MEMBRANE MODIFICATIONS IN NUTRITIONALLY INDUCED FILAMENTOUS *ESCHERICHIA COLI* B

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ABSTRACT

Nutritionally induced filamentous cell forms of *Escherichia coli* B were examined for their morphological and biochemical lesions. The filamentous forms showed no significant alteration in total DNA concentration, RNA synthesis, ability to form β-galactosidase in response to isopropylthiogalactoside, or insensitivity to actinomycin D as compared to the normal cell form. The filamentous cells showed a marked decrease in the ability to incorporate *N*-acetylglucosamine-UL-^{14}C into a phenol-soluble glycoprotein fraction relative to the normal cell form or relative to strain E-26 of *E. coli* grown in the filament-inducing medium. The filaments yielded an envelope-specific phenol-soluble protein fraction markedly reduced in or lacking three proteins as determined by acrylamide gel electrophoresis. Amino acid analysis, and chemical and enzymatic treatments of the envelope-specific phenol-soluble proteins showed striking differences between the fractions obtained from normal and filamentous cells. Electron microscope studies of divalent cation-induced aggregates of the envelope proteins showed different aggregation patterns dependent upon the cell form yielding the protein fraction.

INTRODUCTION

The chemical composition of the cell envelope or cell membrane–cell wall complex of gram-negative bacteria has been established in many laboratories (1-3). It is well known that the envelopes of *Escherichia coli* contain peptidoglycan (4), lipopolysaccharide (5), lipid (1), and protein (6). Recently, in our laboratory, glycoproteins have been isolated from the cell envelope of *E. coli* B (7). We have accomplished the fractionation of the various components of the cell envelope by the phenol-extraction procedure described by Westphal et al. (8). By this technique, the lipopolysaccharide is primarily distributed in the aqueous phase, the peptidoglycan is mainly in the interface and in the residue, the phospholipids are in the phenol phase and the residue, and the protein is distributed at the phenol:water interface, in the residue, and in the phenol phase. The protein fraction that is phenol soluble (7) accounts for 35–45% of the total envelope protein, contains glycoprotein, aggregates in the presence of divalent cations, and shows many characteristics similar to those of the membrane fractions isolated from *Mycoplasma laidlawii* (9) and *Micrococcus lysodeikticus* (10). The use of phenol as an agent for preferentially solubilizing proteins and glycoproteins was described

by Pusztai (11) using model systems. Recently, the technique was modified and used successfully for solubilizing erythrocyte membranes (12).

Since phenol appeared to be a useful agent in solubilizing membrane components, we attempted to use it in fractionating the membrane-like structure originally observed in nutritionally induced filamentous *E. coli* B (13). The fragile, nutritionally induced filamentous cell form of *E. coli* B is capable of cell division as shown by serial subculture in inducing medium (3). However, the filamentous forms of *E. coli* induced by treatment with low concentrations of penicillin are not capable of cell division (14). Penicillin appears to exert its antibiotic action by inhibiting cross-wall formation during peptidoglycan synthesis (15). In contrast, the nutritionally induced filamentous cells appear to have lesions involving inhibition of synthesis of a number of surface macromolecules (16). Synthesis of peptidoglycan is inhibited at least 90%, and the additions of glucose and keto-deoxyoctonate to lipopolysaccharide are inhibited more than 50%. These biosynthetic reactions presumably occur at the level of the cell membrane. Since we previously had data suggesting that the filamentous forms had a morphologically and enzymatically altered cell membrane (13), we attempted to determine if the modified cells also had a chemically altered membrane.

In this paper, we wish to report that the nutritionally induced filamentous cell form of *E. coli* B is deficient in envelope-specific glycoproteins. This deficiency manifests itself in an inability of the phenol-soluble proteins from the filaments to aggregate normally in the presence of divalent cations. It is suggested that this membrane-associated abnormality may be involved in the inhibition of peptidoglycan and lipopolysaccharide syntheses in the filamentous cell form. A portion of this work has been reported previously (17).

MATERIALS AND METHODS

Preparation of Cell Fractions

The normal rod-shaped cell form of *E. coli* B was grown in glycerol-minimal salts synthetic medium containing 4 g of glycerol, 500 mg of NaCl, 410 mg of $MgSO_4 \cdot 7H_2O$, 1 g of NH_4Cl, 6 g of Na_2HPO_4, and 3 g of KH_2PO_4 per 1,000 ml. The filamentous cell form was induced by growing *E. coli* B in a medium containing 24 g of nutrient broth, 50 g of casein hydrolysate (enzymatic or acid hydrolyzed), 10 g of L-lysine, and 7.5 g of NaCl per 1,000 ml. The bacteria were harvested in late logarithmic phase, washed, and envelopes were prepared as described previously (3). The cell supernatant, after envelope isolation, was opalescent and was fractionated further into a 100,000 *g* supernatant and a 100,000 *g* pellet. Partially purified cell membranes were prepared by the method of Kaback (18).

Isolation of Glycoproteins

Whole cells, envelopes, partially purified membranes, cell supernatants, and 100,000 *g* supernatants were all subjected to the phenol-extraction procedure of Westphal et al. (8) as described by Okuda and Weinbaum (7). Under these conditions, the lipopolysaccharide would be extracted into the aqueous phase and the peptidoglycan would be in the residue. Most cell protein would be distributed in the interface, the phenol layer, and the residue. This extraction for glycoprotein is not temperature dependent and was done at 4°C. After the combined interface, phenol layer, and residue was reextracted with glass-distilled water at 4°C, solid sodium dodecyl sulfate was added to the clear phenol layer to a final concentration of 0.4% (w/v) and the solution was dialyzed at room temperature against 0.4% sodium dodecyl sulfate in 30% glycerol. This dialysis system maintains a single phase during the removal of the phenol and prevents marked aggregation which occurs in aqueous dialyses. When the material was maximally solubilized and free of phenol, it was dialyzed against glass-distilled water, and, after dialysis, the remaining insoluble material was removed by centrifugation at 12,500 *g*.

Studies of Glycoprotein Biosynthesis

N-acetylglucosamine-^{14}C (GlcNAc-^{14}C)[1] was prepared from glucosamine-UL-^{14}C (International Chemical and Nuclear Co.) and acetic anhydride by a modification of the procedure of Roseman and Ludowieg (19). The GlcNAc-^{14}C was at least 96% pure as determined by paper chromatography in butanol:acetic acid:water (66:10:27). The specific activity was 11.7 mCi/mmole.

Dobrogosz (20) has shown that GlcNAc could be rapidly incorporated into cell polymers. The GlcNAc-^{14}C was added to log-phase cells (88.4 mμCi/ml culture). The cells were allowed to grow for various periods of time at 37°C. Total incorporation of isotope

[1] Abbreviations used are: GlcNAc-^{14}C, *N*-acetylglucosamine-^{14}C; $GlcNH_2$, glucosamine; IPTG, isopropyl-thiogalactoside; PTA, sodium phosphotungstate; UrAc, uranyl acetate; AmMo, ammonium molybdate; TCA, trichloroacetic acid; ONP, orthonitrophenol; KDO, keto-deoxyoctonate; SDS, sodium dodecyl sulfate. PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)] benzene.

into acid-insoluble products was measured by collecting aliquots of cell suspensions on a Millipore filter (HA, 0.45 μ), washing each three times with cold 5% TCA (10 ml), and counting the dried filter in a Packard liquid-scintillation spectrometer. The scintillation solution used was composed of 150 ml of toluene, 1.5 g of PPO, and 75 mg of POPOP. The remaining cells were extracted with phenol and dialyzed as described above, in order to determine GlcNAc-^{14}C incorporation into the phenol-soluble protein. $\frac{1}{10}$ ml of aliquots of the aqueous sample was measured by liquid scintillation using a solution composed of 208 g of naphthalene, 13 g of PPO, 130 mg of POPOP, 1 liter of xylene, 1 liter of dioxane, and 600 ml of ethanol.

