ELECTRON MICROSCOPY OF ULTRATHIN SECTIONS OF *SPIRILLUM SERPENS*[1]

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Although the various species of the genus *Spirillum* have been under investigation for many years, several cytological questions remain to be answered. For example: there is still disagreement concerning the mode of attachment of the flagella; much remains to be learned about the nature of the nucleus; the question, raised by the excellent micrographs in the report of Houwink (1953), of the number and nature of the layers in the cell wall is yet to be settled.

The recent improvements in the techniques of specimen preparation, which permit bacterial cells to be fixed, embedded, ultrathin sectioned, and examined to advantage in the electron microscope, made it seem worthwhile to apply these methods to the spirilla in the hope of answering one or more of the above questions.

MATERIALS AND METHODS

All of the electron micrographs included in this report are of ultrathin sections of *Spirillum serpens* var. *azotum*. (The culture was kindly supplied by Miss Marion A. Williams, Southern Illinois University, Carbondale, Illinois.) The bacteria were grown on NaCl-free nutrient agar slants or in NaCl-free nutrient broth at 30 C.

Fixation, dehydration, embedding, and microtomy were carried out essentially according to the procedure followed by Chapman and Hillier (1953), except that the fixative consisted of a 2 per cent OsO_4 solution in tap water instead of in distilled water and that polymerization of the monomeric methacrylate embedding medium was carried out at 72 C instead of at 47 C. The former modification was instituted because of a desire to have the fixing solution resemble as closely as possible the last ambient of the living cells. The cells were washed, prior to fixation, in tap water since, in a personal communication (1956), Williams has stated that contact with distilled water causes them to undergo lysis. The need for a procedural alteration, which resulted in the latter modification, was indicated by the fact that cells which had been polymerized at 47 C "exploded," i. e., they had swelled, the cell wall and membrane were ruptured, and the protoplast contents appeared to have been partially extracted. The direction in which attention should be given was suggested by the hypothesis of Borysko (1955) that polymerization damage to cells may be avoided by the use of polymerization temperatures higher than the heretofore generally used 45–47 C.

For sectioning, both glass knives, prepared in the laboratory, and a diamond knife, generously presented to us and described by Dr. H. Fernández-Morán (1953), were used. The diamond knife proved exceptionally valuable as it retained its sharpness after sectioning more than thirty blocks and as it provided a longer straight and flat cutting edge than does glass. The one undesirable characteristic of the knife was the rather large number of knife marks left in the sections. However, these presented no problem in the study of bacteria, and indications are that improved polishing techniques will reduce their number greatly.

The electron micrographs were taken on Kodak Lantern Slide Medium Plates using an RCA, type EMU-2E, electron microscope which had been fitted with a wide field objective lens and a 50 μ objective aperture. The micrographs were taken at an electronic magnification of 9,650× and were enlarged to 38,600×.

[1] The electron micrographic results presented in this paper are included in a thesis submitted by the junior author to the Department of Biology of Princeton University in fulfilment of the requirements for an experimental senior thesis.

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RESULTS AND DISCUSSION

Cell wall and cytoplasmic membrane. The absence of a cytoplasmic membrane from electron micrographs of sectioned bacteria has been a matter of concern to cellular physiologists. As was pointed out in an earlier paper (Chapman and Hillier, 1953), observations of plasmolysis and other light and electron microscope observations had suggested the existence of a morphological entity which was called a cytoplasmic membrane. It was quite natural, then, that the appearance of a number of electron micrographs from several different laboratories, all having higher resolution than the earlier work and all showing no such membrane, should cause some discussion. The obvious possible conclusions were that either there was no morphologically distinct membrane, the preparative techniques were destroying it, or the instruments were incapable of resolving it. (It is inherent in the first alternative that the earlier techniques were producing an artifact when they led to a visible membrane.)

