wet sections of unfixed spores shadowed with palladium supports these



FIGURES 20 THROUGH 22

Electron micrographs of sections of spores fixed 2 hours in 1 per cent OsO_4 in veronal-acetate buffer, pH 7.2 to 7.4, and embedded in methacrylate. Structures identified are outer coat (OC), inner coat (IC), cortex (CX), core wall (CW), plasma membrane (PM), spore core (SC), dissolved spore interior (DSI), and the limits of methacrylate penetration (LMP). Magnification, 50,000.

FIGURES 20 and 21 Spores sectioned dry.

FIGURE 22 Spore sectioned wet.

conclusions (Fig. 27). The thinness of these sections can be gauged from the crescent shadow cast by the methacrylate boundary, and the almost complete absence, through solution, of internal cytoplasm is evident. The position of the methacrylate boundary relative to the two spore coats can also be seen.

UNFIXED SPORES CUT IN OCTANE

Another means of visualizing the anatomy of spores under the electron microscope is to cut unfixed spores in octane. Spore cytoplasm and dipicolinic acid are largely insoluble in this solvent. The octane had a marked tendency to wet the edge of the glass knife and the methacrylate block. Thick sections were with difficulty recovered on grids.

Like the dry sections described above, the octane sections (Fig. 28) were markedly different from the sections floated on water (Figs. 24 and 26). The outer and inner coats are discernible, but the core is diffuse and homogeneous. Neither the limiting membranes of the core nor the cortex is distinct. A thin zone of lower electron opacity, which seems to coincide with the cortex, is present under the coats. It might have originated by expansion of the methacrylate sections in octane or by a selective solution of spore material.

Even when allowances are made for the limited detail, it is apparent that spore anatomy is revealed differently in dry sections of unfixed (Fig. 23) and OsO_4 -fixed spores (Figs. 20 and 21) and in octane sections of unfixed spores (Fig. 28), as compared to sections prepared by conventional procedures. The KMnO_4 fixation and the wet-sectioning applied to spores of *B. megaterium* Texas fixed with OsO_4 appear to have serious limitations. Refinements in dry-sectioning and the development of a technique of wet-sectioning in liquids possessing the requisite surface tension and solvent properties should facilitate the obtaining of a more accurate picture of the internal anatomy of the spore.

DISCUSSION

The original objective of this study was to determine the position of dipicolinic acid in spores of *Bacillus megaterium* Texas. Although electron microscopy of whole resting spores and of spores lacking dipicolinic acid has revealed striking differences, it has not been possible from these studies to deduce the position of dipicolinic acid. Likewise, use of the dry-sectioning technique,

which was prompted by the observed loss of dipicolinic acid from spores during conventional fixation and sectioning procedures, has not provided a clear answer. It is apparent, however, the fixation and wet-sectioning may, under certain conditions, induce marked morphological alterations.

The bacterial spore, due to its large size and opacity to electrons, is ordinarily not a fruitful object for direct study with the electron microscope (Fig. 1; see also Levinson and Wrigley, 1960). However, certain changes resulting from germination and from the induced release of dipicolinic acids (Figs. 2-8) may be observed in *B. megaterium* Texas. The principal germinative changes are the loss of opacity in the peripheral region, the appearance of a discrete double coat, and an enlargement, especially of the opaque core (Figs. 2, 3, and 4). The various other treatments for inducing the release of dipicolinic acid caused similar changes (Figs. 4 through 8). Here, however, a decrease in the size of the opaque central area was evident. Since the material lost in all of these cases consisted chiefly of dipicolinic acid, and since the principal morphological changes were peripheral in the spore, grounds exist for speculating that the dipicolinic acid may have been discharged from a peripheral position.

From differences in thin sections of ungerminated and germinated spores, Mayall and Robinow (1957) considered the cortex to be the likely site of dipicolinic acid. The cortex starts developing with the start of dipicolinic acid synthesis (Hashimoto, Black, and Gerhardt, 1960). However, studies with monochromatic ultraviolet microscopy (Hashimoto and Gerhardt, 1960) were not interpreted in support of a cortical site. Furthermore, boiled spores which have lost their dipicolinic acid appear to retain the cortical structure (Hashimoto, Black, and Gerhardt, 1959; Figs. 14 and 15). The core has also been suggested as the location of the dipicolinic acid (Yoneda and Kondo, 1959) but the evidence is unsubstantive, and this situation is still unresolved.

