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# Outer Membrane Proteins of *Escherichia coli*

## III. Evidence that the Major Protein of *Escherichia coli* O111 Outer Membrane Consists of Four Distinct Polypeptide Species

CARL A. SCHNAITMAN

*Department of Microbiology, The University of Virginia, School of Medicine, Charlottesville, Virginia 22901*

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Previous studies have shown that the outer membrane of *Escherichia coli* O111 gives a single, major, 42,000-dalton protein peak when analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis at neutral pH. Further studies have shown that this peak consists of more than a single polypeptide species, and on alkaline SDS-gel electrophoresis this single peak is resolved into three subcomponents designated as proteins 1, 2, and 3. By chromatography of solubilized outer membrane protein on diethylaminoethyl-cellulose followed by chromatography on Sephadex G-200 in the presence of SDS, it was possible to separate the 42,000-dalton major protein into four distinct protein fractions. Comparison of cyanogen bromide peptides derived from these fractions indicated that they represented at least four distinct polypeptide species. Two of these proteins migrated as proteins 1 and 2 on alkaline gels. The other two proteins migrated as protein 3 on alkaline gels and cannot be separated by SDS-polyacrylamide gel electrophoresis. In purified form, these major proteins do not contain bound lipopolysaccharide, phospholipid, or phosphate. These proteins may contain a small amount of carbohydrate, as evidenced by the labeling of these proteins by glucosamine, and to a lesser extent by glucose, under conditions where the metabolism of these sugars to amino acids and lipids is blocked. All of the proteins were labeled to the same extent by these sugars. Thus, it was concluded that there are at least four distinct polypeptide species with apparent molecular masses of about 42,000 daltons in the outer membrane of *E. coli* O111.

The analysis of the polypeptide composition of the outer membrane of *Escherichia coli* is complicated by the fact that this membrane contains major polypeptides that exhibit unusual properties when examined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Unlike many proteins which have been studied by this technique, the migration of these major polypeptides in SDS-gels is determined both by the conditions of electrophoresis and by the manner in which the proteins are solublized. This has led to considerable confusion in the literature concerning the actual number of major polypeptide species and the molecular masses of these polypeptides (1, 4, 7, 9, 18).

Figure 1 illustrates the different SDS-polyacrylamide gel patterns of the major polypeptides of the outer membrane of *E. coli* O111 that are obtained under different conditions. This figure also summarizes the nomenclature of the various protein bands as described previously (12, 13). The effect of heating of the protein samples in SDS solution prior to electrophoresis is shown in the first two gels in Fig. 1. If the sample of outer membrane is simply solubilized in an SDS solution containing a reducing agent at a temperature of less than 50 C, as in the procedure of Weber and Osborn (16), two major bands designated as A and C in Fig. 1 are observed. If, however, the protein sample is then dialyzed against an SDS-urea solution and boiled briefly (12), these major bands shift to an intermediate position (band B, Fig. 1) with an apparent molecular mass of about 42,000 daltons (12). The protein in bands A and C can also be separated by Sephadex gel filtration in the presence of SDS. Measurement of the intrinsic viscosity of these proteins after separation, and before and after boiling in SDS solution, has demonstrated that the differences between bands A, B, and C are due to the state of aggregation and the conformation of the proteins (12).

Bragg and Hou (1) have observed a similar effect of boiling in SDS, but also observed a very pronounced effect on the pH of the electrophoresis buffers on the gel pattern of the boiled protein. When boiled outer membrane protein from *E. coli* O111 is analyzed in the electrophoresis system of Bragg and Hou, which employs an alkaline (pH 11.4) upper buffer and an acid (pH 4.1) lower buffer instead of the pH 7.2 buffers of the Maizel (6) gel system, the third gel pattern shown in Fig. 1 is obtained. The protein of band B separates into three sharp bands, designated as bands 1, 2, and 3 in Fig. 1. These changes appear to be related to charge density rather than conformation (13). Preliminary evidence has indicated that these are different polypeptide species (13).

In this paper, the techniques utilized in the purification of the colicin E receptor protein from the outer membrane (8) have been combined with analysis of protein fractions by the Bragg-Hou gel system in an attempt to isolate and purify several major polypeptides from the outer membrane of *E. coli* O111.

## MATERIALS AND METHODS

**Strains and culture conditions.** With the exception of sugar-labeling experiments, all studies were carried out with *E. coli* J-5, a galactose epimeraseless derivative of *E. coli* $O111_{B4}$ (17). Glucosamine- and glucose-labeling studies were carried out with strain J-515, which was derived from strain J-5 by mutagenesis with ethyl methane sulfonate followed by penicillin selection. Strain J-515 is presumed to be defective in glucosamine deaminase. It does not require glucosamine for growth, but cannot utilize either glucosamine or *N*-acetyl glucosamine as a carbon source. Except where noted, cultures were grown on minimal medium (10) containing succinate as the carbon source. Inoculum cultures contained 1.5 mM D-fucose to induce the *gal* operon and 20 μg of L-leucine per ml; final cultures contained 1 mM D-galactose, 20 μg of L-leucine per ml, and 150 μCi of L[$^3$H]leucine per liter. This yielded a labeling specific activity of $1.5 \times 10^6$ counts of $^3$H per mg of protein. All cultures were harvested in late log phase, when 60% of the maximal culture density was attained. The time of harvesting was important, since if cultures were allowed to enter the stationary phase the amount of outer membrane protein which could be solubilized with Triton X-100 plus ethylenediaminetetraacetic acid (EDTA) decreased considerably, from 60 to less than 20% of the total outer membrane protein.

**Isolation of outer membrane protein.** Cells were broken and the envelope fraction was obtained as described previously (17). To remove the cytoplasmic membrane, I extracted the envelope fraction twice, at a protein concentration of about 10 mg/ml, with 2% Triton X-100 in 10 mM *N*-2-hydroxyethylpiperazine-piperazine-*N*′-2′-ethanesulfonic acid (HEPES) buffer (pH 7.4). Each extraction was for 10 min at 23 C, followed by centrifugation in the cold at 200,000 × *g* for 45 min. The Triton-insoluble fraction of the envelope (outer membrane plus murein) was then extracted twice with the same HEPES buffer containing 2% Triton X-100 and 5 mM EDTA. These extractions were carried out for 10 min at 23 C, followed by centrifugation at 23 C at 200,000 × *g* for 45 min (8, 11). This resulted in the solublization of about 60% of the outer membrane protein. Two volumes of ice-cold 95% ethanol were added with stirring to the pooled, Triton-EDTA-solubilized outer membrane protein (8), and this was allowed to stand overnight at −20 C followed by centrifugation at 20,000 × *g* to sediment the precipitated protein. The yield from 50 liters of culture was 500 mg of ethanol-precipitated protein.

