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# *Clostridium* Spores with Ribbon-like Appendages

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Spores of *Clostridium* sp. N1 are characterized by numerous broad ribbon-like appendages attached to one end. The appendages are two to three times the length of the spore and, at their maximal dimension, may be two-thirds the width of the spore. They are attached to the spore body by a common trunk which is continuous with the outer spore coat. Each appendage is a multilayered structure and is enclosed in an amorphous material. Details of spore and appendage formation are described, and appendage ultrastructural features are presented. The function of the appendages is not known.

Bacterial spores commonly exhibit surfaces which are sculptured and irregular (2, 3, 16). Although ribbed surface structures have been described (1, 3, 7, 8), neither marked spore protuberances nor appendages had been reported prior to the recent papers by Krasil'nikov, Duda, and Sokolov (10–12). These Russian workers described for soil clostridia elaborate spore protuberances which were sufficiently prominent and varied to be considered taxonomically significant. More recently, Hodgkiss and Ordal (6) described multiple tubular appendages radiating from the surface of *Clostridium botulinum* type E spores.

Although the *Clostridium* which we have examined in the present work was obtained from V. I. Duda, it appears that this organism (*Clostridium* sp. N1) may not have been included in the survey accounts published by the Russian microbiologists. At any rate, we are not aware of an existing published description of this unusual sporeformer.

## MATERIALS AND METHODS

*Organism. Clostridium* sp. N1 was obtained in December 1965 from V. I. Duda, Moscow State University, Moscow, USSR. It was characterized by him as an anaerobe which grows well on ordinary bacteriological media, with the production of spores possessing appendages. Data on culture source, isolation procedures, and species designation have not been available.

*Growth and spore production.* Brain Heart Infusion (Difco), *p*H 7.4, supplemented with 500 μg of sodium thioglycolate per ml and 1.5% agar (Difco), was used for growth and spore production. Petri plates were surface-inoculated and incubated at 30 C in desiccators over wet oats to provide the anaerobic environment. Sporulation was abundant in 6 days. Spore crops, free from vegetative cells, were obtained by conventional procedures of differential centrifugation and repeated washing with demineralized water (15). Spores were stored in demineralized water at 4 C. Germination did not occur under these conditions.

*Spore treatments.* In certain cases, spore suspensions were treated to achieve particular objectives prior to electron microscope study.

Appendages were removed from free spores by sonic treatment with a Branson-Sonifier (model S-75). The spores were chilled in an ice bath during successive 30-sec treatments until the desired results were obtained. Preliminary appraisal of appendage removal was by phase-contrast microscopy; conclusive appraisal was by electron microscopy.

Spores, stripped of appendages and coat structures, were prepared by treatment with 0.1% sodium hypochlorite for periods up to 30 min at 37 C. A different effect of sodium hypochlorite on *Bacillus megaterium* spores has been described (16).

The effects of the proteolytic enzyme, Pronase, and of lysozyme and of ribonuclease on free spores, in particular on the integrity of the appendages, were studied in phosphate buffer (0.016 M, *p*H 7). In each case, enzyme concentrations were 50 μg/ml and incubation was at 37 C for periods up to 6 hr. Appraisals of enzyme effects were by phase-contrast and electron microscopy.

*Specimen preparation for electron microscopy.* Specimens were examined as carbon replicas, as thin sections, or directly with a negative stain.

For preparation of replicas, spore specimens were placed on squares of freshly cleaved mica, allowed to dry, and shadowed with platinum at 35 to 45°. The specimens were then coated with carbon, and the carbon films, removed from the mica by flotation onto water, were treated overnight with 0.5% sodium hypochlorite to remove the cellular material. After thorough washing with demineralized water, the carbon replica films (replicas) were transferred to copper grids for examination (16).

Specimens for thin sectioning were, in most cases, fixed for 1 to 3 hr at room temperature in 2% glu-

taraldehyde (17) at *p*H 6.1 in Kellenberger's Veronal acetate buffer (9). After five rinses in distilled water, specimens were postfixed at room temperature in 1% osmium tetroxide buffered at *p*H 6.1 with the same buffer. After 2 hr, the cells were rinsed five times in distilled water and placed in 0.5% uranyl acetate, at 4 C for 6 hr. At the end of this period, they were rinsed twice in distilled water and dehydrated in an ethyl alcohol series at 4 C. The ethyl alcohol was replaced by acetone, and the cells were returned to room temperature and embedded in Mollenhauer's plastic I (13) at 60 C. In one case (Fig. 24), a fixative containing 2% glutaraldehyde and 2% acrolein buffered at *p*H 6.1 in 0.1 M *S*-collidine buffer containing 0.68% NaCl and 0.11% $CaCl_2$ was used. This fixative replaced the 2% glutaraldehyde used in the previous procedure. Sections were cut on a Sorval Porter Blum MT-1 microtome with a diamond knife. All sections were stained for 4 to 8 min with 0.5% uranyl acetate, then 1 to 2 min with Reynolds' lead citrate (14).

For negative staining, appendage specimens on Formvar films were permitted to dry partially; then a drop of 2% ammonium molybdate (*p*H 8.1) was placed on the grid and the excess stain was removed by selective blotting with filter paper to provide a gradient on the grid. When dry, the preparations were ready for examination.

*Electron microscopy.* Specimens were viewed with an RCA EMU-3G electron microscope equipped with a double condenser, an objective aperture of approximately 30 $\mu$, and an accelerating voltage of 100 kv. Initial magnifications were 5,000 to 43,000 times, and micrographs were taken on Dupont Cronar Ortho S Litho film.

## RESULTS

*Organism.* Vegetative cells of *Clostridium* sp. N1, taken from isolated colonies developing on Brain Heart Infusion plates, and viewed under phase-contrast illumination, varied markedly in size, in both length and width. Cells from young cultures were gram-positive, and many showed marked cytoplasmic granulation. Although the granules did not stain readily with Sudan Black B (4), they persisted throughout the sodium hypochlorite treatment of Williamson and Wilkinson (18) and are presumed to be lipid granules.

Refractile spores were formed terminally and sporangia were not swollen. Spores, like the vegetative cells, were variable in size (see Fig. 2–11). As the spores became free, they retained, attached to one end, packets which appeared to have a diagonal surface pattern. Upon thorough washing or prolonged incubation, these packets separated into numerous individual appendages which then, in wet mount preparations, radiated outward from the spore body. Individual appendages were visible with oil immersion objective and phase-contrast illumination

*Growth and colony characteristics.* Colonies on agar plates of Brain Heart Infusion were flat and slightly raised, the margins were undulating, and the surfaces were smooth and somewhat dull. The colony consistency was butyrous, the colonies were opaque, the growth was gray-white, and under oblique illumination (5) the colonies appeared prismatic. Growth was moderately good on Brain Heart Infusion Agar, and first evidences of sporulation were usually seen in about 4 days. Sporulation was not synchronous and 6-day cultures often contained free spores, nonsporulated vegetative cells, and all intermediate stages.