Electrophoretic Techniques

Acrylamide gel electrophoresis was carried out under a few conditions. The first condition involved Tris-glycine buffer pH 8.6 with 7.5% acrylamide according to the method of Ornstein (21). The second condition involved the same buffer system, but the gels were prepared in 8 M urea (6). The third electrophoretic condition involved acetic acid and urea (22).

Analytical Methods

Protein was determined by the method of Lowry et al. (23). For total carbohydrate, the anthrone procedure (24) was used, and for measurement of reducing sugars after hydrolysis with 1 N HCl for 5 hr on a steam bath, the method of Park and Johnson (25) was used. Phospholipid phosphorus was analyzed by the method of Shibuya et al. (26). Heptose was determined by the method of Osborn (27), and keto-deoxyoctonate was measured by the technique of Weissbach and Hurwitz (28). RNA was determined by the method of Hurlbert et al. (29), and DNA by the method of Burton (30). Lipid extractions were done by the method of Bligh and Dyer (31) as described by Ames (32). Amino acid analyses were performed on a Beckmann Model 120 amino acid analyzer. The data are presented as the average of duplicate 24- and 48-hr hydrolysates. The data are extrapolated to zero time to correct for the destruction of certain amino acids.

Uridine-2-^{14}C (specific activity 1.45 μCi/mg) was obtained from New England Nuclear Corp., Boston, Mass. (See legend of Fig. 8).

Electron Microscopic Techniques

THIN SECTIONING: Bacteria in log-phase growth, in either synthetic (normal) or enriched (filament-inducing) medium, were fixed by adding sufficient phosphate-buffered glutaraldehyde to the culture to give a final concentration of 6% glutaraldehyde and 0.1 M PO_4^{-3}, pH 7. After overnight fixation at 4°C, the cells were washed in PO_4^{-3} buffer, postfixed in 1% OsO_4 (0.1 M PO_4^{-3}, pH 7), then suspended in 1% agar (33), dehydrated in ethyl alcohol, and embedded in Araldite.

Glycoprotein fractions, isolated from normal and filamentous bacteria by the phenol procedure given above, were aggregated at room temperature in 0.01 M $MgSO_4$, 0.001 M PO_4^{-3} buffer, pH 7. After 15 min, the aggregates were fixed in a 2.5% glutaraldehyde solution containing 0.01 M $MgSO_4$, 0.001 M PO_4^{-3} buffer, pH 7, for 1 hr. Following a brief rinse in 0.01 M $MgSO_4$, the aggregates were postfixed in 1% OsO_4, 0.01 M $MgSO_4$, 0.001 M PO_4^{-3} buffer, pH 7, for 1 hr, dehydrated in alcohol, and embedded in Araldite.

All thin sections were stained sequentially with alcoholic uranyl acetate and basic lead citrate (34).

NEGATIVE STAINING: Bacterial cell envelopes from both rod and filamentous bacterial forms were negatively stained with either 1% neutral sodium phosphotungstate (PTA), 1% unbuffered uranyl acetate (UrAc), or 1% neutral ammonium molybdate (AmMo). All negatively stained material was examined on lightly carbon-coated celloidin membranes placed over 300-mesh copper grids.

Glycoproteins, isolated from both normal and filamentous bacteria, were aggregated in 0.01 M $MgSO_4$ (pH 7) and then negatively stained with neutral 1% AmMo. Negative staining of the glycoproteins with either UrAc or PTA proved to be unsatisfactory. In the former case, the aggregates formed large dense clumps, while in the latter case the aggregates were not uniformly stained or fully penetrated. Clearly, AmMo provided the most satisfactory negative staining for this material.

Examination of all preparations was carried out on an AEI EM6B electron microscope. All magnifications were calibrated with a crossed replica grating supplied by Ernst Fullam, Inc., Schenectady, New York.

RESULTS

Physiological Response of E. coli B to Filament-Inducing Medium

We had previously described the aberrant cell structure of *E. coli* B when the organism is grown in inducing medium (3). A representative bacterial cell grown in synthetic medium is presented in Fig. 1. This bacterium illustrates the characteristic morphology of *E. coli* as reported by previous authors (35–38). It is a short rod-shaped cell, approximately 1–2 μ long and 0.5 μ wide, which contains a cell envelope composed of the two trilaminar layers typical of gram-negative organisms (1). The cytoplasm contains densely stained and tightly packed material rich in ribonucleoprotein which surrounds and intermingles with the finely

dispersed nucleoplasm network or nucleoid (39). Under our usual growth conditions in the glycerol minimal salts medium, intracellular membranes or mesosomes are not observed in *E. coli* B, although they have been observed in *E. coli* under other growth conditions (40, 41).

In contrast to the homogeneous population of rod-shaped cells observed in synthetic medium, *E. coli* B develop dramatic alterations in morphology when grown in the enriched medium (Figs. 2–5). Most prominent is the giant filamentous shape seen in Fig. 2. Some bacteria are more than 50 μ long and 1.5–2 μ in diameter. The volume of these cells can be 100 times greater than that of the typical rod-shaped bacterium grown in synthetic medium. Both Figs. 1 and 2 are printed at identical magnifications in order that the size differential can be readily appreciated.

Another feature of the filamentous cell forms is the sparseness of septae within the cells, as reported earlier (13). These bacteria contain a normal ratio of DNA to protein (see below) and thus can apparently synthesize DNA in the usual amounts, but the process by which cells separate after DNA replication is impaired. The filamentous forms are often angulated or bent, and small bulbous protrusions can be seen emerging from the lateral cell surfaces (Fig. 2). In general, these surface irregularities occur at 1.5–2 μ intervals, suggesting that these are regions along the cell where abortive septal formation may have been initiated but prematurely inhibited.

Small vesicles, which are surrounded by a unit membrane, are often attached to the outer surface of the cell wall of bacteria grown in the enriched media (Figs. 2 and 4). The nature of these vesicles is obscure, but they are reminiscent of the endotoxin-containing vesicles in *E. coli* and other gram-negative bacteria (42).

