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Isolation and Characterization of Lipopolysaccharide Protein from *Escherichia coli**

(cell wall/glycoprotein/membrane protein/endotoxin)

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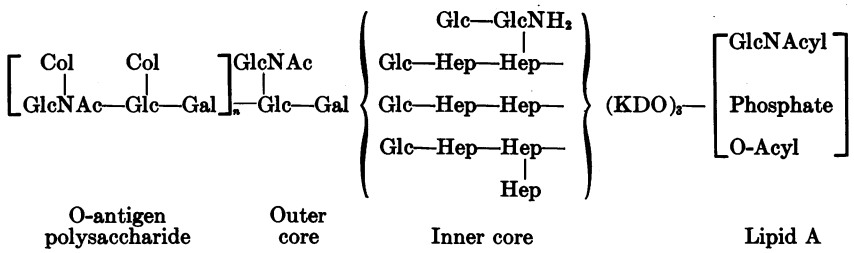
ABSTRACT **Extraction of the cell envelope of *E. coli* with 1% sodium dodecyl sulfate yielded a lipopolysaccharide protein that was purified to homogeneity by conventional techniques. Analysis of the pure protein indicated that it is a complex lipopolysaccharide protein with the following molar ratios of constituents: 3-deoxyoctulosonate, 1.0; glucosamine, 1.3; neutral sugar (glucose + heptose), 1.0; organic phosphate, 2.3; and amino acid, 21. On a quantitative basis, all of the 3-deoxyoctulosonate present in the cell envelope preparation was solubilized in hot sodium dodecyl sulfate and subsequently accounted for in its entirety in the isolated protein component. After incubation of the cell-wall particulate fraction of cells grown on [^{3}H]glucosamine with UDP-[^{14}C]galactose under conditions designed to measure galactosyl transferase activity, the isolated lipopolysaccharide protein contained all of the [^{14}C]galactose that was incorporated during the incubation. We concluded that the lipopolysaccharide of this organism occurs in the outer cell-envelope membrane exclusively in the form of lipopolysaccharide protein.**

The structure, mechanism of biosynthesis, and physiological function of the complex lipopolysaccharide of cell envelopes of Gram-negative bacteria have been studied in several laboratories (2, 3). Our studies have been concerned primarily with the polymer from *Escherichia coli* 0111-B$_4$, the structure of which is outlined as follows:



The general structural components of this polymer include the lipid-A moiety made up of fatty acylated glucosamine residues and organic phosphate; the inner-core region, which is basically composed of glucose (Glc), heptose (Hep), and 3-deoxy-octulosonic acid (KDO); and the outer-core region, which provides a linkage site for the ultimate attachment of the O-antigen polysaccharide. There is a degree of heterogeneity in the inner-core region of this polymer in that there are at least three structural units, each of which constitutes about one-third of this portion of the molecule (4). Mutants of this organism lacking UDP-galactose epimerase, when grown in the absence of exogenous galactose, produce an incomplete lipopolysaccharide in that they are incapable of extending the polymer beyond the glucose residue in the inner core.

Most of the structural studies on lipopolysaccharide have been conducted with preparations extracted from the cell envelope by the conventional hot aqueous phenol procedure (5); polymers prepared by this method may be isolated essentially free of protein. However, in its native state in the outer membrane of the envelope, lipopolysaccharide occurs in close association with protein (6, 7). Others (8, 9) have reported the occurrence of complexes of lipopolysaccharide components and protein, although these complexes were not thoroughly characterized. In our current studies we considered the possibility that lipopolysaccharide may be covalently attached to a protein of the outer membrane in its native state and that the hot phenol extraction procedure results in partial degradation of the native complex macromolecule. In this report, we present evidence that upon mild detergent extraction of the outer membrane of the cell envelope of a mutant of *E. coli* 0111-B$_4$, the lipopolysaccharide can be quantitatively recovered as a lipopolysaccharide protein.

Abbreviations: KDO, 3-deoxy-D-*manno*octulosonate; Col, colitose; Hep, L-*glycero*-D-*manno*heptose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Gal, galactose.

* A preliminary report of this work has been presented (1).

