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Structure of Common Pili from *Escherichia coli*

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Several important properties of the common pili from *Escherichia coli* are discussed. These pili were resistant to the gentle Folin-Ciocalteau reagent methods for protein detection and were not readily solubilized by sodium dodecyl sulfate. They were found to contain a reducing sugar but not peptidoglycan. The pilin had multiple conformations in sodium dodecyl sulfate solution, and the appearance of multiple bands on sodium dodecyl sulfate gels did not necessarily indicate heterogeneity of the preparation. The pilus subunit was found to be a different protein than outer membrane protein III, which has the same apparent molecular weight. In addition, we confirmed the results of Brinton (Trans. N.Y. Acad. Sci. **27**:1003-1054, 1965): that there is a dramatic change in the properties of pili after they are heated at pH values below 2.

The common pili of *Escherichia coli* are very stable protein assemblies. These pili, also called type 1 or type I pili, or fimbriae, are firmly attached to the bacterium. They are more difficult to remove than flagella or F pili (14) and, indeed, vigorous blending is required to detach these pili from the bacteria for purification. In addition to mechanical stability, common pili have been found to resist various disruptive chemicals. Salit and Gotschlich (18) found that they were resistant to 6 M urea, and the present study confirms their resistance to solubilization by sodium dodecyl sulfate (SDS). Preliminary studies in our laboratory and in Brinton's laboratory (personal communication) indicate that these pili also resist hydrolysis by trypsin. Indeed, some of our experiments indicate that the pili were not completely detected by the Lowry method, even after boiling in 1 N NaOH for 20 min.

Because of the refractivity of the pilus towards disaggregation, detection of the protein subunits (pilin) has eluded many of the researchers studying the cell envelope of *E. coli* (1, 6, 11, 13, 19). There appear to be two reasons why detection by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is difficult. First, as shown in this paper, pili are resistant to SDS solubilization procedures that are normally applied to envelope proteins. Second, another protein is present that migrates with nearly the same apparent molecular weight as that of pilin upon SDS-PAGE. This other protein, named protein III by Hindennach and Henning (9), is different from pilin in at least two ways. First, some of our data indicate that this protein has an antigenicity different from that of pilin. Second, protein III does not have the three different conformations upon SDS-PAGE, as do pili that are electrophoresed under different reducing conditions.

The main object of this paper was to describe procedures for handling common pili from *E. coli* and to demonstrate the unusual stability of these pili as glycoprotein assemblies.

MATERIALS AND METHODS

Chemicals and solutions. *N*-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES) was obtained from Sigma Chemical Co., St. Louis, Mo. TES buffer was prepared at a concentration of 5 mM and adjusted to pH 7.0 with NaOH. Dilute TES was prepared as a 10-fold dilution of the 5 mM buffer. $MgCl_2$ and $(NH_4)_2SO_4$ were of American Chemical Society standard and were obtained from Fisher Scientific Co., Pittsburgh, Pa.

Preparation of bacteria and pili. The F^-W1-3 substrain of *E. coli* K-12 was used exclusively. The bacteria were grown in Lennox broth (L-broth) consisting of 10 g of NaCl, 1 g of dextrose monohydrate, 10 g of tryptone (Difco Laboratories, Detroit, Mich.), and 5 g of yeast extract (Difco), made up to 1 liter with distilled water. The bacteria were harvested at mid- to late-log phase by centrifugation at 9,000 × *g* for 5 min.

Pili were prepared by the method of Brinton (4). The details were as follows. The bacteria, harvested by centrifugation from 3 liters of medium, were suspended in 100 ml of TES buffer. In some instances, the cells were washed with TES buffer, but this was not usually necessary. The bacteria were then blended for 2 min in a Waring blender, using 30-s bursts to prevent heating. The bacteria were then centrifuged at 7,000 × *g* for 20 min, and the supernatant was saved. Sufficient $MgCl_2$ was then dissolved in the supernatant to make the solution 0.1 M in $MgCl_2$. After a few minutes at room temperature, the solution became turbid and was then centrifuged at 27,000 × *g* for 40

min. A white pellet consisting almost totally of pili was obtained. This pellet was suspended in 50 ml of dilute TES, and the solution was blended thoroughly with a Vortex mixer. The solution was then centrifuged at 27,000 × *g* for 30 min, and the supernatant was saved. Then 1 M $MgCl_2$ was added to make the supernatant 0.1 M in $MgCl_2$. The pili were recycled twice more in this manner and stored at 4°C in dilute TES buffer plus 0.1 M $MgCl_2$. The pili were used within 2 weeks of their preparation.