Locating the dipicolinic acid in the spore would undoubtedly assist in elucidating its functions. A peripheral deposition has been inferred on the basis of chemical studies (Powell, 1957; Rode and Foster, 1960a). Thus, if localized in the cortex, dipicolinic acid could be visualized as having a role in establishing or maintaining an anhydrous condition (Rode and Foster, 1960a; Lewis, Snell,

and Burr, 1960). The almost instantaneous discharge of all of the spore dipicolinic acid during certain conditions of chemical germination (Rode and Foster, 1961a) argues for a location at or very near the surface of the spore. On the other hand, if dipicolinic acid proves to be more generally distributed through the spore, a different function would seem more likely.

Thin sections of spores appear to have serious limitations in electron microscopy. Potassium permanganate fixation discharged dipicolinic acid from the spores and has been of limited value for visualizing native spore anatomy. However, the outer coat, inner coat, central core, and the limiting membranes of the core are well-preserved (Figs. 9 and 10). The electron-transparent zone between the inner coat and the core wall is attributed to the absence of spore material rather than to electron-transparent material and it appears, therefore, to be an artifact. The excellent thin spore sections of Tokuyasu and Yamada (1959) almost invariably exhibited a similar zone of electron transparency in KMnO_4 -fixed spores which was more often electron-opaque in OsO_4 -fixed spores.

In our hands, osmium tetroxide itself was of limited value as a spore fixative. It did not penetrate the spore as well as KMnO_4 and, under some conditions, it also discharges dipicolinic acid. The discharge does not occur under mild fixation conditions, but here the dipicolinic acid and other soluble components were lost during wet-sectioning, causing a gross distortion of structure.

In contrast to the spores sectioned wet (Figs. 9 through 12) the spores sectioned dry (Figs. 20, 21, 23) exhibit coat structures, cortex, limiting

membranes, and core in intimate association. The quality of such dry sections leaves much to be desired, but they indicate that the cortex may be a compact structure, and that zones of marked electron transparency are not characteristic of these spores.

In connection with the question of a penetration barrier in spores several points may be mentioned: spores can be stained when ruptured mechanically (Fitz-James, 1953; Rode and Foster, 1960a; Lewis, Snell, and Burr, 1960); limited exposure to small, toxic molecules such as phenol, mercuric chloride and hydrogen peroxide does not affect viability (Rode and Foster, 1960d); ungerminated spores remain anhydrous in an aqueous environment (Ross and Billing, 1957). A space technique (Black, MacDonald, Hashimoto, and Gerhardt, 1960) demonstrated that a variety of low molecular weight substances passively penetrate only approximately 40 per cent of the volume of the spores of *Bacillus cereus*. These spores usually have an exosporium. That the entire spore was not penetrated may well be attributable to a barrier.

The ideas of a permeability barrier and internal anhydration have not found, however, general concurrence. For example, the ingenious use by Murrell and Scott (1958) of deuterium oxide led them to conclude that spore water mingles freely with external water, ostensibly unimpeded by a barrier impenetrable to water. The crux of the matter may be, however, whether regions exist in the spore (*e.g.* the core) which are anhydrous and not accessible to external water. Applicability of the "contractile cortex" theory (Lewis, Snell, and Burr, 1960) can better be evaluated when it becomes possible to test it experimentally.

FIGURES 23 THROUGH 28

Electron micrographs of sections of unfixed spores embedded in methacrylate. Structures identified are outer coat (*OC*), inner coat (*IC*), spore core (*SC*), dissolved spore interior (*DST*), limits of methacrylate penetration (*LMP*), shadow cast by palladium (*SH*), and crater (*CR*) formed when internal spore components dissolve. Magnification, 50,000.

FIGURE 23 Thick sections cut dry.

