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Basal Organelles of Bacterial Flagella

GERMAINE COHEN-BAZIRE AND JACK LONDON[1]

Department of Bacteriology and Immunology, University of California, Berkeley, California 94720

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Liberated by enzymatic lysis of the cells, the flagella of *Rhodospirillum rubrum*, *R. molischianum*, and *R. fulvum* all have a similar structure. The hook at the base of the flagellum is connected by a short, narrow collar to a paired disc in the basal organelle. This paired disc is in turn connected to a second paired disc. The disposition of flagella to which fragments of the cell membrane still adhere suggests that the narrow collar at the base of the hook traverses both the wall and the membrane, and that the upper pair of discs in the basal organelle lies just beneath the surface of the membrane.

The discovery that bacterial flagella can be detached mechanically from cells (8) and subsequently purified by relatively simple procedures (17, 22, 23) has made possible detailed studies on the anatomy and chemistry of these organelles (3, 16). Except for the thick, membrane-enclosed flagella of vibrios (12), the extracellular part of bacterial flagella seems to have a relatively simple construction (16). The flagella of a given bacterial strain are composed of a single kind of protein ("flagellin"). In the monomeric state, flagellin exists as isodiametric particles of relatively low molecular weight (approximately 40,000). The flagellum consists of monomeric units polymerized into long, multistranded threads with a regular helicoid form, and with a diameter in the range of 100 to 200 A. Flagellar thickness and wavelength are constant properties of a given bacterial strain or species. The differences between bacteria with respect to these flagellar parameters presumably reflect differences in the primary structures of their specific flagellin, which in turn affect the mode of polymerization.

This pleasingly pythagorean picture becomes obscure near the point where the flagellum penetrates the surface of the bacterial cell. It has been known since the work of Weibull (24) that the flagellum originates from the protoplast and passes through the fabric of the cell wall. However, attempts to ascertain the detailed structure of the basal region have given scanty and inconclusive results. Electron microscopy of sectioned cells (19, 21) has demonstrated that the flagella originate beneath the cell membrane, but has not so far clearly revealed their mode of internal attachment. The examination of partly lysed cells (or of flagella released from them) in shadow-cast or negatively stained preparations has proved more informative. Bacteria examined in this manner include *Vibrio metchnikovii* (12), *Proteus* spp. (1, 13), and *Bacillus* spp. (2). Study of lysed cells has one major drawback: it is not easy to exclude the possibility that some of the observed structures are postmortem artifacts. Nevertheless, the following conclusions seem reasonably well established.

(i) Just external to the point where it enters the cell, the flagellum is characteristically bent and slightly thickened, to form a basal hook. Hooks have also been seen in preparations of mechanically detached flagella (14, 15, 18).

(ii) In partly lysed cells, the hooks frequently appear to be attached to round or disc-shaped basal bodies, some 300 to 500 A in diameter, situated within the cell membrane. The nature of these structures is somewhat controversial; they have been interpreted by some (13) as distinct organelles and by others (2, 12) as anchoring structures surrounded by adherent membrane fragments or cytoplasmic debris.

(iii) A smaller and more regular structure than the basal body has been seen attached to the hooks of occasional flagella in partly lysed cells of *Proteus* spp. (1, 13) and of *Bacillus* spp. (2). Its organization is resolved to different degrees in the various published illustrations. The clearest electron micrograph [Fig.23 of Abram et al. (1)] reveals it as two pairs of discs, separated by a thin connecting strand. Each pair of discs is approximately 200 A wide and 100 A long, the total length of the structure being approximately 300 A. One pair of discs is closely adherent to the base of the book. Both Abram et al. (1) and Hoeniger et al. (13) have interpreted these

[1] Present address: National Institute of Dental Research, Bethesda, Md. 20014.

relatively rare objects as being composed, at least in part, of cytoplasmic debris.

We describe here the basal organization of the flagellum in three species of photosynthetic spirilla, as inferred from the examination in the electron microscope of enzymatically lysed cells.

MATERIALS AND METHODS

The following bacteria were examined: *Rhodospirillum rubrum* strain Sl; a locally isolated strain of *R. molischianum*; and *R. fulvum* strain K.K. (20). Cultures were grown at 30 C in an illuminated water bath. The light intensity at the front window of the water bath was approximately 1,000 ft-c. The growth medium consisted of 0.5% (w/v) NH_4 DL-malate and 0.5% (w/v) Difco yeast extract, dissolved in Hutner mineral base (7). For the cultivation of *R. fulvum* and *R. molischianum*, this medium was supplemented with 0.05% sodium ascorbate. Cultures were constantly gassed with a mixture of 95% N_2 and 5% CO_2.