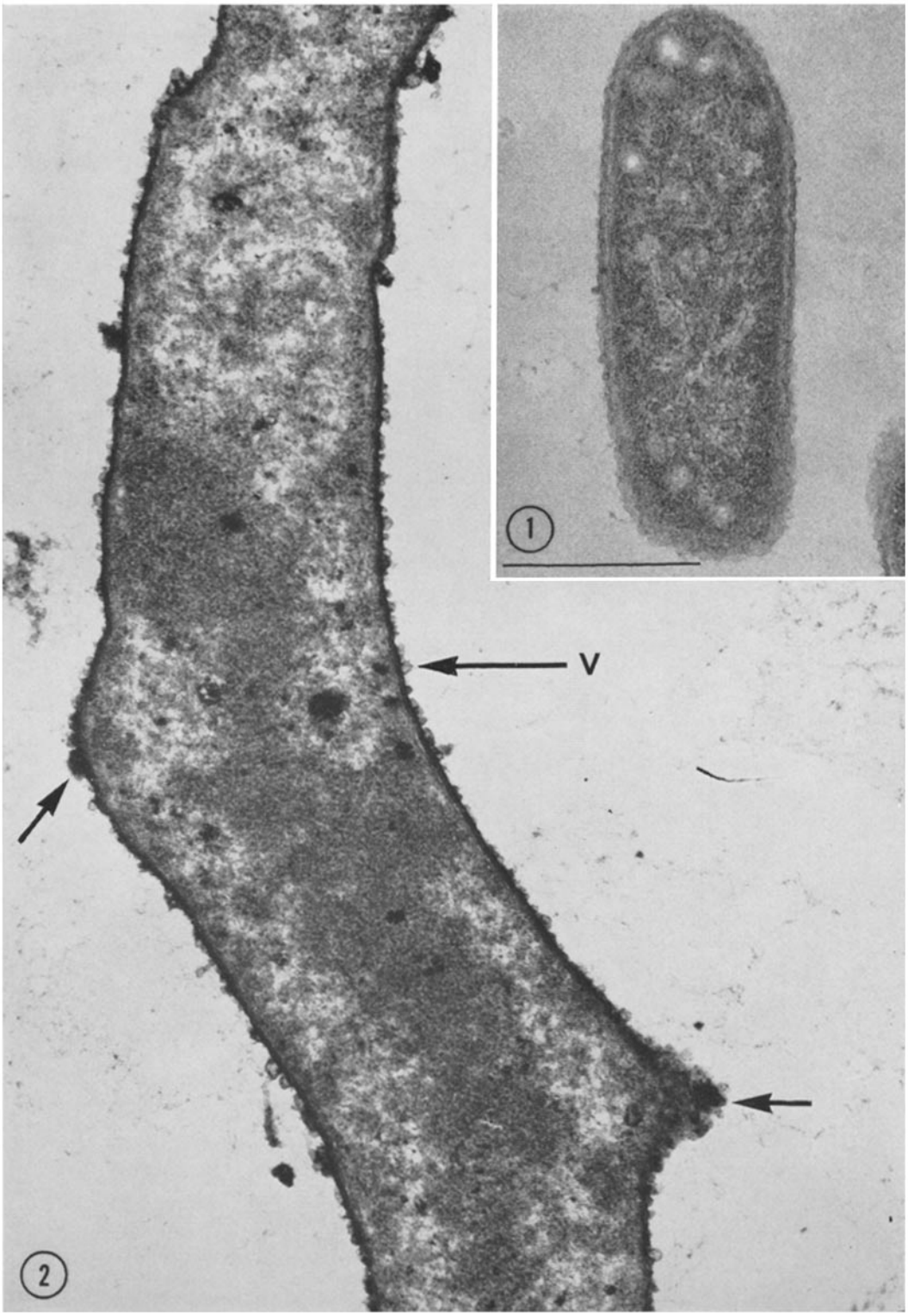
Additional evidence suggesting that cell envelope differentiation is abnormal in the enriched medium is shown in Fig. 3. It is not uncommon to observe, in such cultures, dividing cells in which septae have formed, but in which there has been an unequal partition of the cytoplasmic contents between the daughter cells. These septae are unusually thick and often branched. Besides the filamentous cell forms and abnormal division figures, many bacteria are branched and present a Y-shaped appearance (Fig. 4). When the cell envelope of the filamentous forms is examined at higher magnification (Fig. 5), we have frequently observed multiple trilaminar layers immediately subjacent to the cell membrane (see arrows, Fig. 5). Tentatively, we assume that such trilaminar structures are modified cell membranes which have piled up beneath the cell surface (see Discussion). The dimensions of these trilaminar layers are identical with those of the plasma membrane observed in normal *E. coli* grown on synthetic media, but our biochemical evidence (see below and reference 13) suggests that these membranes are functionally defective.

Cell envelopes have been isolated from both normal and filamentous forms and examined by negative-staining techniques (Figs. 6 and 7). Both of these micrographs are printed at the same magnification, again illustrating the dramatic discrepancy in cell size under the two growth conditions. Besides highlighting this over-all size differential, however, negative staining of the isolated envelopes has not yet provided substantial insight into the structural lesions of the cell surface in the filamentous forms.

The basic biochemical abnormalities are associated with lipid synthesis (43), peptidoglycan and lipopolysaccharide synthesis (16), and distribution of membrane-specific enzymes (13). In order to be certain that the filamentous cells contained their normal amount of nucleic acids and proteins, the normal and filamentous *E. coli* B were fractionated by the method of Schmidt-Thannhauser (44), and the results are given in Table I. These data show that the filamentous cell form has a normal

FIGURE 1 An electron micrograph of a thin-sectioned *E. coli* B cell grown in the synthetic (normal) medium. Note the characteristic rod-shaped appearance of this bacterium. × 60,000; calibration bar = 0.5 μ.

FIGURE 2 Another electron micrograph of a thin-sectioned *E. coli* B cell grown in the enriched (filament-inducing) medium. Note the angulated shape of the cell and the protrusions from the lateral surfaces (arrows). In addition, numerous small vesicles (*V*) appear to be blebbing from the outer surface of the cell wall. × 60,000; calibration bar as in Fig. 1.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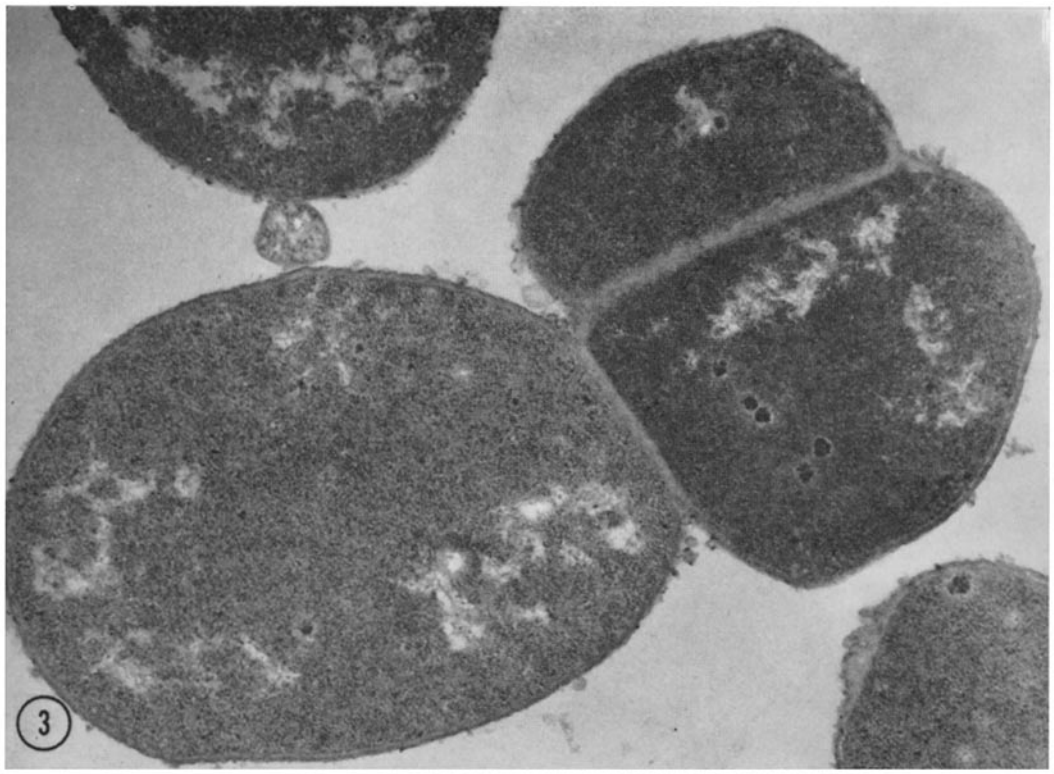
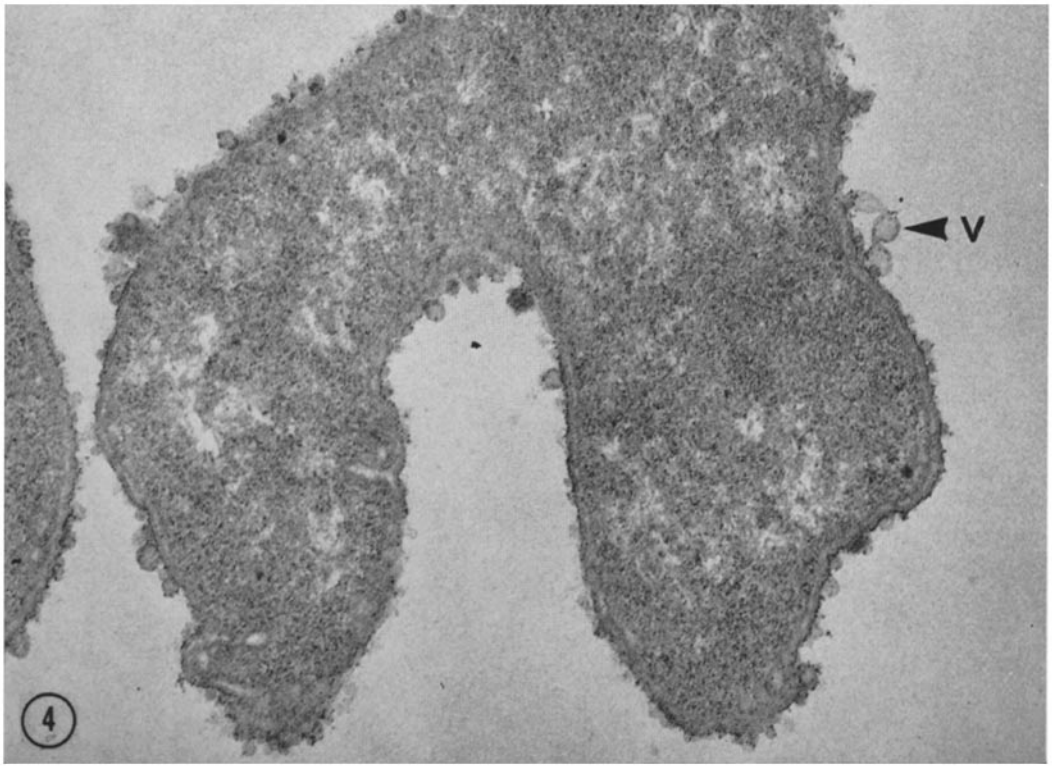




