THE FINE STRUCTURE AND MODE OF ATTACHMENT OF THE SHEATHED FLAGELLUM OF *VIBRIO METCHNIKOVII*

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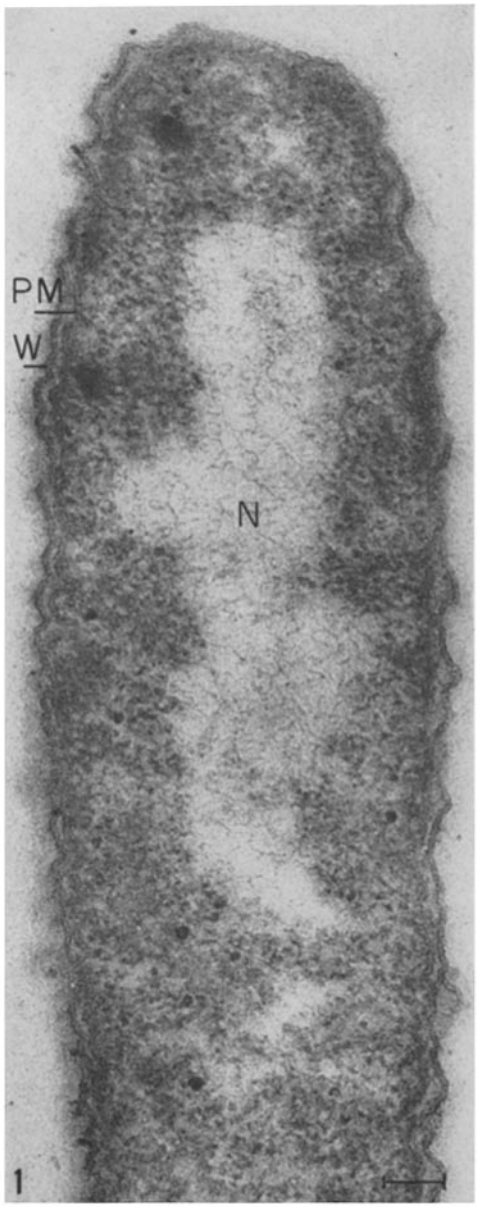
ABSTRACT

The sheathed flagellum of *Vibrio metchnikovii* was chosen for a study of the attachment of the flagellum to the bacterial cell. Normal and autolysed organisms and isolated flagella were studied by electron microscopy using the techniques of thin sectioning and negative staining. The sheath of the flagellum has the same layered structure as the cell wall of the bacterium, and in favourable thin sections it appears that the sheath is a continuation of the cell wall. After autolysis the sheath is usually absent and the core of the flagellum has a diameter of 120 A. Electron micrographs of autolysed bacteria negatively stained with potassium phosphotungstate show that the core ends in a basal disc just inside the plasma membrane. The basal disc is about 350 A in diameter and is thus considerably smaller than the "basal granules" described previously by other workers.

Bacterial flagella were among the first biological specimens to be studied by electron microscopy (*e.g.* Mudd and Anderson, 1942) and the earlier observations on the structure of the flagella of living bacteria (reviewed by Pijper, 1957) and stained bacteria (reviewed by Leifson, 1960) were soon verified and extended. The bundles of flagella typical of *Spirillum* (van Iterson, 1947) and the sheathed flagella of *Bacillus brevis* (De Robertis and Franchi, 1951) and *Vibrio metchnikovii* (van Iterson, 1947, 1953) were described as well as the more common peritrichous flagella of many bacteria (Houwink and van Iterson, 1950). Most authors concluded that the flagella pass through the bacterial cell wall and are attached to specialized structures in the cytoplasm. These "basal granules" were most frequently seen in autolysed cells where they were sometimes as large as 0.1 to 0.2 μ in diameter (Grace, 1954; Tawara, 1957). The existence of these structures has been doubted by Pijper (1957) who has suggested that the basal granules are probably artefacts produced during autolysis of the bacterial cell.

The earlier observations with the electron microscope were made on unsectioned, metal-shadowed bacteria, and the pictures gave no clear indication of the mode of attachment of the flagella, or of the structure of the basal granules. It was therefore decided to study the structure and mode of attachment of bacterial flagella by the newer techniques of thin-sectioning and negative staining. *Vibrio metchnikovii* was chosen since the flagellum of this organism is sheathed with an over-all diameter of about 300 A and is easier to detect in thin sections than the flagella of organisms such as *Salmonella typhimurium* where the flagella are unsheathed and only 120 A in diameter.