Preparation of pilin from pili. Purified pili in the storage solution containing 0.5 mM TES (pH 7.0) plus 0.1 M $MgCl_2$ were centrifuged at 27,000 × *g* for 10 min. The supernatant was discarded, and the pellet was suspended in water which had been adjusted to pH 1.8 with HCl. After the pellet had been completely resuspended, the solution was placed in a boiling-water bath for 5 min, removed, and allowed to cool to room temperature, and then NaOH was added to raise the pH to near 10. At this point, the solution became turbid. It was clarified by centrifugation at 27,000 × *g* for 20 min, and the supernatant was retained. This supernatant was adjusted to neutrality with dilute HCl. The peptide was salted out from the supernatant by using 0.16 to 0.30 ml of saturated $(NH_4)_2SO_4$ per 1 ml of supernatant. The cloudy suspension was centrifuged at 27,000 × *g* for 10 min. The pellet of pilin was saved and suspended in distilled water or buffer. Residual $(NH_4)_2SO_4$ was removed by dialysis against distilled water or buffer.

Protein determinations. The protein determination method of Sutherland et al. (22), which is similar to the method of Lowry et al. (10), was used most often. Because this was an essential technique for this paper, it is given here as we used it. To 0.5 ml of sample was added 5 ml of a solution of 4% $NaCO_3$, 0.02% $CuSO_4 \cdot 5H_2O$, and 0.04% sodium tartrate. This mixture was blended with a Vortex mixer and allowed to react for 45 min at room temperature, 0.5 ml of a threefold dilution of Folin-Ciocalteau phenol solution (Fisher Scientific Co.) was added, and the solution was immediately mixed. The samples were then incubated for 15 min at room temperature and then read either in a Klett-Summerson colorimeter with a red filter or in a Cary model 118 spectrophotometer at 660 nm. Standard curves were obtained with bovine serum albumin for which the concentration had been determined by using a UV extinction coefficient of 5.8 at 280 nm (24).

Two other methods were also used: the Sutherland method, as recommended by Herbert et al. (8), and the biuret method of Gornall et al. (7).

To determine more precisely the concentration of pilus protein by the Sutherland method, the following technique was used. A sample of pilin was prepared as described above. This sample was then dialyzed against 0.1 N NaOH. Portions of the sample were then used to obtain both a UV spectrum and a Sutherland determination, with bovine serum albumin as a standard. Because pilin contains two tyrosine residues and no tryptophan residues (3, 18), the concentration can be determined by using a molar extinction coefficient of 2.33×10^3 for tyrosine (2). By performing a Sutherland determination on this same sample with a bovine serum albumin standard curve, one can calculate a conversion factor for the pilin sample. The average conversion factor which we determined was that when a pilin sample indicated that there was the equivalent of 1 mg of bovine serum albumin present, the actual protein content of the sample was 0.63 ± 0.06 mg of pilus protein, assuming that pilin has a molecular weight of 17,000.

Reducing sugar determination. Reducing sugars were determined exactly as described by Park and Johnson (16). We used only new test tubes to avoid trace sugar contamination which occasionally interferes with this sensitive determination. For maximum detection, samples were hydrolyzed in 4 N HCl for 2 h at reduced pressure. To obtain the data presented in Fig. 2, the samples were heated at various pH values.

UV spectrum shift. Spectra of the samples were determined on a Cary 15 scanning spectrophotometer, using 1-cm path-length quartz cuvettes.

SDS-PAGE. For electrophoresis, the slab gel method of Lugtenberg et al. (11) was used. All reagents were purchased from Bio-Rad Laboratories, Richmond, Calif. Samples were solubilized in a mixture of 1% β-mercaptoethanol (ME), 2% SDS, 3.3% glycerol, and 0.013% bromophenol blue in 0.1 M Tris-hydrochloride buffer at pH 6.8. The samples were heated at 100°C for 5 min in this solution just before being loaded on the gel. Some samples were prepared without ME, and in other experiments 0.1% ME was included in the upper electrode buffer.

Serological studies. Antisera were prepared in New Zealand white rabbits by using subcutaneous inoculations of antigen at 2-week intervals. One week after the last inoculation of antigen, the rabbits were bled. The antigens used to immunize the rabbits were pili (purified as described above), the pilin band cut from an SDS-PAGE gel, and the protein III band cut from an SDS-PAGE gel. The antigens from the gels were homogenized and eluted in normal saline before inoculation. The sera were absorbed with lipoprotein prepared from *E. coli* cell envelope (17) digested with hen egg white lysozyme. The sera were then exposed overnight at 37°C to the antigens in 50-μl disposable micropipettes and examined for precipitates.