No strong effort was made to define optimal growth and sporulation conditions, nor to investigate the marked size variation noted for vegetative cells and spores. A species designation, if any, of this obligate anaerobe is not known to the authors.

*Electron microscopy.* The mature, free spore of *Clostridium* sp. N1 is oval. A typical spore (Fig. 1) is approximately 2.2 $\mu$ long and 1.3 $\mu$ wide, and has a rough surface. Numerous ribbon-like appendages are attached to one end of the spore. The appendages are smooth on one surface and striated on the other. They are relatively thin, may be two to three times the length of the spore body, and are quite wide (as much as 0.8 $\mu$) at the end distal to the attachment site. The attachment site is obscured in replicas of untreated spores (Fig. 1).

Considerable information concerning the sporulation process and the release of the mature spore from the sporangium was obtained with replicas (Fig. 2–11). The vegetative cell is peritrichously flagellated (Fig. 2) and, upon sporulation, the spore may be detected as an elevated (rigid) body occupying a terminal position within the sporangium (Fig. 3 and 4). Appendages are not visible by the replica technique at this stage. However, as the vegetative cell portion undergoes disintegration, the spore body is freed from its sporangial enclosure (Fig. 5) and the appendages become progressively visible as spirally arranged ribbons which seemingly occupy much of the sporangial space not occupied by the spore body itself (Fig. 5–8). When the spore and appendages are completely free, the appendages maintain their spiral orientation for a time (Fig. 9), and then become arranged as a tuft of parallel ribbons (Fig. 10). Eventually, the individual appendages separate in a characteristic flared arrangement (Fig. 11).

A study of the sporulation process and appendage formation in thin sections provided additional information (Fig. 12–19). Vegetative cells were observed both with and without

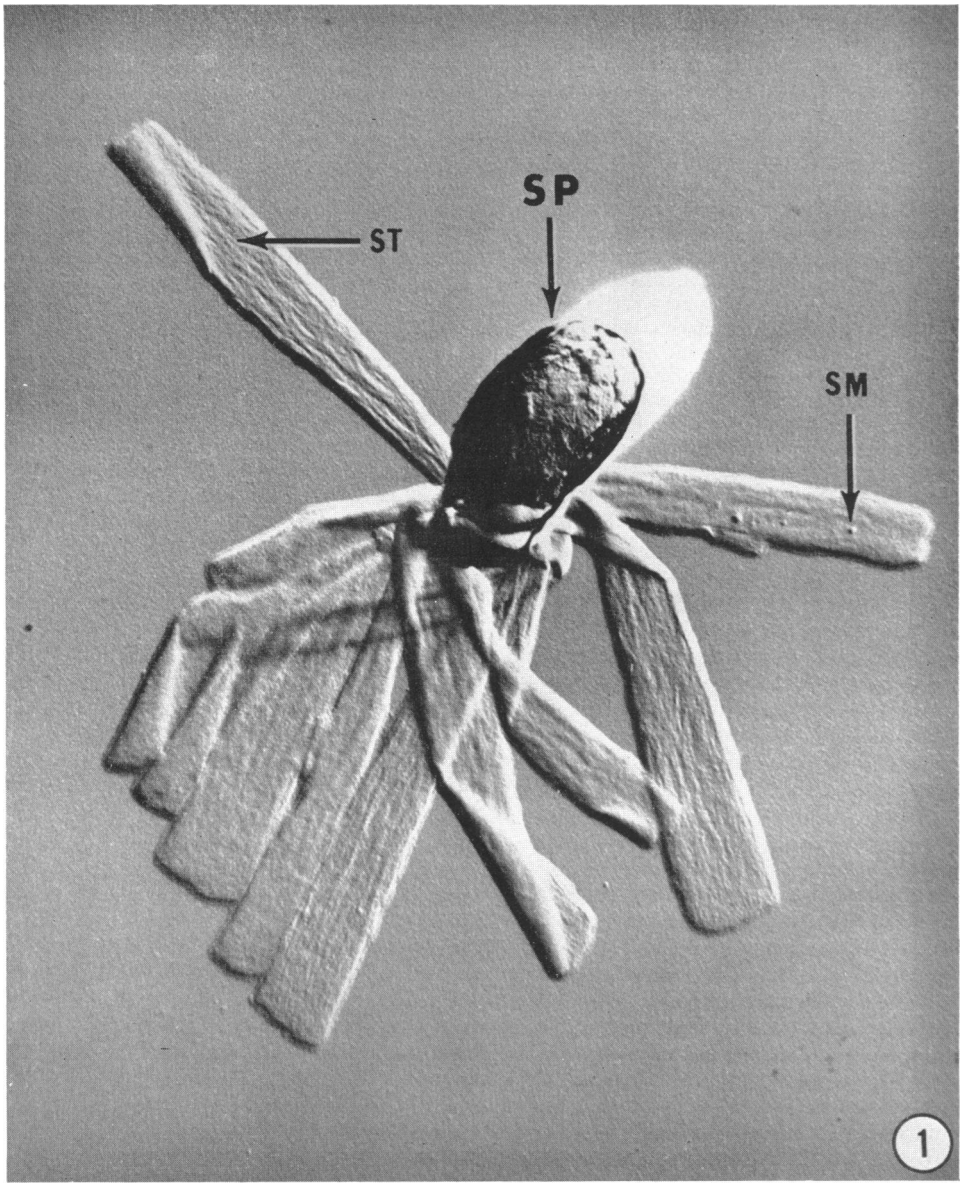


FIG. 1. *Typical mature free spore of Clostridium sp. N1 from a 7-day culture. The spore (SP) surface is rough, and numerous ribbon-like appendages extend from a common origin at one end. The appendages are relatively smooth (SM) on one surface, but appear striated (ST) on the other. Replica.* × *15,000.*

"lipid granules" (Fig. 12 and 13). No appendages were visible in sporangia at very early stages of sporulation, prior to coat formation (Fig. 14). However, coincident with early coat formation, an appendage trunk was formed (Fig. 15), and from this trunk appendages originated and progressively occupied more and more of the sporangial space distal to the spore body as the cortex was laid down and the spore continued to develop (Fig. 16, 17 and 18). Finally, upon maturity, the spore body and its appendages occupied a very significant proportion of the sporangial space (Fig. 19). The appendages within the sporangium were initially oriented upward toward the spore body from their trunk origin, but then assumed a spiralling, downward direction. Multiple layers of appendages were visible in thin sections, and the appendages were enclosed

in an electron transparent substance which separated them from one another (Fig. 19; see below).

Cross sections through sporangia at the level of the spore body revealed nothing unusual. An outer coat, middle coat, inner coat, cortex, and spore wall were demonstrable (Fig. 20). No appendages were present at this level. Other cross sections through sporangia at the trunk level indicated that appendages were not continuous with the trunk over its entire length (Fig. 21). Cross sections through sporangia at a level distal to the spore body and trunk demonstrated that appendages were distributed through the sporangial cytoplasm (Fig. 22).