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EXPERIMENTAL PROCEDURE

Organism. A mutant of *E. coli* 0111-B$_4$, lacking both UDP-galactose 4-epimerase and glucosamine 6-phosphate synthetase, was used in these studies. *E. coli* J-5, a mutant that lacks UDP-galactose 4-epimerase (10), was mutagenized by treatment with nitrosoguanidine (11); glucosamine-re-

quiring mutants were isolated from the surviving population by replica-plating techniques. One of these mutants, *E. coli* M109, was purified and had the following properties: (*a*) an absolute growth requirement for glucosamine (or *N*-acetylglucosamine) at an optimal level of 200 μg/ml; (*b*) cell-free extracts exhibited no detectable amounts of glucosamine 6-phosphate synthetase (12) or UDP-galactose 4-epimerase (13); and (*c*) growth of the mutant on media containing optimal concentrations of glucosamine and galactose resulted in proliferation of organisms that were serologically and chemically identical to wild-type *E. coli* 0111-B_4. In order to avoid induction of glucosamine 6-phosphate deaminase during growth, M109 was routinely grown in Nutrient Broth (Fisher Scientific Co.) supplemented with 200 μg/ml of *N*-acetylglucosamine for 3.5 hr; 0.1 μCi/ml of [^{3}H]glucosamine (specific activity, 3.6 Ci/mmol) was added and growth was continued for the additional 2 hr. Cells were harvested by centrifugation and washed three times with 0.15 M KCl, and finally with 0.1 M Tris·HCl buffer (pH 8).

Extraction of Lipopolysaccharide Protein from Preparations of Cell Envelopes. The washed cells were suspended in 0.1 M Tris·HCl buffer (pH 8) containing 20 μg/ml each of RNase and DNase (Worthington) and then sonicated for 5 min. The sonicated suspension was incubated at room temperature (25°) for 1 hr; then the cell-envelope particulate fraction was sedimented by centrifugation at 78,000 × *g* for 2 hr. The supernatant fraction was discarded. The pellet derived from 100 g of wet-cell paste was suspended in 500 ml of 1% Na dodecyl sulfate, placed in a boiling water bath for 1–5 min, and centrifuged at 40,000 × *g* for 20 min. The Na dodecyl sulfate-soluble fraction contained about one-half of the total radioactivity in the cell envelope, and the radioactivity in this fraction was quantitatively recovered as [^{3}H]glucosamine after hydrolysis for 12 hr in 6 N HCl at 100°.

Purification of Lipopolysaccharide Protein. Dialysis of the Na dodecyl sulfate-soluble fraction of the cell envelope resulted in precipitation of the bulk of the protein and radioactivity in the preparation. Therefore, the initial step in purification of lipopolysaccharide protein was done by fractionation of the solution on Bio-Gel P-100 equilibrated with 1% Na dodecyl sulfate. The crude Na dodecyl sulfate-soluble fraction was concentrated about 10-fold by pressure dialysis. 2.5 ml of the concentrated fraction (about 50 mg of protein) was applied to a column (40 × 2.2 cm) of Bio-Gel P-100, which was then eluted with 1% Na dodecyl sulfate solution. Radioactivity was eluted from this column in two peaks, one at the void volume and another slightly included in the gel. The included material was pooled and subjected to further purification. However, it appears that most of the radioactive material voided from this column constitutes an aggregated form of lipopolysaccharide, since the two peaks described above were obtained if the voided material was again run on an identical column. Thus, after repeated fractionation in the Bio-Gel P-100 column, essentially all of the radioactivity could be recovered in the included peak material. The pooled fractions containing radioactivity were dialyzed against 50 mM Tris·HCl buffer (pH 8); the slightly turbid solution was further desalted by passage through a Sephadex G-25 gel filtration column with the same buffer. The turbid fractions voided from the Sephadex column were pooled and the solution was adjusted to 0.1 N NaOH and heated at 70° for 30 min; the solution was cooled and adjusted to pH 3.5 by addition of glacial acetic acid. The resultant precipitate was collected by centrifugation, washed twice with water, extracted with *n*-butanol, and then dissolved in 0.1 M Tris·HCl buffer (pH 8) without Na dodecyl sulfate. The water-soluble preparation was applied to a column of DEAE–cellulose and fractionated with a KCl gradient. The pooled fractions of lipopolysaccharide protein isolated from the DEAE–cellulose column were dialyzed against 50 mM Tris·HCl buffer (pH 8), concentrated by pressure dialysis, and finally applied to a Sephadex G-150 gel filtration column. The column was eluted with 50 mM Tris·HCl buffer (pH 8) and the bulk of the radioactivity was recovered in the void volume. This material, which appeared to be homogeneous by several criteria, was concentrated by pressure dialysis and used for the remainder of these studies.