FIGURES 3 and 4 Additional bacterial configurations assumed in the enriched medium. Fig. 3 illustrates the aberrant septal formation during cell division, with the unequal partition of cytoplasmic components between daughter cells. In Fig. 4, a branched or Y-shaped form is seen. Again, note the prominent vesiculation (*V*) at the cell surface. × 60,000; calibration bar as in Fig. 1.

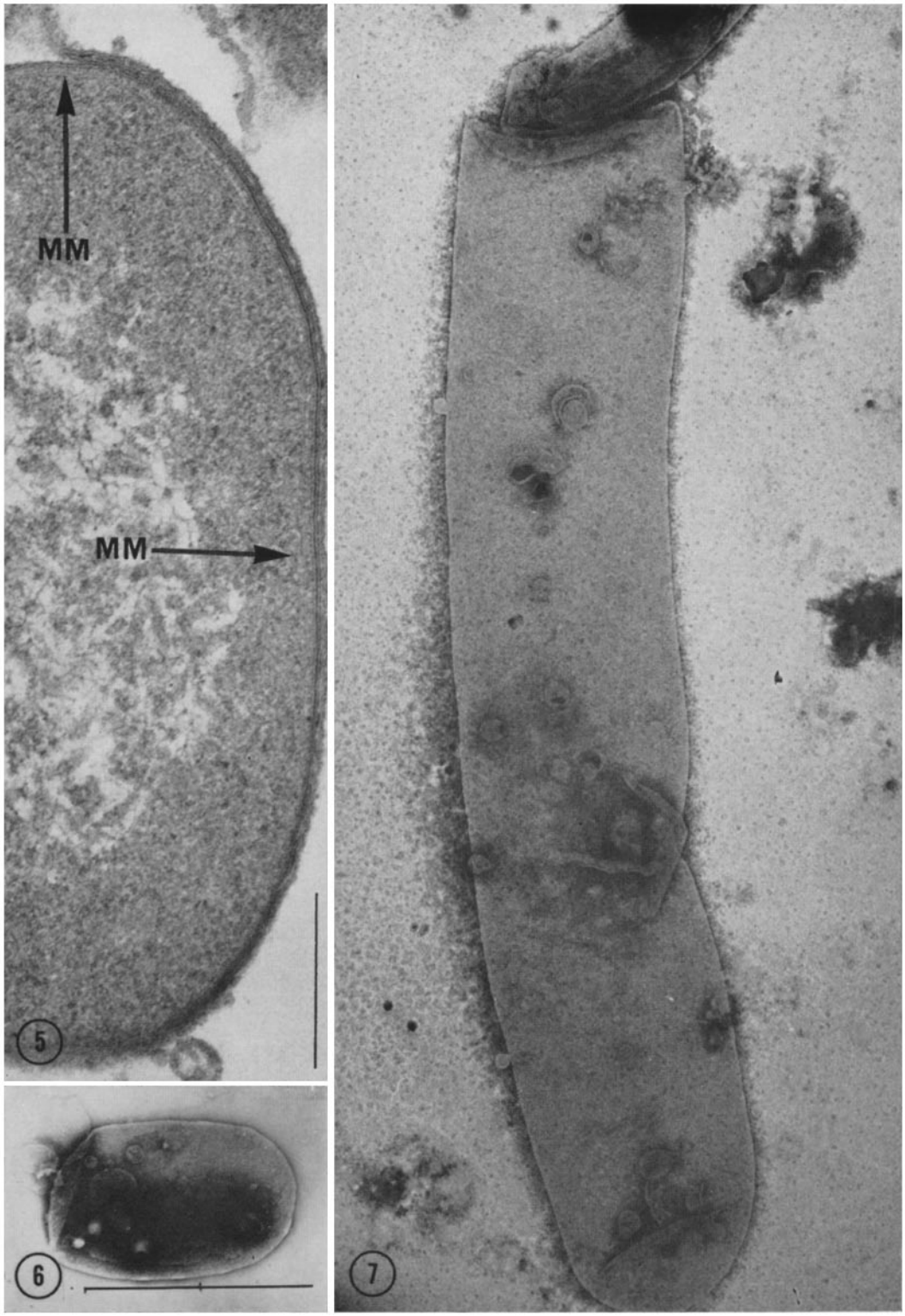


FIGURE 5 A cross-sectioned filamentous bacterium grown in the enriched medium. The multiple membrane-like lamellae (*MM*) beneath the cell envelope can be seen. × 90,000; calibration bar = 0.3 μ.

FIGURES 6 and 7 Two electron micrographs of PTA negatively stained cell envelopes isolated from normal (Fig. 6) and filamentous (Fig. 7) bacteria. The obvious difference in envelope size is apparent. × 35,000; calibration bar = 1 μ.

TABLE I

RNA, DNA, and Protein Contents of Escherichia coli B

Ratios*	Normal form	Filamentous form
RNA/Protein	0.230	0.321
DNA/Protein	0.046	0.047
RNA/DNA	5.020	6.860

* RNA, DNA, and protein concentrations were all calculated as mg per mg dry cells.

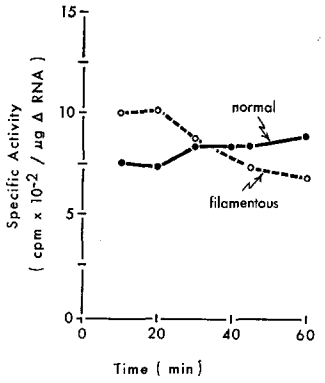


FIGURE 8 RNA synthesis in normal and filamentous *E. coli* B. ½ ml of uridine-2-^{14}C (1 µCi/ml) was added to 10 ml of culture in log phase. Aliquots were removed at the times noted, and radioactivity in the trichloroacetic acid fraction was measured.

ratio of DNA to protein, but an elevated RNA content, as would be expected of cells growing in rich medium (45).

The synthesis of RNA in vivo was determined by uridine-2-^{14}C incorporation, and the data are given in Fig. 8. As can be seen, the specific activities of the nucleoside incorporation into RNA in *E. coli* B growing as short rods or filaments are identical. Therefore, the RNA synthesis of the filamentous cell form appears to be normal. Since the filaments are extremely fragile (3), it was of interest to determine if their permeability toward actinomycin D was altered. It is well known that *E. coli* is normally insensitive to this drug. When actinomycin D was added to normal or filamentous *E. coli* B, there was no diminution in RNA synthesis. Therefore, the filaments maintained their barrier to actinomycin D.

