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Purification and Thermal Stability of Intact *Bacillus subtilis* Flagella

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Flagella were prepared and purified in a relatively intact form from bacterial lysates. Immunochemical tests showed that over 95% of the protein in the final preparation consisted of flagellar antigen. These flagella are more stable to thermal denaturation than flagella filaments obtained by shearing. Their thermal properties more closely resemble those of flagella in the native state on bacteria. The presence of the hook structure is responsible for this extra stability.

When bacterial flagella are examined by electron microscopy (2, 3, 7, 11) they are found to be composed of three morphologically distinguishable sections: a long flagellar filament, a hooklike terminal structure, and a basal region which is attached to the cell membrane. The biochemical properties and composition of the flagellar filament and flagellin, the major filament protein, have been described (4, 14, 19) in a number of bacterial systems. However, relatively little is known about the other morphological sections and their subunits. Further information about these structures and their relationship to the flagellar filament is clearly necessary for understanding both the formation of flagella and the mechanism of motility. However, the usual method for isolating flagella involves shearing the filaments from the bacteria, a process which yields preparations lacking, for the most part, the hook and the basal structures.

In this paper, we describe methods for purifying relatively intact *Bacillus subtilis* flagella after lysis of the bacteria. Flagella prepared in this way maintain the hook structure, the presence of which increases the thermal stability of the flagellar filament. DePamphilis and Adler (8), in an accompanying paper, describe similar methods for the purification of *Escherichia coli* flagella.

MATERIALS AND METHODS

Bacterial strains. The two strains of *B. subtilis* 168 which were used in this study are BD71 (his A-1, ura, arg C-4), obtained from D. Dubnau, and GD1, a flagellar mutant (hag-10) isolated from BD71 by G. Grant (10). All strains were grown on minimal medium prepared by the method of Spizizen and Anagnostopoulos (5).

Measurement of antigenic activity. Flagellar antigen was measured using ^{125}I-labeled antibody as previously described (9). A preparation of sheared flagella extensively purified by differential centrifugation was used to obtain standard curves. Protein concentrations were determined by the method of Lowry et al. (16).

Purification of flagella. The bacteria were grown until late-logarithmic or early stationary phase and then collected by centrifugation. About 3 mg of purified flagella were recovered from 1 liter of culture by the following procedure. The bacterial pellet was resuspended in 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.0) at 1/30 the original volume, and 100 μg of lysozyme per ml and 0.5% Brij-58 were added. Incubation at 4 C was continued until lysis was complete, as determined by the clearing of the solution. Deoxyribonuclease (5 μg/ml) was added in the presence of 0.01 M magnesium chloride to decrease the viscosity of the extract. Bacterial debris was removed by centrifugation at 10,000 × *g* for 10 min, and the resulting supernatant was further centrifuged at 100,000 × *g* for 90 min. The pellet was then resuspended in standard saline citrate (0.01 M trisodium citrate and 0.1 M sodium chloride, *p*H 7.3) and then fractionated by the addition of ammonium sulfate to 20% saturation. The precipitate obtained was further purified by using either cesium chloride gradient or Renografin gradient isopycnic centrifugation. Partially preformed cesium chloride gradients were used; 10 ml of the sample solution (approximately 5 mg of flagellar protein) containing 0.5 g of cesium chloride per ml was layered between equal volumes of 0.6 and 0.4 g of cesium chloride per ml in standard saline citrate. The gradient was centrifuged at 100,000 × *g* for 3 hr in a model 30 Beckman-Spinco rotor. The flagella band was removed and extensively dialyzed against 0.05 M NaCl-0.01 M Tris buffer (*p*H 7.3). For the Renografin gradient, 1.0 ml of the sample solution was layered onto a 30-ml, linear, 7.6% (v/v) to 53.2% (v/v) Renografin gradient. After centrifugation at 60,000 × *g* for 5 hr, the gradient tube was punctured, and samples were collected and assayed for flagellar protein. Sheared flagella were prepared as previously described (9).