Examination of figures 1 and 2 will reveal that *Spirillum serpens* appears to possess both a cell wall, *CW*, and a cytoplasmic membrane, *CM*. The wall is about 130A thick. The membrane usually seems to be of nearly equal thickness, but occasionally, as in figure 5, it appears slightly thinner than the wall. These dimensions are considerably smaller than the 200A thickness reported for the wall of *Bacillus cereus* by Chapman and Hillier (1953) and the 150–300A thickness reported for the wall of *Bacillus megaterium* by Piekarski and Giesbrecht (1956). The difference is quite expected, however, when one considers the much more active wriggling and flexing motions exhibited by the spirilla (Pijper *et al.*, 1953). (The space between wall and cytoplasmic membrane, of density equal to that of the supporting methacrylate, and therefore presumably filled with methacrylate, was very likely occupied by water in the living cell. Preparative procedures may, of course, have altered the size of this space.)

From the above observations, it would seem that, when a cytoplasmic membrane exists, it will be demonstrable by the techniques here employed unless it is extraordinarily delicate or possesses nearly the same degree of osmiophilia as the cytoplasm. It is difficult, although not impossible, to conceive of a bacillary "cytoplasmic membrane" which so differs from the cytoplasmic membrane of spirilla.

Figure 7 reveals a rather rarely occurring phenomenon, *viz.*, an invagination, *I*, of the cytoplasmic membrane. Such an invagination could arise as the response of a small, weakened area of membrane to a decreased internal or increased external pressure. However, its actual method of origin and significance are unknown.

Unfortunately, practically nothing can be said about the fine structure of the wall and membrane. Several micrographs were obtained which showed 50A fibrils associated with the wall. However, it was impossible to distinguish them from flagellar fibrils of adjacent cells.

Nuclear apparatus. Despite extensive study, the nuclear apparatus of bacteria remains an enigma. The latest work supports a long-standing indication that the reason for this is that the nuclear material is capable of assuming a wide range of configurations and that any given configuration depends on the particular conditions in effect at the moment of observation.

Thus, it was shown by Johnson and Gray (1949) that reversible changes in the appearance of the nuclear material of luminous bacteria could be effected by changes in the salt concentration of the media as well as by changes in other environmental factors. It was noted by Delaporte (1950) and Bisset (1950) that aging would lead to an aggregation of chromatin and the formation of an axial filament. Whitfield and Murray (1956) have extended and elaborated the above observations, and have concluded that the state of the chromatin (dispersed or aggregated) depends on the concentration of electrolytes in the cell environment—a superfluity of cations acting to neutralize the negative charges on the mutually repulsive chromatin units and so bring about their aggregation. It is obvious, as the experiments of Whitfield and Murray show, that any factors acting to disrupt metabolic functions associated with ion regulatory mechanisms will, under the appropriate environmental conditions, result in chromatin aggregation or dispersion. Thus, it has been shown that changes in chromatin configuration may be induced by exposure of the cells to cold, ultraviolet irradiation, starvation, metabolic inhibitors and by the aging process.

Figures 1 to 6 are arranged in order of the age of the culture from which the cell was obtained, from 5 to 22 days. It should be pointed out that, while some cells in the older cultures resembled the majority of cells in the young cultures, no cells