To determine if the nutritionally induced filamentous cell form was capable of utilizing its protein-synthesizing system effectively, we examined the ability of these cells to form β-galactosidase. The data are presented in Table II.

TABLE II

Induction of β-Galactosidase with IPTG

Induction period	Δ ONP formed/Δ Protein increase	
	Normal cells	Filamentous cells
min	*mµmoles/µg*	*mµmoles/µg*
0	—	—
5	81.5	15.1
10	117.0	24.9
20	118.0	43.2
30	134.0	118.0
40	154.0	130.0

The table shows that the filamentous cell form is capable of taking in the inducer, IPTG, forming messenger RNA, and utilizing ribosomes to make functional enzyme. The kinetics of induction are slightly slower, but reach the same specific activity by 40 min of induction.

It appears, then, that the general cell machinery of the filamentous cell form is operating and that the lesions of importance center around abnormalities of the cell membrane. Therefore, we investigated the proteins of the cell surface of normal and filamentous *E. coli* B.

Yield of Membrane Proteins

Partially purified membranes (18) were prepared from normal and filamentous cells. This membrane fraction accounted for 15–25% of the total protein of the normal cells. On the other hand, this membrane fraction accounted for 35–40% of the total protein of the filamentous cell form. These data are in agreement with the morphological observation of multiple membranes in the filamentous cell (Fig. 5). Approximately 50–70% of both types of membranes were phenol soluble. Therefore, the phenol-soluble fraction accounted for about 15% of the total cell protein from normal cells and about 25% of the total cell protein of the filamentous cell form. These results were essentially the same, whether the extraction was done on whole cells, envelopes, or partially purified membranes.

Incorporation of N-Acetylglucosamine-^{14}C

When GlcNAc-^{14}C was administered to a log-phase culture of normal or nutritionally induced filamentous *E. coli* B, it was readily incorporated

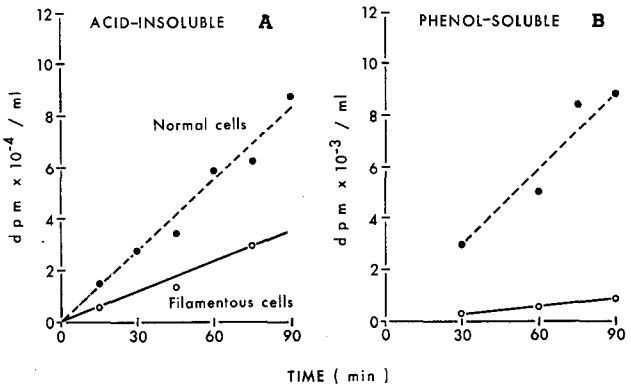


FIGURE 9 Incorporation of *N*-acetylglucosamine-UL-^{14}C into acid-insoluble (*A*) and phenol-soluble (*B*) products of normal and nutritionally induced filamentous *E. coli* B.

into the cells. The distribution of the radioactivity in acid-insoluble or phenol-soluble products is seen in Fig. 9 *A* and *B*. It is well known that GlcNAc is a major component of two surface macromolecules in *E. coli:* the peptidoglycan (4) and the lipid portion of the lipopolysaccharide (46). The acid-insoluble products would include these polymers as well as other components derived from metabolic products of GlcNAc. The phenol-soluble fraction includes proteins and phospholipids (7). The protein fraction contains covalently linked GlcNAc as well as other sugars (7). At 90 min after GlcNAc-^{14}C incorporation, the specific activity of GlcNAc in the phenol-soluble fraction (Fig. 9 *B*) is 1.62×10^5 dpm/mg protein in the normal cells and 2.01×10^4 dpm/mg protein in the filamentous cells, showing almost 90% inhibition of glycoprotein synthesis in the filamentous cells. In the normal cells, the total amount of carbohydrate and reducing sugar measured after acid hydrolysis is approximately 2–4% of the protein. This fraction shows similarities to the recent observation of carbohydrates associated with proteins in the solubilized membranes of *Mycoplasma laidlawii* (9). Since the incorporation of GlcNAc-^{14}C into the phenol-soluble fraction of the filamentous *E. coli* B was inhibited 90% (Fig. 9 *B*), it was necessary to determine if this inhibition was due to the rich inducing medium diluting the incorporation or if it was due to physiological aberration occurring during filamentous formation. This question was answered in two ways. First, a second strain of *E. coli*, strain E-26, was grown in glycerol-minimal salts medium and in the filament-inducing medium. The *E. coli* E-26 grows normally in the rich medium and does not form filamentous cells (3). In the filament-inducing medium, it incorporates GlcNAc-^{14}C into phenol-soluble protein at least five times better than *E. coli* B grown in the same medium. In synthetic medium, both strains incorporate GlcNAc-^{14}C into phenol-soluble protein at the same rate. Second, a mutant (C_1) of *E. coli* B was isolated which cannot utilize either GlcNAc or $GlcNH_2$ as a sole source of carbon owing to a lesion in glucosamine-6-phosphate deaminase.[2] This C_1 mutant still forms filamentous cells in the rich medium. Since GlcNAc-^{14}C cannot be randomly metabolized by C_1, it is a good organism in which to check the direct incorporation of GlcNAc-^{14}C into the phenol-soluble fraction. In the C_1 mutant of *E. coli* B in synthetic medium, at least 10% of the total GlcNAc-^{14}C incorporated into the cell is found in the phenol-soluble glycoprotein fraction. In the rich medium, less than 1% is incorporated into this fraction. Therefore, these data suggest that there is a lesion in envelope-specific glycoprotein synthesis in the nutritionally induced filamentous cell form of *E. coli* B. For determining the specificity of the lesion, it was necessary to characterize the envelope proteins electrophoretically, chemically, and electron microscopically.

Electrophoretic Analysis of the Phenol-Soluble Glycoprotein Fraction

Since 35–45% of the envelope protein was soluble in phenol, it was desirable to determine whether in this fraction there were marked differences between the normal and filamentous cells. As was observed in the previous section, $GlcNH_2$-^{14}C incorporation into the phenol-soluble fraction was markedly inhibited in the filamentous cells.

[2] Pressman and Weinbaum, manuscript in preparation.