The outermost appendages within the sporangium lay in close proximity to the cell wall, but were separated from it by a thin layer of cytoplasm. A cytoplasmic membrane has not been observed. Underlying layers of appendages were spaced closely (Fig. 23–24). The appendages radiated outward in spiral fashion from the trunk (Fig. 24), the trunk was continuous with the outer spore coat (Fig. 25), and the appendages curved upward from their trunk attachment (Fig. 25). A bulbous structure, located beneath the site of appendage attachment, appeared to be an integral part of the trunk (Fig. 25).

Each appendage was enclosed in an amorphous material approximately 300 to 400 A thick (Fig. 26). The appendage is a multilayered structure which presents a different pattern in longitudinal section and cross section (Fig. 27–30). One appendage surface is smooth, and this is oriented toward the sporangial cell wall (Fig. 28 and 29). The other surface is very irregular (Fig. 27–29), which accounts for the striated appearance of replicas of the ribbon-like appendages (Fig. 1). Structural details of an appendage in longitudinal section and in cross section are depicted in Fig. 30. Direct demonstration of the spherical subunits depicted in Fig. 30B has not been accomplished, but this interpretation seems preferable to an alternative hypothesis that the structure is indeed honeycomb in nature.

Appendages were removed from the spore body by sonic treatment. The native state of the trunk was thereby revealed (replicas) to be bulbous (Fig. 31 and 32). Appendages removed by sonic treatment were often intact, and were frequently characterized by a hook at the end proximal to the trunk attachment and by a 90° twist adjacent to this hook (Fig. 33). The appendages are presumed, therefore, to be somewhat rigid in the region proximal to trunk attachment. By contrast, the distal and broader regions of the appendages appeared to be flexible as evidenced by a marked tendency to fold (Fig. 1, 11, 34). Details of the appendage at its point of attachment to the trunk are visible in Fig. 34. The distal margin of the appendages is very irregular. The ultrastructural repeating patterns of appendages were visualized with negative stains (Fig. 35) in spite of their multilayered structure (Fig. 27, 28, and 29).

Additional features were revealed by treating spores with 0.1% sodium hypochlorite. Short exposure (5 min) caused the trunk and appendages to lose rigidity and become flaccid. This permitted a good view of the trunk in an expanded condition and of the appendage attachment sites (Fig. 36). More prolonged treatment with 0.1% sodium hypochlorite (20 min) resulted in dissolution and loss of the spore coats and appendages. Such "stripped" spores were smooth (Fig. 37). Thin sections revealed that the coats and appendages had been selectively removed, that the cortex was now the outermost structure, and that the spore wall and central body were well preserved (Fig. 38). Such spores did not, however, contain dipicolinic acid.

## DISCUSSION

The chemical nature of the appendages is not known. They are unaffected by Pronase, by lysozyme, or by ribonuclease; such enzyme-treated spores, viewed as replicas, appear structurally unaltered. Relatively pure preparations of free appendages, judged by electron microscopic appearance, have been prepared. Crude spore suspensions were freed from much of the contaminating vegetative debris by sequential treatment with Pronase, lysozyme and ribonuclease, followed by thorough washing with demineralized water. After sonic treatment, which did not affect the spore body, the removed appendages were readily separable from the spore bodies by differential centrifugation. Chemical studies of such appendage preparations are planned.

The apparent selective removal of coats and appendages by treatment with dilute sodium hypochlorite solutions requires additional study. Although the smooth spores which result from this treatment lack dipicolinic acid, the possibility has not been excluded that appropriate modifications of the treatment may yield spores devoid of coat and appendage structures but still possessing dipicolinic acid. Such studies could yield information concerning the localization of dipicolinic acid in bacterial spores.

No systematic germination studies have been carried out. The spores do, however, darken and lose refractility and rigidity upon germination without obvious associated appendage changes.

Experimental evidence which bears on the function of the appendages is not available. One

might speculate that the appendages facilitate spore dissemination in nature, assist in spore nutrition during formation, or have no function and result from a deranged metabolism. It seems fruitless to speculate on these and other possibilities until supporting evidence is available.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. BRADLEY, D. E., AND D. J. WILLIAMS. 1957. An electron microscope study of the spores of some species of the genus *Bacillus* using carbon replicas. J. Gen. Microbiol. **17**:75–79.
2. FITZ-JAMES, P. C. 1959. Morphology of spores of *Bacillus apiarius* Katznelson. J. Bacteriol. **78**: 765–768.
3. FITZ-JAMES, P. C., AND I. E. YOUNG. 1959. Cytological comparison of spores of different strains of *Bacillus megaterium*. J. Bacteriol. **78**:755–764.
4. HARTMAN, T. 1940. The use of sudan black B as a bacterial fat strain. Stain Technol. **15**:23–28.
5. HENRY, B. S. 1933. Dissociation in the genus *Brucella*. J. Infect. Diseases **52**:374–402.
6. HODGKISS, W., AND Z. J. ORDAL. 1966. Morphology of the spore of some strains of *Clostridium botulinum* type E. J. Bacteriol. **91**:2031–2036.
7. HOLBERT, P. E. 1960. An effective method of preparing sections of *Bacillus polymyxa* sporangia and spores for electron microscopy. J. Biophys. Biochem. Cytol. **7**:373–376.
8. HOOF, A. VAN DEN, AND S. ANINGA. 1956. An electron microscope study on the shape of the spores of *Bacillus polymyxa*. Antonie van Leeuwenhoek J. Microbiol. Serol. **22**:327–330.
9. KELLENBERGER, E., A. R. RYTER, AND J. SÉCHAUD. 1958. Electron microscope study of DNA-containing plasms. II. Vegetative and mature phage DNA as compared with normal bacterial nucleoids in different physiological states. J. Biophys. Biochem. Cytol. **4**:671–678.
10. KRASIL'NIKOV, N. A., V. I. DUDA, AND A. A. SOKOLOV. 1963. Outward growths on spores of anaerobic bacteria of the genus *Clostridium*. Proc. Acad. Sci. USSR Microbiol. Sect. **152**:735–736.
11. KRASIL'NIKOV, N. A., V. I. DUDA, AND A. A. SOKOLOV. 1964. New type of sporulation in anaerobic bacteria. Proc. Acad. Sci. USSR **159**:434–435.
12. KRASIL'NIKOV, N. A., V. I. DUDA, AND A. A. SOKOLOV. 1964. Protrusions on the surface of spores of anaerobic bacteria of the genus *Clostridium*. Mikrobiologiya **33**:454–458.
13. MOLLENHAUER, H. H. 1964. Plastic embedding mixture for use in electron microscopy. Stain Technol. **39**:111–114.
14. REYNOLDS, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. **17**:208–212.
15. RODE, L. J., AND J. W. FOSTER. 1960. Mechanical germination of bacterial spores. Proc. Natl. Acad. Sci. U.S. **46**:118–128.
16. RODE, L. J., AND M. G. WILLIAMS. 1966. Utility of sodium hypochlorite for ultrastructure study of bacterial spore integuments. J. Bacteriol. **92**:1772–1778.
17. SABATINI, D. D., F. MILLER, AND R. J. BARRNETT. 1964. Aldehyde fixation for morphological and enzyme histochemical studies with the electron microscope. J. Histochem. Cytochem. **12**:57–71.
18. WILLIAMSON, D. H., AND J. F. WILKINSON. 1958. The isolation and estimation of the poly-β-hydroxybutyrate inclusions of *Bacillus* species. J. Gen. Microbiol. **19**:198–209.