Cells in the course of exponential growth were harvested by centrifugation, washed, and suspended in a mixture of 2.5×10^{-3} M tris(hydroxymethyl) aminomethane (Tris) chloride buffer (*p*H 9.0) and 5×10^{-4} M ethylenediaminetetraacetic acid (EDTA). Samples of such suspensions containing 0.5 to 1.0 mg of cells (dry weight) per ml were mixed with the purified bacteriolytic enzyme of *Myxobacterium* AL1 (9), at a final concentration of 100 μg/ml. The suspensions were incubated at 35 C until the optical density at 680 mμ had fallen to approximately 30% of its initial value (approximately 1 to 2 hr). Deoxyribonuclease (Calbiochem, Los Angeles, Calif.) was added at a concentration of 10 μg/ml to reduce the viscosity of the lysates; residual cells and coarse debris were removed by low-speed centrifugation. The deeply pigmented supernatant liquid was then centrifuged at 100,000 $\times$ *g* for 30 min, and the pellet was collected and suspended in 0.03 M Tris chloride (*p*H 7.6). Drops of this suspension were diluted in distilled water containing 0.04% (w/v) sucrose and placed on carbon-stabilized, Formvar-coated, 300-mesh copper grids. The preparations were negatively stained (4) with either 2% (w/v) potassium phosphotungstate (*p*H 7.0) or 1% (w/v) uranyl acetate (*p*H 4.4) and were immediately examined in a Siemens Elmiskop I electron microscope.

The purified bacteriolytic enzyme of *Myxobacterium* AL1 was kindly provided by J. C. Ensign.

RESULTS

The bacteriolytic enzyme of *Myxobacterium* strain AL1 is of unusually low molecular weight (8,700). It is capable of hydrolyzing peptide bonds of both proteins and mureins (10). As a consequence, it can lyse living cells of many gram-positive bacteria, but is ineffective in lysing most gram-negative bacteria, with the exception of spirilla (9). Ensign and Wolfe (9) demonstrated rapid lysis of *R. rubrum*, *Spirillum serpens*, and *S. itersonii*, and we have found that *R. molischianum* and *R. fulvum* are also highly sensitive to the enzyme.

Electron microscopic examination of the particulate fractions isolated from lysates of the three *Rhodospirillum* species reveals the presence of membrane and wall fragments, together with flagella, either free or attached to these fragments.

The particulate fraction isolated from enzymatic lysates of *R. rubrum* contains many typical cup-shaped membrane fragments (chromatophores; Fig. 1 and 2) derived from the vesicular infoldings of the cell membrane characteristic of this species (5). The particulate fractions from enzymatic lysates of *R. molischianum* and *R. fulvum* contain larger, usually multilayered membrane fragments (Fig. 3 and 6), presumably derived from the stacked lamellar infoldings of the membrane evident in thin sections of both these species (6, 11).

In lysates of *R. rubrum*, isolated and very long flagella are often observed. Each flagellum is approximately 120 A wide, and terminates in a somewhat thicker hook, approximately 150 A wide. On many flagella, the hook is attached to an organelle with a very regular structure (Fig. 1, 2). A narrow collar, approximately 80 to 90 A in diameter, connects the rounded extremity of the hook to a pair of discs, 250 A wide and 140 A thick; this pair of discs is in turn connected by a second narrow collar to a second pair of discs of similar dimensions. Occasionally, membrane fragments within which the basal elements of several flagella remained associated were observed. Such a configuration is shown in Fig. 4; the double pair of discs at the bases of six flagella can be seen with reasonable clarity. Both the upper and the lower pair of discs appear to be joined to their neighbors by membranous material. Fragments of this membranous material are sometimes observed attached to one of the pairs of discs in isolated flagella (see Fig. 1).

The flagella of *R. molischianum* and *R. fulvum* are considerably thicker than those of *R. rubrum*. An isolated flagellum in a lysate of *R. molischianum* is shown in Fig. 3. The width is approximately 180 A, and the hook is slightly thicker. The attached basal organelle is structurally homologous with that of *R. rubrum*, and their dimensions are identical. A fragment of membranous material is attached to the disc adjacent to the hook.