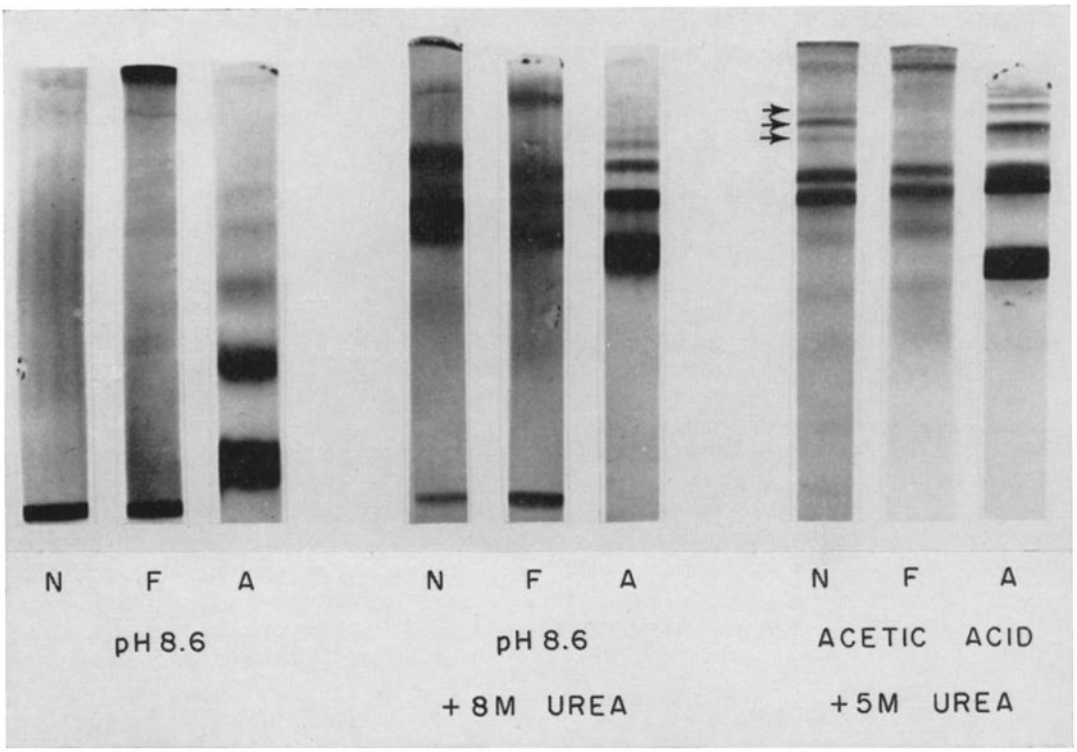


FIGURE 10 Acrylamide gel electrophoresis of phenol-soluble proteins from normal and filamentous *E. coli* B. *N*, normal cell fraction; *F*, filamentous cell fraction; *A*, bovine serum albumin standard. These fractions were all dialyzed against sodium dodecyl sulfate and water before electrophoresis. Electrophoresis in acetic acid + 5 M urea shows the presence of three protein bands in the normal cell fraction (arrows) which are lacking or markedly reduced in the filamentous cell fraction.

When the phenol was dialyzed away against 0.4% SDS in 30% aqueous glycerol, the protein remained soluble when the excess SDS was removed by dialysis against water. This aqueous solution obtained from normal cells contained protein, phospholipid, and carbohydrate in a ratio of approximately 65:33:2, and aggregated completely in the presence of divalent cations as described previously (7). This fraction obtained from filamentous cells had a ratio of 74:25:0.5 and, therefore, had less phospholipid and less carbohydrate associated with the protein.

That the phospholipid is associated with the protein by weak interactions and not covalently linked can be easily demonstrated. If the whole cells or envelopes were first extracted by the method of Bligh and Dyer (31) to remove essentially all the phospholipids, subsequent phenol extraction gave the same glycoprotein fraction with no phospholipids. Such a preparation had electrophoretic characteristics identical with those described below for the fraction containing phospholipid. The filamentous cell form yielded a lipid-free fraction which still showed a very low carbohydrate:protein ratio (less than 1%), suggesting that the lipid binding did not determine the solubility of this fraction in phenol.

The soluble protein fractions after dialysis against water were subjected to acrylamide gel electrophoresis under various conditions, and the patterns are seen in Fig. 10. At pH 8.6 in 7.5% gels, both the normal and filamentous cell fractions run as single sharp bands and move close to the front ahead of serum albumin. When 8 M urea is added to the gels, partial disaggregation takes place. The disaggregation is more complete in the normal cell sample than in the filamentous cell sample, and the patterns appear to be different between the two cell types. When the electrophoresis is performed in acetic acid plus 5 M urea,

a marked difference is observed between the normal and filamentous envelope proteins. Three of the slow moving proteins appear to be absent or significantly reduced in concentration in the filamentous cell form. An identical difference in patterns is observed if the phospholipids are removed prior to phenol extraction. Also, the patterns are identical if 8 M urea replaces SDS as the disaggregation agent during removal of phenol by dialysis. It is apparent that neither phospholipid association nor SDS binding plays a role in the protein banding patterns observed.

When the C_1 mutant of *E. coli* B is used to specifically incorporate $GlcNH_2$-^{14}C into the phenol fraction, aggregation with 0.02 M Mg^{++} or Ca^{++} precipitates 98–100% of the radioactivity, protein, phospholipid, and carbohydrate. If the precipitate is solubilized by EDTA or phenol and analyzed by gel electrophoresis in acetic acid plus 5 M urea, the same pattern is obtained as with the nonaggregated material. If the gels containing the radioactive material are sliced into 1 mm discs and counted in a scintillation counter, a portion of the radioactivity from $GlcNH_2$-^{14}C is associated with the three slow bands (see arrows, Fig. 10) of the normal cell fraction. These data suggest that there are at least three glycoproteins and that the filamentous cells are strongly inhibited in their ability to synthesize these envelope-specific glycoproteins. This inhibition of glycoprotein synthesis is essentially complete. It is the most striking finding we have made in the filamentous cells, even more complete than the observed inhibition of peptidoglycan synthesis (16), though the two may be related as will be described in the Discussion. The low specific activity of the proteins from the filamentous cell form (less than 10% of the normal) precludes identification of the proteins with which the small amount of carbohydrate is associated. Radioautographic analysis of the acetic acid urea gel from the normal cell fraction showed that at least one (the lower) of the two dense protein bands was radioactive and that there may be some isotope associated with the slower moving dense band. We are presently determining if this slow band is a glycoprotein or if C_1 allows a small but significant amount of radioactive glucosamine to be randomly metabolized to amino acids.

To be assured that the medium inducing the filamentous cell form of *E. coli* B did not cause an artifact, we examined the glycoprotein fraction of *E. coli* E-26. This strain does not form filaments and grows as a normal cell form in the inducing medium. Electrophoresis of strain E-26 glycoproteins in acetic acid–urea gels showed identical protein patterns, independent of the growth medium (i.e., synthetic or complex). Therefore, it appeared that only the filamentous cell form was lacking certain membrane-protein components.

Further Differences in the Glycoprotein Fractions from Normal and Nutritionally Induced Filamentous E. coli B

Amino acid analyses were performed on the whole envelopes and glycoprotein fractions from the normal and filamentous *E. coli* B. The average data of duplicate samples are presented in Table III. The total amino acid compositions of envelopes isolated from normal and filamentous cell forms of *E. coli* B are very similar. The glycoprotein fractions were also analyzed for their total amino acid compositions, even though the fractions are mixtures of a number of proteins. The glycoprotein fraction from the normal cell form appeared to be enriched in aspartic acid, tyrosine, and perhaps lysine when compared to normal envelopes. The glycoprotein fraction from the filamentous cell form was also enriched in tyrosine and lysine, but it lacked the marked increase in aspartic acid. These differences may become even more significant as the individual proteins of this fraction are purified and further characterized.

Additional chemical and enzymatic treatments showed more differences between glycoprotein fractions from the normal and filamentous cell forms. These studies are described in Table IV. The carbohydrate portion of the glycoproteins was released in 1 N HCl, but not in dilute acid or alkali. The clearest difference is the apparent resistance to pronase of the glycoprotein fraction from the filamentous cell form. The normal fraction released 70% of the radioactive *N*-acetylglucosamine, while the filamentous fraction released only 11%. This release was independent of the length of time of enzyme digestion (overnight vs. 6 days), daily addition of fresh enzyme, or addition of papain after pronase digestion.

Differences in Glycoprotein Fractions as Observed by Electron Microscopy

Glycoproteins, isolated from both normal and filamentous forms, have been aggregated in 0.01 M Mg^{++} and examined by electron microscopy with

TABLE III

Amino Acid Analyses of E. coli B Envelopes and Glycoproteins

Amino acid	Normal cells Envelope*	Normal cells Glycoprotein‡	Filamentous cells Envelope*	Filamentous cells Glycoprotein‡
Aspartic acid	7.8	10.5	7.3	7.7
Threonine	2.5	2.9	2.6	2.6
Serine	2.0	2.5	2.4	1.6
Glutamic acid	6.6	6.5	7.1	6.1
Proline	1.5	1.7	1.8	2.1
Glycine	3.1	3.9	3.1	4.6
Alanine	4.3	4.0	4.8	4.6
Valine	3.7	3.7	4.2	4.6
½ Cystine	–0–	–0–	–0–	–0–
Methionine	1.4	1.4	1.5	1.7
Isoleucine	2.7	2.6	3.1	3.0
Leucine	4.9	4.8	5.2	5.5
Tyrosine	3.5	5.2	2.8	4.7
Phenylalanine	3.1	3.1	2.7	3.0
Lysine	3.5	4.3	3.8	5.4
Histidine	1.1	0.7	1.2	1.1
Arginine	4.0	3.7	4.4	4.9

* g/100 g envelopes.
‡ g/100 g glycoprotein.