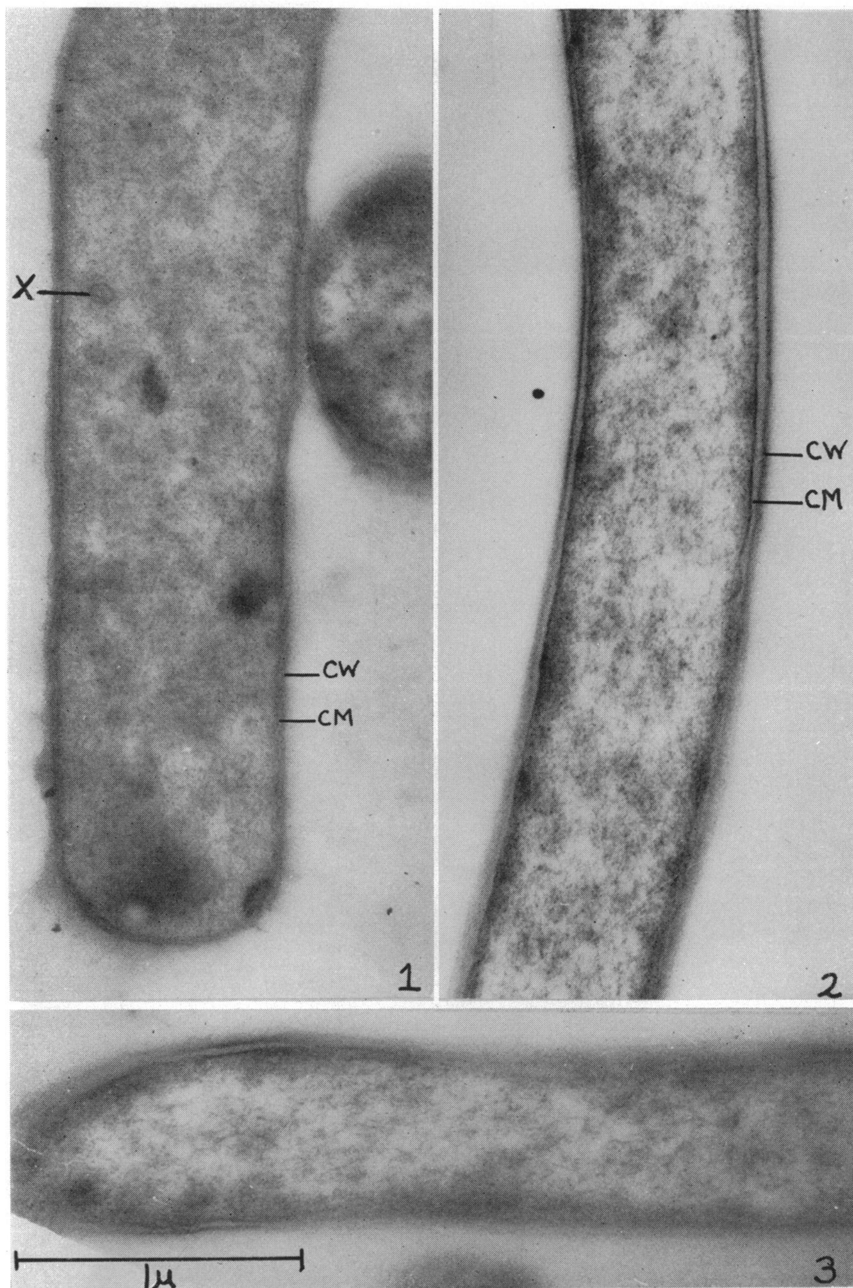
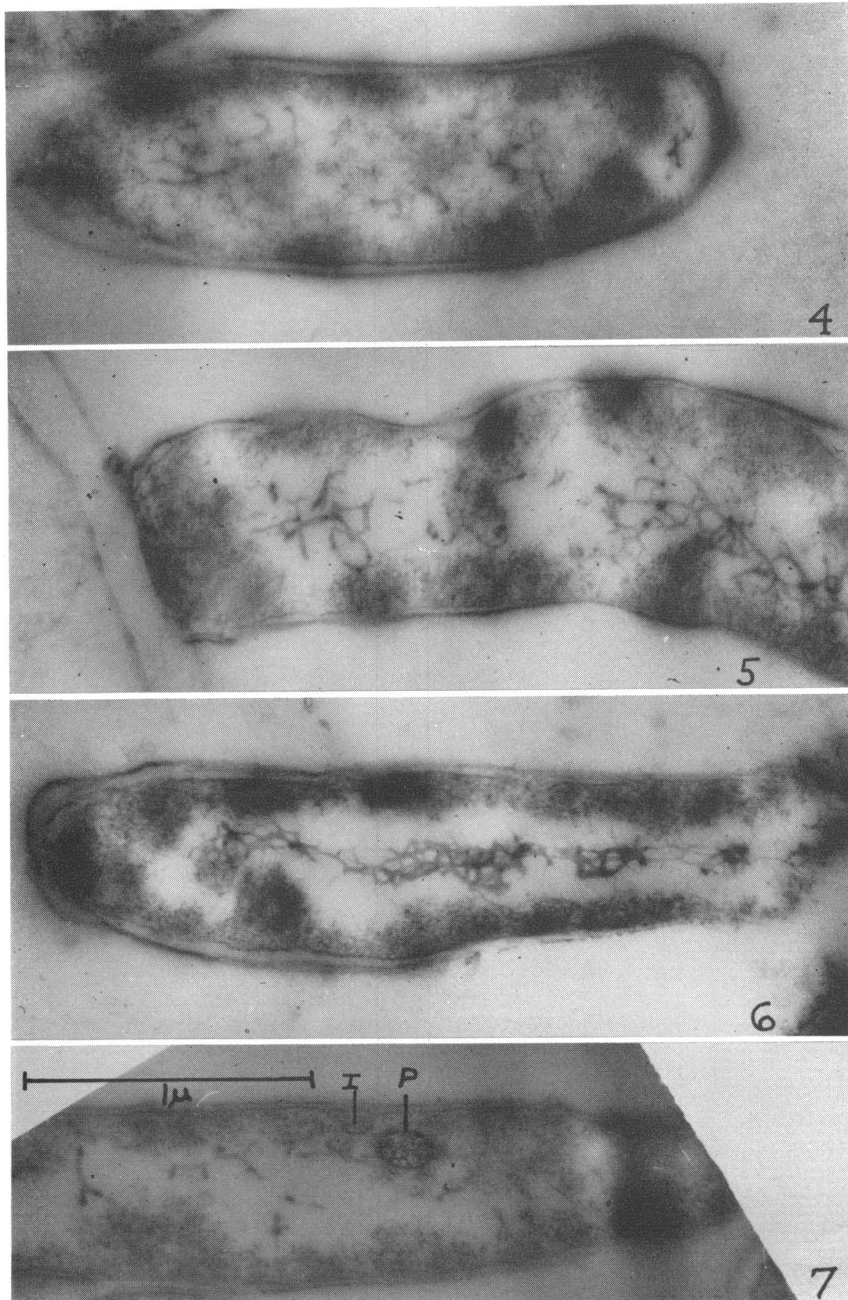