**Chromatography on DEAE-cellulose and Sephadex G-200.** Diethylaminoethyl (DEAE)-cellulose chromatography was carried out, as described previously (8), on Whatman DE-52 equilibrated with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (pH 7.2) containing 5 mM EDTA and 2% Triton X-100 (Tris-Triton buffer). All columns were run at room temperature. The ethanol-precipitated outer membrane protein was dissolved with gentle homogenization in Tris-Triton buffer at a final concentration of 20 mg of protein/ml, and applied to the DE-52 columns without further treatment. Elution was carried out with NaCl gradients in Tris-Triton buffer. Samples from column fractions were analyzed by gel electrophoresis after precipitation of the protein by the addition of 2 volumes of cold 95% ethanol, as described above. In cases where the protein concentration was less than 0.5 mg/ml, 1 mg of calf serum protein per ml was added as carrier to insure complete precipitation. Pooled fractions from columns were concentrated to about 1 mg of protein per ml with an Amicon pressure filtration apparatus containing a PM-30 filter, and the concentrated sample was then precipitated with ethanol as described above.

Sephadex G-200 chromatography was carried out in 0.1 M sodium phosphate buffer containing 0.1% SDS,

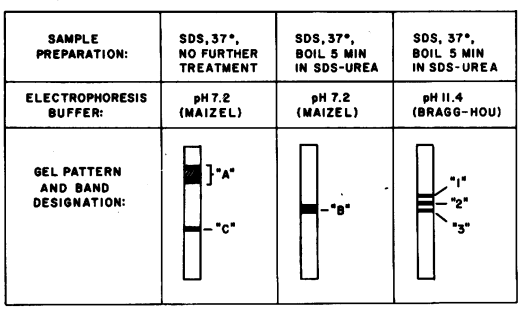


FIG. 1. *Schematic illustration of the major bands observed under various conditions when outer membrane protein of E. coli O111 is analyzed by SDS-polyacrylamide gel electrophoresis. The conditions and nomenclature of the bands are as described previously (12, 13).*

0.5 mM EDTA, and a small amount of azide to prevent bacterial growth. Ethanol-precipitated fractions from DEAE-cellulose columns were dissolved at a protein concentration of about 15 mg/ml in 0.066 M sodium phosphate buffer (pH 7.2) containing 3% SDS, 5 mM EDTA, and 0.1% 2-mercaptoethanol. Solubilization was carried out at 37 C. Peak fractions were pooled and concentrated to about 10 mg of protein per ml with an Amicon apparatus containing a PM-10 filter. The concentrated sample was made 1% SDS and 0.1% 2-mercaptoethanol and boiled for 10 min. The sample was then chromatographed again on the same Sephadex G-200 column. Final samples were concentrated to 1 mg of protein per ml and stored frozen.

**Cyanogen bromide cleavage.** Final samples from SDS-Sephadex columns were reduced fully and carboxymethylated. Reduction was accomplished by the addition of 50 mM Cleland reagent followed by incubation at 37 C for 30 min. Samples were carboxymethylated by the addition of excess iodoacetic acid followed by incubation for 30 min at 37 C. During this step, the pH was maintained at 8.0 by the addition of 2 M Tris base. The samples were then dialyzed overnight against 0.1 M phosphate buffer (pH 7.2) containing 0.1%, followed by dialysis for 24 h against the same buffer diluted 10-fold (to reduce the amount of SDS and phosphate in the sample). The samples were then lyophilized and dissolved to the same volume in 70% formic acid. An excess of cyanogen bromide (about 4 mg/ml) was added, and the samples were allowed to stand for 24 h at room temperature in the dark. The samples were diluted with 10 volumes of water and lyophilized. The dried samples were dissolved in a small amount of water and lyophilized again to remove all formic acid. It should be noted that care must be taken to prevent thawing during any of the lyophilization steps, since this results is an insoluble protein precipitate. The samples were then dissolved in the solubilizing solution described by Swank and Munkres (15), and electrophoresis in highly cross-linked gels was carried out as described previously (13).

**Polyacrylamide gel electrophoresis.** Particulate protein samples (membrane fragments, ethanol-precipitated protein) were solubilized in SDS solubilizing solution (0.066 M sodium phosphate buffer [pH 7.2] containing 2% SDS, 5 mM EDTA, and 0.1% 2-mercaptoethanol) at 37 C, followed by dialysis against 8 M urea-SDS solution and boiling as described by Schnaitman (reference 12, method III). In both the Maizel (5) and Bragg-Hou (1) gel systems, the gels contained 7.5% acrylamide, 0.2% bis-acrylamide, and 0.5 M urea, and were prepared in 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS. In the Maizel gel system, the upper and lower buffers consisted of 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% SDS. In the Bragg-Hou gel system, the upper buffer consisted of 0.1 M sodium phosphate buffer (pH 11.4), and the lower buffer consisted of 0.1 M sodium phosphate buffer (pH 4.1), with both buffers containing 0.1% SDS. With the Bragg-Hou gel system, the upper buffer was allowed to stand on the top of the gel for at least 1 h prior to application of the sample. Electrophoresis on gels (5 by 100 mm) was carried out for 6 to 8 h at 5 mA/gel. The Swank-Munkres (15) gel system for cyanogen bromide peptides contained 12.4% acrylamide, 1.25% bis-acrylamide, and 8 M urea-phosphate-SDS buffer. Gels were stained or sliced and counted as previously described (12, 13).

## RESULTS

**Nomenclature of protein fractions.** The general scheme illustrated in Fig. 1 and described in the introduction has been used throughout and is based on the system used in two preceding papers (12, 13). The designations A, B, and C refer to large, intermediate, and small proteins, respectively, from either Maizel gels or SDS-Sephadex G-200 columns. B always refers to protein with an apparent molecular mass of 42,000 daltons obtained after boiling in SDS, whereas A and C refer to proteins dissolved in SDS without boiling. The numbers 1, 2, and 3 refer to the three major bands observed when protein B is analyzed on the alkaline Bargg-Hou gel system. The designations 3a and 3b refer to the two fractions of protein 3 that are eluted from DEAE-cellulose columns at low and high salt, respectively.

**Preliminary fractionation of solubilized outer membrane protein with DEAE-cellulose.** It has been shown previously (11) that extraction of the outer membrane fraction of the *E. coli* envelope with Triton X-100 plus EDTA solubilized about half (or slightly more than half) of the outer membrane protein, and that both the solubilized and insoluble fractions contained similar amounts of the 42,000-dalton major protein (B). It was also shown that protein B from the solubilized and insoluble fractions had the same polypeptides (13), both as determined by the analysis of this protein in the alkaline Bragg-Hou gel system and as determined by comparison of the cyanogen bromide peptide patterns. Thus, as far as the major proteins are concerned, the Triton-EDTA-solubilized protein is representative of the bulk of the outer membrane protein and can be used as a starting material in the isolation and purification of the major polypeptides.

