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Purification of Intact Flagella from *Escherichia coli* and *Bacillus subtilis*

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A procedure is described for the purification of bacterial flagella in the form of a filament-hook-basal body complex (intact flagella) free from detectable cell wall, membrane, or cytoplasmic material. Spheroplasts produced with lysozyme and ethylenediaminetetraacetic acid were lysed with Triton X-100, and the flagella were purified by $(NH_4)_2SO_4$ precipitation, differential centrifugation, and CsCl gradient centrifugation. As much as 40% of the flagella were recovered, and they contained about one basal body per 4 to 6 μm of flagella. The same procedure developed for *Escherichia coli* was also successful for purifying intact flagella from *Bacillus subtilis*.

The bacterial flagellum is composed of three structurally defined parts: the filament, the hook, and the basal body (3, 13, 17). The filament is the long helical structure, composed of flagellin, external to the cell. The hook is a morphologically distinct unit, generally hook-shaped, attached to the proximal end of the filament and terminating at the cell wall. The filament and hook are further distinguished from each other immunologically (11, 18) and by their different solubilities in acid and other reagents (3). The basal body is the part of the flagellum attached to the hook and bound into the cell wall and membrane (2, 7, 24, 25).

A detailed analysis of the hook and basal body in whole or degraded cells (2, 7, 24, 25) is difficult because of their small size and close association with the cell envelope. Flagella purified after being mechanically removed from cells ("sheared flagella") have been extensively studied (3, 17) but these have few hooks and no basal bodies. As a result, many questions dealing with the structure, chemistry, synthesis, and function of the base of the flagellum remain unanswered.

This paper describes a procedure for the purification of the flagellum as a filament-hook-basal body complex ("intact flagella") that should permit a more comprehensive definition of its base. A similar procedure is described by K. Dimmitt and M. Simon in an accompanying article (12). The two following papers discuss the detailed structure of the basal end of the flagellum (9) and the basal body's specific interactions with the cell wall and cytoplasmic membrane (10).

MATERIALS AND METHODS

Bacteria. AW330 (4), an *Escherichia coli* K-12 strain, is F$^-$, *gal*$^-$, *ara*$^-$, *lac*$^-$, *xyl*$^-$, *thr*$^-$, *leu*$^-$, *thi*$^-$, and resistant to phages T1 and T5 and to streptomycin. It is piliated, and motile, and has about six flagella per cell. *Bacillus subtilis* 168 Marburg strain is a motile tryptophan auxotroph.

Media. *E. coli* was grown on either tryptone broth (1% Bacto-Tryptone plus 0.3% NaCl) or glycerol-Casamino Acids medium [0.2% glycerol, 1% Casamino Acids, 1 μg of thiamine per ml, 0.1 M potassium phosphate (*p*H 7), 0.005 M $(NH_4)_2SO_4$, 0.001 M $MgSO_4$, and 10^{-6} M $Fe_2(SO_4)_3$]. The salts were autoclaved separately from the glycerol and Casamino Acids. The thiamine was sterilized by filtering through a membrane filter (Millipore Corp., Bedford, Mass.). *B. subtilis* was grown on 1% Penassay Broth. The tryptone, Casamino Acids, and Penassay Broth were all Difco products.

Reagents. All reagents were made from analytical grade chemicals in double-distilled water. When tris-(hydroxymethyl)aminomethane (Tris) was used, it was adjusted to *p*H 7.8 at 26 C with HCl; the *p*H was then 8.2 at 5 C. This will be referred to as Tris-hydrochloride, *p*H 7.8. Egg white lysozyme (three times crystallized) was purchased from Mann Research Laboratories. Deoxyribonuclease I (one time crystallized) was purchased from Worthington Biochemical Corp. Triton X-100 (octylphenoxypolyethoxyethanol) was purchased from Sigma Chemical Co.

Assays of flagella. A modification of the method of Grant and Simon (15), using radioactive flagella antibody, was employed to assay flagella on whole cells or in samples contaminated with other proteins. The antibody was produced in rabbits by injecting sheared flagella purified through the CsCl step. The serum gamma globulin was purified by using diethylaminoethyl cellulose (5) and then labeled with I^{125} in the pres-

ence of chloramine-T at 5 C (19). A 25-fold dilution of the labeled antibody was used to prepare a standard curve with known amounts of sheared flagella. The flagella and antibody were incubated for 30 min at 26 C. The antigen-antibody complex was then collected on a Gelman cellulose acetate filter, pore size 100 nm, presoaked in 0.1 M potassium phosphate (*p*H 7), and the excess antibody was washed through with 5 ml of this buffer. Millipore filters could not be used because they bound the free antibody. The filters were dried under a heat lamp, placed in 10 ml of scintillation fluid [3 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene in 1 liter of toluene], and counted. The counts on the filter were proportional to the amount of flagella in the range 0.03- to 0.6-μg flagella. The background was about 4,300 counts/min.