TABLE IV

*Treatment of N-Acetylglucosamine-UL-^{14}C-Labeled Glycoprotein Fraction with Various Agents**

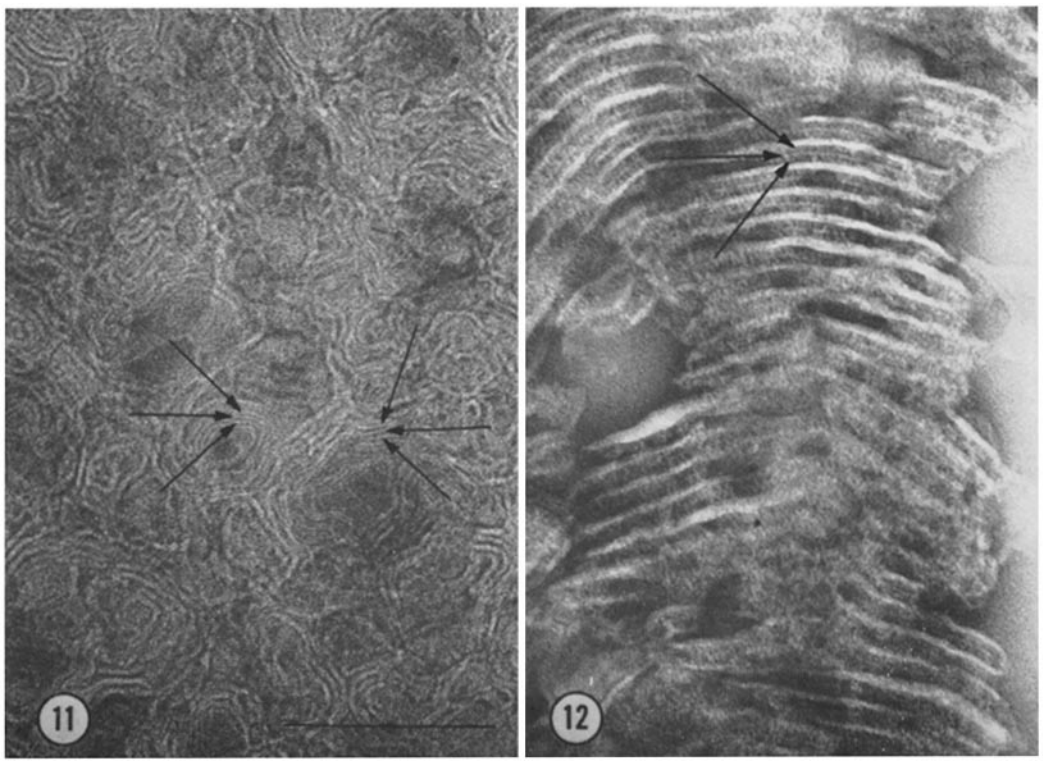
Treatment	Radioactivity solubilized in 5% TCA: Normal	Radioactivity solubilized in 5% TCA: Filamentous
	%	%
NaOH, 0.5 N at 22°, 30 min	6	10
HCl, 1.0 N at 100°, 5 hr	61	49
H_2SO_4, 0.05 N at 80°, 1 hr	10	10
Pronase (1 mg/ml), 37°		
Overnight	70	11
2 days	66	8
6 days with fresh addition	65	0
6 days, then Papain	65	0

* In all treatments, 20 μg of radioactive protein was incubated under the various conditions in a total volume 100 μl. At the end of each incubation, 120 μg of cold carrier glycoprotein was added and the reaction was terminated by addition of 1 ml of cold 5% TCA. The specific activity of the normal glycoprotein was 1.62×10^5 dpm/mg protein, and that of the filamentous material was 2.01×10^4 dpm/mg protein.

both negative-staining and thin-sectioning methods. Figs. 11 and 12 are examples of the aggregates formed by glycoprotein from the normal (Fig. 11) and abnormal (Fig. 12) cells and examined by AmMo negative staining. Both have been printed at identical magnifications. The characteristic appearance of the glycoprotein aggregates from normal *E. coli* is a series of concentric whorls reminiscent of disorganized myelin figures. The distance between the thinnest layers of these whorls (see arrows, Fig. 11) is 35–40 A, although a great deal of variability between concentric layers is apparent. In contrast, the glycoprotein aggregates from the filamentous cells form a series of stacked plates which in Fig. 12 are viewed end-on. The distance between the majority of broad plates is approximately 190–200 A. Between these broad plates a smaller period (see arrows, Fig. 12) can be seen; the space between the smaller layers is 35–40 A. The fine structure of the Mg^{++}-induced aggregates is better seen in fixed, embedded, and thin-sectioned material (Figs. 13 and 14). In both glycoprotein preparations, the aggregates possess a clear myelin-like configuration, the major difference being the larger size of the aggregates and the presence of a more prominent wide period in the glycoprotein isolated from the abnormal cell forms. In both preparations, the distance between the fine lamellae is 35–40 A. The wide period seen most clearly in the abnormal glycoprotein measures 90–95 A. This larger period is also observed in the normal glycoprotein preparation, but much less frequently than in the abnormal material.

DISCUSSION

The data presented in this paper describe the morphological and chemical aberrations present in the cell envelopes of nutritionally induced filamentous forms of *Escherichia coli* B. The original observations of Fischman and Weinbaum (13) of multiple membrane-like lamellae in the filamentous *E. coli* B showed that these envelopes were altered in the distribution of certain membrane-associated enzymes and suggested that such a condition might induce an abnormal physiological state in the bacterial cell. The evidence obtained from thin-sectioned whole bacteria supports this hypothesis that cell envelope biosynthesis or assembly is altered from the normal when *E. coli* B are grown in the enriched (filament-inducing) medium. The main support is the following: (*a*) enormously increased cell size by defective cell



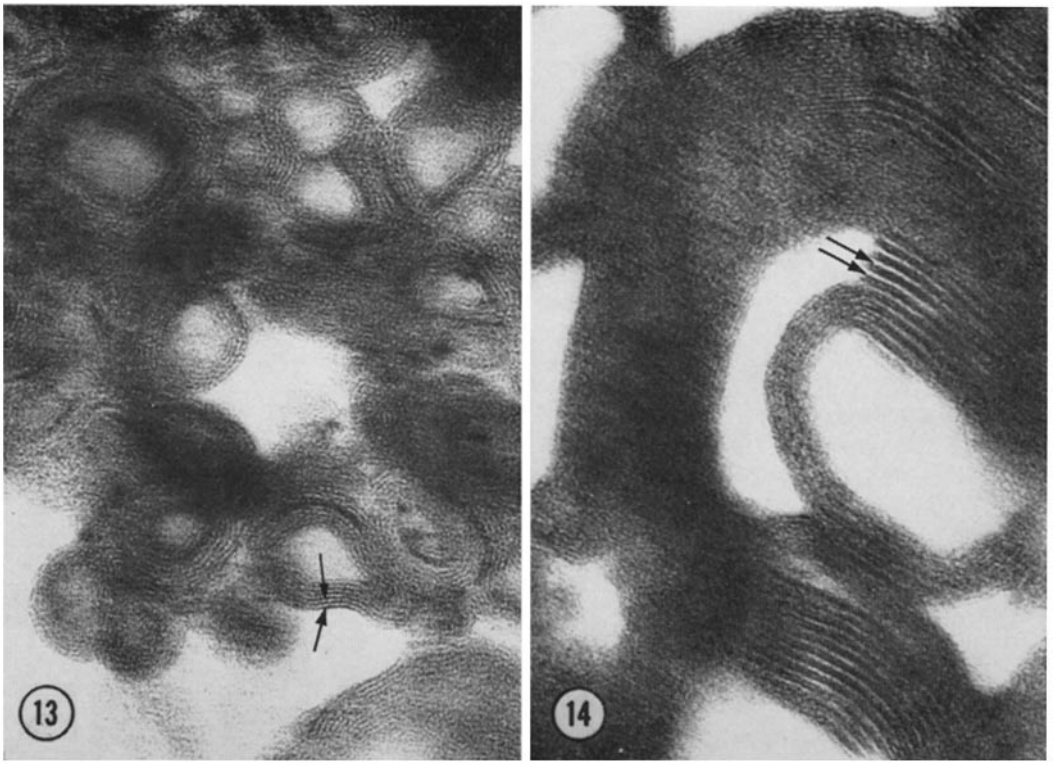
FIGURES 11 and 12 Electron micrographs of negatively stained (AmMo), divalent cation-induced, glycoprotein aggregates. × 160,000; calibration bar = 0.2 μ. Fig. 11 is the glycoprotein from normal cells. The spacing* between the finest lamellae (arrows) is 35–40 A. Fig. 12 is the glycoprotein from filamentous bacteria. The prominent major spacing is 190–200 A, while the small period (arrows) between this major period is 35–40 A.