We have also studied the structure of the sheath of the flagellum of *Vibrio metchnikovii* and the relationships of the sheath to the core of the flagellum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N
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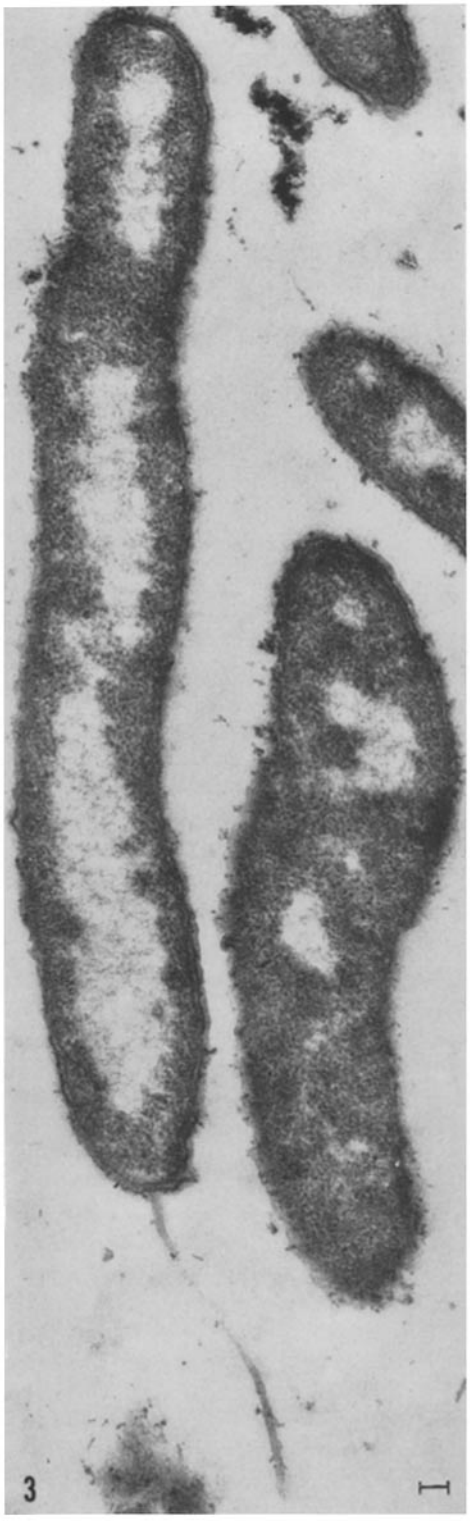
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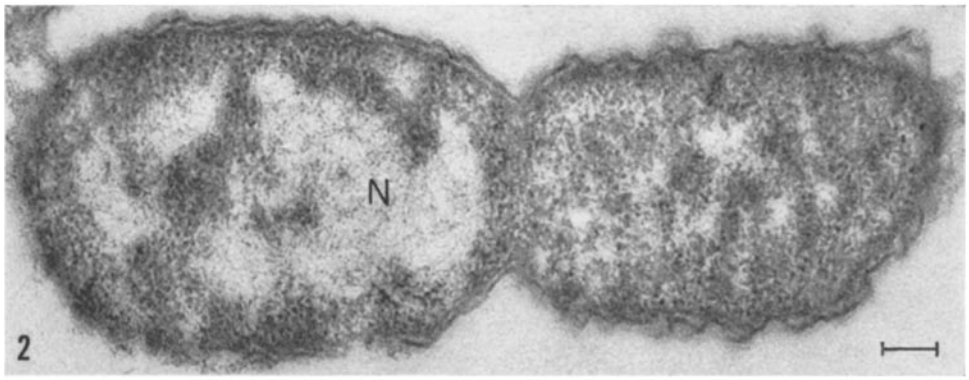
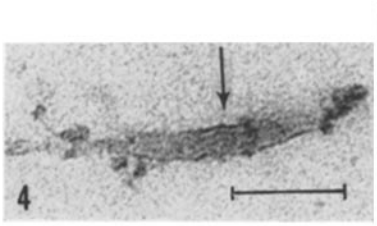
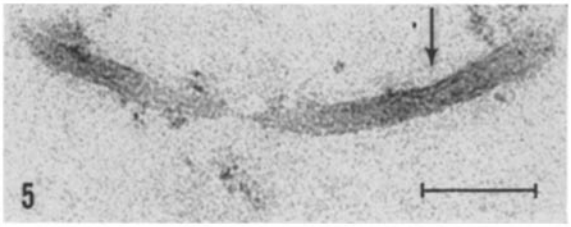






FIGURES 1 TO 5 Electron micrographs of thin sections of *Vibrio metchnikovii*. The scale marks represent 0.1 micron.