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FIG. 2–11. *Replica series, showing sequential steps in the development of the free spore of Clostridium sp. N1. Culture age varied from 6 days (Fig. 2–6) to 7 days (Fig. 7–11). Replicas.* × *7,000.*

FIG. 2. *Vegetative cell with peritrichous flagella.*

FIG. 3. *Early indication of spore (SP) formation (arrows).*

FIG. 4. *Sporangium with maturing spore (SP); the spore body is rigid. This is indicated by the prominent shadow it casts compared with that cast by the vegetative portion of the sporangium. Spores at this stage are refractile under phase-contrast illumination.*

FIG. 5. *Spore body (SP) is free and it has a rough surface; the vegetative (V) portion of the cell is undergoing lysis and disintegration.*

FIG. 6–7. *First evidence of spirally arranged ribbon-like appendages (AP) in the disintegrating vegetative cell.*

FIG. 8. *Spore body with exposed spiral of attached appendages (AP, arrows).*

FIG. 9. *Free spore with some appendages (AP) still spirally arranged; last vestiges of vegetative cell have disappeared.*

FIG. 10. *Free spore with appendages (AP) appearing now as a tuft of parallel ribbons.*

FIG. 11. *Free spore with ribbon-like appendages (AP) now separated and flared. The connection between appendages and spore body (SP) is obscured.*

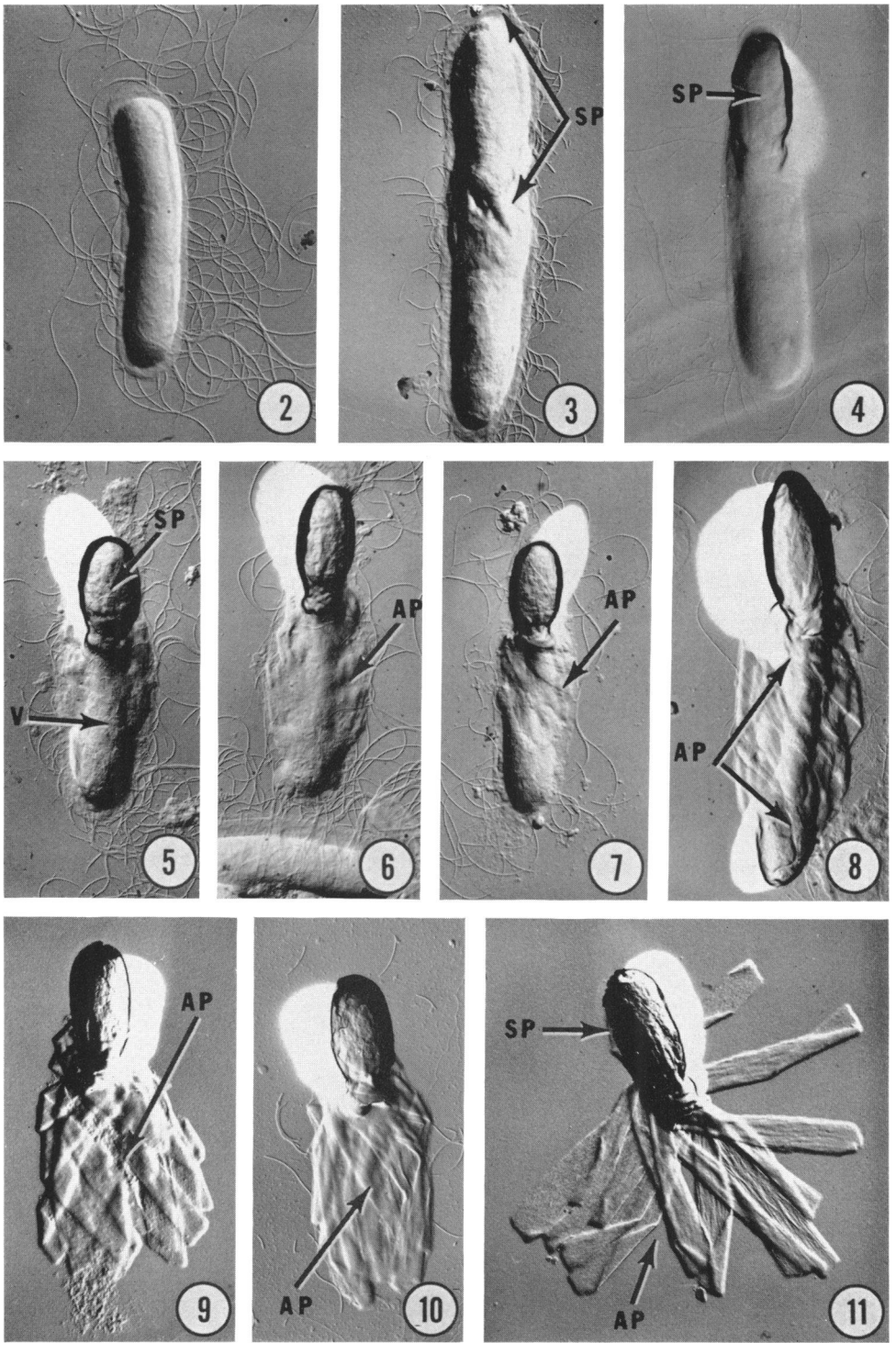



FIG. 2–11

FIG. 12–19. *Thin section series showing sequential steps in spore and appendage formation by Clostridium sp N1. Culture age varied from 5 days (Fig. 12–15) to 6 days (Fig. 16–19).* × *15,000.*

FIG. 12. *Vegetative cell lacking inclusion bodies.*

FIG. 13. *Vegetative cell with lipid granules (LG) and partially separated cell wall (CW).*

FIG. 14. *Sporangium with an early, immature spore (SP); no evidence of appendages.*

FIG. 15. *Sporangium with immature spore; the spore coat(s) and the trunk (T) from which the appendages originate have formed, and early appendage formation proximal to the trunk is faintly visible.*

FIG. 16. *Sporangium with maturing spore; the cortex (CX) has developed and appendages (AP) are forming near the trunk.*

