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Differentiation Within the Bacterial Flagellum and Isolation of the Proximal Hook

DINAH ABRAM,[1] J. R. MITCHEN, HENRY KOFFLER, AND A. E. VATTER

Department of Biological Sciences, Purdue University, Lafayette, Indiana, and Department of Pathology, the University of Colorado Medical Center, and the Webb-Waring Institute for Medical Research, Denver, Colorado 80220

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Purified and crude flagellar isolates from cells of *Bacillus pumilus* NRS 236 were treated with acid, alcohol, acid-alcohol, or heat, and were examined electron microscopically in negatively stained and shadow-cast preparations. Under certain conditions, each of these agents causes the flagella to break between the proximal hooks and the spiral filaments. In such preparations, filaments are seen in various stages of disintegration, whereas hooks of fairly constant length retain their integrity and morphological identity. When crude isolates of flagella are treated under these conditions, the hooks remain attached to membrane fragments or bear basal material. These findings substantiate previous structural observations that led to the view that the proximal hook is a distinct part of the bacterial flagellum and further confirm that the hook is tightly associated with basal material and the cytoplasmic membrane. It appears that the hook is a polarly oriented structure, and that the interactions between the hook and the basal material or the cytoplasmic membrane are different from those between the hook and the filamentous portion of the organelle. Moreover, both types of interaction apparently differ still from those by which the flagellin subunits are held together in the flagellar filament. Hooks were isolated by exploiting the differences in relative stability shown by the various morphological regions of the bacterial flagellum.

The bacterial flagellum consists of the following three morphological components: a basal region (3, 4, 9, 12–16, 18, 21, 28–37) clearly associated with the cytoplasmic membrane (3, 4); a proximal hook (3, 4, 9, 11, 14, 20, 21, 23, 27, 29–31, 36, 37); and the filamentous portion of the organelle. The hooks can be easily recognized by their gross and fine structure, which are distinct from that of the flagellar filament (3, 4, 23). Specialized "surface" structures that are confined to hooks have been described in flagella from certain organisms (4). In the case of the flagellum of *Salmonella typhimurium*, the hook and the filament have been shown to differ immunologically (20).

In this paper we will show that exposure of flagella from cells of *Bacillus pumilus* to various relatively mild chemical and physical treatments results in the disintegration, to varying degrees, of the flagellar filaments, whereas the hooks remain intact. The hooks are of fairly constant length and, under certain conditions, are still connected to membrane fragments. This observation substantiates previous findings that the hooks are different entities from the flagellar filament and are firmly attached to the cytoplasmic membrane. A combination of several of these treatments resulted in a procedure for the separation and purification of hooks. Preliminary reports of parts of this study have been presented on two previous occasions (Abram et al., Bacteriol. Proc., p. 39, 1967; Mitchen and Koffler, Bacteriol. Proc., p. 29, 1969). Recently these have been supported by work of Nauman et al. (27), who arrived at comparable enrichment conditions for the isolation of the terminal knobs of *Leptospira*, which appear to be structurally analogous to flagellar hooks.

MATERIALS AND METHODS

B. pumilus NRS 236 was used in this study. The growth medium and the procedures for the isolation of flagella were previously described (2). The isolates were stored at 2 to 5 C and used within 5 days. The final concentration of the flagella used was 0.5 to 1.5 mg/ml. Partial or complete disintegration of the flagella in aqueous suspension was accomplished with:

[1] Present address: Department of Microbiology, University of Pittsburgh, School of Medicine, Pittsburgh, Pa. 15213.