To assay purified flagella, the absorption at 280 nm, corrected for light scattering (14), was used after relating this measurement to protein content in the following way. Total nitrogen was measured in purified samples of sheared flagella at different concentrations by using a modified Kjeldahl procedure (16). A plot of (milligrams of nitrogen $\times$ 6.25) against [corrected optical density (OD) at 280 nm] gave the relationship: 1 OD_{280} = 1.85 mg of flagella protein.

Since the basal body has a strong affinity for cellular materials, it was necessary to evaluate the purification at each step with an electron microscope rather than relying on the above assays. In this way, each step was judged by its ability to purify the basal body rather than just the filament.

Measurement of CsCl density. CsCl gradients without flagella present were fractionated, and the refractive index was measured at 25 C with an Abbe-56 refractometer. Alternatively, 0.2-ml samples were weighed on an analytical balance.

Electron microscopy. Samples were negatively stained and then examined with a Siemens Elmiskop I electron microscope as described in an accompanying paper (9).

Disc-gel electrophoresis. Polyacrylamide-gel electrophoresis of acidic proteins was done by a modification of the Davis method (8). Basic proteins were electrophoresed by the method of Reisfeld et al. (20). The gels were 7% acrylamide and 0.14% *N*,*N'*-methylenebisacrylamide. Electrophoresis was carried out at 5 C by using a constant current power supply delivering 2 ma/gel. The reservoir buffer included 1 mM mercaptoacetate. The gels were stained with 0.5% Amido Black in 7% acetic acid for 1 hr and then destained at a current of 5 ma/gel. Flagella were either applied directly to the gels or first depolymerized by collecting the flagella on a 13-mm Gelman GA membrane filter (100 nm pore size) and resuspending them in either 0.003 M HCl or 8 M urea for 30 min at room temperature or else in 0.05 M KCl for 13 min at 61 C.

Chemical analyses. Total inorganic phosphorous was determined with a sensitivity of 1 μg by the method of Chen et al. (6). Anthrone reagent was used as described by Scott and Melvin (22) to determine total carbohydrate present as glucose equivalents. The assay is sensitive to 5 μg of glucose.

Spectrophotometry. The ultraviolet absorption spectrum of purified flagella was determined with a Zeiss PMQII spectrophotometer. The samples were in 0.1 M Tris-hydrochloride, *p*H 7.8, and were corrected against the same solution minus flagella. Corrections for light scattering were made by using the method of Englander and Epstein (14).

Purification of sheared flagella from E. coli. The procedure was based on methods previously described (1, 23). Motile *E. coli*, grown with rotary shaking in 6 liters of tryptone broth (1 liter of medium per 6-liter flask) to an OD_{590} of 1.2 (about 9×10^8 cells/ml), were centrifuged and suspended in 0.1 M Tris-hydrochloride buffer, *p*H 7.8, to a density of 5×10^{10} cells/ml and then sheared at 4 C in a Waring Blendor at 19,500 rev/min to remove their flagella. In 45 sec the cells lost 99% of their motility, with no loss of viability. The suspension was diluted sixfold in the Tris buffer and subjected to two cycles of differential centrifugation at 12,000 $\times$ *g* for 10 min to sediment cell debris and 55,000 $\times$ *g* for 1 hr to sediment the flagella. Flagella pellets were soaked overnight in the Tris buffer at 4 C and then gently suspended. This preparation formed a single band of flagella on a CsCl gradient. CsCl-purified flagella, viewed in the electron microscope, were free of pili and cell debris, and the acid-dissociated flagella gave a single band (flagellin) on polyacrylamide-gel electrophoresis. The yield was 0.6 to 0.7 mg of flagella per 10^{12} cells, a recovery of about 35%.

Purification of intact flagella from E. coli. The procedure for purification of intact flagella from *E. coli* is diagrammed in Fig. 1, which also shows recoveries at each step.