Figure 1. Longitudinal and portion of a transverse section of cells in the logarithmic growth phase.
Figures 2 and 3. Longitudinal sections of cells early in the resting phase. Note protoplasmic fibrils in figure 3. *CW*, cell wall; *CM*, cytoplasmic membrane; *X*, inclusion body.

in the young cultures resembled the old culture cells in having axial networks. Furthermore, an attempt has been made to have each micrograph selected present an appearance typical of cells in a culture of that age. (Stages of development of the cultures were approximated by inspection of the turbidity of the culture tubes.)

Figure 1 shows a longitudinal and a portion of a transverse section of cells in the logarithmic growth phase. The granular nature of the cyto-



Figures 4 to 7. Longitudinal sections of cells successively later into the resting phase. The compaction of the fibrillar network into an axial chain may be seen, with subsequent fragmentation of the chain. *I*, cytoplasmic membrane invagination; *P*, high density particle.

plasm is readily discernible. Figures 2 and 3 are cells from cultures early in the resting phase. The first appearance of fibrils in the protoplast is noted and early stages in the organization of the fibrils into a delicate reticulum may be detected, especially in figure 2. Figure 4 illustrates the transformation of the reticulum into a network. In cells from this stage on, the appearance of the

dense threads in low density areas makes it increasingly apparent that the threads are forming at the expense of the surrounding protoplasm, i. e., the threads are the result of an aggregation of an element which was dispersed in the protoplasm. Figures 5 and 6 illustrate the culmination of the network forming process. The network now appears as a dense mesh of axially arranged threads extending nearly the entire length of the cell.

The threads attain a maximum diameter of about 400A. At irregular intervals along their length occur very dense areas which are nearly circular in section and which are approximately the same diameter as the threads. The significance of these areas is unknown.

It appears that further aging of the cultures results in the breaking up of the network into short U-shaped, Y-shaped and rod-shaped fragments.

The process depicted in the above series of micrographs may be summarized as follows: The protoplast, which is of a fine granular texture in young cells, becomes interspersed with delicate fibrils in those cells from cultures over five days in age. The fibrils come together forming a reticulum as the cells progress into the resting phase. As cultures age, the thin fibrils coarsen and form a dense network or assemblage of fibrils in a low density milieu, the whole being axially disposed in the cell. Further aging results in the fragmentation of the network.

It now remains to establish the nature of the dense, thread-like material. In their work on *Escherichia coli*, Birch-Andersen *et al.* (1953) observed high density threads in vacuoles in the cytoplasm. Further observations by Maaløe *et al.* (1954) revealed that phage particles showed the same high electron density as the vacuolar threads. Since it was known that the cores of free phage particles consist largely of DNA, the authors concluded that the threads were composed of nuclear material. These threads resemble in size and density the threads in the axial network of spirilla.