The flagella of *R. fulvum* are of the same width as those of *R. molischianum*, and show little or no enlargement in the hook region. In this species, isolated flagella with attached basal organelles were not observed. However, many of the isolated flagella possessed hooks which terminated in nar-

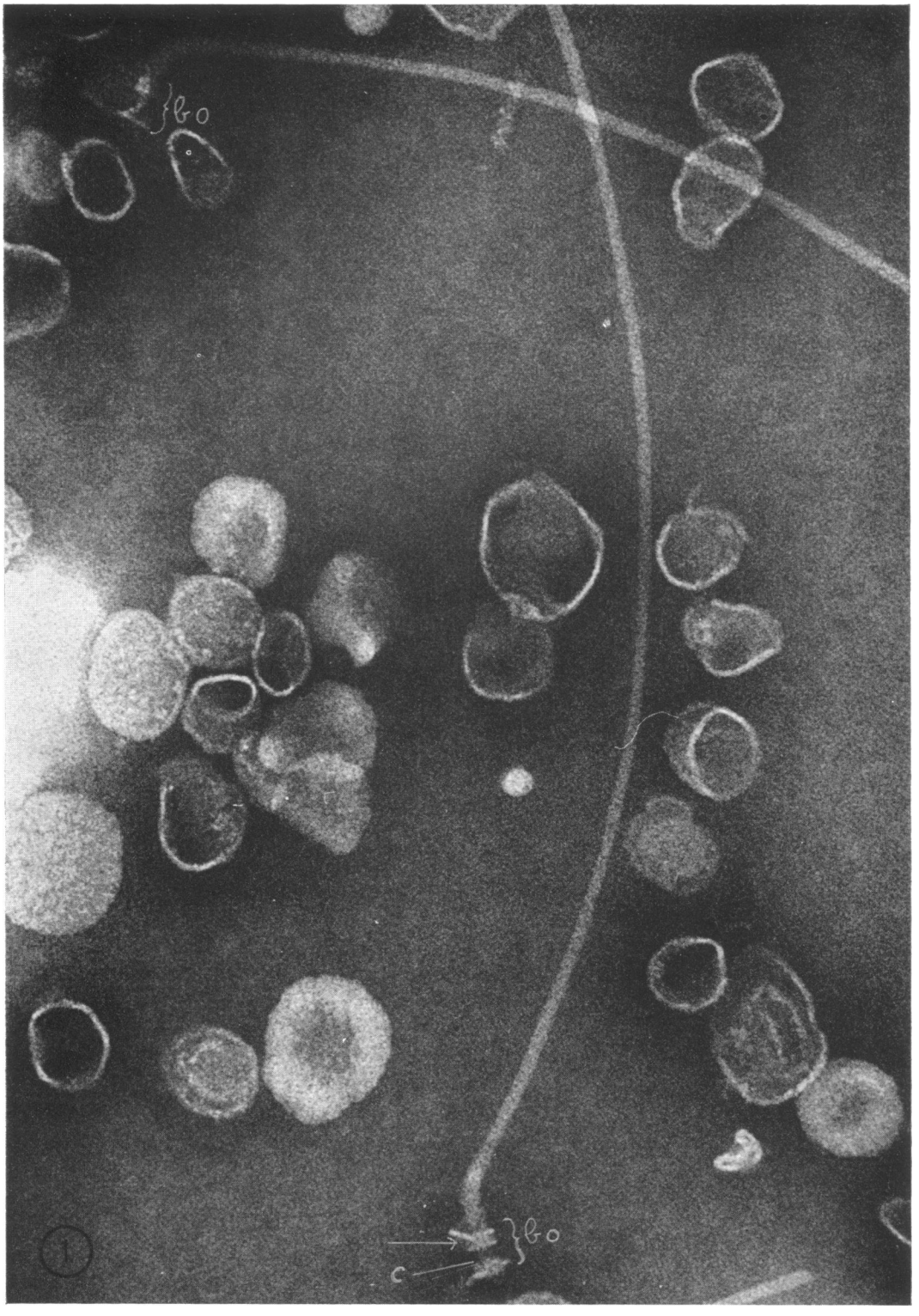


FIG. 1. *Flagella and chromatophore fragments liberated by enzymatic lysis of Rhodospirillum rubrum. Both flagella are terminated by a basal organelle (bo). This organelle consists of two paired discs connected to each other and to the flagellar hook by a narrow collar (c). A small piece of membranous material (arrow) is attached to one of the paired discs of the basal organelle of one flagellum. Negatively stained with potassium phosphotungstate.* × *200,000.*