Step I: growth and harvest of cells. *E. coli* cells taken from a stab were grown overnight on a rotary shaker at 35 C in 10 ml of tryptone broth. This served as the inoculum for 1 liter of the same medium in a 6-liter flask agitated on a rotary shaker. Just after divergence from exponential growth, at an OD_{590} of 1.2 (about 9×10^8 cells/ml), 2 liters of cells was harvested by batch centrifugation at 5,000 $\times$ *g* for 15 min. Continuous centrifuges, such as the Sharples, effectively sheared off the flagella. The pellets were suspended in 50 ml of 20% (w/w) sucrose (final volume about 70 ml) by gently swirling for 1 hr in a rotary shaker containing ice water. The pellets were suspended gently to prevent loss of flagella from shear. These cells, before centrifugation, had 2.4 mg of flagella per 10^{12} cells as determined with the flagella antibody assay.

Tryptone broth or glycerol-Casamino Acids medium was superior to minimal media in producing highly motile, well-flagellated cells that were suitable for the purification procedure described below.

The quantity of cells was limited by the need of avoiding continuous centrifugation, as well as the need to harvest cells at an OD_{590} of 1.2. Cells in stationary phase of growth were very sensitive to the lysing procedure but had fewer flagella per cell. Intact flagella isolated from cells prior to their point of divergence from exponential growth were difficult to purify from material adhering to the basal bodies (see step V). This appears to be the result of incomplete degradation in the lysozyme treatment. Osmotic shock lysed fewer of these spheroplasts and yielded larger cell fragments than spheroplasts made from cells between exponential and stationary phases of growth.

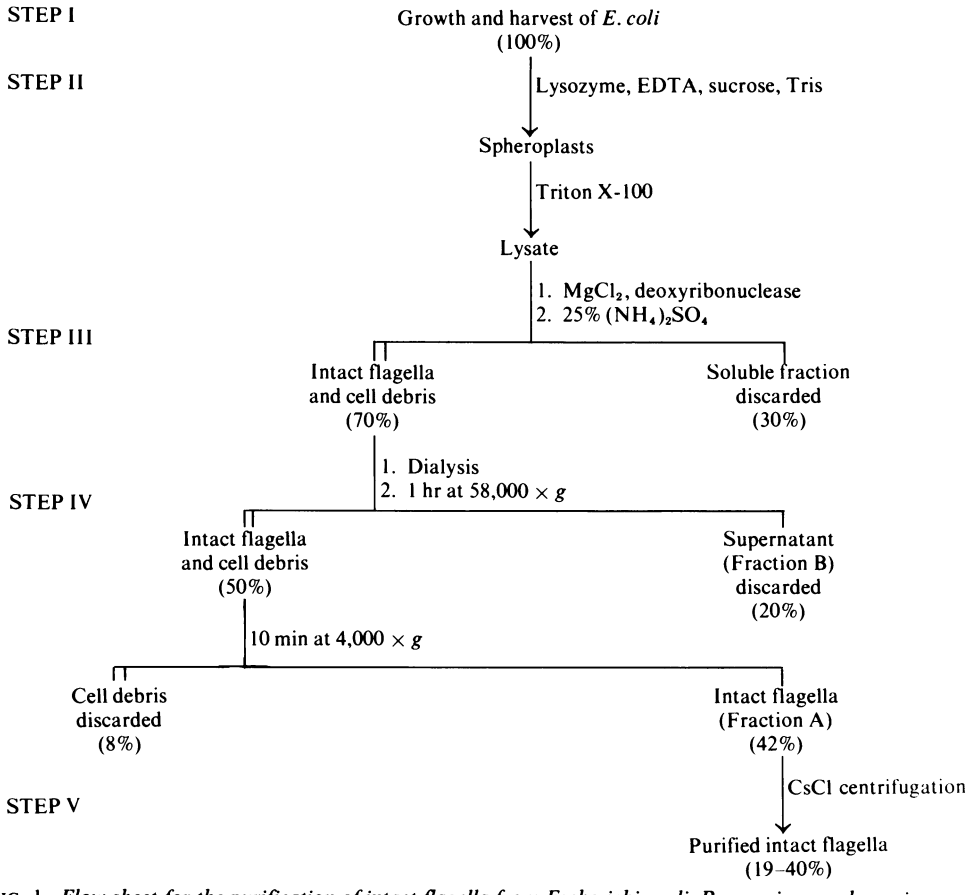

FIG. 1. *Flow sheet for the purification of intact flagella from Escherichia coli. Recoveries are shown in parentheses. The original amount of flagella, 2.4 mg/10^{12} cells, is defined as 100%.*