Circular dichroism. Circular dichroism was meas-

ured by using a J-10 modification of the Durrum-Jasco UV/ORD/CD-5. The kinetics of disaggregation of flagella was measured in a 1-cm water-jacketed cell at a protein concentration of 50 μg/ml in 0.01 M Tris-hydrochloride buffer (*p*H 7.3). The sample was equilibrated at 53 C, the temperature was then raised to 62 C, and, after equilibration, ellipticity $[\theta]$ was observed at 22 nm. The per cent ellipticity remaining was calculated by measuring $[\theta]$ after incubation at 62 C for 1.5 hr $[\theta]_f$, at the start of heating $[\theta]_i$, and at any time during incubation $[\theta]_t$, and applying the formula, $([\theta]_t - [\theta]_f)/([\theta]_i - [\theta]_f) \times 100$ = per cent of ellipticity.

Thermal stability. Thermal stability was followed by heating a 1.0-ml solution containing 2 μg of flagellar antigen in 0.01 M Tris-hydrochloride buffer (*p*H 7.3) and 0.05 M sodium chloride at the desired temperature for 15 min. A sample was then removed and diluted with cold buffer. Antigenic activity was measured, and the per cent of antigenic activity remaining was calculated as the antigenic activity at any temperature divided by the antigenic activity at room temperature. The assay conditions were chosen so that flagellin gave no measurable residual antigenic activity. We can define a temperature (Td) at which 50% of the antigenic activity of the flagella remains after 15 min of incubation.

Electron microscopy. Samples were placed directly on carbon-coated Formavar-covered grids. They were negatively stained with 1% phosphotungstic acid (*p*H 7.3) or 1% uranyl formate (*p*H 4.0). An electron microscope (Phillips model 200) was used with an accelerating voltage of 60 kv.

Phosphorus assay, Phosphorus was measured by modification of the procedure of Lowry et al. (17). The final volume which we used was 1.0 ml, and 5 μmoles of phosphorus could be detected.

Chemicals. Egg-white lysozyme was obtained from Calbiochem, Los Angeles, Calif. Brij-58 [polyoxyethylene (20) Cetyl Ether] was obtained from Atlas Chemical Industries, Inc., Wilmington, Del. Deoxyribonuclease was obtained from Worthington Chemical Corp., Freehold, N.J. Cesium chloride was obtained from Research Organic/Inorganic Chemical Co., Sun Valley, Calif. Renografin-76 (Squibb Methylglucamine Diatrizoate) was obtained from E. R. Squibb and Sons, Inc., New York, N.Y.

RESULTS

Purification of flagella. To obtain flagella, the bacteria were treated with lysozyme and detergent. A number of detergents were tried, including Nonidet, sodium dodecyl sulfate, Brij-58, and sodium deoxycholate. Brij treatment was found to free flagella from the cell debris without affecting their antigenic activity. The flagella were collected by centrifugation, and 25% of the protein in the resuspended pellet was found to be flagellar antigen. After ammonium sulfate fractionation, 80% of the protein was flagellar antigen. The final step in the purification involved density gradient centrifugation, and 95% of the protein in the major band was flagellar antigen. Figure 1A and 1B show electron micrographs of purified flagella. When the entire length of the flagellum could be observed, the hook structure was generally present. There was some heterogeneity in the appearance of the hooks. At the terminus of many of them (Fig. 1D), basal structures with amorphous membrane-like material were found. Others (Fig. 1C) lacked the basal structure, and a short rodlike structure protruded from the base of the hook. A more complete description of purified hooks will be presented elsewhere.

Figure 2 shows the distribution of flagellar antigen in cesium chloride and Renografin gradients. Minor bands of antigenic activity appeared in both gradients. However, when the material in the minor band was rebanded, the activity was found at the position of the major band. Thus, the minor bands may result from aggregation of flagella or from binding of extraneous material to the flagella.