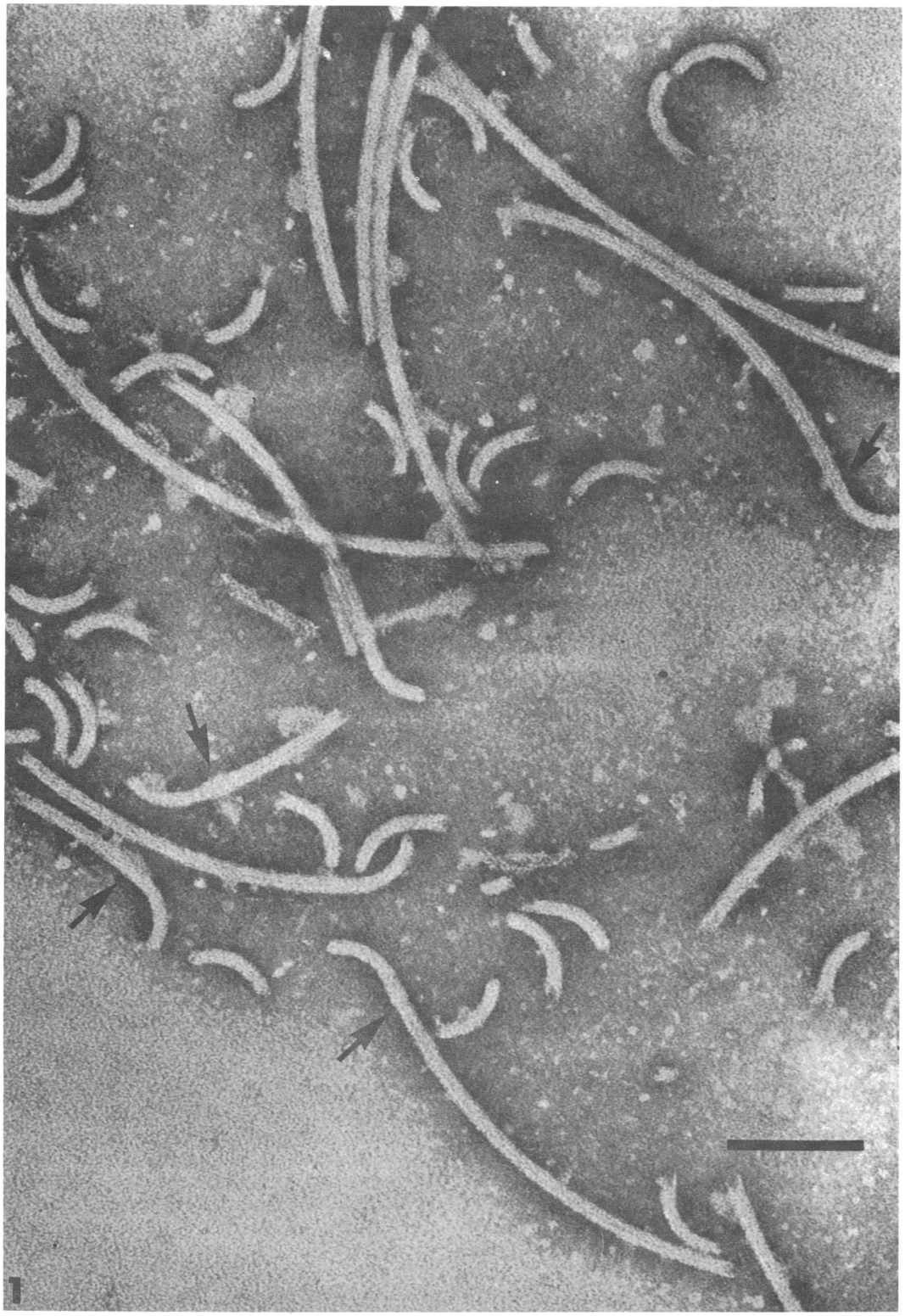
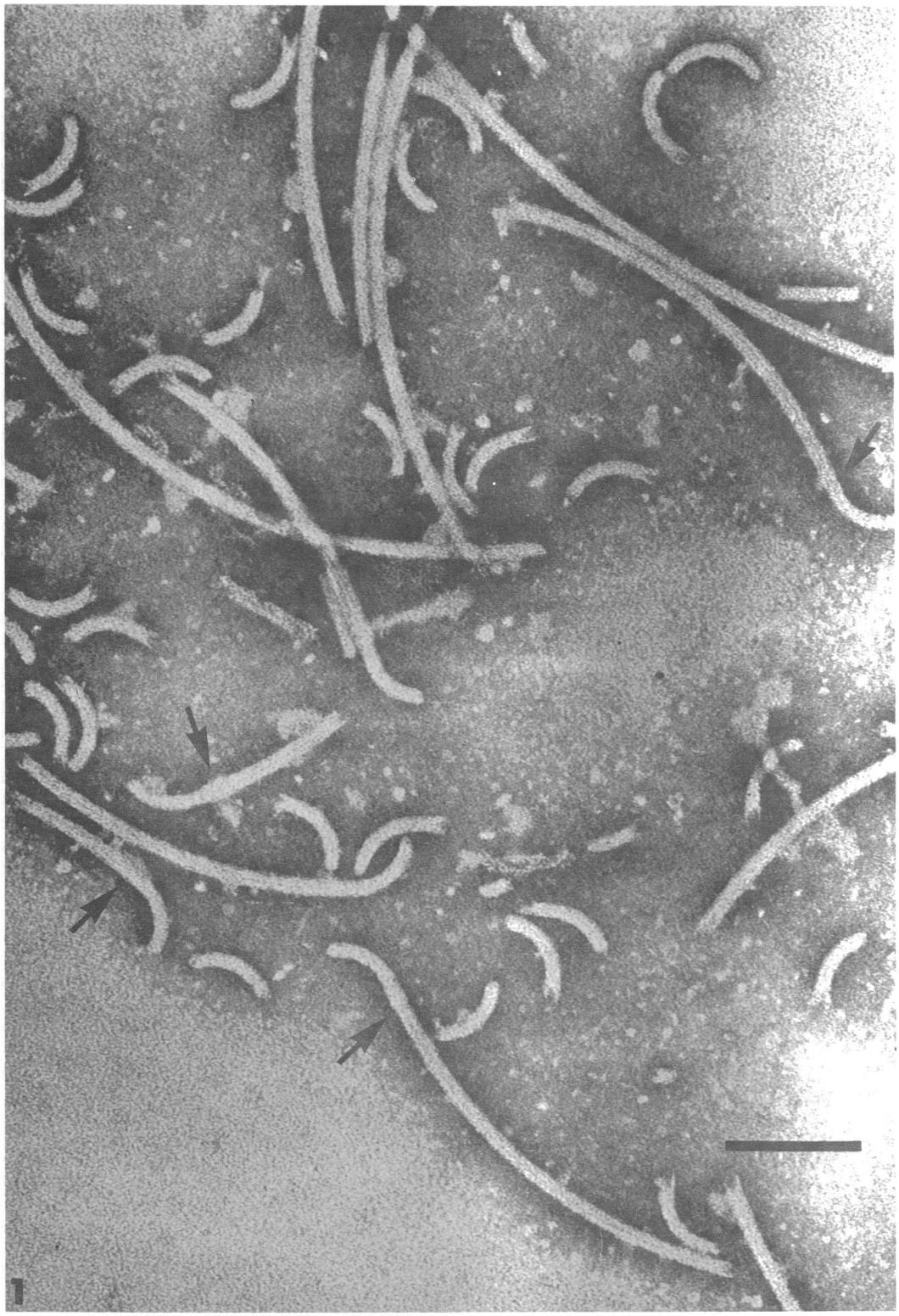