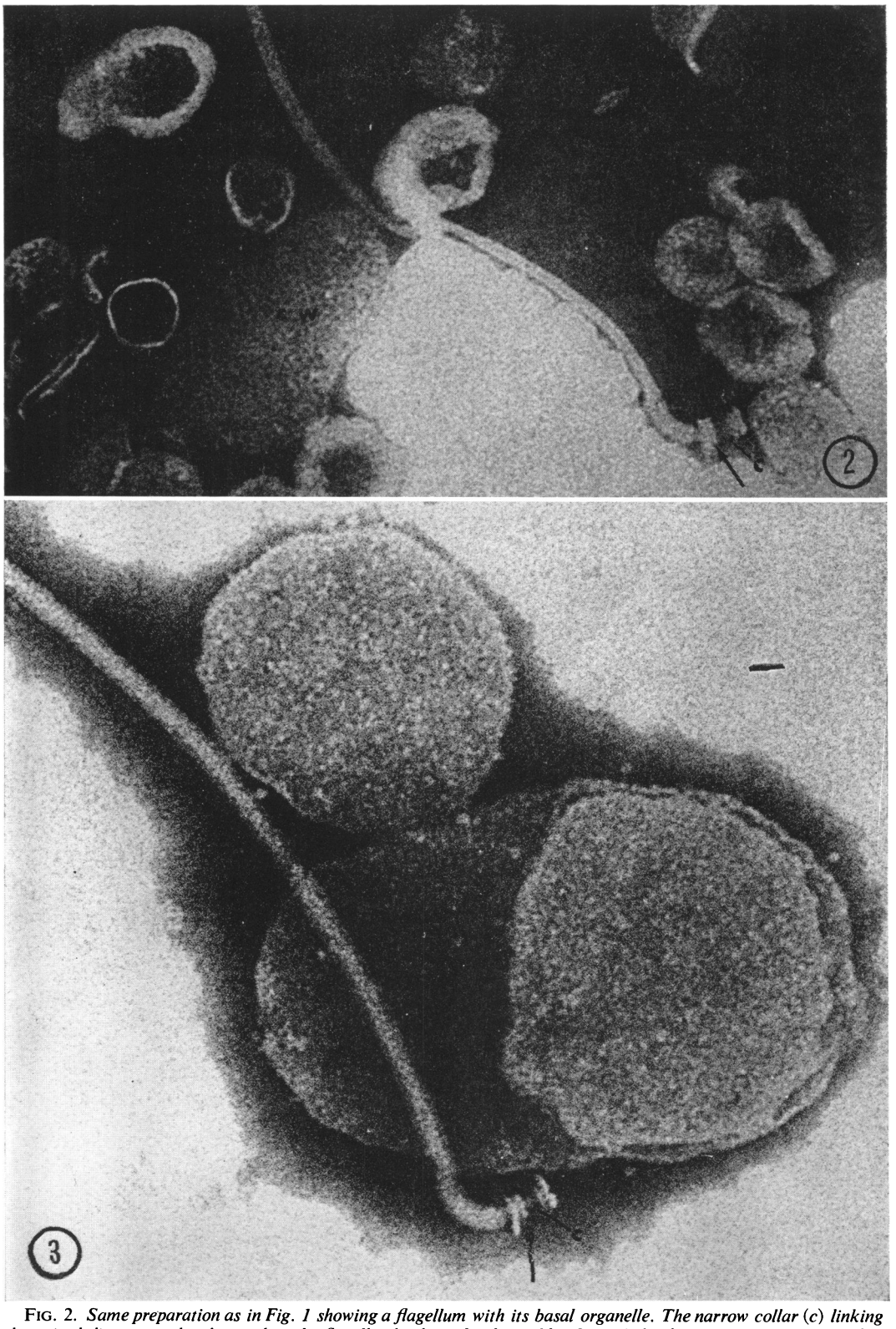


FIG. 2. *Same preparation as in Fig. 1 showing a flagellum with its basal organelle. The narrow collar (c) linking the paired discs to each other and to the flagellar hook is clearly visible. One of the discs (arrow) is composed of subunits 35 A in apparent diameter. A fragment of cell wall (cw) is visible in this preparation. Negatively stained with potassium phosphotungstate.* × *200,000.*

FIG. 3. *Flagellum and membrane fragments liberated by enzymatic lysis of Rhodospirillum molischianum. The basal organelle consists of two pairs of discs connected by a narrow collar (c) to each other and to the flagellar hook. A membranous fragment is attached to the disc closest to the hook. One of the inner discs (arrow) is formed by the juxtaposition of 50 A globular subunits. Negatively stained with potassium phosphotungstate.* × *240,000.*