Analysis of the phosphorus content of the purified preparations showed that less than 0.05% (by weight) of the preparation was phosphorous.

Measurement of thermal stability. When flagella are heated (15, 18) above a specific temperature, they disaggregate. A convenient way to follow this process is to measure the loss of flagellar antigenic activity. However, these measurements cannot be made at the ambient temperature; samples must be cooled before they are measured. It is possible that during the cooling some reaggregation may occur. To test this possibility, another parameter, circular dichroism, was used to follow disaggregation at the ambient temperature. When the flagella disaggregate, there is a dramatic change in ellipticity at 22 nm. This reflects the transition of flagella to flagellin (6, 12; Simon, *unpublished data*) and the loss of α-helical structure in the flagellin subunit. When the kinetics of disaggregation were followed by measuring antigenic activity and ellipticity, both methods gave the same results (Fig. 3).

Thermal stability of flagella. To further characterize the purified flagella, their thermal stability was compared to that of flagella obtained by shearing and to flagella found on intact bacteria. Flagella obtained by lysing the bacteria are more stable than sheared flagella, and their thermal stability profile is similar to that of flagella on intact bacteria (Fig. 4A). This increased stability does not result from the purification procedure since it is observed with crude lysates of bacteria before CsCl or ammonium sulfate treatment. It is not a function of the presence of extraneous material or cells since there is no stabilization of