FIG. 1. *Specimens prepared from material that upon standing had settled from a flagellar suspension in 65% ethyl alcohol. Many hooks of fairly constant length are surrounded by fragmented flagellar filaments and other material without distinct morphological features. Note that the hooks and some fragments of flagellar filaments appear split only at one end; since this occurs at the end opposite from the hook, the "split" characterizes the distal end of the flagellum. Also note the altered organization just beyond the bend of the hook (arrows), negatively stained with potassium phosphotungstate.* × *210,000. Marker indicates 0.1 μm.*



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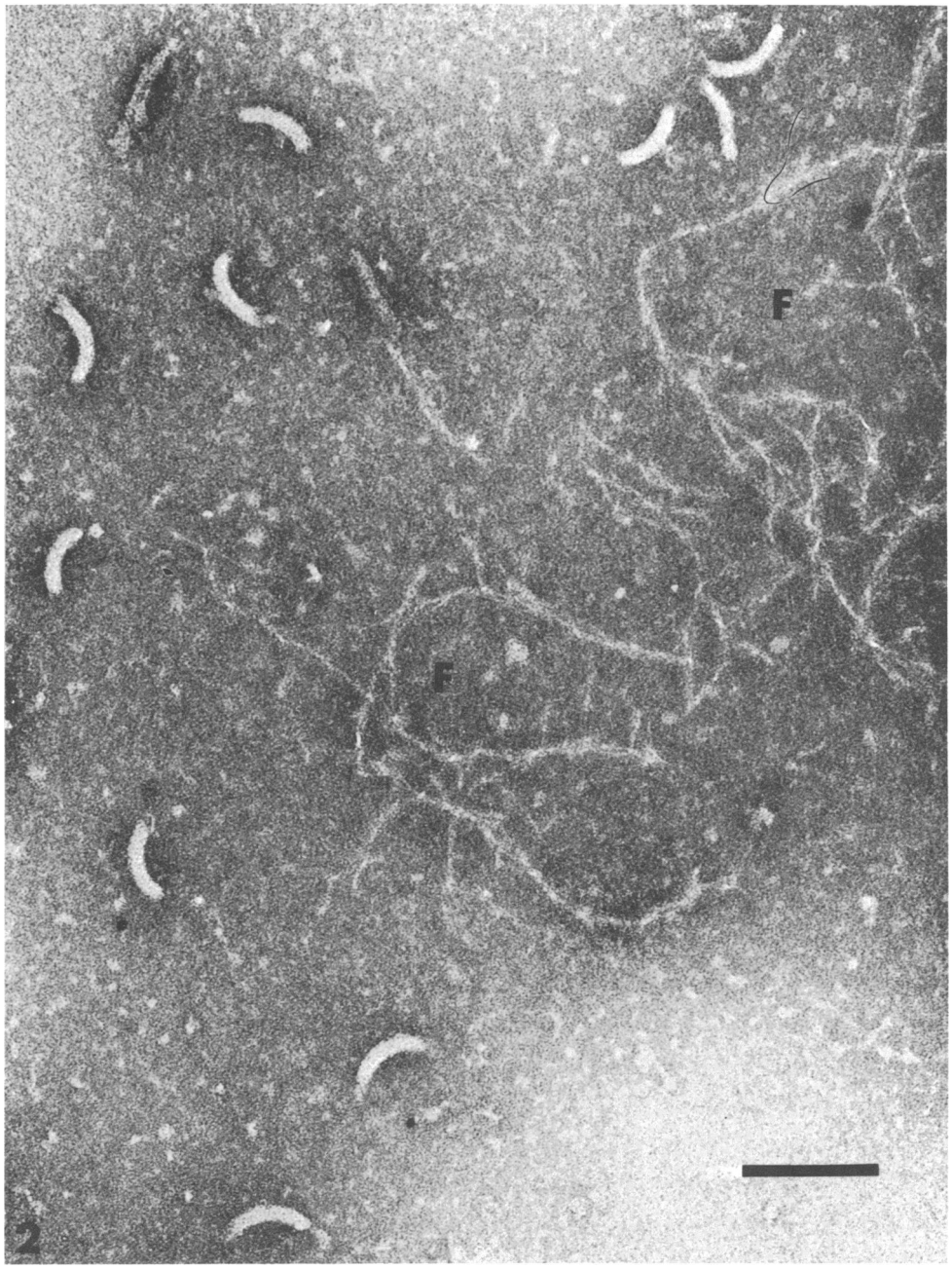

FIG. 2. *Hooks of fairly even length, surrounded by fine fibers (F) and other material without distinct morphological features, present in flagellar suspension in 85% ethyl alcohol. Negatively stained with potassium phosphotungstate. × 180,000. Marker indicates 0.1 μm.*

also disintegrate, but under more drastic conditions (for example, at *p*H 1 to 2). Since detachment of hooks and the disintegration of flagellar filaments proceed simultaneously but at different rates, hooks still attached to filaments can also be observed.

Until recently, we failed to notice the hooks in preparations of flagella after acid treatment