FIGURE 1 The bacterium is bounded by a thin cell wall (*W*) and plasma membrane (*PM*). The nuclear material (*N*) is in the form of fine fibrils. Araldite embedding. Section stained with uranyl acetate. × 70,000.

FIGURE 2 A dividing cell has a simple constriction at the point of division. The nuclear region (*N*) is less dense than the cytoplasm. Araldite embedding. Section stained with uranyl acetate. × 70,000.

FIGURE 3 The single polar flagellum is curved and passes in and out of the plane of the section. Methacrylate embedding. × 40,000.

FIGURE 4 At higher magnification the layered structure of the sheath of the flagellum is visible (arrow). Methacrylate embedding. Section stained with uranyl acetate. × 150,000.

FIGURE 5 The sheath appears as a "unit membrane." Methacrylate embedding. Section stained with lead hydroxide. × 150,000.

and to the bacterial cell. Recently, information about the nature of the sheath has been provided by Gordon and Follett (1962) who used the negative staining technique to investigate the changes produced by treatment of the flagellum of *V. metchnikovii* with hydrochloric acid or urea, and after autolysis.

MATERIALS AND METHODS

ORGANISM AND CONDITIONS OF GROWTH: *Vibrio metchnikovii* NCTC 8443 was used throughout this study. The organism was maintained by weekly subculture on Oxoid nutrient agar. Cultures grown in Oxoid nutrient broth at 37°C for 16 hours were used for the examination of the normal organisms. Auto-

lysed preparations of *V. metchnikovii* were obtained by washing bacteria from nutrient agar slopes with distilled water and incubating the bacterial suspension at 37°C for several days.

ISOLATED FLAGELLA: Flagella were isolated from bacteria grown on Oxoid nutrient agar in Roux bottles at 37°C. The bacteria were washed from the surface of the agar with distilled water and the flagella were then removed mechanically and purified by centrifugation as described by Kerridge, Horne, and Glauert (1962).

FIXATION: Some cultures were fixed, before being washed, by adding sufficient 40 per cent formaldehyde to the growth medium to give a final concentration of formaldehyde of 1 per cent. After 16 to 24 hours the preparations were washed with distilled water.

For thin sectioning the bacteria were fixed in buffered osmium tetroxide by the method of Ryter and Kellenberger (1958), treated with uranyl acetate, dehydrated in ethanol, and embedded in *n*-butyl methacrylate or Araldite by the standard techniques.

THIN SECTIONING: Thin sections were cut on an A. F. Huxley microtome with a glass knife. Some sections were stained with uranyl acetate or lead hydroxide.

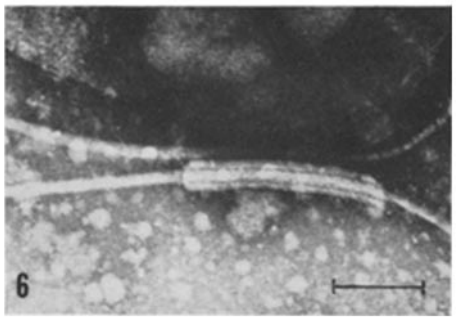
NEGATIVE STAINING: A suspension of bacteria or flagella in distilled water was mixed with an equal volume of 2 per cent (w/v) potassium phosphotungstate. A small drop of this mixture was placed on an electron microscope grid coated with collodion and carbon, left for about 1 minute, and then dried with filter paper (Thornley and Horne, 1962).