When solubilized protein in Tris-Triton buffer was applied to a DEAE-cellulose column in the same buffer and eluted with a series of NaCl gradients, the results shown in Fig. 2 were obtained. The first protein eluted was a breakthrough peak (fractions 12–25) eluted with the first column volume of buffer. The breakthrough peak did not contain any of the major

polypeptides and consisted primarily of a mixture of high-molecular-weight polypeptides plus the lipopolysaccharide and phospholipid present in the preparation. This was followed by three peaks (I, II, and III, Fig. 2) that consisted primarily of the major polypeptides. A Bragg-Hou gel was run on protein from every third fraction, and these were used to determine which fractions were to be pooled. The gel scans shown in the top part of Fig. 2 are Bragg-Hou gels of the sample that was applied to the column and of the pooled samples from peaks I, II, and III. When the salt gradient was terminated, more than 95% of the protein applied to the column had been eluted.

The first peak (peak I) contained proteins 2 and 3 plus some minor proteins, and protein 1 was entirely absent from this peak. From the gels on individual fractions, it was determined that peak I contained about 60% of the total amount of protein 2 present in the initial sample and about half of protein 3. Protein 1 did not elute from the column until the beginning of the first salt gradient, and about 70% of protein 1 was eluted in peak II. There was some protein 2 and protein 3 eluted at the beginning of the first salt gradient, but this decreased and the final fractions from the first salt gradient contained almost no protein 2 or protein 3. As the scan illustrates, there was a considerable enrichment of protein 1 in peak II. When the second salt gradient was started, protein 3 again began to be eluted from the column, and peak III consisted almost entirely of protein 3. About 40% of the total protein 3 was eluted in peak III. Thus, there was clearly a bimodal elution of protein 3, with part of it coming off the column without salt and the remainder coming off at high salt. Since this suggested that there might be two species of protein 3, the protein 3 eluted without salt (designated as protein 3a) and the protein 3 eluted at high salt concentration (designated as protein 3b) were each purified further.

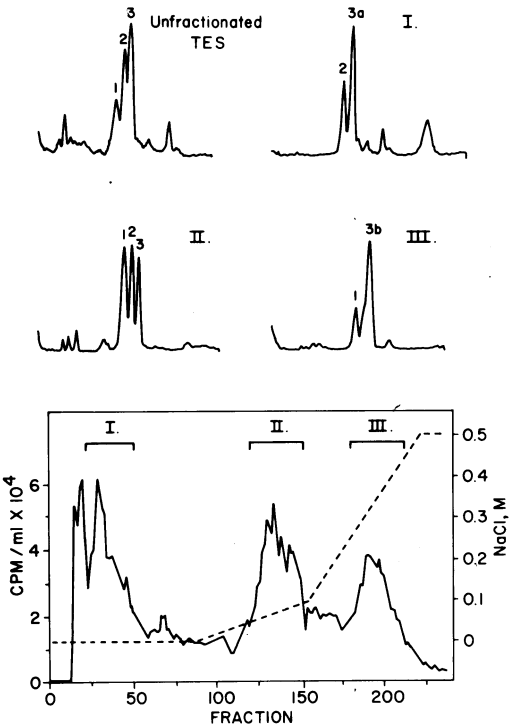


FIG. 2. *Elution of outer membrane protein from DEAE column I. Sample solution (100 ml) containing 596 mg of ethanol-precipitated, Triton-EDTA-solubilized outer membrane protein in Tris-Triton buffer was applied to a 2.5-cm column containing 400 ml of Whatman DE-52 DEAE-cellulose equilibrated with Tris-Triton buffer. Elution was with 1,200 ml of Tris-Triton buffer followed by 1,000 ml each of the two salt gradients (indicated by the dashed line) in Tris-Triton buffer. Fractions (5 ml) were collected. The brackets indicate the pooled fractions I, II, and III. The gel scans at the top of the figure show the appearance of the unfractionated sample and fractions I, II, and III on Bragg-Hou gels.*

**Further purification of proteins 2 and 3a.** The further purification of these proteins was simplified by the fact that peak I from the DEAE-cellulose column contained both of these proteins but did not contain protein 1. The pooled sample from peak I was precipitated with ethanol and dissolved in SDS solution at 37 C. When this material was electrophoresed in the Maizel gel system, there was no band B, but very heavy A and C bands. When this sample dissolved in SDS solution at 37 C was chromatographed on an SDS-Sephadex G-200 column, two major peaks were observed, as indicated by the solid line in Fig. 3. Peak A from this column was analyzed on Bragg-Hou gels after boiling in SDS, and was found to consist entirely of protein 2 plus some large minor peptides. Peak C consisted entirely of protein 3a plus some small minor peptides. The final purification of proteins 2 and 3a consisted of boiling the protein from peaks A and C in SDS solution and rechromatographing these protein samples on the same SDS-Sephadex G-200 column. When protein from peak A was boiled and rechromatographed, it eluted as indicated by the dashed line in Fig. 3. Peak B was now free from the minor polypeptide contaminants and consisted almost entirely of protein 2. When protein from peak C was boiled and

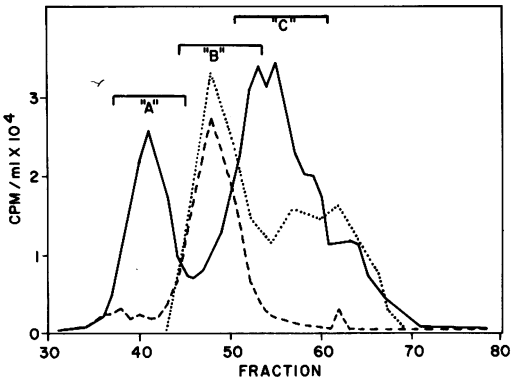


FIG. 3. *Chromatography of peak I from DEAE column I on Sephadex G-200 in the presence of SDS. Pooled fractions from peak I (see Fig. 2) were precipitated with ethanol, and the precipitate was dissolved at 37 C at a concentration of 10 mg of protein per ml in SDS solubilizing solution. This was applied to a column (2.5 by 100 cm) of Sephadex G-200 equilibrated and eluted with 0.1 M sodium phosphate buffer (pH 7.2) containing 0.5 mM EDTA and 0.1% SDS. The elution profile is shown by the solid line. When peak A (indicated by the bracket) was concentrated, boiled, and rechromatographed on the same column, most of the protein shifted to peak B (dashed line), and this peak B protein consisted almost entirely of protein 2 (see Fig. 7). When the peak C protein was concentrated, boiled, and rechromatographed on the same column, the bulk of the protein also shifted to peak B (dotted line) and this consisted primarily of protein 3a (see Fig. 7).*

rechromatographed, it eluted as indicated by the dotted line in Fig. 3. The protein which eluted at peak B consisted almost entirely of protein 3a, with one minor contaminant which amounted to less than 5% of the final protein. (The purification scheme for proteins 2 and 3a is summarized in Fig. 6, and Fig. 7 shows the protein profile of the purified preparations of proteins 2 and 3a on both Maizel and Bragg-Hou gels.)