Step II: preparation and lysis of spheroplasts. Spheroplasts were formed by adding 7 ml of 1 M Tris-hydrochloride (*p*H 7.8), 2 ml of 0.25% lysozyme in 0.1 M Tris-hydrochloride (*p*H 7.8), and 0.2 M NaCl, and 6 ml of 0.1 M ethylenediaminetetraacetate (EDTA) in 0.1 M Tris-hydrochloride (*p*H 7.8), in that order. The suspension was mixed after addition of each reagent, and the EDTA was added within 30 sec after the lysozyme. Incubation at 30 C for 1.5 hr with gentle shaking yielded 99% spheroplasts. Then, addition of 7 ml of 20% (w/w) Triton X-100 gave a clear viscous lysate in 15 sec. In the lysate, the basal bodies had a strong affinity for deoxyribonucleic acid, and the flagella could not be separated from the deoxyribonucleic acid by sedimentation. To free the flagella of deoxyribonucleic acid, we added 0.8 ml of 1 M $MgCl_2$ followed by 1.5 mg of deoxyribonuclease I, and the mixture was incubated at 30 C for 20 min. The Mg^{2+} must not be added before the Triton X-100, since Mg^{2+} prevents solubilization of the outer (lipopolysaccharide) membrane by Triton X-100, with the result that the basal bodies remain encapsulated in this material (10).

Tris is the most effective buffer for preparing spheroplasts with lysozyme and EDTA (21). In addition, the optimum *p*H for purifying the intact flagella was between 8.1 and 8.4 (see preparation of *p*H 7.8 Tris under Reagents). At higher *p*H values, the flagella began to fragment. At lower values particulate material adhered to the basal bodies.

Triton X-100, a nonionic detergent, serves to lyse the cells by solubilizing the cytoplasmic membrane. In addition, in the presence of EDTA, it also solubilizes the outer membrane. The Triton X-100 does not damage the flagella as judged by the fact that the motility and growth of *E. coli* were normal in 1.5% Triton X-100, and no effect on the structure of the purified intact flagella was observed after treatment in 10% Triton X-100.

Step III: ammonium sulfate fractionation. The lysate was immediately diluted to 260 ml with cold (5 C) 0.1 M Tris-hydrochloride (*p*H 7.8) containing 5×10^{-4} M EDTA (Tris-EDTA buffer) in prechilled glassware. A solution of $(NH_4)_2SO_4$ (87 ml), saturated at 5 C in Tris-EDTA buffer, was rapidly poured into the diluted lysate to give 25% of saturation, and the suspension was stirred for 2 hr at 5 C. When the $(NH_4)_2SO_4$ was added slowly at a rate of 0.2 ml/sec with stirring, the final purity and yield were poor. At $(NH_4)_2SO_4$ concentrations higher than 30% of saturation, the flagella were difficult to purify.

Centrifuging the suspension at 12,000 × *g* for 25 min in an angle head rotor gave a viscous white material, floating at the meniscus and adhering to the side of the tube, which was collected with a flat spatula. (The use of a swinging bucket rotor allows all of the material to float at the surface in a dense, easily removed layer.) The fluid was discarded and the tube was rinsed with Tris-EDTA buffer. The rinse fluid was combined with the recovered material to give a final volume of 40 ml. After 1.0 ml of 20% Triton X-100 was added, the material was gently dispersed. It was then dialyzed twice for 12 hr at 5 C against 1 liter of Tris-EDTA buffer containing 5 ml of 20% Triton X-100 and 1 ml of toluene as a bactericide, with stirring that was rapid enough to create a vortex to disperse the toluene. The initially turbid suspension completely cleared on dialysis. Beside removing the low-molecular-weight compounds, dialysis allows the EDTA and Triton X-100 to solubilize outer membrane aggregates which formed when Mg^{2+} was added. This prevents that material from adhering to the basal bodies.

Step IV: differential centrifugation. The dialyzed preparation was diluted to 50 ml with Tris-EDTA buffer. In each of two Spinco SW-25 tubes, 25 ml of the diluted material was placed on top of 2 ml of 20% (w/w) sucrose which had been layered over 3 ml of 60% sucrose. The sucrose solutions were prepared in Tris-EDTA buffer. The use of sucrose layers was necessary when sedimenting intact flagella; otherwise, tightly packed pellets formed which were difficult to resuspend without breaking off about 70% of the basal bodies. After centrifugation in a Spinco model L SW-25 rotor at 20,000 rev/min for 1 hr, the liquid above the sucrose layers (fraction B) was removed with a pipette and discarded. To the sucrose layers, 5 ml of Tris-EDTA buffer was added. After mixing gently, the sucrose was removed by dialysis overnight against Tris-EDTA buffer. The suspension was then diluted to 25 ml with the buffer, and 0.15 ml of 20% Triton X-100 was dissolved. This was centrifuged at 4,000 × *g* for 10 min; the pellet, containing unlysed cells and large aggregates, was discarded and the supernatant material (fraction A) was used in the next step.