FIG. 17–18. *Later stages; the appendages now extend progressively farther into the vegetative cytoplasm.*

FIG. 19. *Sporangium with mature spore body (SP) and appendages (AP) extending from their trunk (T) origin. These structures occupy a large portion of the sporangial space.*

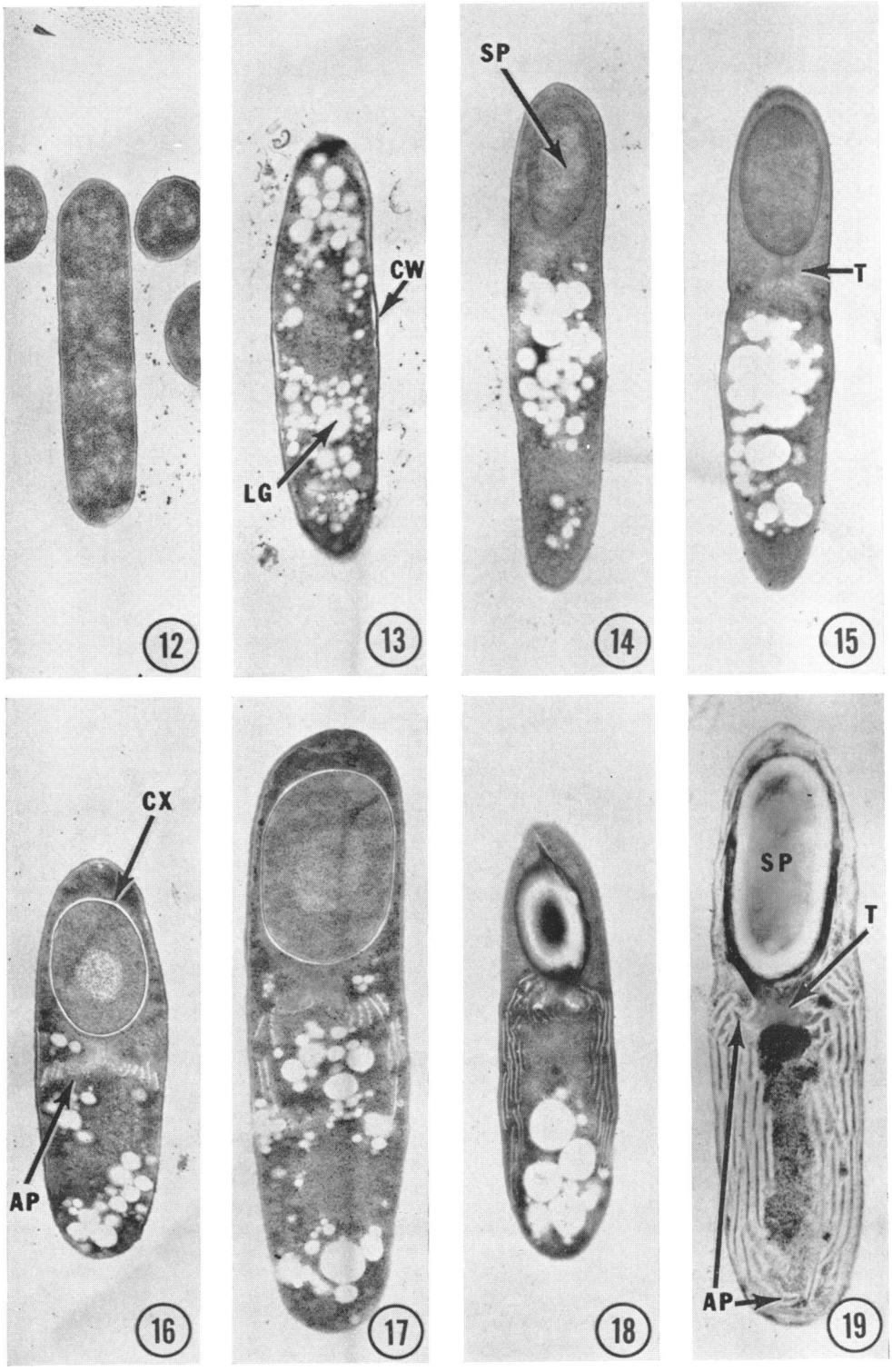
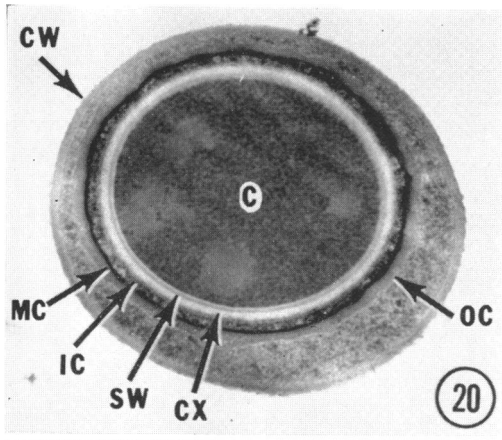
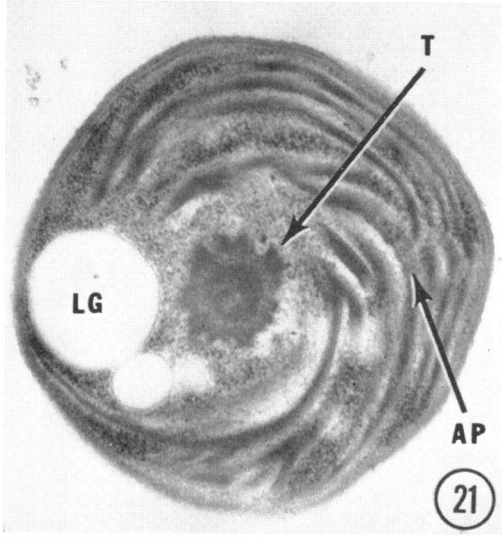



FIG. 12–19



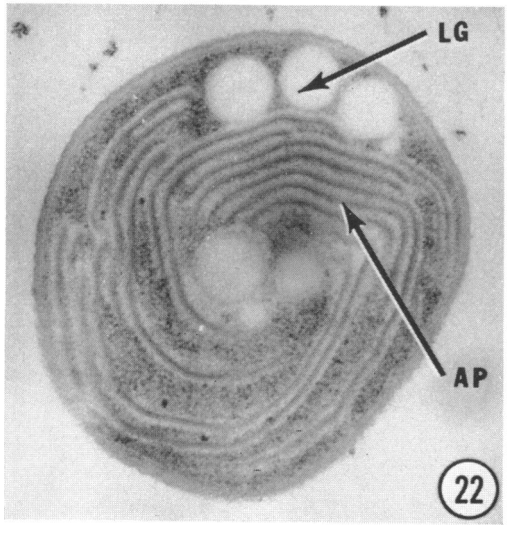




FIG. 20–22. *Cross sections through mature sporangia.* × *34,000.*

FIG. 20. *Section through sporangium at the level of the spore body. Structures visible include the sporangial cell wall (CW), an outer spore coat (OC), a middle spore coat (MC), an inner spore coat (IC), the spore cortex (CX), and the spore core or central body (C) with its spore wall (SW).*