Williams (1956, *personal communication*) has described in *Spirillum cohnii* a condensation of the chromatin into a common element which extends the length of the cell. This common element subsequently divides into shorter units. Although a difference in species and culture age must be taken into account when comparing the above observations with those reported herein, one must be struck by the similarities in the two instances. It should be realized, of course, that the "common element" of Williams probably corresponds to the low density zone *and* the included network of the present report and not to the network alone.

In consideration of the already discussed observations by Whitfield and Murray (1956), the findings of the workers in Stockholm, the report of Williams, and the identification as chromosomal structures of similar threads in *Bacillus megaterium* by Piekarski and Giesbrecht (1956), the authors are of the opinion that there is ample evidence to suggest that the dense network represents a considerable portion of the chromatin of *S. serpens*. The observed aggregation can reasonably be explained as due to the accumulation of cations in the aging culture with a concomitant loss of osmoregulative faculties by the aging cells, according to the system proposed by Whitfield and Murray (1956). If this opinion is correct, it would then seem that the chromatin of *S. serpens* must be quite dispersed in the cells of young cultures and that, therefore, *S. serpens* might be said to possess a diffuse nucleus. (Lewis, 1941, p. 193, defines a diffuse nucleus as consisting of "fine particles of chromatin dispersed uniformly in the cytoplasm.") It is indeed interesting that use of a relatively new research technique should suggest a conclusion long held by many workers in the field. (It should be realized that observations of phenomena occurring in spirilla need not necessarily be expected to occur in bacteria in general.)

Should the conclusions herein suggested be supported by further work, it would then be possible to explain why no nuclear membrane has been observed—none is required or expected for a diffuse nucleus.

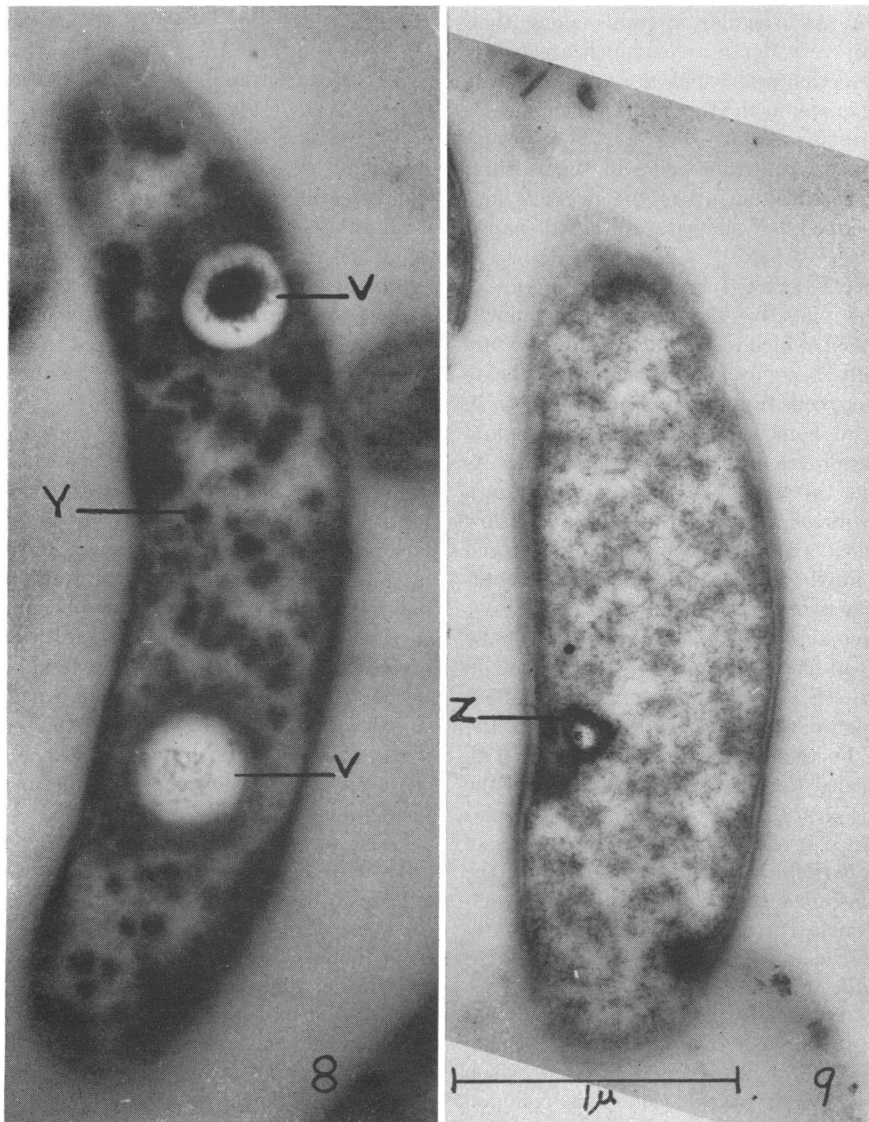
Of course, it is also possible that the apparent absence of discrete nuclei from the cells of young cultures of this spirillum could be a result of the destruction or disorganization of existent but delicate nuclei due to the preparative procedures. Such nuclei might be homologous to the "primary nuclei" described by Knaysi (1955) in the actively growing cells of young cultures of bacilli. These "primary nuclei" appear much less robust than the compound nuclei to which they may give rise.