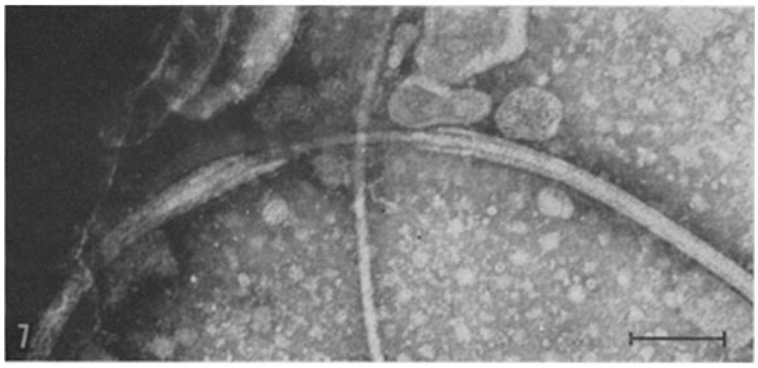
ELECTRON MICROSCOPY: Electron micrographs were taken with a Siemens Elmiskop I electron microscope, operating at 60 kv with a 50 μ objective aperture, at instrumental magnifications of 6,000 to 42,000.

RESULTS

The Fine Structure of the Bacterium

Cells of *Vibrio metchnikovii* examined in thin sections have the typical fine structure of Gram-negative bacteria. Each organism is bounded by a cell wall (Fig. 1, *W*) and a plasma membrane





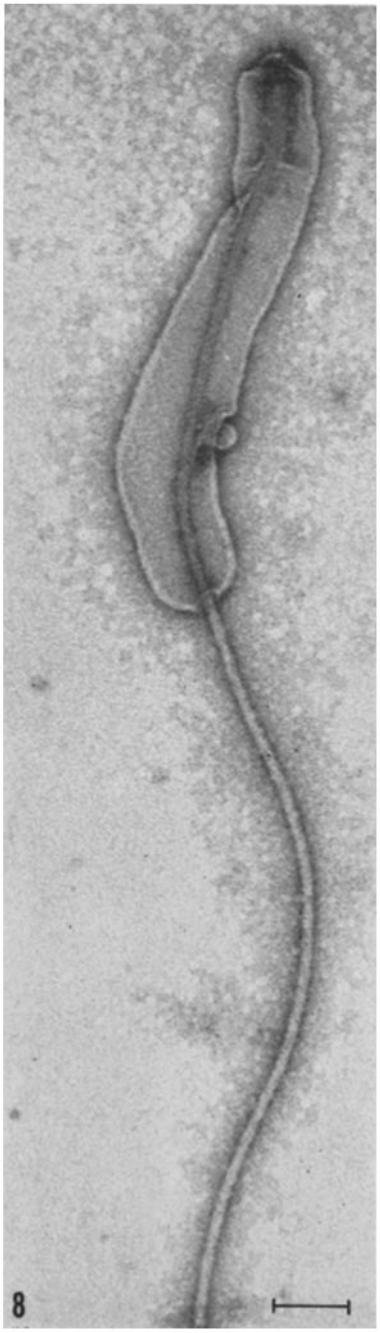
FIGURES 6 TO 9 Electron micrographs of flagella of *Vibrio metchnikovii* negatively stained with potassium phosphotungstate. The scale marks represent 0.1 micron.

FIGURES 6 AND 7 In places the sheath of the flagellum has broken to reveal the inner core. × 120,000.

FIGURE 8 Part of the swollen membranous sheath still surrounds the core of a flagellum. Fixed with formalin. × 100,000.

FIGURE 9 Isolated flagella have lost their sheaths and are about 120 A in diameter. A central dense line is visible in some of the flagella, and one flagellum has a terminal hook (arrow). × 100,000.

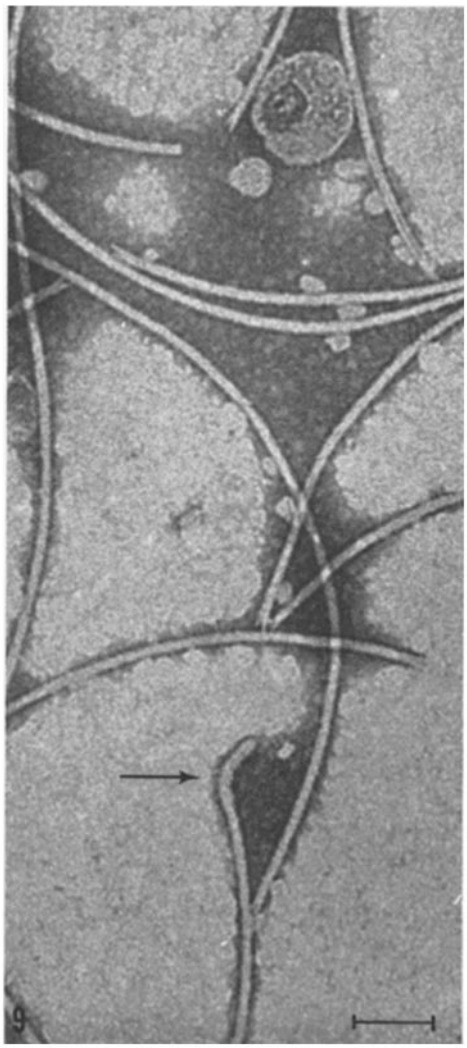
(Fig. 1, *PM*); both wall and membrane appear as two dense layers separated by a less dense layer and each has a total thickness of about 75 A. The cells divide by a simple "pinching off" process (Fig. 2) without the formation of a cross-wall or cross-plate.