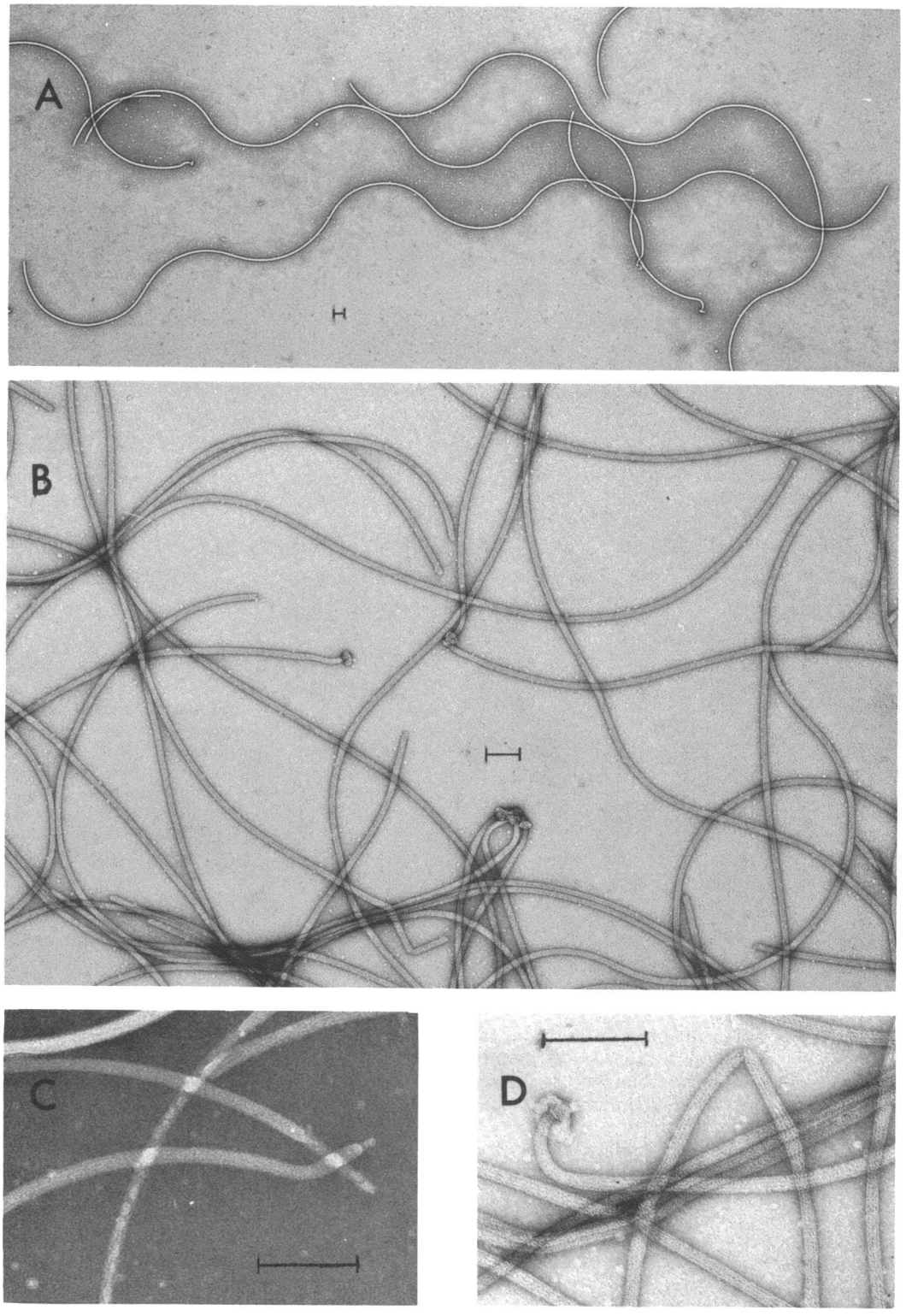

FIG. 1. *Flagella after purification by ammonium sulfate fractionation. A, Negative staining with PTA. B, Negative staining with uranyl formate. C, Stained with uranyl formate, showing the basal region and membrane-like material. D, Stained with PTA, showing shaft-like protrusion at the base of the hook. The bar equals 100 nm.*

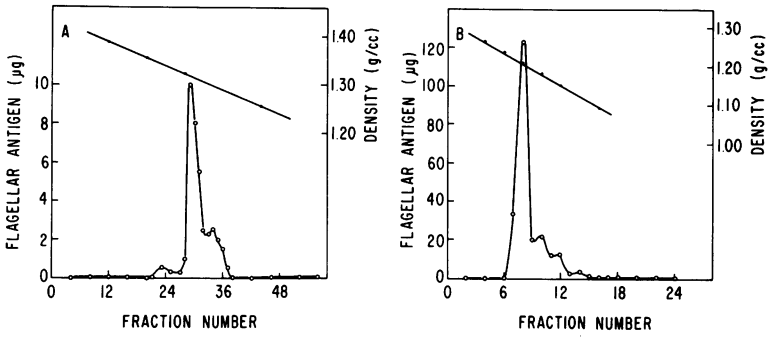

FIG. 2. *Density gradient centrifugation of flagellar antigen. A, Analytical cesium chloride gradient. The density of the flagella solution was adjusted to 1.31 g/cm² with cesium chloride and the gradient was formed by centrifugation for 24 hr at 130,000 × g in a Spinco model L SW39 rotor. B, Renografin gradient.*

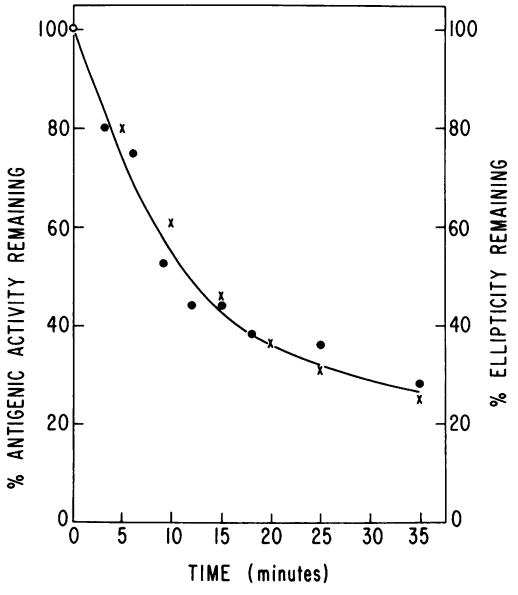

FIG. 3. *Kinetics of thermal disaggregation. Symbols: ×, measured by following ellipticity at 222 nm; ●, measured by following antigenic activity.*