Although serial sections were not employed in this study, the possibility that discrete nuclei were overlooked is extremely remote, as a single microscope field always presented cells cut at

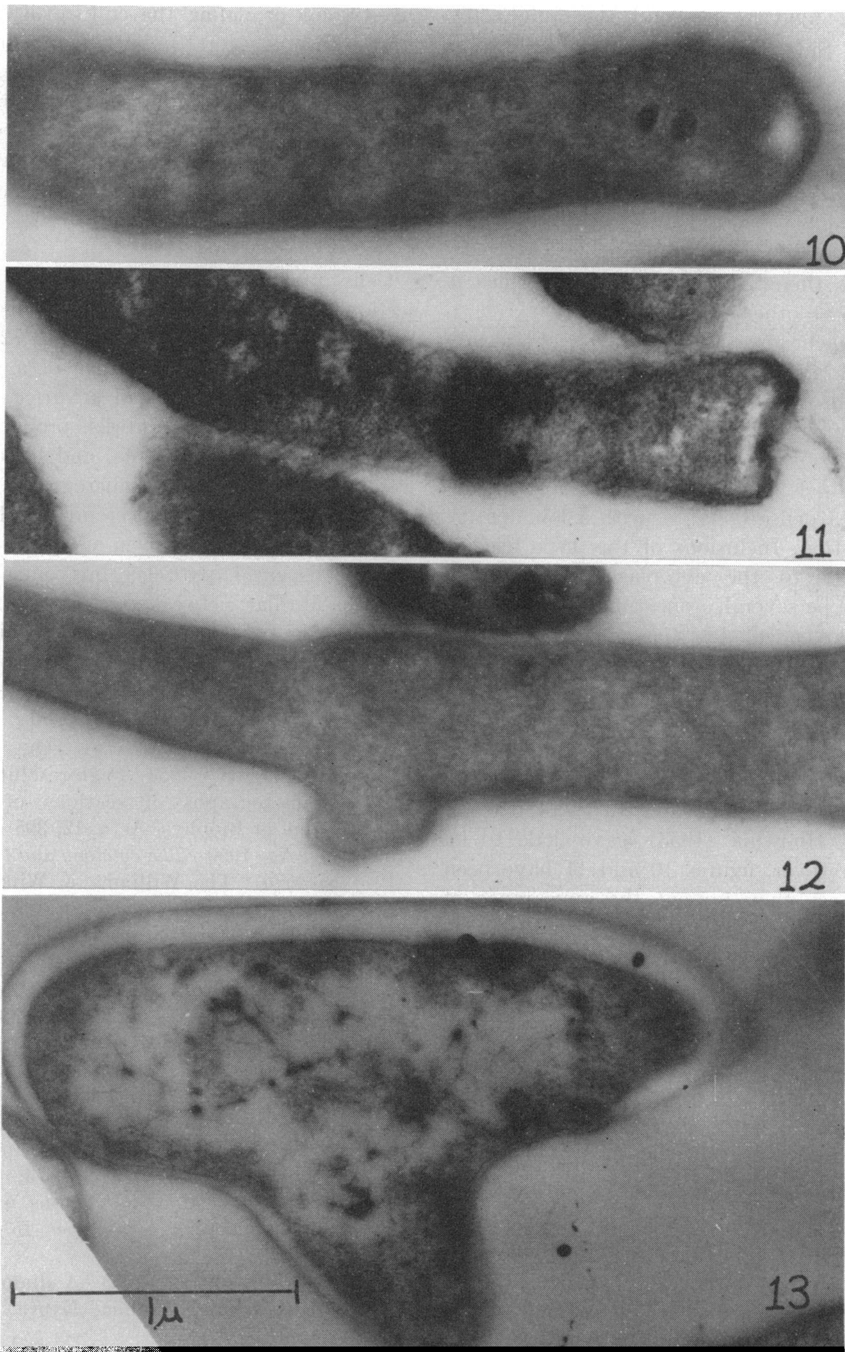
many levels. Such a population of sections provides information comparable to that supplied by serial sections in so far as the structures under discussion in these cells are concerned.