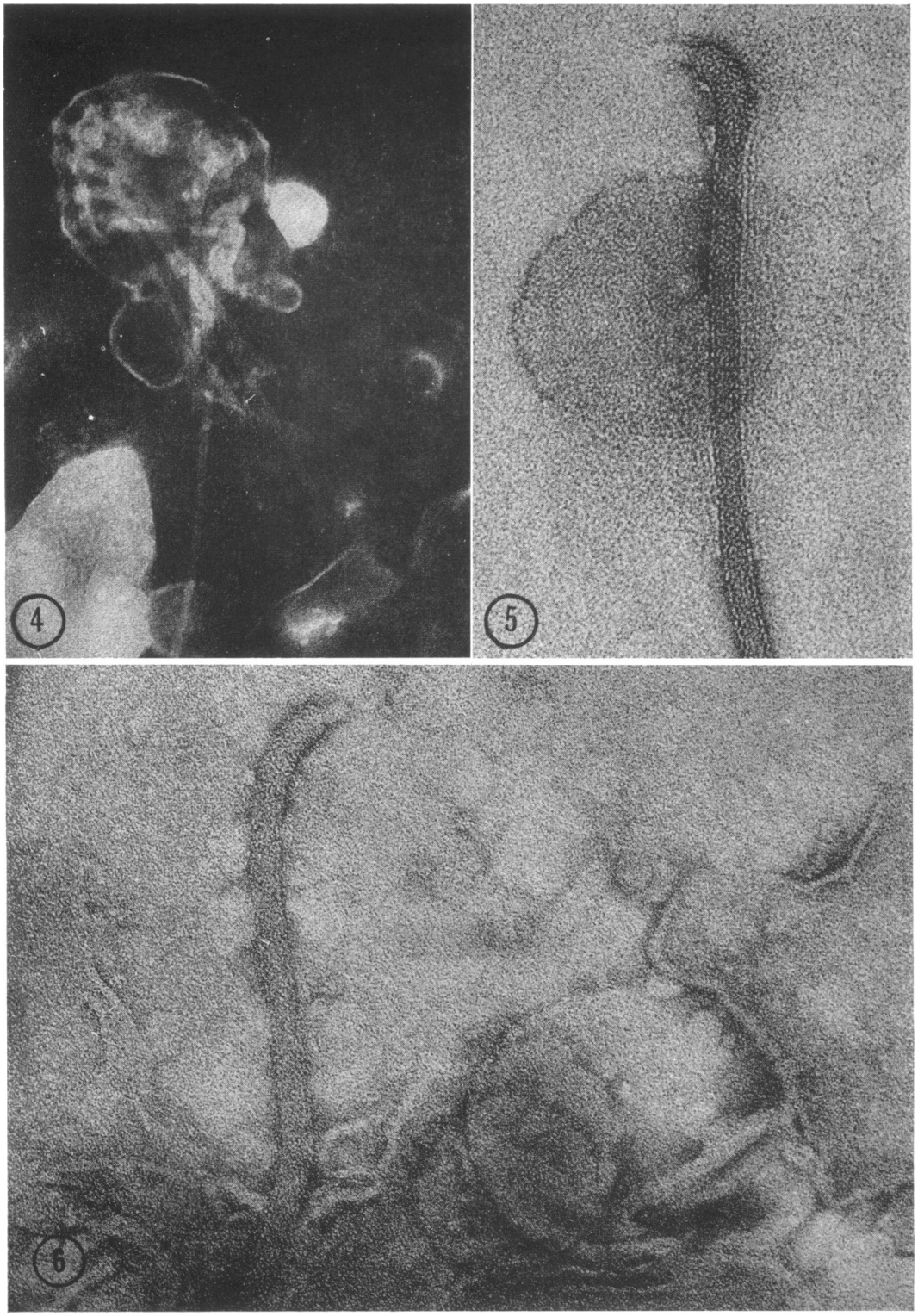


FIG. 4. *Flagellar tuft liberated by enzymatic lysis of Rhodospirillum rubrum. The basal organelles of six flagella are linked to each other by membranous material attached to both pairs of discs. Negatively stained with potassium phosphotungstate.* × *160,000.*

FIG. 5 and 6. *Flagella and membrane fragments isolated by enzymatic lysis of Rhodospirillum fulvum. Each flagellum is terminated by a short narrow cylindrical element continuous with the hook. This element is probably a portion of the narrow collar connecting the basal organelle with the flagellar hook. Negatively stained with uranyl acetate.* × *240,000.*

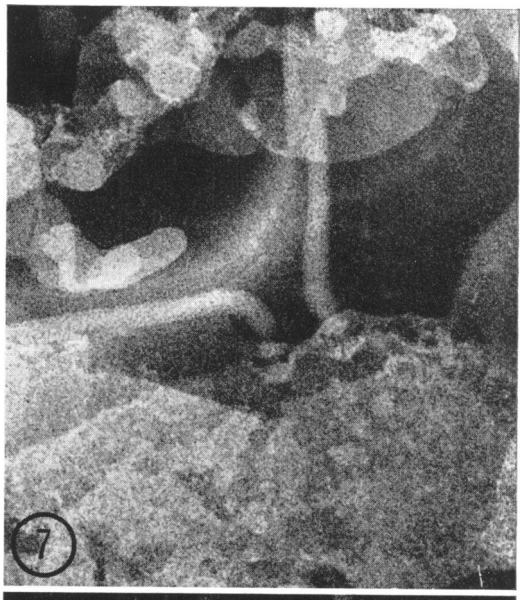
row collars, 80 A wide and 100 A long (Fig. 5 and 6); apparently in this strain, the connection between the collar and the basal organelle is easily broken. Typical basal organelles of *R. fulvum* with flagella still attached were frequently observed in association with fragments of the cell membrane (Fig. 7, 8). The connection between the flagellar hook and the basal organelle is particularly clear in Fig. 8: the narrow linking collar can be seen emerging from the fragment of the membrane which is wrapped around the basal organelle.