RESULTS

Protein determinations. The difficulty in measuring the protein content of common pili was the impetus for this paper. Table 1 lists some of the methods used to prepare the pili for application of the Sutherland (22) version of the Lowry method. The pilus samples which had been heated in acid solution consistently indicated higher protein contents than did those treated by other means. The effect of pH on detection of protein by the Sutherland method was determined more exactly by heating samples at various pH values for 5 min, cooling, adjusting the pH to neutrality, and proceeding with the protein determination (Fig. 1 C). Detection of protein was sharply increased after the pili had been heated at pH values of 2.1 or less. The addition of urea raised the pH at which the

TABLE 1. *Effect of alterations of the Sutherland et al. (22) method for detecting protein*

Sample prepn[a]	Degree of detection[b] (%)
pH 1.8 HCl, 100°C, 5 min	100
pH 1.8 HCl, room temp	4
Water, 100°C, 5 min	2
Water, room temp	2
pH 2.4, 8 M urea, 100°C, 5 min	99
0.1 N NaOH, 100°C, 2 min[c]	20
1.0 N NaOH, 100°C, 20 min	69

[a] Controls which lacked pili were subtracted.

[b] 100% was defined as the protein detected in the sample prepared in HCl (pH 1.8) that was heated at 100°C for 5 min.

[c] Protein determined by the method of Lowry et al. (10).

protein could be detected (Table 1, Fig. 1).

Because the Sutherland method is largely dependent on the presence of aromatic amino acids, we used the biuret method for some protein determinations, since it is considered to be less dependent on specific amino acids. The samples heated for 5 min at pH 7 consistently yielded protein contents lower than those heated for 5 min at pH 1.8. The amount detected in the higher-pH samples averaged 85% of the amount detected at the lower-pH treatment; i.e., for a 17,000-dalton pilin, about 16 amino acids were masked in the high-pH samples.

Reducing sugar determinations. Two methods were used to disrupt pili for the reducing sugar determinations. First, hydrolysis was performed by adding 4 N HCl to a lyophilized sample of pili and heating at 100°C for 2 h under reduced pressure. Using this procedure, we were able to detect 0.87 ± 0.24 reducing sugar as glucose per peptide, based on the Sutherland protein. Second, reducing sugars could be detected by heating the pili samples a low pH for 5 min or less (Fig. 1 B). The coincidence of pH at which the reducing sugar and the pH at which the Sutherland method became an effective method of protein detection should be noted. An average of 1.28 ± 0.41 reducing sugars as glucose per peptide were detected by using the low-pH heating step to expose the sugar. The difference between the two methods for exposing the reducing group would seem to reflect the relative potential of the methods for hydrolyzing the pili. What is surprising was the ease of disruption.

The reducing sugar content was found to remain associated with the peptide fraction purified as described in Materials and Methods. This fraction had been first salted out from solution and then dialyzed against two changes of distilled water. Thus, it would appear that the sugar remained strongly associated with the peptide

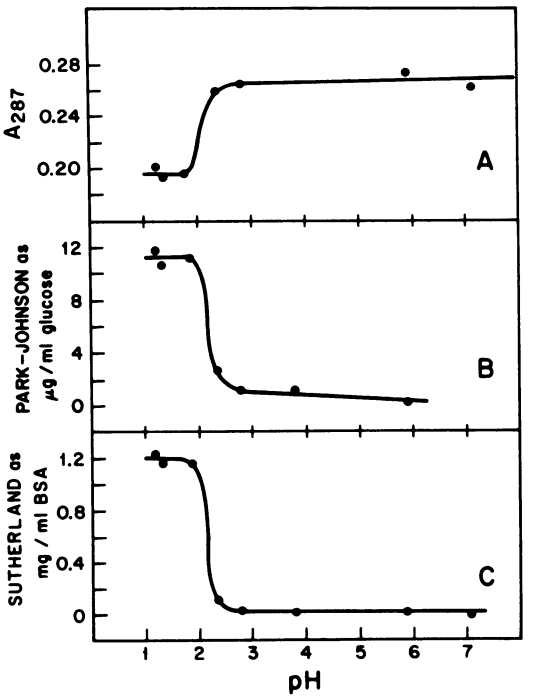

FIG. 1. *Effect of pH on the response of pili to three different procedures. Samples contained 1.16 mg of pili (as bovine serum albumin [BSA]) and were heated at 100°C for 5 min in various concentrations of HCl. (A) Shift in absorption at 287 nm; (B) reducing sugar as measured by the method of Park and Johnson (16); (C) protein detected by the Sutherland method. A_{287}, Absorbance at 287 nm.*