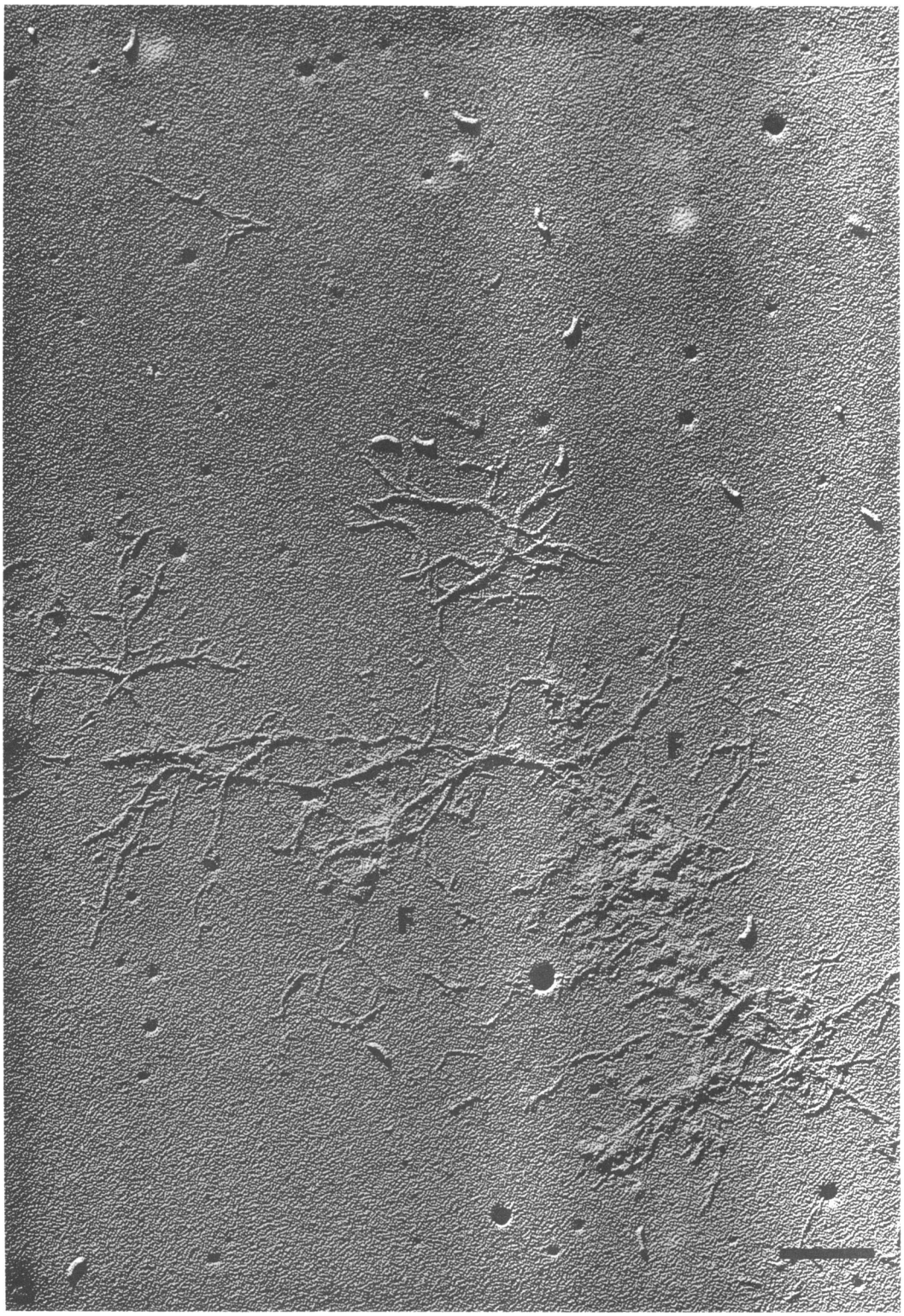

FIG. 3. *Same preparation as shown in Fig. 2. Shadow cast.* × *70,000. Marker indicates 0.2 μm.*