Step V: CsCl gradient centrifugation. Fraction A was diluted to 27 ml with Tris-EDTA buffer, and 12.1 g of CsCl was added in one portion and rapidly dissolved. After centrifugation in a Spinco SW-25 rotor at 22,000 rev/min for 50 hr, the tube appeared as shown in Fig. 2A. The viscous oily material at the meniscus was removed with a pipette, and the gradient was then fractionated with a siphon. The band of flagella in the center of the gradient, at a density of 1.30 g/ml, was collected and the CsCl was removed by dialysis against Tris-EDTA buffer. Purified sheared flagella had the same density as intact flagella; this was not altered by the presence of 0.5% Triton X-100. RbCl gave consistently poor results when substituted for CsCl.

Usually fraction A gave a single translucent band (Fig. 2A) which contained about 98% of the flagella in the tube, or 40% of the original amount of flagella. However, sometimes fraction A gave, in addition, a white band at a lighter density (Fig. 2B). A quantitative description of the gradient in Fig. 2B is seen in Fig. 3. The majority of the flagella were restricted to

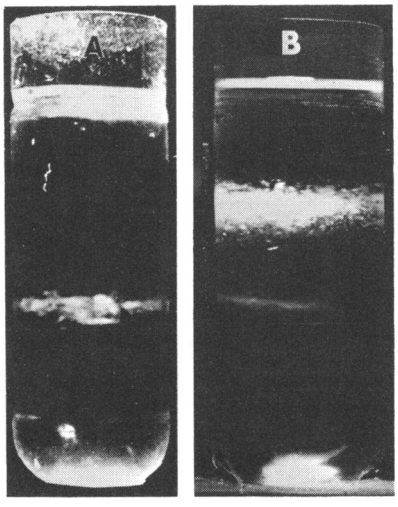

FIG. 2. *CsCl gradients at equilibrium containing: (A) fraction A, the one band visible contains flagella; (B) fraction A, two visible bands.*

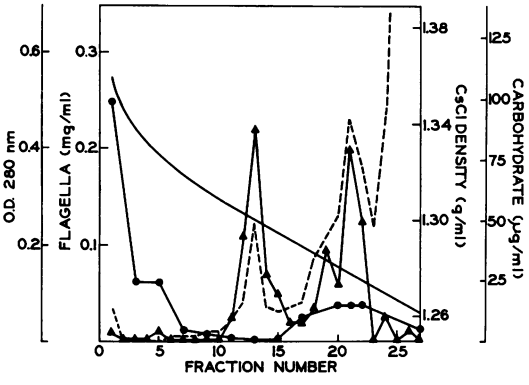

FIG. 3. *Fractionation and chemical analysis of the gradient shown in Fig. 2B. The broken line is the OD at 280 nm. The OD values of fractions 19 to 25 were adjusted for light-scattering (14). Fractions were assayed for carbohydrate, i.e., glucose equivalents (●), and flagella antigen (▲). The density was measured by refractive index on a duplicate gradient run simultaneously but without fraction A (solid line).*

two regions. The band at a density of 1.30 g/ml contained about 45% of the flagella in the tube, or 19% of the original amount of flagella, and was relatively free of carbohydrate. The band centered at 1.28 g/ml contained most of the remainder of the flagella but was contaminated with carbohydrate-containing material. Electron microscopy showed that the basal bodies of flagella in this band were embedded in cell material.