and was not liberated into the solution. In some of the samples of the purified peptide, the sugar appeared to be labile. After a week of storage at 4°C, there was a loss of the reducing sugar as detected by this method. When the Park and Johnson method was applied after the pili had been exposed to 0.1 and 0.3 N NaOH for 16 h at room temperature, an average of 0.32 ± 0.05 sugar per peptide was detected.

UV spectrum shift. The data presented in Fig. 1A follow the decrease in the absorption peak at 287 nm. This agrees with the results obtained by Brinton (3). The change in absorption of samples heated at the various pH values also correlated with the changes that occurred for the protein determinations and the reducing sugar determination.

SDS-PAGE. A peptide derived from the pilus samples used to obtain the data of Fig. 1, which were heated at a pH value below 2, entered the SDS gel (Fig. 2). Solubilization of the pili in SDS required two heating steps for the pilin to enter the gel (Table 2). The first heating step must be done at a pH below 2. The second must

be done in the presence of SDS (2%). Another property of this peptide was that its conformation in SDS was variable, as evidenced from its migration in the gels. For the samples seen in Fig. 2, the method of Lugtenberg (11) was used. On these gels, the pilus samples gave two bands, one migrating with an apparent molecular weight of 17,000 and the other migrating with an apparent molecular weight of 19,500. The larger species appeared to be labile since, after storage of the purified pilin for a week or more, only the 17,000-dalton species was detected. (Note that on these gels the molecular weight marker tobacco mosaic virus protein was especially useful. The relative migration of this protein on the gels remained quite constant under all reducing conditions, and since it has a molecular weight of 17,500, one could clearly observe the differences in the migratory pattern of the pilin under different reducing conditions.) In the Lugtenberg system, ME was included only in the solubilization mixture. When ME was left out of the mixture, the pilin migrated as a single band with an apparent molecular weight of 15,500. On the other hand, when the peptide was freshly prepared and run on a 4% urea gel with ME included in the upper electrode buffer as well as in the solubilization mixture by the method of Dreesman et al. (5), the peptide again gave only one band but with an apparent molecular weight of 19,000. The fact that there were three possible molecular weights indicated that there were at least three conformational or charge states for pilin in SDS solution after heating of the low-pH material. These different states apparently were the result of two different factors: the degree of reduction by ME and the age of the preparation.

The fact that the peptide did possess three

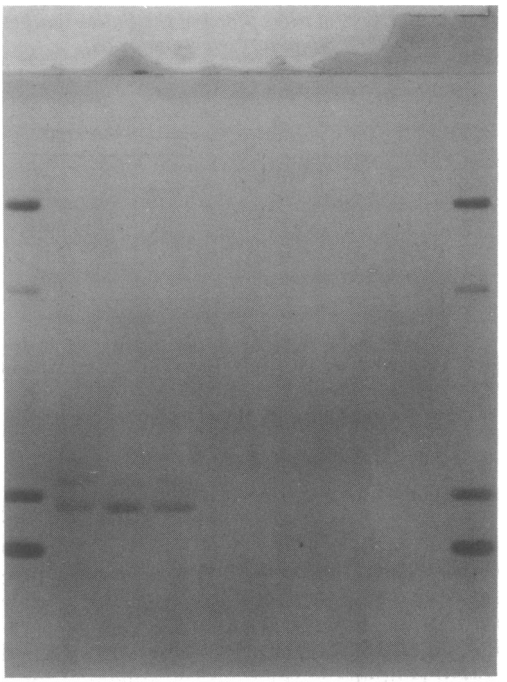

FIG. 2. *Effect of pH on the ability of pili to enter an acrylamide gel during SDS-PAGE. The samples of the pili used to obtain the data of Fig. 1 were solubilized in a solution of 2% SDS, 1% ME, 3.3% glycerol, and 0.013% bromophenol blue in 0.1 M Tris-hydrochloride buffer (pH 6.8). They were then heated at 100°C for 5 min before being loaded on the gel. Wells 1 and 10 contained the molecular weight markers bovine serum albumin, egg albumin, tobacco mosaic virus protein, and hen egg white lysozyme. Wells 2 through 9 in ascending order contained pili initially heated at pH values of 1.2, 1.32, 1.85, 2.35, 2.8, 3.8, 5.9, and 7.1.*