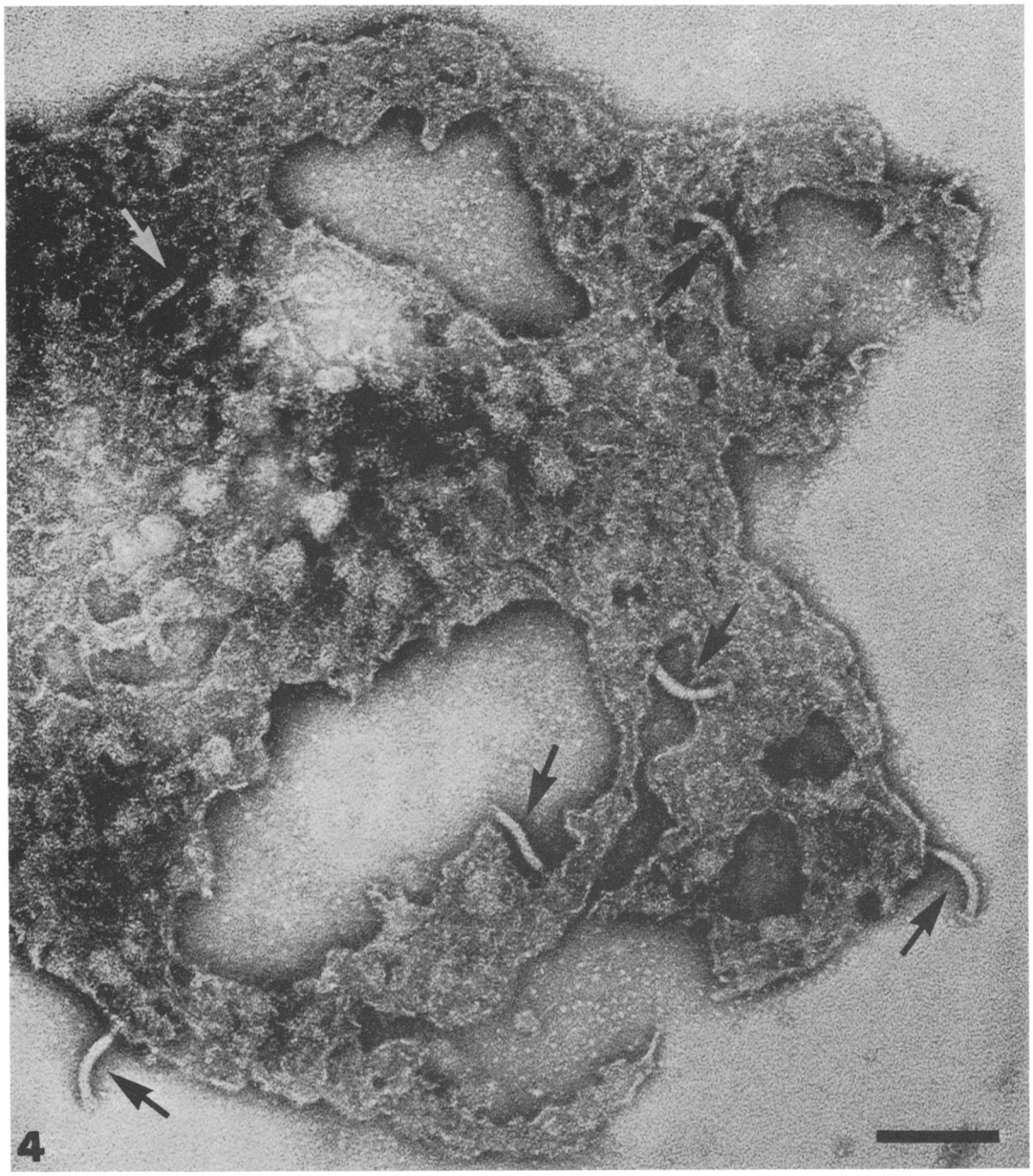

FIG. 4. *Large membrane fragment to which hooks (arrows) are attached in a suspension of a crude flagellar isolate treated with acid alcohol (50% ethyl alcohol; 5 × 10^{-5} N HCl). Negatively stained with potassium phosphotungstate. × 165,000. Marker indicates 0.1 μm.*

(0.1 N HCl to *p*H 2.6, for 30 min at 26 C), because the acid-insoluble material was usually removed from the reaction mixture by high-speed centrifugation (100,000 × *g* for 30 to 60 min at 4 C) in the form of a well-packed precipitate. Forcing such precipitates through a pipette several times or suspending them with the aid of a Teflon homogenizer still results in a suspension that contains clumps. Such preparations do not yield satisfactory specimens for electron microscopy. The acid-insoluble material was found to be unsuitable for the purification of hooks, since it did not prove possible to separate intact hooks from the precipitate to allow further purification.