* In all cases, the spacings presented in Figs. 11–14 refer to the center-to-center distances between layers and not the measured thickness of white or dark lines in the micrographs.

partition; (*b*) the presence of angulated and branched bacteria; (*c*) the presence of multiple membrane-like lamellae beneath the cell envelope; and (*d*) the occurrence of many membrane-enclosed vesicles protruding from the cell surface (Figs. 1–7).

In order to be certain that the multiple membranous structures behaved like membranes, partially purified membranes were prepared from normal and filamentous *E. coli* B by the method of Kaback (18). The filamentous cells yielded more membrane than the normal cells as calculated on a dry weight or starting protein basis, and at least 50% of the protein is phenol soluble. Therefore, the filamentous cell form yields more phenol-soluble protein than the normal cell form.

The phenol-soluble or glycoprotein fractions of normal and filamentous cells have been compared; filamentous cells are markedly inhibited in their ability to incorporate *N*-acetyl-glucosamine-^{14}C into this fraction (Fig. 9 *B*). These data are substantiated by use of a glucosamine-6-phosphate deaminase negative mutant of *E. coli* B. In this organism, the labeled glucosamine cannot be randomly metabolized and can only be incorporated into glucosamine-containing polymers. This mutant showed about 90% inhibition of glycoprotein synthesis in the filamentous condition, similar to the wild-type parent *E. coli* B. The small amount of glycoprotein that is made in the filamentous cells is appreciably different from normal cell glycoprotein in its solubilization by pronase



FIGURES 13 and 14 Electron micrographs of thin-sectioned, divalent cation-induced, glycoprotein aggregates from normal and filamentous bacteria. × 160,000; calibration bar as in Fig. 11. In Fig. 13, the glycoprotein aggregates from normal *E. coli* B are shown. The spacing between the fine lamellae (arrows) is 35–40 A. Glycoprotein from filamentous bacteria are presented in Fig. 14. The minor period measures 35–40 A, while the distance between major bands (arrows) is 90–95 A.

(Table IV). Another modification of the phenol-soluble fraction from filamentous bacteria relative to the fraction from normal cells is the lack of increased aspartic acid content in the filaments (Table III). Since there is evidence that aspartic acid or asparagine is involved in the linkage of glucosamine to other similar types of glycoproteins (7), this lack of aspartic acid seems to parallel the lack of glycoprotein synthesis. The causal relationship between these two observations is not established, but merely suggested.

The general macromolecular syntheses in the filamentous cell form appear to be quite normal (Tables I and II; Fig. 8). Indeed, the cell adapts to IPTG by forming β-galactosidase and the cell adapts to its rich medium environment by inducibly forming large quantities of lysine decarboxylase (47).

In contrast, all those reactions studied which involve membrane-directed syntheses showed a marked inhibition in the filaments as compared to the normal cell. For example, cyclopropane fatty acid synthesis is inhibited 50–70% (43), peptidoglycan synthesis is inhibited approximately 90% (16), and additions of glucose and KDO to lipopolysaccharide are inhibited more than 50% (16). Inhibition of peptidoglycan and lipopolysaccharide syntheses may be directly related to a lack of glycoprotein synthesis in the filamentous cell form. Our present hypothesis is that there are multiple glycoproteins each of which is the anchor or point of initiation or attachment for the synthesis of various amino sugar-containing surface polymers of *E. coli* B. The isolation of a glucosamine-requiring mutant would be of inestimable use in this regard.

When the glycoproteins are isolated from either normal or abnormal bacteria and aggregated in

the presence of Mg^{++}, they form prominent myelin-like figures (Figs. 13 and 14). The size of the aggregates is much greater in the preparation isolated from the filamentous bacteria, but the substructure of the aggregates seems basically similar in both samples. This differential in aggregate size and shape cannot be accounted for by differences in glycoprotein concentration during aggregation for, in all cases, the protein concentrations were equalized before Mg^{++} addition. Whether this difference in aggregation pattern is due to the altered protein composition (Fig. 10 and Table III) in the phenol-soluble fraction from the abnormal bacteria or to a difference in phospholipid concentration is difficult to establish. The protein:phospholipid ratio in the glycoprotein fraction differs in normal vs. filamentous cells (65:33 vs. 74:25). A number of workers (48–52) have demonstrated that purified phospholipid preparations can form patterns of aggregation similar to those we have observed with the phenol-soluble material. To differentiate between the phospholipid and protein effects, we have prepared phospholipid-free glycoprotein fractions from both normal and filamentous cells. These phospholipid-free fractions do aggregate in the presence of divalent cations, but preliminary electron microscopic evidence suggests that these aggregates differ in morphology from aggregates which contain phospholipid. No Mg^{++}-induced aggregation occurs in pronase-treated preparations. Apparently, protein is essential for aggregation to occur, but phospholipid has an important influence on the final morphology of the aggregates.

The electrophoretic patterns observed in Fig. 10 suggest that certain envelope proteins are lacking or markedly reduced in concentration in the filamentous cell form. Since the differences observed are identical whether we use samples prepared by dialysis against sodium dodecyl sulfate, dialysis against 8 M urea, or electrophoresis of the phenol fraction directly, we suggest that the differences observed are not due to differential binding of SDS. A number of strains of *E. coli* have been phenol extracted, and the electrophoretic pattern of the extracts looks remarkably similar to the ones observed for *E. coli* B. On the other hand, other gram-negative organisms (i.e. *Salmonella*) give quite different patterns. Since the phenol-soluble fraction from the filamentous *E. coli* B still aggregates in the presence of divalent cations, the proteins in this fraction have the required binding protein essential for induction of myelin-like figures (Figs. 12 and 14). Whether one of the two heavy bands observed is the organizer of aggregation is still unresolved.

Insufficient evidence is at hand to suggest a model for the assembly of the glycoprotein monomers into these membrane-like structures. However, additional high-resolution electron microscope studies of dilute, nonaggregated preparations might shed some light on the shape of the individual molecules and provide some insight into the mechanism of this salt-induced polymerization of the glycoprotein. It may well be that this in vitro aggregation system will provide a model system for establishing the mechanism of membrane coalescence in vivo. Perhaps this system will also be useful in characterizing the protein:divalent cation interaction, and aid in determining if there is a single divalent cation-binding protein.

In summary, the data presented suggest that the nutritionally induced filamentous cell form of *E. coli* B is markedly altered in its ability to synthesize certain membrane-specific proteins. This alteration manifests itself in unusual aggregation patterns in the presence of divalent cations. The lack of these proteins may aid in explaining the severe inhibition in the synthesis of certain macromolecules observed in the filamentous cell form. Finally, these data do not suggest the mechanism of induction of the aberrant growth state by the environment, nor do they elucidate the sequence of events in the organism culminating in the filamentous cell form. These questions will require additional examination of the system.

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