The nuclear regions (Figs. 1 and 2, *N*) are less dense than the cytoplasm and contain the networks of fine fibrils characteristic of bacteria fixed by the method of Ryter and Kellenberger (1958). The cytoplasm is packed with granules; the largest are about 150 A in diameter and presumably correspond to the ribosomes that can be isolated from preparations of broken cells.

The Fine Structure of the Flagellum

Each bacterium has one or two polar flagella (Figs. 3 and 14). After fixation and embedding the flagella appear to retain the sinusoidal shape that is seen in fixed and stained preparations examined by light microscopy (Leifson, 1960) and in consequence pass in and out of the plane of the



section (Fig. 3). In favourable sections it is seen that the outer sheath of the flagellum consists of two dense layers separated by a less dense layer and has a total thickness of about 75 A, (Figs. 4 and 5, arrows) and is thus similar in appearance to the cell wall or plasma membrane of the bacterium.

Thin sections reveal little structure in the core region of the flagellum within the sheath. The diameter of this region is about 120 A and the whole flagellum is about 270 A thick.

When bacteria are washed in distilled water in preparation for negative staining, many of the flagella lose their sheaths, either wholly or partially (Figs. 6 and 7, *cf* van Iterson, 1953; Gordon and Follett, 1962) and the remnants of this sheath often appear swollen (Fig. 8) even after fixation with formalin. The core of the flagellum has a diameter of about 120 A and in this respect is similar to the unsheathed flagella of *Salmonella typhimurium* (Kerridge, Horne, and Glauert, 1962) and *Proteus vulgaris* (Rogers and Filshie, 1963). A few of the flagella removed mechanically from the bacteria have terminal hooks (Fig. 9, arrow) similar to those originally described in shadowed preparations of *Agrobacterium radiobacter* (Houwink and van Iterson, 1950), *Spirillum* sp. (Houwink, 1953) and *P. vulgaris* (van Iterson, 1953) and recently observed in negatively-stained preparations of *S. typhimurium* (Kerridge, Horne, and Glauert, 1962).

The flagella in these preparations were not clean enough to make possible a study of their structural subunits at high resolution as was successfully achieved for *Salmonella typhimurium* (Kerridge, Horne, and Glauert, 1962). Remnants of the sheath (Fig. 7) and other materials are present and partially obscure the surface of the flagellum. An adequate procedure for removing this adsorbed layer has not yet been devised. It is noticeable, however, that the phosphotungstate appears to penetrate into a central region of the flagellum so that a fine dark line is seen (Figs. 9 and 15).