Flagellar filaments also disintegrate when

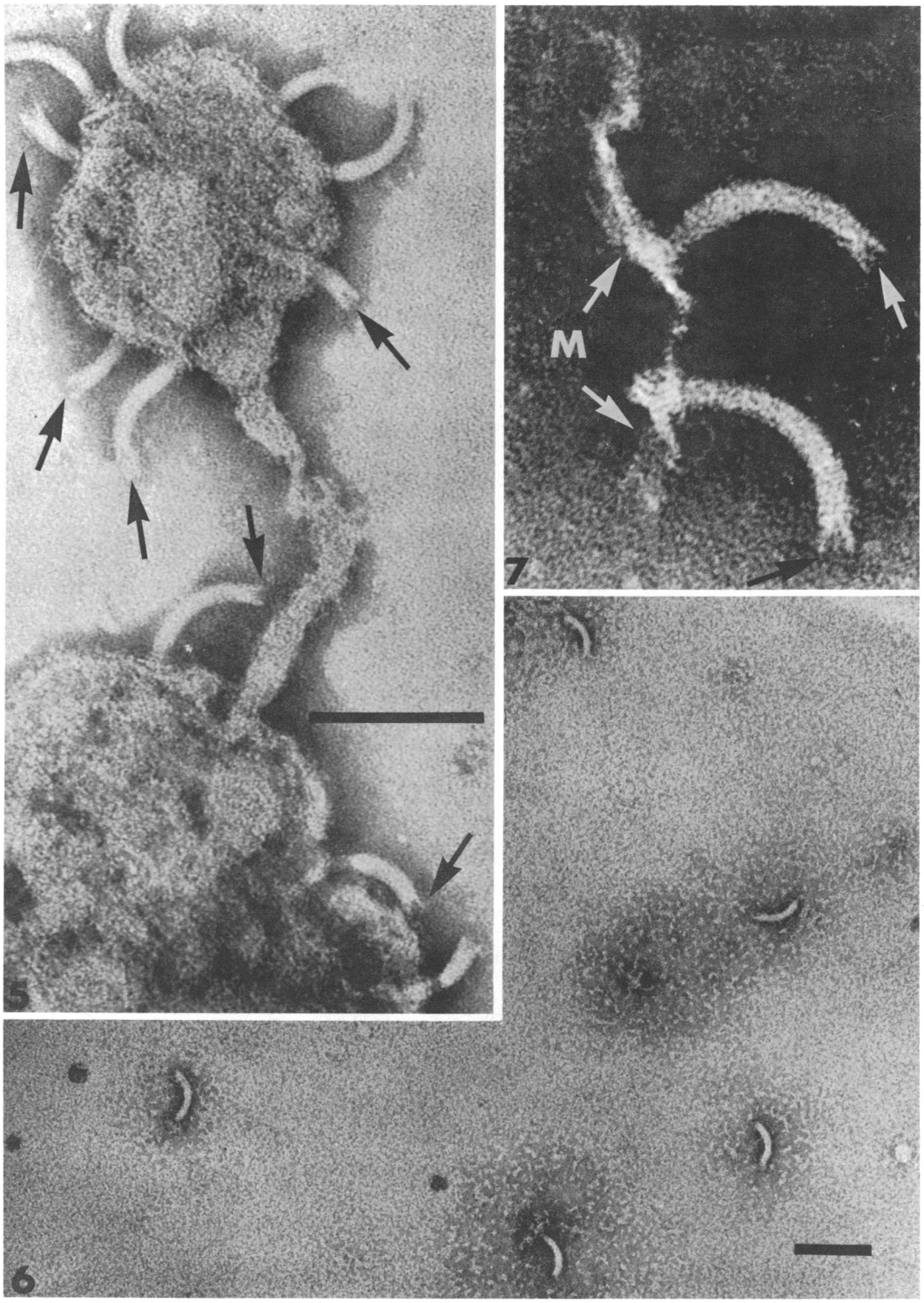

FIG. 5–7. *Fig. 5. Preparation similar to that shown in Fig. 4. The hooks are still attached to the membrane, showing that the end which appears split is the distal one (arrows), as in the case of flagellar filaments (Fig. 1). × 260,000. Fig. 6. Hooks of fairly constant length surrounded by material without distinguishable morphological features in a suspension of flagella in HCl at pH 2.6. × 110,000. Fig. 7. Hooks attached to basal material (M) in a suspension of flagella at pH 2.6. Note the split distal end of the hooks (arrows). × 520,000. Negatively stained with potassium phosphotungstate. Markers indicate 0.1 μm (Fig. 5 and 6) and 0.05 μm (Fig. 7).*

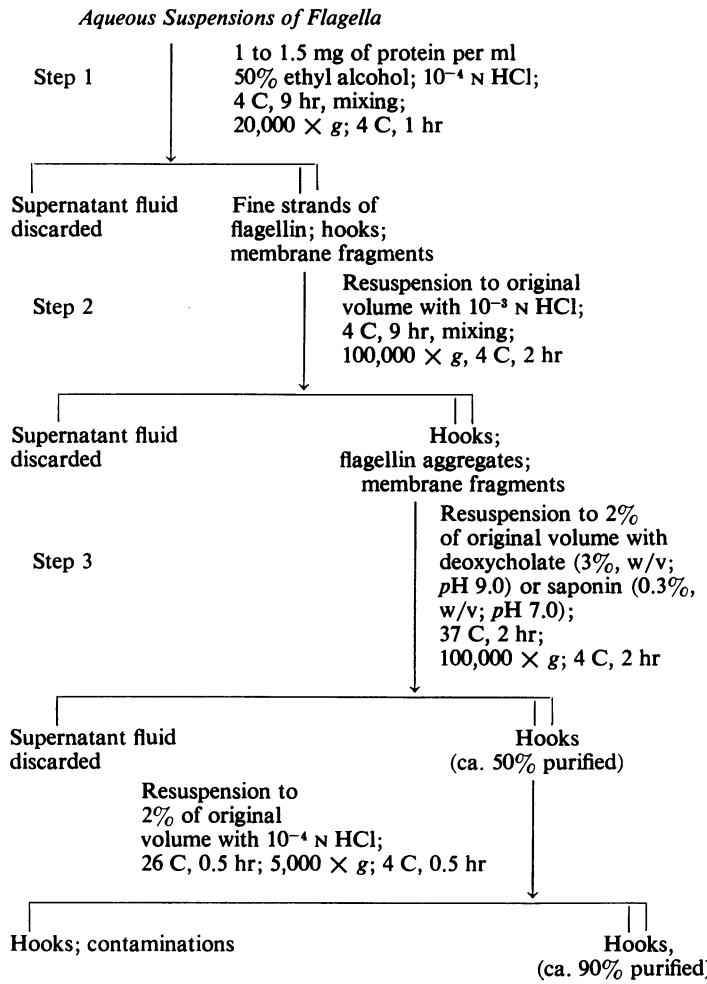

FIG. 8. *Procedure for the isolation of flagellar hooks. All concentrations are the final ones for each step of the procedure.*

heated for 5 to 20 min at 59 C, and one can observe hooks associated with basal materials during various stages of the process, which also are present in the heat-insoluble material collected by low-speed centrifugation.

The greater stability of hooks to acid alcohol and acid relative to flagellar filaments and their relative resistance to detergents in concentrations sufficient to remove membrane and cytoplasmic components were used to advantage in a three-step procedure for the purification of hooks from flagellar isolates (Fig. 8).

First, an aqueous suspension of a relatively purified isolate of flagella is mixed with cold ethyl alcohol and HCl to give final concentrations of 1 to 1.5 mg of protein per ml, 50% ethyl alcohol (v/v), and 10^{-4} N HCl. The mixture, which becomes very viscous, is stirred rapidly with a magnetic stirrer for 9 hr at 4 C to accomplish complete disintegration of the filaments. However, the hooks remain intact and are detached from the filaments that have disintegrated into fine strands (as in Fig. 1 to 3). Centrifugation at 20,000 × *g* for 1 hr at 4 C results in the collection of all of the protein in the preparation.

In step 2, the sedimented material is suspended in 10^{-3} N HCl at 4 C to its original volume, and is incubated at 4 C for 9 hr, again with thorough mixing. Hooks are stable to the acid, but almost all of the flagellin strands further disintegrate to a soluble form. Hooks are then separated by centrifugation at 100,000 × *g* for 2 hr at 4 C. The sediment contains approximately 5% of the