The appearance and number of the bands on CsCl and the purity and quantity of flagella recovered were markedly influenced by the rate of addition of the CsCl to the flagella and by the age of the cells at harvesting. Dissolving the CsCl in 0.5-g quantities resulted in a wide and continuous density distribution of material with a faint, poorly resolved flagella band. Adding

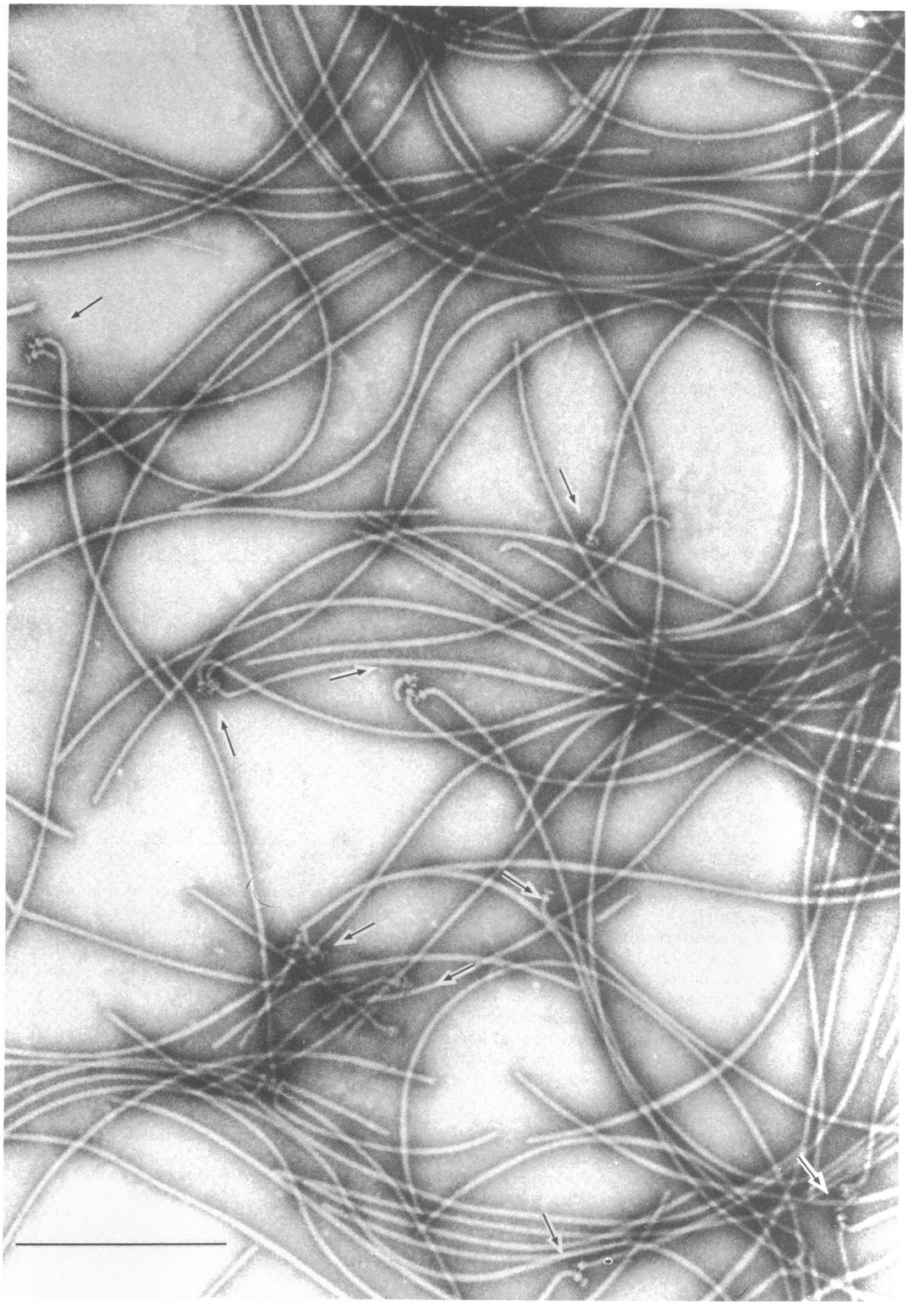

FIG. 4. *Purified intact flagella from Escherichia coli negatively stained with uranyl acetate, pH 4.5. A hook-basal body complex is visible at one end of many of the filaments (arrows).* × *66,000. Bar represents 0.5* μm.