The Relationship of the Flagellum to the Bacterium

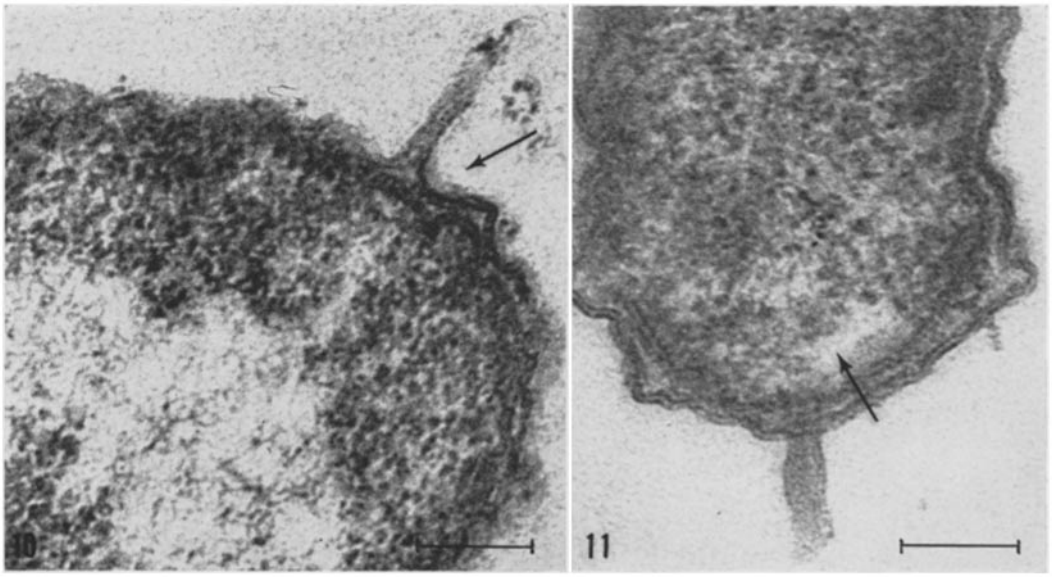
An examination of thin sections through the point of attachment of the flagellum indicates that the sheath is continuous with the cell wall (Fig. 10, arrow), and that the core of the flagellum is attached to the plasma membrane or just within it. In some bacteria a clear area of cytoplasm, free of granular elements, is present near the base of the flagellum (Fig. 11, arrow). Similar clear areas have been observed by Murray and Birch-Andersen (1962) in sections of *Spirillum serpens* near the bases of the tufts of flagella, but the significance of these regions is unknown. Murray and Birch-Andersen also observed "an extra membrane about 200 A inside the cytoplasmic membrane, forming a ring around the area of insertion (of the flagella), and joined to it by fine bars." A modified membrane has not been seen associated with the base of the flagellum in *Vibrio metchnikovii*; the plasma membrane has the same appearance near the base of the flagellum (Fig. 11) as elsewhere (Fig. 1, *PM*).

In negatively stained preparations of autolysed bacteria the sheath is usually absent and the point of attachment of the core of the flagellum is seen. There is often a bend in the flagellum at the surface of the bacterium (Fig. 14) and it seems likely that these bent ends correspond to the terminal hooks observed in some isolated flagella (Fig. 9, arrow).

Pictures of intact bacteria cannot show conclusively that the cores of the flagella pass through the cell walls, but the electron micrographs of autolysed cells suggest that they do and that they end in small basal structures associated with the plasma membrane (Figs. 12 to 14). The basal structure is visible in the majority of the bacteria examined and appears to consist of a small disc or cup, 300 to 350 A in diameter, which is usually seen from the side (Figs. 12 and 13, arrows). In face view (Fig. 14, inset) the disc appears as a light ring with a light central dot, and similar structures are occasionally found attached to the ends of fragments of flagella detached from the bacteria (Fig. 15, arrow). The end of the flagellum near the basal disc often appears cross-banded (Fig. 13).

DISCUSSION

The three components of the flagellum of *Vibrio metchnikovii*, the sheath, the core, and the basal structure, were first observed by van Iterson (1953) who found that the core is more resistant to autolysis than the sheath and concluded that the sheath is a loose membrane. Similar observations were made by Gordon and Follett (1962) on negatively stained cells. They also found that the sheath rapidly disintegrated on treatment with hydrochloric acid and became swollen on treatment with 6 M urea, while the core was relatively resistant. In this respect the core appears to differ



FIGURES 10 AND 11 Electron micrographs of thin sections of *Vibrio metchnikovii*. Methacrylate embedding. Sections stained with uranyl acetate. The scale marks represent 0.1 μ.

FIGURE 10 The sheath of the flagellum appears to be continuous with the cell wall of the bacterium (arrow). × 150,000.

FIGURE 11 A clear area, free of granules, is present near the base of the flagellum (arrow). × 150,000.

from the unsheathed flagella of *Proteus vulgaris* and *Salmonella typhimurium* which disintegrate in 6 M urea and under acid conditions (Weibull and Tiselius, 1945; Koffler, Mallett and Adye, 1957); the *Vibrio* flagella were pretreated with formalin and potassium iodide, however, which may have affected the stability of the cores. Gordon and Follett (1962) concluded that the core and sheath of *V. metchnikovii* are chemically different and that the sheath is more closely related to the cell wall than to the protein flagellin. They presented no evidence that the cell wall is sensitive to the same treatments that disintegrate the sheath of the flagellum, but their suggestion is supported by the present observations that the sheath and the cell wall have the same structure when seen in thin sections and appear to be continuous with each other.