DISCUSSION

The organized structure of the basal organelles attached to flagellar hooks, coupled with their homology in the three *Rhodospirillum* species examined, leads us to conclude that they are the intracytoplasmic anchoring elements of bacterial flagella. In support of this conclusion, it should be noted that homologous organelles have been observed previously attached to the flagellar hooks of peritrichously flagellated bacteria, *Proteus* spp. (1, 13) and *Bacillus* spp. (2). If our interpretation is correct, the somewhat larger, structureless, "basal bodies" which have been so frequently observed at the base of the flagella in partly lysed cells contain basal organelles, the presence of which is concealed by enclosure within folded fragments of membrane.

Electron micrographs of thin sections of *S. serpens* (19) and *P. mirabilis* (21) show the flagellar hooks external to the cell wall, but closely appressed to it at their bases. In view of this fact, the narrow collar at the base of the hook, which we have observed both on detached flagella and on flagella bearing basal organelles, can be plausibly interpreted as the region of the flagellum which traverses the wall and the membrane, and which is anchored immediately within the membrane to the basal organelle. This location could explain why the collar has never been seen on the hooks of mechanically detached flagella, which would tend to be sheared off at the outer surface of the wall.

In favorable thin sections of *S. serpens*, Murray and Birch-Andersen (19) observed a differentiated structure, the "polar membrane," 200 A thick, immediately adjacent to the inner surface of the cytoplasmic membrane at the pole of the cells. They noted that the polar membrane did not extend into the region of insertion of the flagellar tuft itself, but surrounded it. Over this region, the



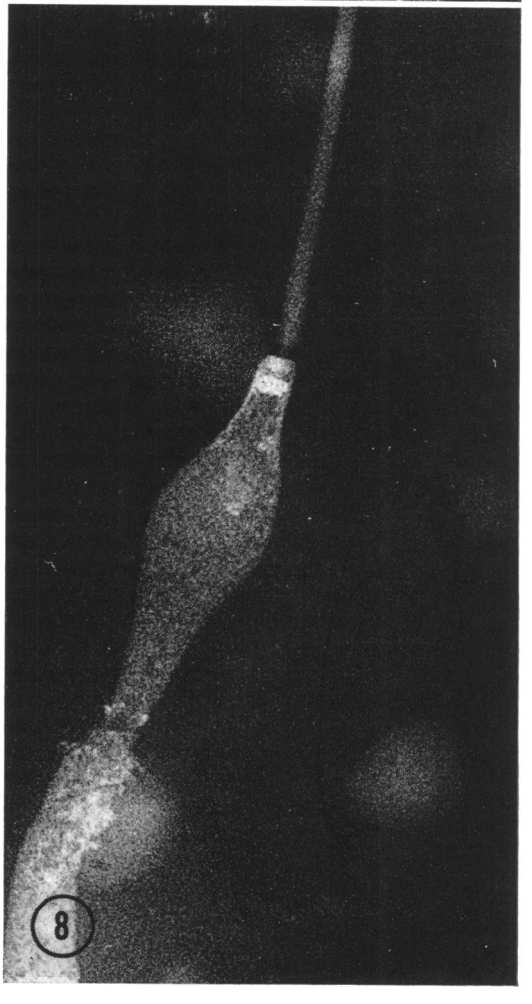


FIG. 7 and 8. *Flagella of Rhodospirillum fulvem with their basal organelle still attached to membrane fragments. The narrow collar and the system of paired discs is particularly clear in Fig. 8. Negatively stained with potassium phosphotungstate.* × *160,000.*

wavy profile of the wall characteristic of the cell surface was smooth. Favorable sections in the polar region showed that the flagella penetrate through the cell wall and the plasma membrane, and seem to terminate in a kind of button immediately inside the plasma membrane. No connection between these buttons and the polar membrane was observed.

A similar polar membrane has also been observed in thin sections of photosynthetic spirilla, such as *R. rubrum*, *R. molischianum*, and *R. fulvum* (5), and has been termed the "polar cap."