**Purification of protein 1.** Additional steps of DEAE-cellulose chromatography were required to obtain a preparation of protein 1 that was free from protein 2. As in the purification of the colicin E receptor (8), it was necessary to precipitate the protein with ethanol and redissolve it in Tris-Triton buffer before each successive DEAE-cellulose column step to obtain additional purification. This suggests that DEAE-cellulose chromatography is actually separating protein aggregates probably associated with detergent micelles, and precipitation and redissolution are necessary to allow these aggregates to break down and then reform.

The second DEAE-cellulose column (DEAE column IIA) step in the purification of protein 1 is shown in Fig. 4. The salt gradient was modified slightly since only the low-salt fraction was of interest. As in the first DEAE-cellulose column, material eluted before the salt gradient was started, and this contained much of the contaminating proteins 2 and 3. The fractions that were pooled contained the greatest amount of protein 1, and a scan of the pooled fraction run on a Bragg-Hou gel is shown in the upper part of Fig. 4.

The pooled fraction from column IIA was precipitated with ethanol and run on a third DEAE-cellulose column (column IIIA, Fig. 5). Again, contaminating proteins were removed prior to the start of the salt gradient. The gel scan in the upper part of Fig. 5 shows that the amount of protein 2 has been reduced even further, although it is not entirely absent. To remove the small amount of protein 3 and the contaminating minor polypeptides, I precipitated the pooled fraction from column IIIA with ethanol, dissolved it in SDS solution, and subjected it to two cycles of chromatography on SDS-Sephadex G-200 exactly as described previously for protein 2 and illustrated in Fig. 3. The entire purification scheme for protein 1 is

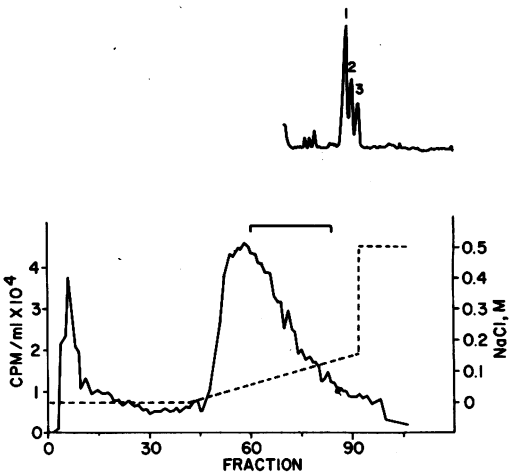


FIG. 4. *Elution profile of DEAE column IIA. For the further purification of protein 1, peak II from DEAE column I (see Fig. 2) was precipitated with ethanol, dissolved in Tris-Triton buffer, and applied to a 30-ml column of Whatman DE-52 as described in Fig. 2. Samples (5 ml) were collected. The bracket indicates the final fractions that were pooled, and the gel scan shows the appearance of this pooled sample on a Bragg-Hou gel. The dashed line indicates the NaCl gradient.*

shown in Fig. 6, and Fig. 7 shows Maizel and Bragg-Hou gels of the final product. Although the final preparation of protein 1 was free from minor contaminants and protein 3, Bragg-Hou gels indicated that it still contained significant amounts of protein 2. By slicing Bragg-Hou gels and measuring the amount of label, I estimated that the final preparation contained 85% protein 1 and 15% protein 2. For purposes of comparison this was satisfactory since the purified preparation of protein 2 did not contain any significant amount of protein 1 (Fig. 7).

**Purification of protein 3b.** The purification scheme for protein 3b is shown in Fig. 6. The general procedure was similar to that employed with protein 1, but consisting of two additional DEAE-cellulose column steps (columns IIB and IIIB, Fig. 6). The DEAE-cellulose columns were similar to those shown in Fig. 4 and 5, except that a second salt gradient from 0.1 to 0.5 M NaCl was employed as in Fig. 2. After the final DEAE-cellulose column, the protein was precipitated with ethanol, dissolved in SDS solution, and subjected to two cycles of SDS-Sephadex G-200 chromatography as described previously for protein 3a. The final preparation of protein 3b (Fig. 7) contained one minor contaminant that was seen moving just ahead of the major band on both Maizel and Bragg-Hou gels. This minor component accounted for 10% of the protein in the final preparation.

**Cyanogen bromide peptide patterns of the purified proteins.** One of the major reasons to purify the major proteins was to obtain definative cyanogen bromide peptide "fingerprints" for each of the proteins (Fig. 8 and 9). Figure 8 shows the migration of the cyanogen bromide peptides derived from proteins 1 and 2 in the Swank-Munkres gel system. Seven distinct peptide peaks can be resolved. Peaks 3 and 6 are clearly unique to protein 1, and peak 1 is clearly unique to protein 2. These are the major peaks, and it is these which were observed previously (13) when the cyanogen bromide peptide pattern of the band A protein eluted from gels was examined. Some of the smaller peaks also appear to be unique to each of the proteins although they overlap. Peak 2 appears to be primarily a product of protein 2. Small amounts of peaks 1 and 2 were observed with the purified preparation of protein 1, but these can be accounted for by the small amount of protein 2 present as a contaminant of protein 1 (Fig. 7). Peaks 4 and 5 overlap, but they appear to be derived from proteins 1 and 2, respectively. Peak 7 is not well resolved and may not be an actual peptide. The pattern shown in Fig. 8 has been reproduced in three separate cleavage experiments and is supported by data from other strains of *E. coli* that lack protein 2 (14).