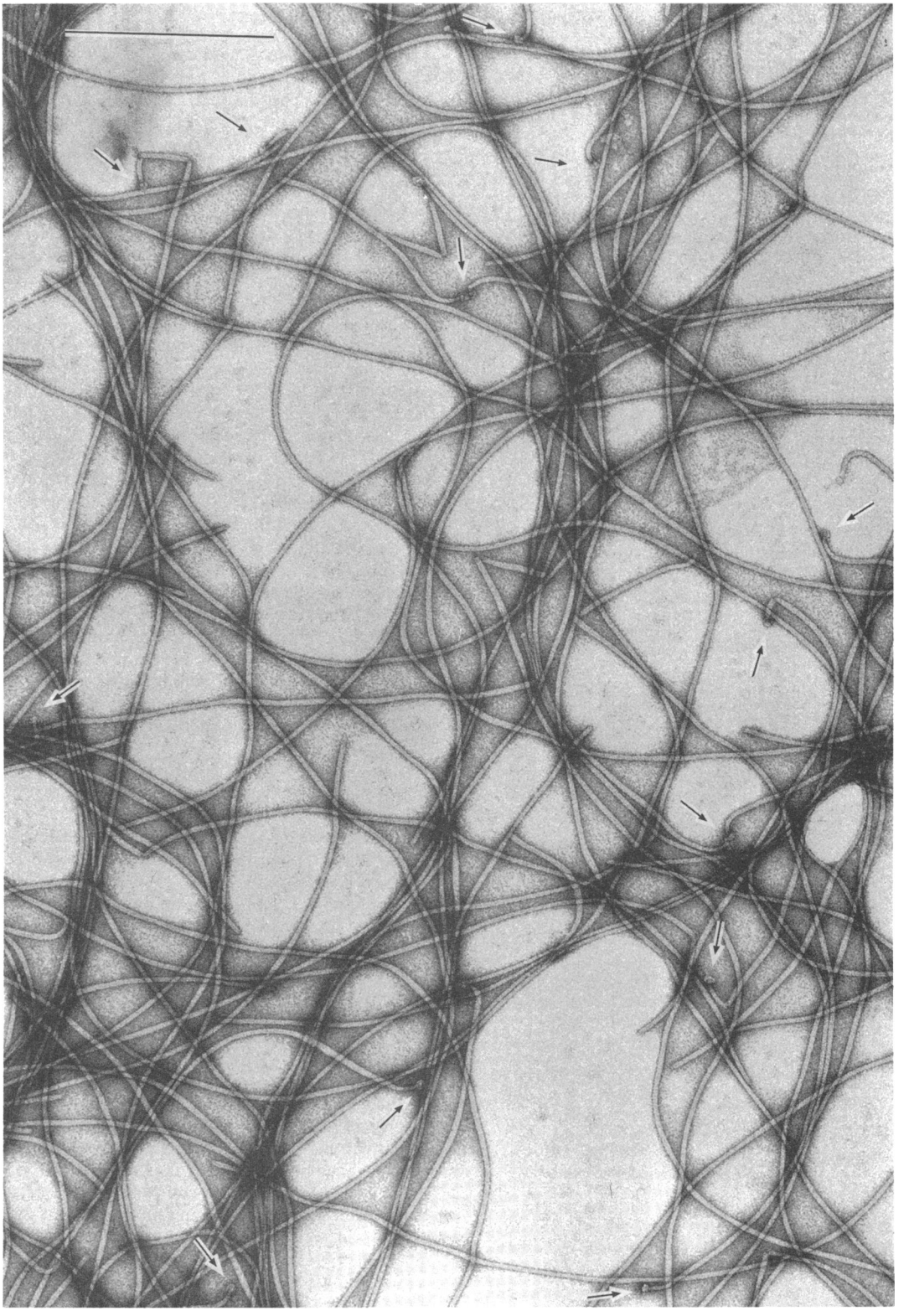

FIG. 5. *Purified intact flagella from Bacillus subtilis negatively stained with uranyl acetate, pH 4.5. Many of the filaments have a hook-basal body complex at one end (arrows).* × *66,000. Bar represents 0.5 μm.*

all the CsCl at once to an identical sample gave a single visible band containing four times as much purified flagella. Under our conditions, cells departed from exponential growth on tryptone broth at an OD_{590} of 1.0. Cells harvested at an earlier time (OD_{590} of 0.75) did not yield pure, well-defined bands in the CsCl gradient.

When fraction B was subjected to a CsCl gradient, flagella free of other cellular materials could not be recovered. Fraction B was therefore discarded.

Purification of intact flagella from B. subtilis. Intact flagella were prepared from *B. subtilis* by using exactly the same procedure developed for *E. coli*. The critical harvest period for *B. subtilis* was at an OD_{590} of 0.55, just before the long, motile filaments began to separate into smaller, motile rods which were resistant to the lysing procedure. Lysozyme and EDTA caused the long filaments to lyse within 45 min, despite the presence of sucrose, and Triton X-100 was added at that time to complete the lysis. The appearance of the CsCl gradient was comparable to that for *E. coli*; the flagella banded at 1.30 g/ml.