TABLE 2. *Summary of SDS-PAGE migration behavior of the pilus peptide by several solubilization methods*[a]

Deviation	Migration behavior
1. First step was done at pH 7	No bands, did not enter gel
2. First step not done	No bands, did not enter gel
3. Second step not done	No bands, did not enter gel
4. No changes in procedure	Two bands: one at 17,000 and one at 19,000
5. No ME in second step	One band at 15,500
6. ME added to upper electrode buffer	One band at 19,000
7. Stored for 2 weeks at 4°C in neutralized solution, between steps 1 and 2	One band at 17,000
8. Replaced ME with 0.01 M dithiothreitol in step 2	Two bands: one at 17,000 and one at 19,000

[a] This table lists deviations from the following solubilization procedure. First step: Samples of pili pelleted by centrifugation at low speed were suspended in HCl (pH 1.8), heated at 100°C for 2 min, and then allowed to cool. Second step: The samples were neutralized by the addition of a high-pH Tris buffer and then made 1% in SDS and 1% in ME, after which they were heated at 100°C for 5 min. Third step: After the solution cooled, bromophenol blue and glycerol were added to concentrations of 0.013% and 3.3%, respectively. SDS-PAGE was then performed in the normal way, using the Lugtenberg procedure (11).

conformational states upon SDS-PAGE provided us with a novel approach for discerning whether certain protein moieties were identical. As stated earlier, protein III has an apparent molecular weight of 17,000. This molecular weight has also been the accepted value for pilin upon SDS-PAGE. Examination of Fig. 3 shows the migration pattern for pilin and for an outer membrane fraction obtained from the same strain of *E. coli*. The change in the migration pattern observed in the case of pilin peptide in the presence and the absence of ME was not observed to occur for protein III. This provided one indication that these two proteins were different entities. It is interesting that the major outer membrane proteins were relatively insensitive to the presence or absence of ME. (Even the shift in migration rate of protein d can be ascribed to metal binding rather than to reduction [12]). This whole set of proteins appeared to be cystine poor or reduction-proof, or both, but the isolated pilin was very sensitive to the presence of ME.

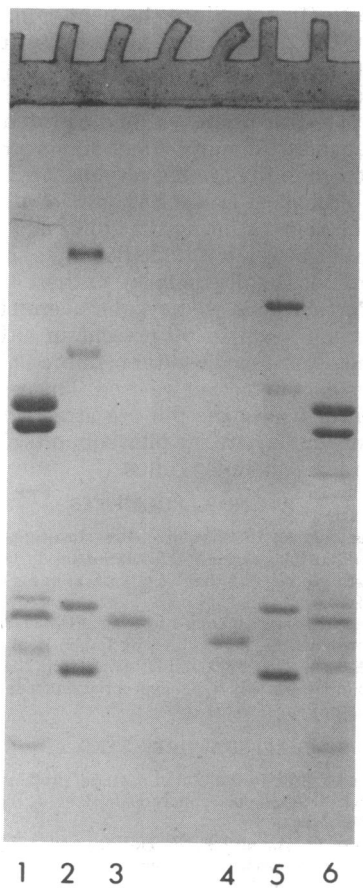

FIG. 3. *Effect of ME, upon SDS-PAGE, on pilus peptide (pilin) and on outer membranes (OM) from F⁻W1-3. The peptide preparation was prepared as described in the text and was stored for 2 weeks at 4°C. The OM preparation was prepared by the method of Osborn et al. (15) and stored at −20°C for 2 months. The solubilization procedure and molecular weight markers (MW) were the same as for Fig. 2, except for presence or absence of ME. Well 1, OM + ME; well 2, MW + ME; well 3, pilin + ME; well 4, pilin − ME; well 5, MW − ME; well 6, OM − ME.*

Serological study. The results of the serological study are presented in Table 3. These data indicate that pilus and protein III have different antigenicities. Another observation was that pilin did not express itself in the inner or outer membranes isolated from *E. coli*.

DISCUSSION

The protein assembly of *E. coli* pili has been observed to be extremely stable and difficult to disrupt into subunits. The method we developed brings about the most complete disruption of these pili. Our results confirm some of Brinton's observations (3), but we have added additional information concerning other changes in the properties of pili treated at low pH.