Figure 9 represents our present interpretation of the structural relationship between the basal portion of the flagella and the cortical layers in the polar region of a spirillum cell. It is a slightly modified version of the schematic drawing of Murray and Birch-Andersen (19). Specifically, we suggest that the flagella are anchored in a series of interconnected paired discs (*see* Fig. 4) which would in turn be connected to the surrounding polar membrane. From each paired disc, a narrow collar passes through the overlying plasma membrane and wall and connects immediately exterior to the wall to the flagellar hook (Fig. 10).

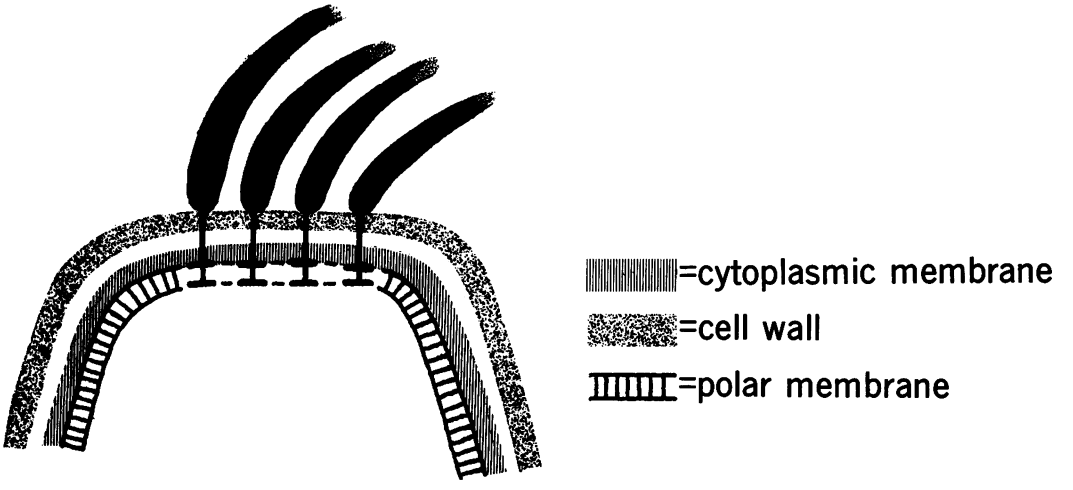


FIG. 9. *Schematic representation of the structural relationship between the cortical layers of the polar region of a Spirillum and the basal region of the flagella.*

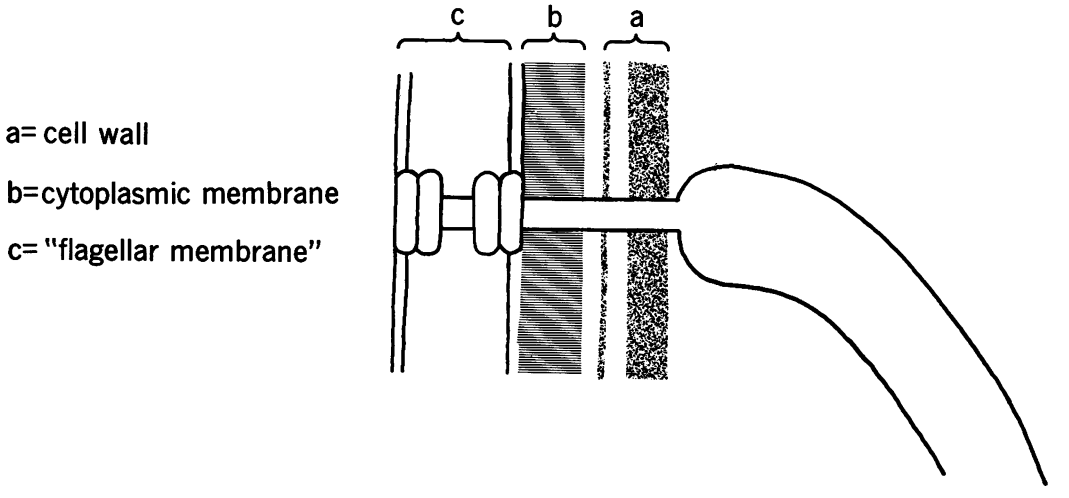


FIG. 10. *Schematic representation of the various components of the basal organelle of a flagellum and their position relative to the cell membrane and cell wall. A "flagellar membrane" is represented connecting the paired discs of the basal organelles to each other and to the polar membrane.*