RESULTS

Purification of intact flagella. The procedure for the purification of intact flagella from *E. coli* and *B. subtilis* has been presented. Figure 1 summarizes this procedure and shows the recovery of flagella at each step.

Electron microscopy. Observations of the purified material from *E. coli* revealed that many of the flagella have a hook and basal body at one end (Fig. 4). The preparation contained about one basal body per 4 μm of flagella. Since the average length of flagella in vivo was roughly 5 μm, no significant loss of basal bodies occurred during the purification. No pili or cell debris was visible with either uranyl acetate or phosphotungstate negative staining. Electron microscopy of the purified material from *B. subtilis* revealed about one basal body per 6 μm of flagella and no contaminants (Fig. 5).

The basal bodies of purified intact flagella from *E. coli* tended to aggregate (Fig. 4). This phenomenon became extensive when concentrated solutions of flagella (0.8 mg/ml) were stored in a refrigerator for 1 week. The flagella could not be disaggregated by extensive dialysis against Tris-EDTA buffer or such buffer containing 2.0 M KCl. The presence of 0.1% Triton X-100 caused partial disaggregation. We found the best remedy was to store the flagella in dilute solutions of 0.1 mg/ml. The aggregation phenomenon was caused by the extraction of the Triton X-100, which floats on the CsCl gradient, from the flagella and the concentration of the flagella during the CsCl centrifugation. No aggregation of the basal bodies was observed before the CsCl step as long as Triton X-100 was present. The basal bodies of intact flagella from *B. subtilis* (Fig. 5) showed less tendency to aggregate than did those of *E. coli*.

A detailed study of the structure of the basal end of the flagella is presented in an accompanying paper (9).

Electrophoresis. Polyacrylamide-gel electrophoresis of the purified intact flagella from *E. coli* at *p*H 8.9 (Fig. 6A) or *p*H 4.5 showed no protein bands. Therefore, based on a limit of detection of 1 μg and a gel exclusion limit of 10^5 daltons, proteins of this size or smaller constituted less than 1% of the preparation. When the purified intact flagella from *E. coli* were first treated with either heat, urea, or acid, a single band, flagellin, was seen with an R_F of 0.39 (Fig. 6B, C, D).

Chemical and spectral analysis. Analysis of purified intact flagella from *E. coli* showed less than 0.15% inorganic phosphate and 1.8 to 2.2% carbohydrate. The ultraviolet absorption spectrum of both purified intact flagella and purified sheared flagella from *E. coli* was typical of a pure protein with a maximum absorbance at 278 nm, a minimum at 255 nm, and a 280 nm/260 nm ratio of 1.35 to 1.40.

According to the above criteria of purity, our preparation of intact flagella from *E. coli* contains small or insignificant amounts of nucleic

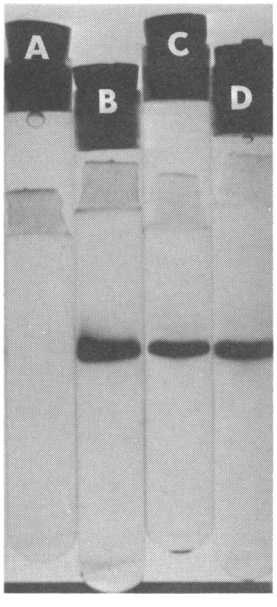

FIG. 6. *Polyacrylamide-gel electrophoresis of the acidic proteins in purified intact flagella from Escherichia coli. A 110-μg amount of protein was applied to each gel. A, No treatment; B, 13 min in 0.05 M KCl at 61 C; C, 30 min in 8 M urea; D, 30 min in 0.003 N HCl. Position of the bromophenyl blue dye band was marked by cutting off the gel at that point.*

acids, phospholipids, carbohydrates, or small proteins. The only detectable components were the flagella and their monomer flagellin. However, these data would not reveal the composition of the hook or basal body since these comprise only 2% of the length of the flagellum.

DISCUSSION

The procedure described in this paper yields flagella in the form of a filament-hook-basal body complex, free of detectable cell wall, membrane, or cytoplasmic material. The method gives substantial product recovery and is adaptable to large-scale preparations from which the hook and basal body could be isolated and characterized. Finally, the techniques are applicable to a variety of lysozyme-sensitive bacteria, despite strikingly different cell envelopes, as demonstrated with *E. coli* and *B. subtilis*.

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