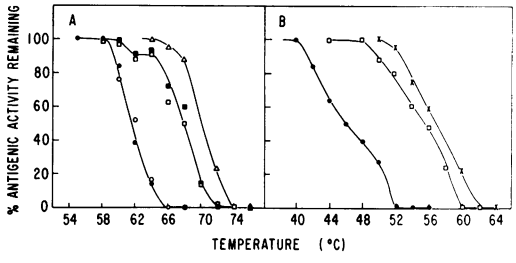

FIG. 4. *Thermal stability of various forms of flagella. A, Flagella from wild-type bacteria. Symbols: ●, sheared flagella obtained by shearing washed bacteria (16,000 rev/min in Virtis omnimixer for 30 sec) and removing bacteria by centrifugation; ○, sheared flagella were purified by ammonium sulfate fractionation and cesium chloride density centrifugation; □, intact flagella obtained by lysis and centrifugation; ■, intact flagella after ammonium sulfate and cesium chloride gradient purification; △, whole bacteria washed once in buffer. B, Flagella from GID mutant. Symbols: ●, sheared flagella; ×, whole bacteria; □, intact flagella.*

sheared flagella when they are measured in the presence of cell extracts. The same thermal stability profiles are also found after sheared flagella are purified by ammonium sulfate and CsCl treatment.

The difference in thermal stability is even more dramatically observed with a flagellar mutant GD1 (Fig. 4B). Its flagellin differs from wild type in a single peptide, and this difference results in destabilization of the flagellar filament (Emerson and Simon, *unpublished data*). The sheared flagella disaggregate at temperatures which are 10 C lower (Td, 46 C) than those at which flagella purified from lysed bacteria (Td, 56 C) disaggregate.

There is a variety of possible explanations for the relative stability of the flagella obtained by the lysis procedure. Some of the explanations which we considered are the following. (i) The stability may be a function of the length of the filament, and sheared filaments might be shorter than those obtained by lysis, (ii) the shearing process could damage or distort the filament structure and thus destabilize it, (iii) the presence of the hook structure could stabilize the filament—the sheared flagella for the most part lack the hook structure and would therefore disaggregate more readily. To distinguish among these possibilities, flagella obtained by the lysis procedure were sheared by passing them through a 24-gauge needle 10 times. Figure 6A shows that the flagella were broken into relatively short pieces. When these flagella were heated they showed a biphasic thermal stability profile (Fig. 5). Eighty-five per cent of the flagellar antigen showed a thermal stability profile characteristic

of sheared flagella; the temperature stability of the remaining 15% of the antigen was similar to that of the original intact flagella. On the basis of the first two proposed explanations, we would predict that the 15% of the flagellar antigen which remains relatively stable represents flagella which have escaped shearing and are therefore long or intact. On the other hand, if the hook structure stabilizes the adjacent filament, we would expect the residual fraction to be enriched in filaments carrying the hook structure. Table 1 shows the results of an experiment in which samples were removed at various stages in the heating process and examined by electron microscopy. Fields were randomly chosen, and all of the particles in these fields were counted and classified. Initially, most of the flagella do not have hook structures; as the temperature is increased, the fragments lacking the hook disappear. The ratio of flagella fragments without hooks to those with hooks ranges from 9.4 after incubation at 30 C to 0.34 after incubation at 52 C. There was no apparent enrichment for relatively long fragments. After heating at 60 C, almost all of the observable flagella filaments are disaggregated and only the hook structure remains (Fig. 6C,D). These results suggest that the thermal stability reflects the presence of the hook on the filament.