It has been possible to obtain a rough estimate of the molecular mass of these peptide peaks by comparison of the migration of these peptides to a standard mixture containing chymotrypsinogen, myoglobin, lysozyme, cyto-

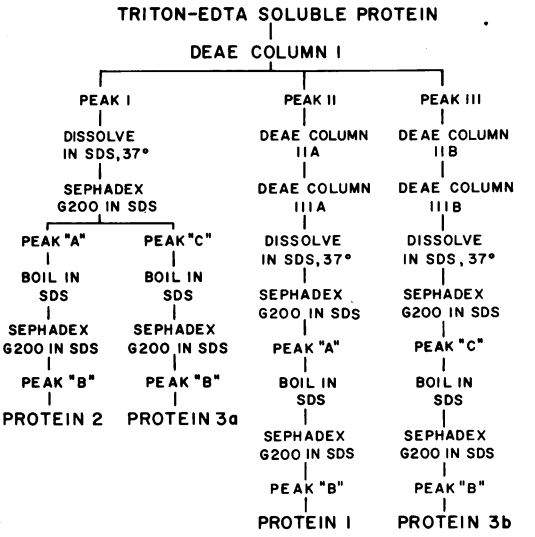


FIG. 6. *Overall purification scheme of proteins 1, 2, 3a, and 3b. A final yield of 1 to 2 mg of each of the purified proteins was obtained.*

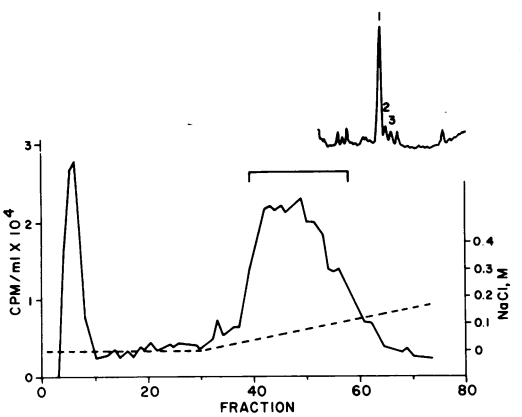


FIG. 5. *Elution profile of DEAE column IIIA. The pooled sample from column IIA (see Fig. 4) was precipitated with ethanol, dissolved in Tris-Triton buffer, and chromatographed as described in the legend of Fig. 4. The bracket indicates the samples which were pooled, and the gel scan shows the appearance of this pooled sample on a Bragg-Hou gel. The dashed line indicates the NaCl gradient.*

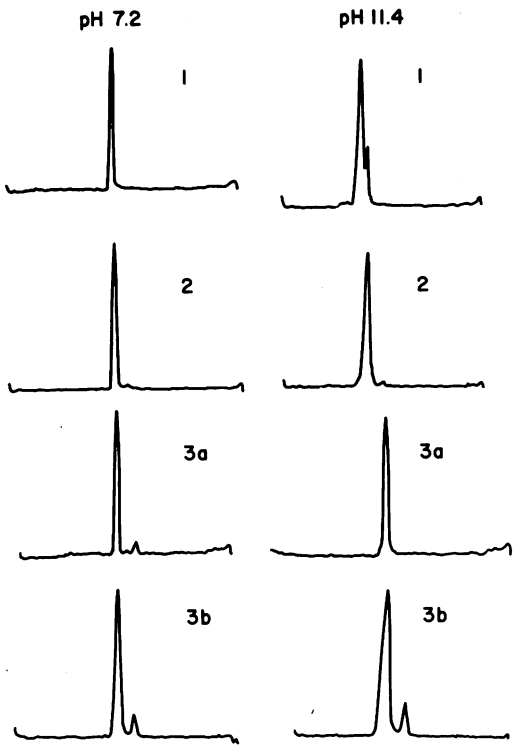


FIG. 7. *Appearance of the final purified samples of proteins 1, 2, 3a, and 3b on SDS-gels. The left-hand scans show proteins analyzed on Maizel (pH 7.2) gels, and the right-hand scans show the same samples analyzed on Bragg-Hou gels (upper buffer pH 11.4). The tops of the gels are to the left in all cases.*

chrome *c*, and insulin. The apparent molecular mass of peptide 1 shown in Fig. 8 is >30,000 daltons and that of peptide 3 is about 23,000 daltons. The mass of peptide 6 is 12,000 to 15,000 daltons. Unfortunately, there is no obvious relationship of the apparent molecular masses of the peptides to the apparent 42,000-dalton uncleaved proteins 1 and 2. If proteins 1 and 2 are pure and homogeneous polypeptide species, then some of the larger peptides must be the result of either partial cleavage or some modification of the methionine residues that prevents cleavage.

The cyanogen bromide cleavage products of proteins 3a and 3b are shown in Fig. 9. The first point which is evident from Fig. 9 is that proteins 3a and 3b are obviously different polypeptides, even though both are components of band C (Fig. 1) and they cannot be separated on either Maizel or Bragg-Hou gel systems. Of the major peaks in Fig. 9, peaks 1, 2, and 4 are unique to protein 3a, and peaks 3 and 5 are unique to protein 3b. The apparent molecular mass of peak 2 is about 22,000 daltons, and that of peak 4 is about 12,000 daltons. The peptide profiles of proteins 3a and 3b are much simpler than those of proteins 1 and 2, and can be more easily reconciled with the molecular mass of the uncleaved proteins. In several different cleavage experiments with the same protein samples, the amounts of peptides 1 and 2 and the ratio of these peptides have varied considerably. The gel shown in Fig. 9 shows the maximal amount of these peptides that has been observed, and in some experiments they were very minor components. Hence, it can be assumed that they represent partial-cleavage products. In the case of protein 3a, three peptides of 12,000 daltons

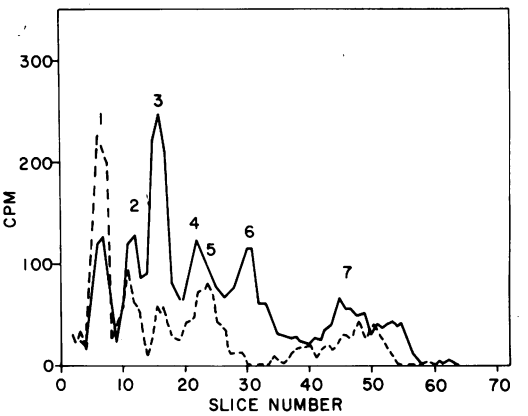


FIG. 8. *Analysis of $^3$H-labeled cyanogen bromide peptides derived from protein 1 (solid line) and protein 2 (dashed line) in the Swank-Munkres SDS-gel system. The tops of the gels are to the left, and the numbers refer to the major peptide peaks in each sample.*

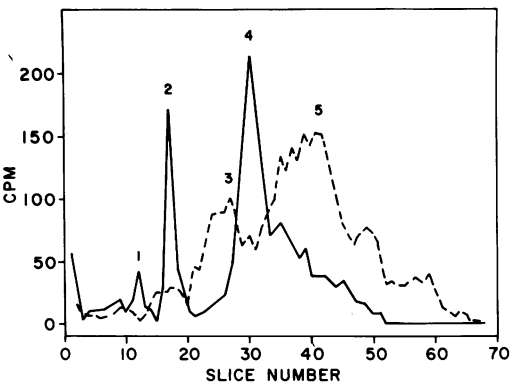


FIG. 9. *Analysis of $^3$H-labeled cyanogen bromide peptides derived from proteins 3a (solid line) and 3b (dashed line) in the Swank-Munkres SDS-gel system. The tops of the gels are to the left, and the numbers refer to the major peptide peaks in each sample.*

plus one smaller peptide (the leading edge of peak 4) would add up to about 42,000 daltons. In the case of protein 3b, two peptides the size of peak 3 plus two peptides the size of peak 5 would add up to about 42,000 daltons.