The data presented in Fig. 1 and 2 document the importance of the low-pH heating step on the change in four properties of these pili. A sharp transition occurred near pH 2. Above this pH, the pili had an absorption peak at 297 nm, appeared to lack a reducing sugar, were not readily detected by the Sutherland method, and did not enter the gel upon SDS-PAGE. Below this pH, the quaternary structure was disrupted, but only after heating. The disruption of the interpeptide bonds permitted the unmasking of the reactive groups involved in the Sutherland

TABLE 3. *Comparison of the antigenicity of pili and protein III by the precipitin test in micropipettes*

Sera[a]	Antigen		
	Inner membrane[b]	Outer membrane[b]	Pili
Normal	0[c]	0	0
Antipilus	0	0	+++
Antipilin from SDS gel	0	0	++
Antiprotein III from SDS gel	++	+++	0

[a] Sera were absorbed twice against *E. coli* lipoprotein.

[b] Inner and outer membranes were prepared by the method of Osborn et al. (15).

[c] 0, No precipitate visible; ++, flocculent precipitate less than 2 mm in length; +++, flocculent precipitate longer than 2 mm.

method and reducing sugar tests. Simultaneously, the spectrum changed from an "aberrant" spectrum to one expected from a protein lacking tryptophan. Also, the peptides became amenable to entering a 10% gel upon SDS-PAGE. Taken together, these data indicate a generalized disruption of the bonds between the pilin subunits.

The tyrosine residues seemed to be particularly affected by the disruption of the pilus. The shift in UV spectrum originally observed by Brinton (3) appeared to be superimposed on the tyrosine spectrum. Since the Sutherland protein determination is largely dependent on the aromatic amino acid content (8), the shift in the Sutherland determination may also reflect an unmasking of this amino acid. The involvement of the pilin tyrosine residues in the stabilization of the pilus suprastructure would appear to be an attractive idea.

The unmasking of a reducing group (presumably a reducing sugar) as determined by the Park and Johnson technique may also offer an insight into the forces stabilizing the pilus. Whether the availability of this sugar was the result of simple unmasking or of hydrolysis of glyco-linkages remains unclear. At present, we believe that the reducing sugars detected upon alkaline hydrolysis formed a portion of those detected by low-pH hydrolysis. The reducing sugars detected by alkaline hydrolysis were probably linked to the peptide via O-linkages, as specified by Spiro (20, 21). The fact that a reducing sugar became available for detection after weak alkaline hydrolysis would also indicate that at least some of the reducing sugars were linked to a threonine, a serine, or possibly a tyrosine residue.

Other groups have studied the sugar content of pili. Brinton and co-workers (personal communication) have observed approximately one hexose residue per peptide, using the phenol-sulfuric acid assay. Salit and Gotschlich (18) examined these pili for amino sugars and came to the conclusion that there were none. We have confirmed this last result. In addition, we also examined the pili for the presence of diaminopimelic acid, using the radioactive labeling method of Osborn et al. (15). We discovered that its presence in preparations of pili was a function of purity. When only two cycles of purification were used, diaminopimelic acid was present. After three cycles, its presence was no longer detectable. Since diaminopimelic acid and amino sugars are both components of the peptidoglycan of *E. coli*, this possible source of carbohydrate in the pilus preparation was eliminated.

The results of SDS-PAGE were complex. There appeared to be three conformational states for the pilus peptide. Depending on the degree of reduction and age of a peptide preparation, the peptide migrated with apparent molecular weights of 15,500, 17,000, or 19,000. If the most reduced form of a peptide is necessary to estimate the molecular weight by the method of Weber and Osborn (23), then the molecular weight of the pilus subunit should be 19,000 rather than the presently accepted value of 17,000. The fact that the preservation of the 19,000-dalton conformational state required the continual presence of ME during SDS-PAGE (by addition of ME to the cathode buffer chamber) indicated that there was a particularly reactive group in the peptide derived from the low-pH treatment. Since the peptide only contains two cystine residues, we have found it difficult to explain the existence of three conformational states. Presently, we postulate the existence of a third group within the protein which would be more difficult to maintain in a reduced condition than the cystine residue.

Our serological study has provided another means of demonstrating that pilin and protein III may not be related. Thus, we have shown by two methods the dissimilarity of these two proteins. Furthermore, we have discovered that pilin does not seem to be present in either the inner membrane or the outer membrane, at least at the level of detection we used. This raises the question of where the pili are attached to the bacterium and where the pilin subunits are processed to form the intact pilus.

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