FIG. 21. *Section through sporangium at the level of the appendage trunk (T). "Lipid granules" (LG) and appendages (AP) occupy a large portion of the sporangium. This section does not show continuity between the appendages and the trunk from which they originate (see Fig. 24), but the starlike appearance of the trunk suggests that the section is near the attachment site.*

FIG. 22. *Section through sporangium at a level distal to the spore body. "Lipid granules" (LG) and multiple layers of appendages (AP) occupy a large portion of the cellular space.*

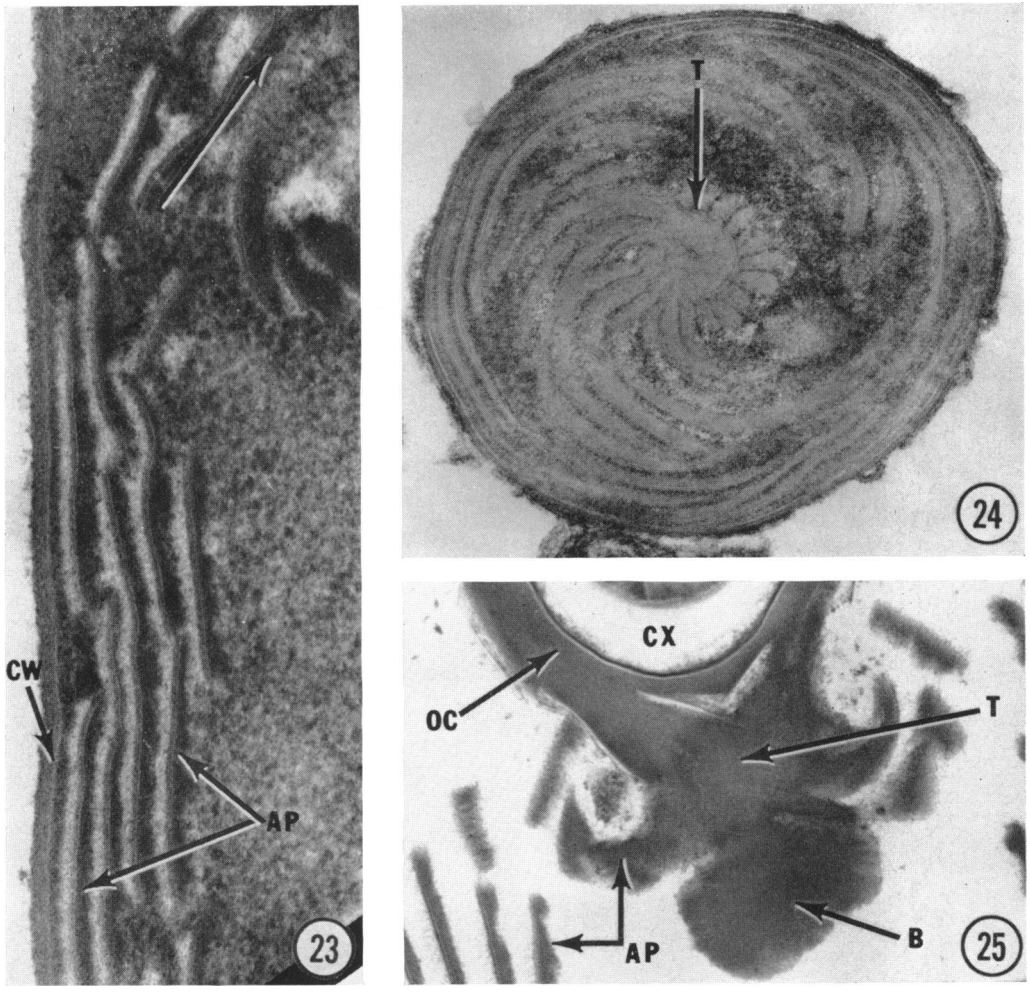



FIG. 23. *Longitudinal section through sporangium. The relationship of the multiple layers of appendages (AP) to the cell wall (CW) is apparent. The unlabeled arrow points in the general direction of the spore body and trunk.* × *87,000.*

FIG. 24. *Cross section through sporangium at the level of the appendage trunk (T). The appendages, 15 to 20 in number, radiate outward from the trunk in spiral fashion.* × *40,000.*

FIG. 25. *Longitudinal section which shows the relationship of the appendage trunk to the spore body. The trunk (T) is continuous with the outer coat (OC) of the spore body. The appendages (AP) originate at the trunk and are oriented upwards near their origin. The trunk has a bulblike protuberance (B) beneath the site of appendage attachment.* × *47,000.*