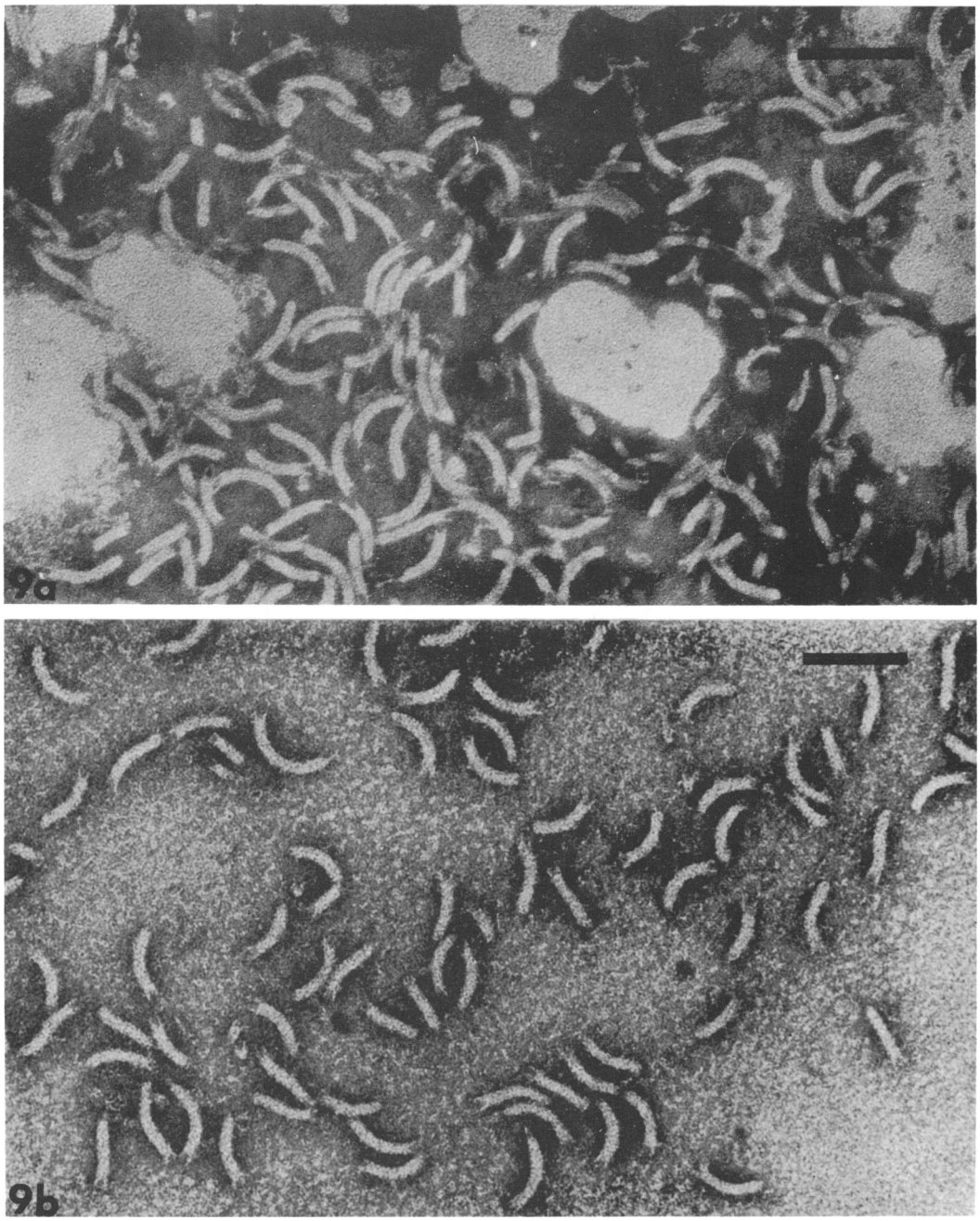

FIG. 9. *Purified preparations of hooks: (a) after step 3 (b) after the final treatment described in the procedure of purification (Fig. 8). Negatively stained with potassium phosphotungstate. (a) × 160,000; (b) × 142,000. Markers indicate 0.1 μm.*

original dry weight. Although this material is rich in hooks, it contains aggregates of what most likely is flagellin, since more than 98% of the starting material was flagellin. The sediment also contains cell fragments that are present in the original flagellar isolate (Fig. 6). Unlike the insoluble material from flagella, obtained when they are disintegrated by acid without pretreat-

ment with acid ethyl alcohol (step 1), this material can be readily suspended in 10^{-3} N HCl.

In step 3, the crude hook preparation from step 2 is purified further by suspension in a detergent. The volume is kept small (about 2% of the original) to allow efficient recovery of hooks by centrifugation. The pellet is suspended in either sodium deoxycholate (3%, w/v; *p*H 9.5) or saponin (0.3%, w/v; *p*H 7.0). After incubation at 37 C for 2 hr (continuous mixing is not necessary), the hooks are collected by centrifugation at 100,000 × *g* for 2 hr at 4 C and suspended with deionized water to 2% of the original volume. The concentrated material is rich in hooks but still contains some cell fragments that were not disintegrated by the detergent. This material is resuspended and washed twice with deionized water to 2% of the original volume, and the material is centrifuged at 100,000 × *g* for 2 hr at 4 C. This material is rich in hooks, but as much as 50% consists of nonhook material (Fig. 9a).

The following optional step has been found to yield nearly pure hooks, unfortunately, only in minute yields. The bulk of the nonhook material can be precipitated by adjustment of the *p*H to 4 to 5. Most of the hooks also precipitate and, when centrifuged at 3,000 × *g* for 10 min at 4 C, come down with the contaminants. However, those remaining in the supernatant liquid appear to be ca. 90% pure (Fig. 9b).

The procedure used to isolate flagella results in filaments that average 5.5 μm in length on the curve. Only ca. 20% of these filaments have hooks; some hooks remain on the cells and some of the filaments are broken. Since the length of the hook is 58 to 74 nm, the hook represents ca. 1.2% of the average filament contained in the isolate. In terms of the original flagellar isolate, a theoretical yield for hooks of 0.24% is expected. On the basis of protein determination (after disintegration of the hooks at *p*H 1 to 2 for 30 min at 26 C), the actual yield of hooks obtainable after step 3 is ca. 0.2%; if the selective precipitation at *p*H 4 to 5 is used after step 3, the yield is less than 0.01% of the starting material.