It does not appear that any major polypeptide species were lost during purification. If the four cyanogen bromide peptide profiles shown in Fig. 8 and 9 are added together in the correct proportions (a ratio of 1:2:2:1 for proteins 1, 2, 3a and 3b, respectively), the composite profile is identical to that shown previously (13) for unfractionated protein from band B from gels. Similarly, a composite of the peptide patterns of proteins 1 and 2 is identical to that reported for band A, and a composite of the peptide patterns of proteins 3a and 3b is identical to that reported for band C.

**Carbohydrate and phosphate content of the major proteins.** Two properties of these proteins are difficult to explain, namely, the differences in migration on Bragg-Hou gels and the apparent poor cleavage of protein 2 with cyanogen bromide. Therefore, these proteins were examined for the presence of bound carbohydrate or phospholipid which could account for these properties. Since it is difficult to obtain large amounts of protein for direct analysis, specific labeling techniques were employed.

A culture was heavily labeled with [$^{14}$C]galactose and [$^{3}$H]leucine to test the major proteins for the presence of galactose-containing carbohydrates such as lipopolysaccharide (LPS) or colanic acid (3). Strain J-5 preinduced with D-fucose (17) was used in this experiment. The outer membrane fraction obtained by Triton X-100 extraction was solubilized in SDS solution and centrifuged to remove murein fragments (12), and chromatographed on SDS-Sephadex G-200. The elution profile of this column (Fig. 10A) shows that the bulk of the galactose label eluted after peak A, as would be expected for complete LPS molecules (12). Fractions from peak A were pooled, concentrated, boiled, and chromatographed again on the same column (Fig. 10B). Only a small amount of the galactose label was present in peak B, and to show that this label was not associated with proteins 1 and 2 the protein from peak B was analyzed with the Bragg-Hou gel system (Fig. 11). Only a few $^{14}$C counts were detected in the gel, and these did not coincide with proteins 1 and 2. Thus, it was concluded that the protein bands on the gels are free from LPS and do not contain any significant amount of galactose. It should be noted that the $^{14}$C peak seen in Fig. 10A represents only a small

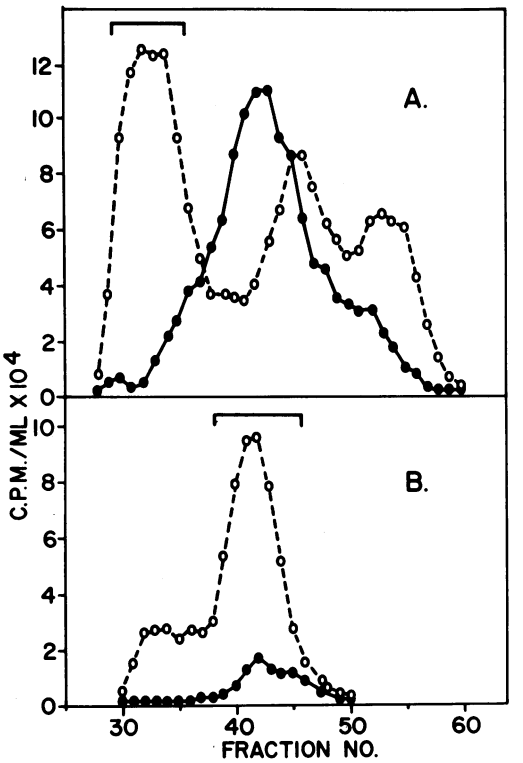


FIG. 10. *Chromatography of a culture labeled with [$^{14}$C]galactose and [$^{3}$H]leucine on Sephadex G-200 in the presence of SDS. The column and conditions are as in Fig. 3. (A) Elution profile when the Triton-insoluble envelope fraction was dissolved in SDS solubilizing solution at 37 C, centrifuged to remove murein fragments, and applied to the column. (B) Elution profile when the protein of peak A (bracket, Fig. 10A) was concentrated, boiled, and rechromatographed on the same column. The solid line indicated $^{14}$C and the dashed line indicates $^{3}$H. The bracket in Fig. 10B indicates the final sample which was analyzed on Bragg-Hougels.*

fraction of the total LPS of the cell envelope, since the bulk of the LPS is removed by the Triton X-100 extraction used to prepare the outer membrane fraction (11).

None of the major proteins detected on Bragg-Hou gels contains any bound phosphorus, and this provides further evidence that they do not contain bound LPS. This was tested in the following way. A culture of strain J-5 was grown on the Tris-based medium of Garen and Levinthal (2) containing 0.6 mM phosphate. The culture was heavily labeled (1 $\mu$Ci/ml) with $^{32}$P (orthophosphate), and the incorporation of the label was great enough to permit detection of 0.01 mol of phosphate per mol of 42,000-dalton protein. This culture was grown without

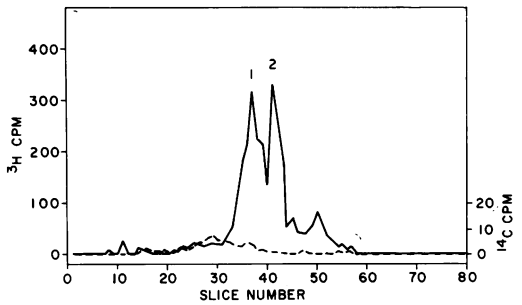


FIG. 11. *Bragg-Hou gel profile of protein from peak A (Fig. 10A and B) after two cycles of SDS-Sephadex G-200 chromatography. The numbers indicate proteins 1 and 2; the solid line indicates $^3$H counts; and the dashed line indicates $^{14}$C counts from [$^{14}$C]galactose.*

added galactose, which permits peak C to be separated from the LPS "core" by SDS-Sephadex chromatography. When the outer membrane protein fraction from this culture was dissolved in SDS solution and chromatographed as shown in Fig. 10A, all of the $^{32}$P counts eluted in a broad peak slightly after peak C (data not shown). Peak A, which was shown by Bragg-Hou gel electrophoresis to contain proteins 1 and 2, contained no detectable $^{32}$P counts. Since there was some overlap between peak C and the $^{32}$P-labeled peak, the protein from peak C was boiled and rechromatographed on the same SDS-Sephadex G-200 column. The bulk of the protein now eluted as peak B, which was shown by Bragg-Hou gel electrophoresis to consist primarily of protein 3. The elution of the $^{32}$P counts did not shift after boiling, so peak B contained almost no $^{32}$P counts. The $^{32}$P counts in peak B represented less than 0.05 mol of phosphate per mol of 42,000-dalton protein, so it was concluded that protein 3 was also free from bound LPS or phosphate.