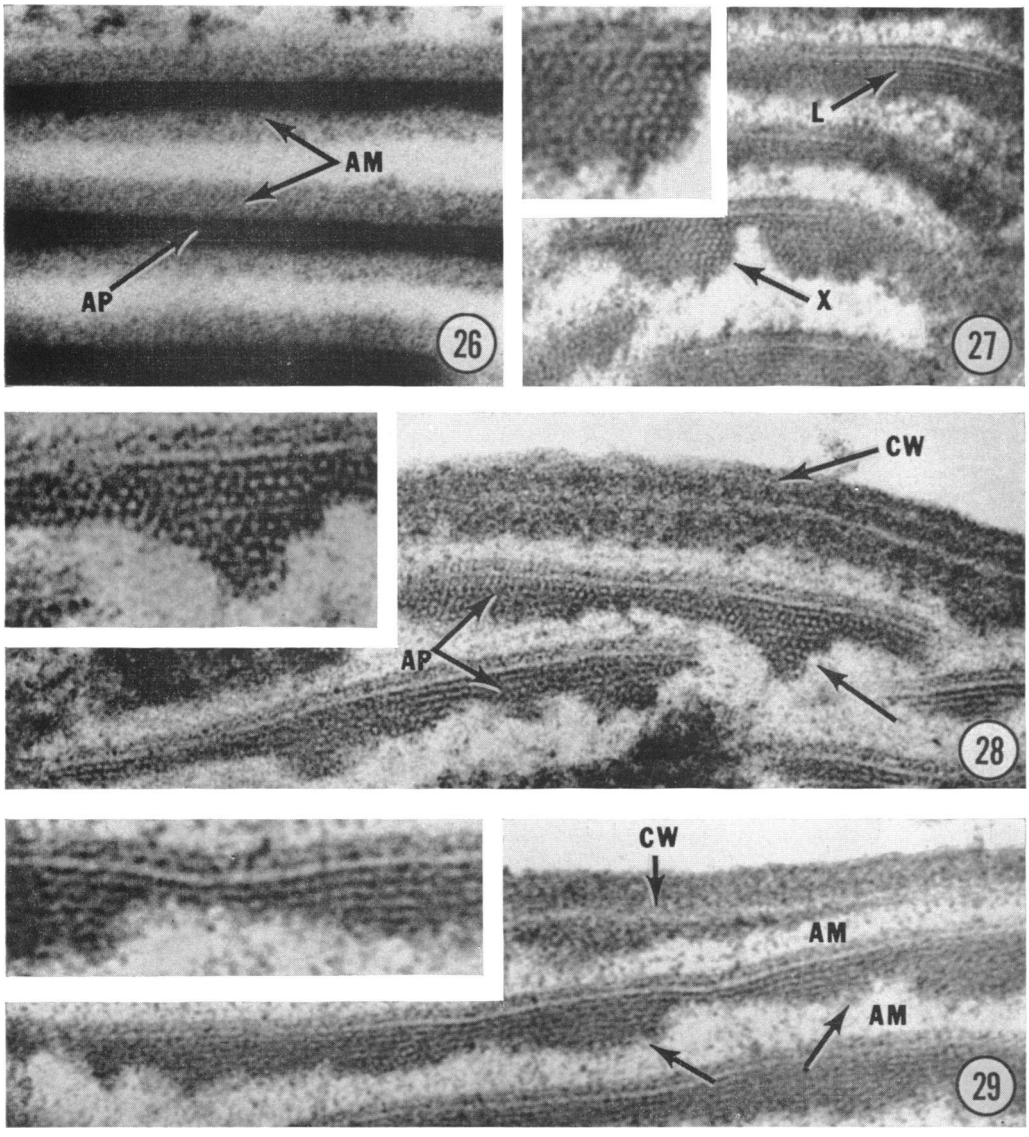



FIG. 26. *Section through appendages. Each appendage (AP), black as printed here, is enclosed by an amorphous (AM) layer. The thickness of these layers is approximately the same as the thickness of the appendage.* × *124,000.*

FIG. 27. *Section through mature sporangium near the trunk site of appendage attachment. The multilayered appendages are seen in both longitudinal section (L) and cross section (X). The inset is an enlargement of the area indicated by (X).* × *175,000; inset, X 330,000.*

FIG. 28. *Cross section through mature sporangium. The two prominent multilayered appendages (AP) are cut in cross section over much of their width. The upper surface of these appendages is relatively smooth [i.e., the surface oriented toward the cell wall (CW) or cell exterior]; the lower surface is irregular. The inset is an enlargement of the area indicated by an arrow.* × *180,000; inset,* × *340,000.*

FIG. 29. *Longitudinal section through mature sporangium. The two appendages are cut longitudinally over much of the length shown, and structural details are evident. The surrounding amorphous (AM) layers are electron-transparent. The inset is an enlargement of the area encompassed by the two arrows.* × *180,000; inset,* × *330,000.*

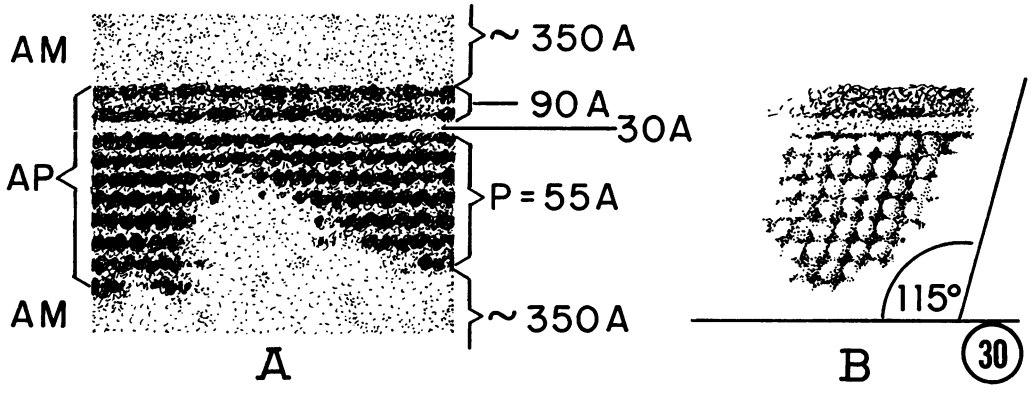



FIG. 30. *Sketches illustrating longitudinal (A) and cross section (B) detail of appendages. (A) The appendage is enclosed by an amorphous material (AM) approximately 300 to 400 A in thickness. The uppermost appendage layer, which corresponds to the smooth (SM) surface in Fig. 1, has a dimension of approximately 90 A. The underlying electron-transparent layer is 30 A, and the lowermost multilayered region of varying thickness has a periodicity (P) of about 55 A. (B) The 55 A multilayered appendage region has crystal-like order and the "subunits" are arrayed linearly at an angle of approximately 115°.*