To test this hypothesis, further flagella were disaggregated and the hook structures were separated from flagellin by centrifugation. The resulting flagellin was repolymerized into long flagellar filaments which lacked hook structures (1). These were then tested for thermal stability and were found to show exactly the same profile as sheared flagella. We conclude, therefore, that the hook stabilizes the adjacent flagellar filament.

DISCUSSION

Under a given set of conditions (18), the thermal stability of the flagellar filament is a function of the composition of the flagellin subunit (15). This is further illustrated by the properties of sheared flagella from GD1, a mutant which differs from the wild type in a single peptide (Emerson, *in preparation*) but disaggregates at temperatures 14 C lower than the wild type. In addition to the composition of flagellin, the presence of the hook has a marked effect on the thermal stability of the filament.

There are a number of ways in which we can rationalize the stabilizing effect of the hook. It may directly influence the structure of the filament. Since high-resolution electron microscope studies have shown that there are no gross structural differences between flagella on intact bacteria and sheared or reaggregated

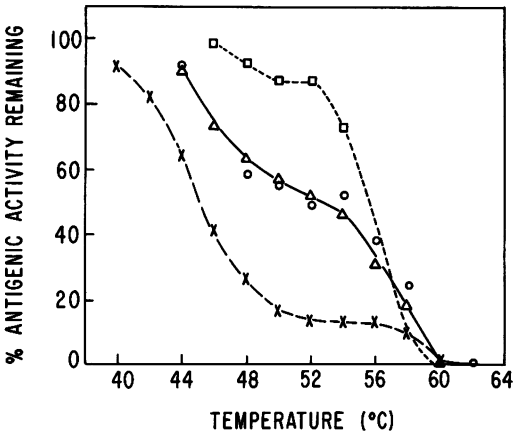

FIG. 5. *Thermal stability of GID flagella after shearing. Symbols:* □, *thermal stability of intact flagella;* ×, *intact flagella after shearing by passing through a 24-gauge needle;* ○, *a mixture of intact and sheared flagella.* △, *curve calculated for thermal stability of mixture of sheared and intact flagella.*

flagella, any effect of the hook would be relatively subtle. However, just such subtle effects were postulated in models to explain the mechanism of motility (13, 20). Since the filament itself is devoid of adenosine triphosphotase activity, it has been suggested that the basal structures, the hook, or the cell membrane may contain the appropriate enzymes, and the hook may transduce the chemical activity into a physical change which can be propagated down the flagellar filament. If such a mechanism is correct, we would expect the hook to be able to influence and stabilize the filament structure.

Another way of looking at the stabilizing effect of the hook is from the point of view of the mechanism of thermal disaggregation. Koffler and his co-worker (1) showed that the hook structure is relatively stable to detergents, heat, and other agents which disaggregate flagella. We have shown (*in preparation*) that the hook does not lose its characteristic structure or antigenicity until it is heated above 72 C. If one of the mechanisms for thermal dissociation of the flagella is involved in the preferential denaturation of flagellin on the proximal end of the filament, the presence of the hook and its connection to that end would protect it, thus stabilizing the filament structure.

The purified "intact flagella" can also be used to separate the other flagella components. By heating GD1 flagella, the filament can be removed, and relatively pure preparations of hook and basal structures can be obtained. These procedures open the possibility for investigating