Since the results above indicated that proteins 1, 2, and 3 were free from LPS and phosphate, it was of interest to determine whether they could be labeled with glucosamine. These experiments were done with a derivative of strain J-5, strain J-515, which is a mutant that cannot utilize glucosamine as a carbon source and is presumably blocked in the conversion of glucosamine to glucose. Since the neutral sugar *N*-acetyl glucosamine is taken up more readily than the charged amino sugar and is rapidly deacetylated inside the cell, the culture was labeled with *N*-acetyl [1-$^{14}$C]glucosamine (Amersham-Searle, Inc., Des Plaines, Ill.) and [$^3$H]leucine. The inoculum was grown in the presence of 10 mM unlabeled *N*-acetyl glucosamine, centrifuged, and washed prior to inoculation of the final culture. The culture was grown in minimal medium with succinate as the carbon source.

In contrast to the phosphate-labeling experiments described above, when SDS-solubilized outer membrane protein from this culture was chromatographed on SDS-Sephadex G-200, there were $^{14}$C counts in both peak A and peak C as well as in a broad peak which eluted after peak C. Protein samples from peak A and peak C were each boiled and rechromatographed on SDS-Sephadex G-200, and in both cases a small but significant amount of the $^{14}$C counts shifted into peak B with the bulk of the protein. Protein from the B peaks derived from both peak A and peak C were analyzed by gel electrophoresis in the Bragg-Hou gel system (Fig. 12). The $^{14}$C label coincided exactly with the $^3$H protein label, both in the case of proteins 1 and 2 (Fig. 12A) and in the case of protein 3 (Fig. 12B). The ratio of $^{14}$C to $^3$H was the same in proteins 1, 2, and 3.

*N*-acetylneuraminic acid is derived from glucosamine and is present in *E. coli* as a homopolymer, colominic acid. McGuire and Binkley (5) have observed that a portion of the carboxyl groups of this polymer is not titratable until the polymer has been treated with alkali (pH 11). They suggested that the carboxyl groups were linked to the hydroxyl groups of neighboring monomer units by alkali-labile ester bonds. Since the proteins appear to label with glucosamine and exhibit differences in migration in an alkaline gel system, such an alkali-labile bond might be present in these proteins. To test for this I treated the samples of protein from the A and C peaks, at pH 11.4, with a solution identical to the upper buffer of the Bragg-Hou gel system for 8 h at 40 C to simulate the most severe conditions which might occur during electrophoresis. The pH of the samples was then adjusted back to 7.2 with 0.1 M phosphoric acid, and the samples were dialyzed overnight against 0.1 M sodium phosphate buffer containing 0.1% SDS. These alkali-treated samples and identical control samples that were not treated with alkali were compared in both the Maizel and Bragg-Hou gel systems. Both the alkali-treated and the control samples gave only band B in the Maizel gel system and bands 1, 2, and 3 in the Bragg-Hou gel system, indicating that treatment at pH 11.4 did not cause an irreversible alteration in the structure of the proteins. This experiment also tends to rule out beta-elimination of carbohydrate linked to serine residues as being the phenomenon causing the

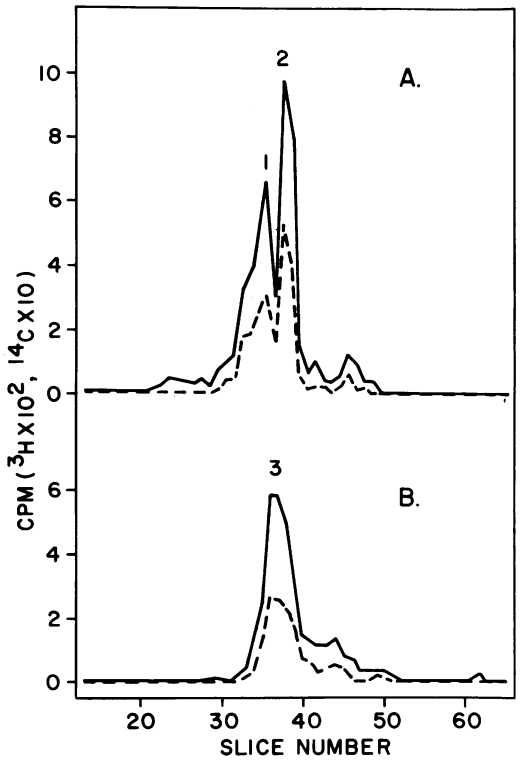


FIG. 12. *Labeling of proteins 1, 2, and 3 with $^{14}$C from N-acetyl glucosamine. The Triton-insoluble envelope fraction from a culture labeled as described in the text and in Table 1 was dissolved in SDS and chromatographed as in Fig. 10A. Protein peaks A and C were boiled and rechromatographed on Sephadex G-200, and the protein in both cases that shifted to peak B was analyzed on Bragg-Hou gels. (A) Gel pattern of protein from peak A containing proteins 1 and 2; (B) gel pattern of protein from peak C containing protein 3. The solid line indicates $^3$H label from leucine, and the dashed line indicates $^{14}$C counts from N-acetyl glucosamine.*

difference in migration observed in the Bragg-Hou gel system.

The major proteins were also labeled with glucose under conditions where conversion of glucose to amino acids, lipids, or glucosamine was minimized. The labeling was performed as follows. A culture was grown on minimal medium with glycerol as the carbon source. Strain J-515 was used in this experiment, and 10 mM *N*-acetyl glucosamine was added to compete out glucosamine derived from labeled glucose. Amino acids which could be derived from glucose or glycolytic intermediates were added as follows (per milliliter): L-serine, 60 μg; L-alanine, 40 μg; L-lysine, L-isoleucine, L-leucine, and L-valine, 20 μg each; and L-tyrosine, L-phenylalanine, L-tryptophan, and histidine, 10 μg each. The culture was labeled with [$^3$H]leucine and 10 μCi of D-[3,4-$^{14}$C]glucose. The rationale of this labeling procedure is that C3 and C4 of glucose are lost as $CO_2$ during the oxidation of pyruvate, and this would prevent incorporation of label into lipid or amino acids derived from citric acid cycle intermediates. No significant level of $^{14}$C could be detected in soluble "cytoplasmic" protein prepared as described previously (8).