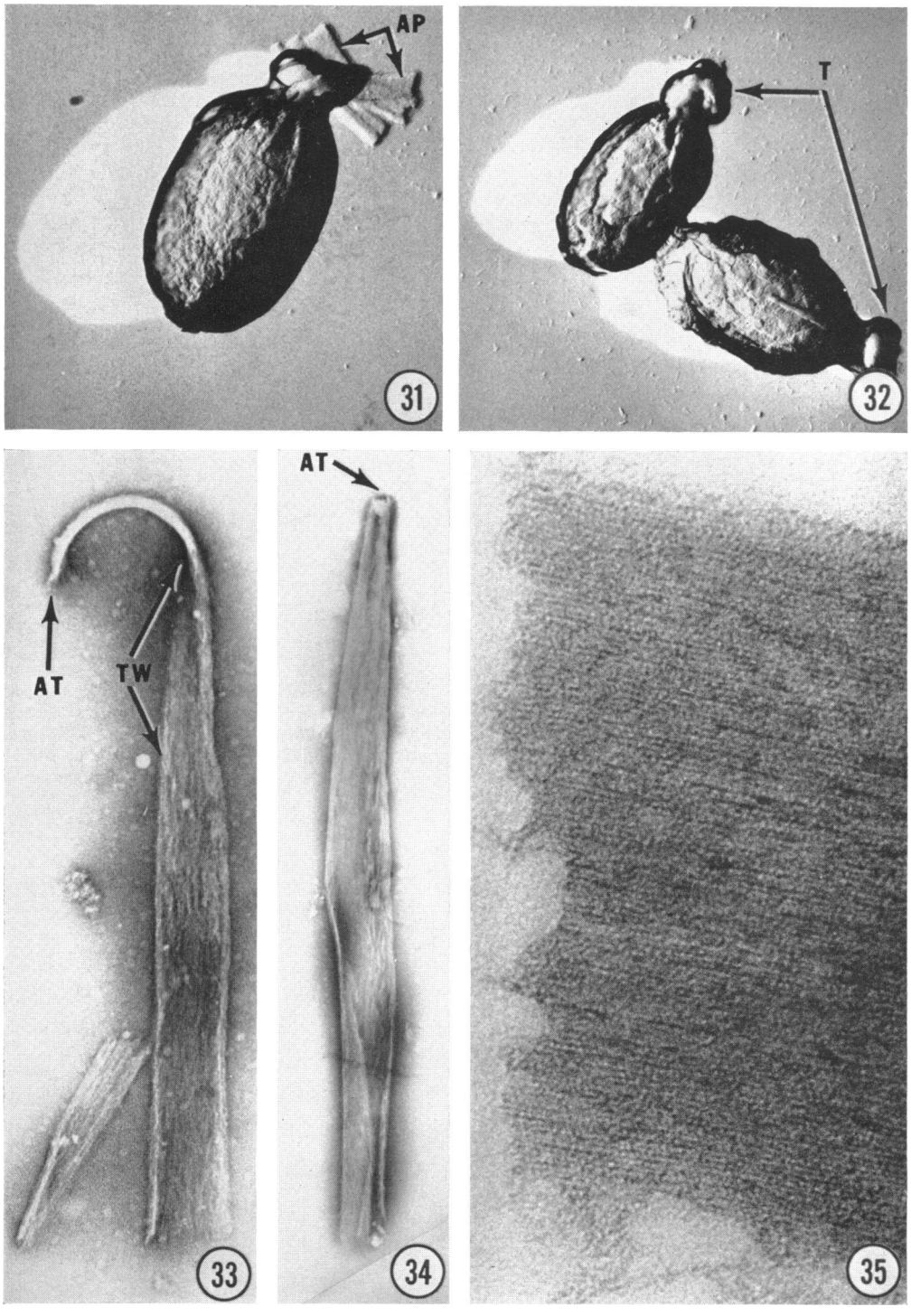



FIG. 31–35

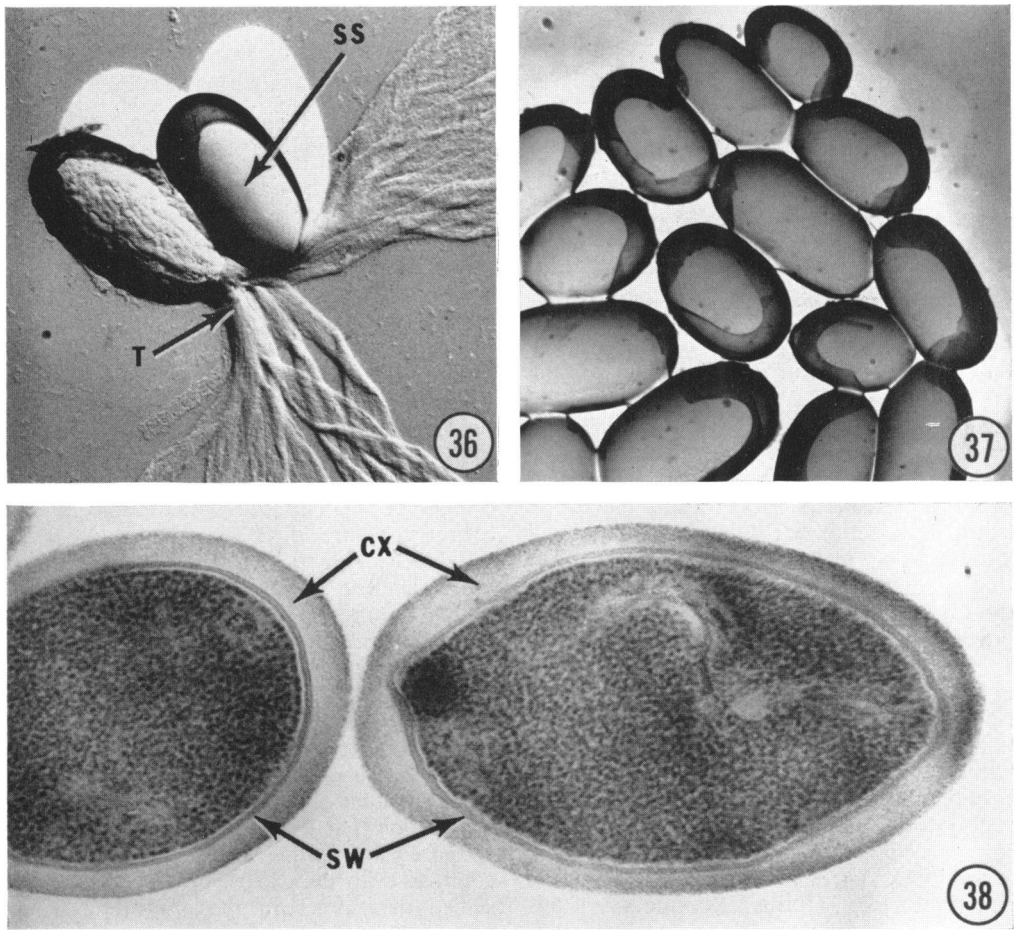



FIG. 36. *Two spores treated with 0.1% sodium hypochlorite at 37 C; one (left) for 5 min, the other (right) for 20 min. The 5 min spore retains its rough coat, the appendages have disintegrated somewhat but retain their overall form, and the trunk (T) has expanded from its native bulbous state and the appendage attachment sites are visible. The 20-min spore is smooth and stripped of both appendages and the rough coat (stripped spore, SS). Replica. × 17,000.*

FIG. 37. *Smooth (stripped) spores, lacking appendages, resulting from treatment with 0.1% sodium hypochlorite for 20 min at 37 C. Replica. × 17,000.*

FIG. 38. *Thin section of spores treated for 20 min with 0.1% sodium hypochlorite at 37 C. The spore coats and appendages have been selectively removed by the treatment. The cortex (CX) is now the outermost structure; the spore wall (SW) and the central core body are well preserved. × 53,000*

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FIG. 31. *Free spore from suspension after 7-min sonic treatment. Some of the appendages (AP) have been broken near their attachment site, leaving appendage stumps. Replica. × 20,000.*

FIG. 32. *Spores with appendages removed by 7-min sonic treatment. The characteristic bulbous appearance of the appendage trunk (T) is apparent. Replica. × 14,000.*

FIG. 33. *Isolated appendage removed from spore body by sonic treatment. The characteristic hook appearance of the appendage at the end proximal to its attachment site (AT) is characteristic, and the plane of the appendage in this hook region is perpendicular to that of the remainder of the appendage, a consequence of a 90° twist (TW) in the region indicated (arrows). The typical striated appearance of the appendage surface is apparent. Negative stain. × 35,000.*

FIG. 34. *Isolated appendage lacking the hook. The nature of the attachment site (AT) is visible. Negative stain. × 20,000.*

FIG. 35. *Enlarged view of the distal end of an appendage. Note the irregular margin, the parallel fibrous arrangement, and the overall beaded appearance. The fibers run parallel to the long axis of the appendage. The periodicity of the parallel fibers is 40 to 45 A. Negative stain. × 210,000.*