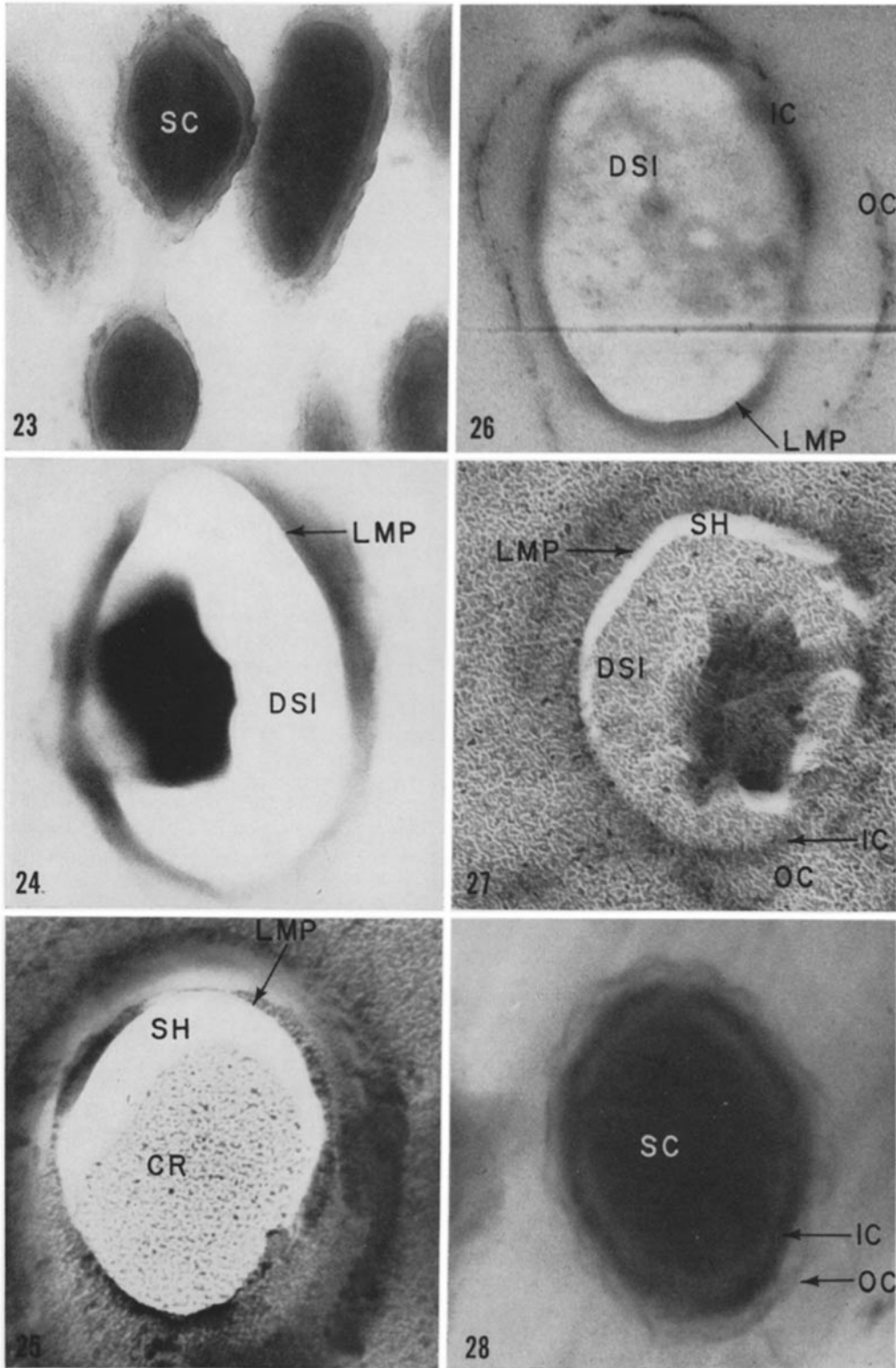
FIGURE 24 Thick section cut wet.

FIGURE 25 Thick section cut wet and shadowed with palladium at an angle of 50°.

FIGURE 26 Thin section cut wet.

FIGURE 27 Thin section cut wet and shadowed with palladium at an angle of 50°.

FIGURE 28 Thick section cut in octane.



The existence in spores of a structural barrier to hydration and solutes could account for the failure of methacrylate to penetrate unfixed spores beyond a sharply delineated boundary at the juncture of the cortex and the inner coat (Figs. 26 and 27). Spores fixed with KMnO_4 (Figs. 9 and 10) apparently no longer possess this barrier since methacrylate penetrates to all parts of the spore, as verified with sections shadowed with palladium.

Both of the spore coats of unfixed spores appear to embed in methacrylate. Since dipicolinic acid dissolves in the cutting liquid coincident with erosion of the internal structures, this suggests that dipicolinic acid is not in the coats of intact spores unless one assumes that embedded dipicolinic acid can dissolve. Unfortunately, these procedures do not settle whether the cortex or the

core (Hashimoto and Gerhardt, 1960) contains the dipicolinic acid. Further developments in the dry sectioning technique and the application of differential solubilities to such sections should aid in the resolution of this question.

Spores of the Texas strain of *B. megaterium* possess a thick inner coat, a much thinner outer coat, and are devoid of an equatorial fold (Figs. 9 and 10), a situation opposite to that of the Penn and L strains of *B. megaterium* (Fitz-James and Young, 1959). Nor have the polar knobs described by Levinson and Wrigley (1960) for their strain, and confirmed by us, been detected.

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