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LITERATURE CITED

1. ABRAM, D., H. KOFFLER, AND A. E. VATTER. 1965. Basal structure and attachment of flagella in cells of *Proteus vulgaris*. J. Bacteriol. **90**:1337–1354.
2. ABRAM, D., A. E. VATTER, AND H. KOFFLER. 1966. Attachment and structural features of flagella of certain bacilli. J. Bacteriol. **91**:2045–2068.
3. ASTBURY, W. T., E. BEIGHTON, AND C. WEIBULL. 1955. The structure of bacterial flagella. Symp. Soc. Exptl. Biol. **9**:282–305.
4. BRADLEY, D. E. 1962. A study of the negative staining process. J. Gen. Microbiol. **29**: 503–516.
5. COHEN-BAZIRE, G., AND R. KUNISAWA. 1963. The fine structure of *Rhodospirillum rubrum*. J. Cell Biol. **16**:401–419.
6. COHEN-BAZIRE, G., AND W. R. SISTROM. 1966. The procaryotic photosynthetic apparatus, p. 313–341. *In* L. P. Vernon and G. R. Seely [ed.], The chlorophylls. Academic Press, Inc., New York.
7. COHEN-BAZIRE, G., W. R. SISTROM, AND R. Y. STANIER. 1957. Kinetic studies of pigment synthesis by non-sulfur purple bacteria. J. Cell. Comp. Physiol. **49**:25–68.
8. CRAIGIE, J. 1931. Studies on the serological reactions of the flagella of *B. typhosus*. J. Immunol. **21**:417–531.
9. ENSIGN, J. C., AND R. S. WOLFE. 1965. Lysis of bacterial cell walls by an enzyme isolated from a *Myxobacter*. J. Bacteriol. **90**:395–402.
10. ENSIGN, J. C., AND R. S. WOLFE. 1966. Characterization of a small proteolytic enzyme which lyses bacterial cell walls. J. Bacteriol. **91**:524–534.
11. GIESBRECHT, P., AND G. DREWS. 1962. Elektronenmikroskopishe Untersuchungen über die Entwicklum der Chromatophoren von *Rhodospirillum molischianum* Giesberger. Arch. Mikrobiol. **43**:152–161.
12. GLAUERT, A. M., D. KERRIDGE, AND R. W. HORNE. 1963. The fine structure and mode of attachment of the sheathed flagellum of *Vibrio metchnikovii*. J. Cell Biol. **18**:327–336.
13. HOENIGER, J. F. M., W. VAN ITERSON, AND E. N. VAN ZANTEN. 1966. Basal bodies of bacterial flagella in *Proteus mirabilis*. II. Electron microscopy of negatively stained material. J. Cell Biol. **18**:603–618.
14. HOUWINK, A. C., AND W. VAN ITERSON. 1950. Electron microscopical observations on bacterial cytology. II. A study of flagellation. Biochim. Biophys. Acta **5**:10–44.
15. KERRIDGE, D., R. W. HORNE, AND A. M. GLAUERT. 1962. Structural components of flagella from *Salmonella typhimurium*. J. Mol. Biol. **4**:227–238.
16. KOBAYASHI, T., J. N. RINKER, AND H. KOFFLER. 1959. Purification and chemical properties of flagellin. Arch. Biochem. Biophys. **84**:342–362.
17. KOFFLER, H., AND T. KOBAYASHI. 1957. Purification of flagella and flagellin with ammonium sulfate. Arch. Biochem. Biophys. **67**:246–248.
18. LOWY, J. 1965. Structure of the proximal ends of bacterial flagella. J. Mol. Biol. **14**:297–299.
19. MURRAY, R. G. E., AND A. BIRCH-ANDERSEN. 1963. Specialized structure in the region of the flagella tuft in *Spirillum serpens*. Can. J. Microbiol. **9**:393–401.
20. PFENNIG, N., K. E. EIMHJELLEN, AND S. L. JENSEN. 1965. A new isolate of the *Rhodospirillum fulvum* group and its photosynthetic pigments. Arch. Mikrobiol. **51**:258–266.
21. VAN ITERSON, W., J. F. M. HOENIGER, AND E. N. VAN ZANTEN. 1966. Basal bodies of bacterial flagella in *Proteus mirabilis*. I. Electron microscopy of sectioned material. J. Cell Biol. **31**:585–601.
22. WEIBULL, C. 1948. Some chemical and physicochemical properties of the flagella of *Proteus vulgaris*. Biochim. Biophys. Acta **2**:351–361.
23. WEIBULL, C. 1949. Chemical and physico-chemical properties of the flagella of *Proteus vulgaris* and *Bacillus subtilis*. A comparison. Biochim. Biophys. Acta **3**:378–382.
24. WEIBULL, C. 1953. The isolation of protoplasts from *Bacillus megaterium* by controlled treatment with lysozyme. J. Bacteriol. **66**:688–695.