DISCUSSION

The findings reported in this paper support previous structural observations suggesting that the proximal hooks are specialized parts of bacterial flagella. Agents that apparently affect noncovalent bonds, such as dilute acid, alcohol, acid-alcohol, or heat, cause the breakage of flagella between the hook and the spiral filament. In preparations of disintegrated flagella, the hooks still retain their integrity and morphological identity. The observations reported here on flagellar isolates further support our previous view that the proximal end of the hook is tightly associated with basal material and fragments of cytoplasmic membrane (3, 4). Under certain conditions, the filamentous part of the flagellum can be removed from the hook, but the firm attachment of the hook to membrane fragments or basal material is not disrupted. It thus appears that the hook is a polarly oriented structure that participates in two distinctly different interactions: first with the basal material or the cytoplasmic membrane, or both, and second, with the filamentous portion of the organelle. Both associations apparently differ still from the interactions by which the flagellin subunits are held together in the flagellar filament. These findings may also explain the observation by Martinez (25), who by column chromatography separated a ribonucleic acid (RNA)-containing fraction from fragmented flagellar filaments of *Spirillum serpens*. Because the purified fractions no longer contained the hooks that were originally present in the flagellar preparations, he surmised that they were present in the RNA-containing fraction, and that in intact flagella RNA is associated with the hooklike structures. However, since it appears that under certain conditions the hook has a stronger attachment to the cytoplasmic membrane than to the flagellar filament, it is possible that the RNA-containing material is derived from the basal material attached to the hooks. Moreover, Martinez' starting preparations were crude, and it is likely that they contained membranous elements to which ribosomes may have adhered (7).

The isolated hooks of *B. pumilus* described in this study are of fairly constant length (58 to 74 nm); hooks still attached to flagellar filaments, recognized by their fine structure, are of similar length (4). In flagella of most of the other organisms studied so far (3, 4, 23), the hooks, as defined by their fine structure or surface structure (*B. brevis* and *B. circulans*), also are of fairly constant length for a given bacterial strain. Two exceptions are the flagella of *Pseudomonas rhodos* (23) and *B. stearothermophilus* 194 (4). In *P. rhodos* two types of flagella, sheathed and sheathless, were found on the same organism. The lengths of the proximal end showing globular unit structure are 200 nm and 70 nm in the sheathed and the sheathless flagella, respectively; however, for each type of flagellum the length of the proximal region is fairly constant. Previously, in the case of *B. stearothermophilus* 194, we described a "hook region" of varying length. Recently, we found that in acid- or acid alcohol-treated preparations only the proximal portion of

the "hook region" maintains its integrity and it is of fairly constant length. In this case, the distal part of what we previously referred to as the "hook region" appears to represent a part of the flagellar filament, which consists of two regions that differ in their fine structure, as was previously shown to be true for flagella of *B. stearothermophilus* 2184 (4).

The nature and function of the hooks are unknown. It is possible that they serve a role in the assembly of flagellin or in motility. The distal ends of hooks and of flagellar fragments still attached to hooks appear to be split. This difference in appearance enables one to distinguish the distal end of the fragments from the blunt proximal end. Pye (Australian Biochem. Soc. Annu. General Meeting, Programme Abstr., p. 68 1967) and Asakura et al. (8) observed that the in vitro addition of soluble flagellin to primer pieces of filaments proceeds unidirectionally. Moreover, Asakura et al. (8) found that this addition takes place at the "split," i.e., the distal end. On the other hand, Pye was unable to polymerize flagellin onto flagellar "stumps" of mechanically deflagellated cells and tentatively interpreted this to mean that monomers could not add to the distal end. Metani and Iino (26) found that deflagellated cells of *S. typhimurium*, upon growth in a regeneration medium containing *p*-fluorophenylalanine, which causes the formation of curly flagella, produced the curly portions of some flagellar filaments near the cell bodies, whereas the distal portions remained normal. However, in spite of these discrepancies, the most recent experiments of Iino (17) and Kerridge (*personal communication*), in which curliness brought about by *p*-fluorophenylalanine was used as a morphological marker, indicate that lengthening of mechanically shortened *Salmonella* flagella in vivo also proceeds from the distal end. In any case, on the assumption that flagellin, like other proteins, is synthesized within the protoplast, probably near or in the basal structure of the flagellum, this protein most likely travels through the hook and perhaps the filament before it polymerizes. In this hypothetical view, the distal end of the hook serves as the initiation site for the polymerization of flagellin. Although it is conceivable that the flagellin molecules travel along the hook and the flagellum or even diffuse through the medium, it is more likely that they migrate through the central hole in these structures. Such holes can be readily observed to exist in flagellar filaments of some organisms, though they have escaped observation in others (24). We have been able to observe a central core in hooks only occasionally, but its presence may be obscured by the curvature of the hooks or their surface patterns. In connection with the possible role of the hook in motility, such a function is plausible even though the mechanism is not understood yet, since the primary input of energy probably occurs at the level of the cytoplasmic membrane to which the hook is firmly attached. The procedure used for the purification of hooks is empirical. The agents and conditions used at each stage of the enrichment procedure were chosen because the resulting material after differential centrifugation contains morphologically intact hooks and can be resuspended, a step prerequisite to retreatment or further treatment. In essence, this procedure results in the stepwise disintegration of flagellar filaments first into fibers and then into soluble flagellin. As determined by electron microscopy, the hooks themselves can be disintegrated by acid (*p*H 1 to 2 at 26 C for 30 min). The yield of highly purified hooks soluble under such conditions, as determined by protein analysis, represents less than 0.01% (dry weight) of the original flagellar isolates. Of course, this value does not reflect the natural occurrence of hooks, since losses occur at each step, and some hooks remain on the cells during the isolation of the flagella. However, the final preparation represents approximately a 500-fold enrichment over the initial isolate of flagella in which only 20% of the filaments contained hooks. Yields must be improved before the chemical nature of hooks can be studied extensively. It is hoped that the work reported here will be useful in further pursuit of the still unanswered questions concerning the composition and function of the flagellar hooks and the molecular organization of the flagellum.

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