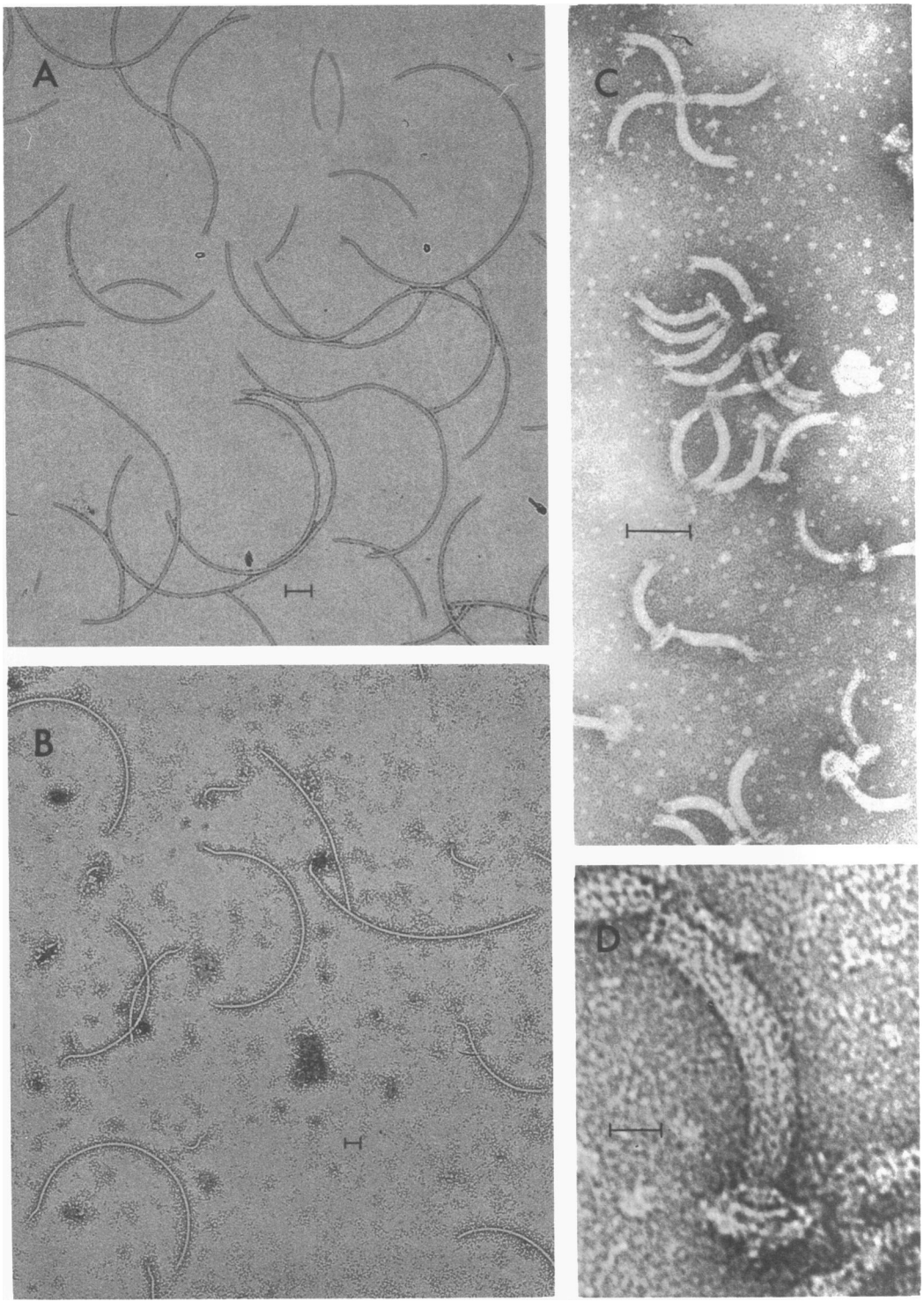

FIG. 6. *Sheared flagella before and after heating. A, Before heating, stained with phosphotungstic acid; B, after heating at 50 C for 15 min, phosphotungstic acid-stained; C, heated at 60 C for 15 min, stained with uranyl formate; D, hook structure stained with uranyl formate. The bar equals 100 nm in A, B, and C and 20 nm in D.*

TABLE 1. *Comparison of the effect of temperature on flagellar fragments with and without hook structures*

Temp	Free hooks	Filaments with hook	Filaments without hook	Ratio of fragments without hooks to those with hooks
C				
30	0	42	396	9.4
46	0	42	153	3.6
50	6	116	62	0.53
52	105	245	84	0.34

the nature of these other structures and their roles in flagella formation and motility.

ACKNOWLEDGMENT

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