The fragments of network material in figure 7 are in an old cell. They do not, therefore, correspond temporally to the primary nuclei of Knaysi. However, their appearance as organized bodies consisting of several terminal beads connected by material of lower density suggests that they might represent an approximation of what Knaysi designates primary nuclei. One might then postulate that the network (possibly homologous to the compound nuclei) broke up into fragments which represent equivalents of the primary nuclei.

Cytoplasmic inclusions. Several morphologically different cytoplasmic inclusions were observed in the cells. The most interesting type appears in sections as two nearly concentric rings, the inner



Figures 8 and 9. Longitudinal sections of cells included to illustrate types of inclusions, *Y* and *Z*. *V*, vacuoles, which appear to be sites of formation or accumulation of a dense particulate matter.



Figures 10, 11, 12, and 13. **Figures 10 and 11 illustrate the delicate flagellar fibrils which occur as a tuft at the cell ends.**

Figures 12 and 13 illustrate the phenomenon of budding.

being about 400A in diameter, the outer 850A in diameter. Since these particles have only been observed in circular or slightly elongated profile, it is likely that they represent a sphere within a sphere. Although some sections show none of the particles, others show as many as three. Therefore there may be as many as 10 to 20 in a cell. Such a particle is labeled X in figure 1.

Figure 7 contains a rather high density particle, P, which is thought to be an artifact since it appeared in no other section.

Another inclusion type, Y, in figure 8, appears as a small globule about 850A in diameter. Figure 8 is also of interest because of the vacuoles, V, which appear to be sites of formation or accumulation of a dense particulate matter.

Still another type of inclusion appears in figure 9 as a dense body, Z, with a low density central portion. Inclusions of this type usually appear close to the cytoplasmic membrane. There may be several, appearing slightly differently, in a given cell.

Although the significance of the above inclusion types is unknown, they have been presented to illustrate the wide variety of structures, some very likely artifacts, seen in these cells.

Flagella. The electron micrographs of flagella which have been published by Van Iterson (1947) and Houwink (1953) leave little to be desired. However, figures 10 and 11 have been included since they present the appearance of unshadowed flagella. Unfortunately, these flagella have been partially destroyed by the processing. Nevertheless, one can easily make out the flagellar tuft at the end of each cell. There is a suggestion, in the original prints, that the individual flagella penetrate through the wall to the membrane. No information relative to the flagellar structure was obtained.

Budding. Figures 12 and 13 illustrate the process of branching or budding and suggest that this phenomenon occurs independent of the state of chromatin condensation. This suggestion becomes less surprising when one considers a report from Williams (1956, *personal communication*) that no correlation was found between the various nuclear phenomena and cell division.

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SUMMARY

Cells of *Spirillum serpens* were ultrathin sectioned with diamond and glass knives following methacrylate embedding at 72 C. The sections indicated the existence of a cell wall, cytoplasmic membrane, and a variety of cytoplasmic inclusions. Fine fibrils, probably representing flagellar components, and budding cells were observed. A series of micrographs has been included which is thought to represent the change in chromatin distribution on aging—from small, widely dispersed particles in young cells to delicate fibrillar networks in cells early in the resting stage to coarse axial chains (which subsequently fragment) in old resting cells.

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