Proteins from peaks A and C were isolated from this glucose-labeled culture as described previously for the *N*-acetyl glucosamine labeling experiment, and these were examined with the Bragg-Hou gel system. Low levels of $^{14}$C counts were detected when the gels were sliced and, as in the case of the *N*-acetyl glucosamine labeling experiment, all of the $^{14}$C counts coincided exactly with the $^3$H counts in proteins 1, 2, and 3. These gels are not shown, since they were qualitatively identical to those shown in Fig. 12.

The data from the three sugar labeling experiments are summarized in Table 1. In the

TABLE 1. *Incorporation of labeled sugars into envelope fractions*[a]

| Fraction | $^{14}$C counts/10$^3$ $^3$H counts | | |
|---|---|---|---|
| | [$^{14}$C] galactose | [$^{14}$C] *N*-acetyl glucosamine | [$^{14}$C] glucose |
| Crude envelope | 1,272 | ND | ND |
| Triton X-100 soluble | 3,544 | ND | ND |
| Triton X-100 insoluble (outer membrane plus murein) | 701 | 435 | 1,425 |
| SDS soluble (after removal of murein) | 682 | 52 | 147 |
| Protein 1 (Bragg-Hou gel) | 61 | 49 | 28 |
| Protein 2 (Bragg-Hou gel) | 12 | 52 | 19 |
| Protein 3 (Bragg-Hou gel) | ND | 45 | 21 |

[a] ND, Not determined. The cultures were grown in medium containing 20 μg of L-leucine per ml and 200 μCi of [$^3$H]leucine per liter. In the galactose-labeling experiment, the inoculum contained 1.5 mM D-fucose, and the final culture contained 0.5 mM D-galactose and 75 μCi of [U-$^{14}$C]galactose per liter. In the *N*-acetyl glucosamine experiment, the culture contained 25 μCi of [$^{14}$C]*N*-acetyl glucosamine (labeled in C1 of the glucosamine) per liter and no unlabeled carrier. In the glucose-labeling experiment, the culture contained 10 μCi of [$^{14}$C]glucose (labeled in C3 and C4) per liter and no unlabeled carrier. The Triton X-100-insoluble fraction was dissolved in SDS solubilizing solution at 37 C at a protein concentration of 5 mg/ml, and then centrifuged at 200,000 × *g* for 2 h to remove insoluble murein fragments. In all cases the supernatant, designated as the SDS-soluble fraction, contained about 90% of the $^3$H counts present in the Triton X-100-insoluble fraction.

galactose-labeling experiment, there was a considerable amount of label in the Triton-insoluble outer membrane fraction, even though much of the label had been removed by the Triton X-100 extraction. Most of this galactose label was solubilized by SDS, but the ratio of $^{14}C$ to $^{3}H$ was reduced more than 10-fold in the protein 1 peak on the gel and 50-fold in the protein 2 peak. The [$^{14}C$]galactose label remaining after SDS-Sephadex G-200 chromatography did not coincide with the $^{3}H$-labeled peaks of proteins 1 and 2 (Fig. 11). However, in the case of the *N*-acetyl glucosamine- and glucose-labeling experiments, much of the $^{14}C$ label present in the Triton-insoluble outer membrane fraction was not solubilized by SDS and is presumed to be in the SDS-insoluble murein fraction. The murein sugars are derived from glucosamine and the *O*-lactyl group of muramic acid would contain the C3 and C4 from glucose, so heavy labeling of the murein was anticipated in both cases.

Because of limitations in the amount of material and difficulties associated with the analysis of sugars in the presence of a large excess of SDS and protein, no attempt was made to characterize the labeled products. Hence, the glucose- and *N*-acetyl glucosamine-labeling experiments are not conclusive in showing the the major outer membrane proteins are in fact glycoproteins. However, some preliminary conclusions can be drawn from these experiments. First, the proteins contain no bound phosphate. Second, unlike the outer membrane glycoprotein recently described by Wu and Heath (19), these proteins do not contain galactose and hence do not contain lipopolysaccharide outer core or side-chain carbohydrate. Third, if the counts incorporated from glucose and *N*-acetyl glucosamine are still in the form of sugar, they represent only a small amount, at most a few sugar molecules per molecule of protein. Proteins 1, 2, and 3 all contain about the same amount of label from these sugars. Thus, it is unlikely that carbohydrate bound to these proteins accounts for the differences in their mobility in the Bragg-Hou gel system.

## DISCUSSION

SDS-polyacrylamide gel electrophoresis has been one of the major techniques for examining the composition of membranes, and this technique has been widely applied to the study of the outer membrane proteins of gram-negative bacteria. Although refinements such as the use of discontinuous buffer systems have improved the resolving power of this technique, it still suffers the limitation that polypeptides are separated on the basis of size more than on the basis of charge. As this study demonstrates, this limitation has been particularly troublesome in the analysis of the major polypeptides of the outer membrane of *E. coli* O111.

When the outer membrane proteins of *E. coli* O111 were first examined by SDS-polyacrylamide gel electrophoresis (9, 10, 11), it was observed that as much as 70% of the outer membrane protein was present in a single, sharp band on the gels, with an apparent molecular mass of about 42,000 daltons. In this study, the protein of this major, 42,000-dalton component has been fractionated into four distinct subcomponents. This fractionation has been accomplished by ion exchange chromatography in the presence of the non-ionic detergent Triton X-100, which is capable of separating proteins on the basis of charge, and by Sephadex G-200 chromatography in the presence of SDS, which separates these proteins by virtue of the unusual conformation that these proteins exhibit when exposed to SDS under mild conditions.

Each of the subcomponents of this 42,000-dalton protein has a unique cyanogen bromide peptide profile, which provides direct chemical proof that these subcomponents represent at least four distinct polypeptide species. Although the cyanogen bromide peptide profiles of each of these subcomponents are fairly simple, it is by no means certain that there are only four species. Further genetic and chemical studies will be necessary to establish the maximal number of individual polypeptides present in this mixture.

The alkaline gel system described by Bragg and Hou (1) is capable of resolving the 42,000-dalton major polypeptide band of *E. coli* O111 into three sharp, distinct bands, presumably because of some effect of the alkaline pH on the net charge of the protein-SDS complexes (16). However, one of these bands (band 3) still consists of two distinct polypeptide species. These two species, designated in this study as proteins 3a and 3b, have very similar properties and apparently identical molecular masses. They can be separated by charge in Triton X-100 solution and can be identified by their characteristic cyanogen bromide peptide profiles, but they cannot be separated by any of the SDS-polyacrylamide gel systems tested in our laboratory.

The situation of multiple protein components with very similar properties and molecular masses is probably not unique to the outer

membrane of *E. coli*, and should be anticipated in other biological membranes as well. It is possible that the structure of the membrane or the mode of biosynthesis of the membrane proteins in some way restricts the size of the proteins, as if each protein must fit a certain kind of "niche" in the finished membrane.

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