The core of the flagellum has the same diameter as the unsheathed flagella of *Salmonella typhimurium* and *Proteus vulgaris* and is probably composed of protein, although chemical analyses have not yet been made since it has not been possible to obtain clean preparations of the core. The fine dark line observed in the centre of the core of the flagellum after autolysis and negative staining is not seen in the untreated flagella of *S. typhimurium*, although the model suggested for the structure of the flagellum by Kerridge, Horne, and Glauert (1962) has a central hole about 30 A in diameter into which the negative stain might be expected to penetrate under suitable conditions. It seems likely that there are similarities in structure between the flagella of the two organisms and that the core of the *Vibrio* flagellum may be composed of a number of parallel subfibrils.

The function of the hook at the base of the flagellum is not known, although it is possible that its role is to provide a firm attachment of the flagellum to the bacterium (van Iterson, 1953) since the hook appears to represent the part of the flagellum within the cell wall. In *Proteus vulgaris* the hooks easily break away from the main part of the flagellum; these fragments have been described as "rootlets" by Rogers and Filshie (1963). After negative staining, the "rootlets" show

a hexagonal pattern of globular units which is more distinct than on the surface of the flagellum, where it is presumably obscured by adsorbed material.

The study of autolysed cells by the negative staining technique indicates that the core of the flagellum of *Vibrio metchnikovii* is attached to a basal disc just inside the plasma membrane. The observation of a similar structure attached to the ends of isolated flagella supports the view that this structure is not an artefact. Ideally it is necessary to show this structure in the cytoplasm of actively growing bacteria, but in such cells the cytoplasm is so densely packed with granular material that it is impossible to resolve a structure of similar density that is only 350 A in diameter. It seems certain, however, that the larger basal granules described by previous workers in shadowed preparations are agglomerations of cytoplasmic debris surrounding the basal disc.

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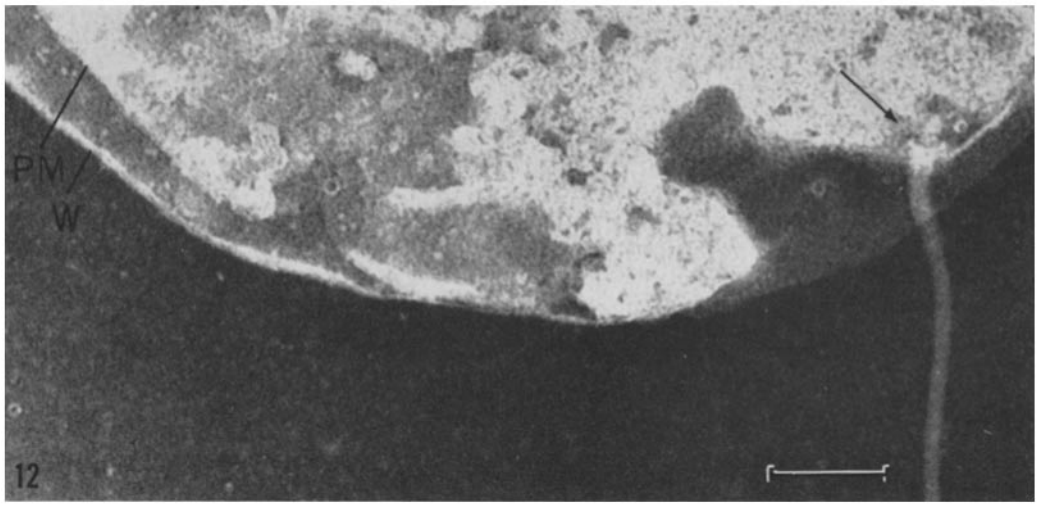
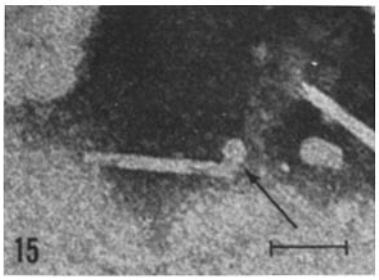




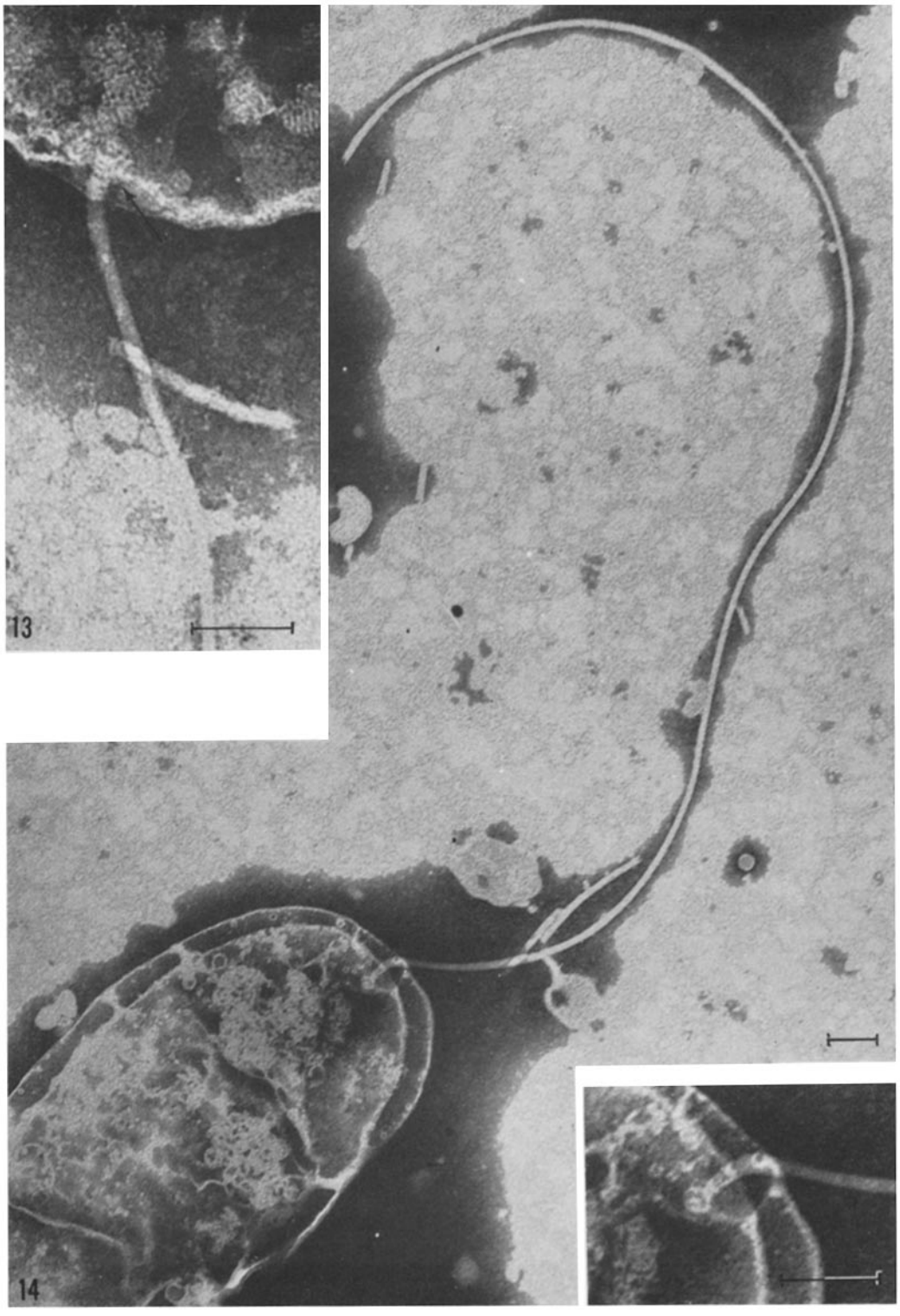
FIGURES 12 TO 15 Electron micrographs of negatively stained cells of *Vibrio metchnikovii* after autolysis. The scale marks represent 0.1 μ.

FIGURE 12 A spheroplast in which the plasma membrane (*PM*) has retracted from the cell wall (*W*). The flagellum has lost its sheath and the core ends in a small basal disc (arrow). × 150,000.

FIGURE 13 A side view of the basal disc (arrow). The end of the flagellum near the basal disc appears cross-banded. × 150,000.

FIGURE 14 The negative stain outlines the bacterium and its single polar flagellum. × 70,000. Inset. An enlargement of the basal disc seen in face view. × 150,000.

FIGURE 15 A structure similar to a basal disc (arrow) is attached to one end of an isolated fragment of a flagellum. × 100,000.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