Electrophoresis. Phenol–urea–acetic acid polyacrylamide-gel electrophoresis was done (14). Samples were dialyzed exhaustively and dissolved in phenol–acetic acid solution before application to the gel. Na dodecyl sulfate–polyacrylamide gel electrophoresis was done (15) with 0.5% Na dodecyl sulfate. Proteins were detected in polyacrylamide gels by staining with Coomassie Blue; carbohydrate was detected by the periodate-Schiff reagent (16). Radioactivity profiles of gels were obtained by slicing the entire gel into uniform discs. Each disc was then digested (17), and a sample was finally diluted in Triton–toluene scintillation fluid and counted. Cellulose acetate strip electrophoresis was conducted with 1% sodium borate buffer (pH 9). Protein was detected on cellulose acetate strips by staining with Ponceau Red (18) and carbohydrates, by staining with Alcian Blue (19). For determination of radioactivity after cellulose acetate electrophoresis, strips were cut into segments, each of which was suspended in Triton–toluene fluid and counted.

Proteolytic Digestion of Lipopolysaccharide Protein. Purified protein preparations dissolved in 50 mM Tris·HCl buffer (pH 8) were treated with trypsin (2% w/w, Worthington, twice crystallized). The solution was incubated at 37° for 18 hr and then heated in a boiling water bath for 3 min. Pronase (2% w/w, Calbiochem) was added and the mixture was incubated for 40 hr at 30°; the incubation mixture was again heated in a boiling water bath for 3 min. Finally, subtilisin (5% w/w, Sigma) was added to the solution and incubation was continued at 37° for 18 hr. During the entire proteolysis procedure, chloramphenicol was included in the solution at 20 μg/ml.

Analytical Methods. The following procedures were used for quantitative determination of individual components of lipopolysaccharide protein: KDO by the thiobarbituric acid method (20); glucosamine by the method of Elson and Morgan (21); neutral sugar by the anthrone reagent (22); organic phosphate by the method of Ames and Dubin (23); and protein by the method of Lowry *et al.* (24). Lipopolysaccharide protein preparations were hydrolyzed as follows: for amino-acid analysis, 6.7 N HCl (sealed ampule) at 100° for 24 hr; for free KDO determination, in 0.1 N HCl at 100° for 3 min; and for liberation of neutral sugars, in 2 N HCl at 100° for 2 hr. Amino-acid analyses were conducted in a Spinco model 120C amino-acid analyzer by the method of Spackman *et al.* (25). Routine paper chromatographic analysis of hydrolysates was conducted on Whatman no. 1 paper with *n*-butanol–pyridine–water 9:5:4 as the solvent; for separation of

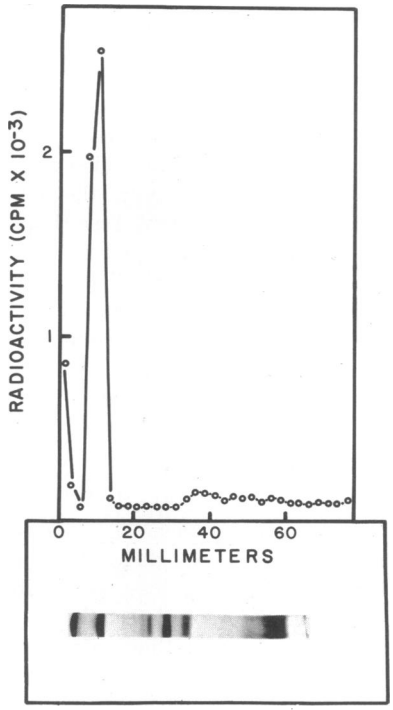

FIG. 1. Gel electrophoresis profile of crude Na dodecyl sulfate-soluble extract of cell envelope. A 50-μl aliquot (200 μg of protein, 7000 cpm) of the crude Na dodecyl sulfate-soluble fraction of the cell envelope was dissolved in 100 μl of phenol–25 μl of glacial acetic acid and then applied to a urea–acetic acid polyacrylamide gel. Electrophoresis was conducted for 8 hr at 2 mA per gel.

glucose and L-*glycero*-D-*manno*heptose, 95% acetone in water was used.

Determination of Galactosyl Transferase. Cell-envelope particulate fractions were prepared and assayed for galactosyl transferase (26). After incubation, the washed 70% ethanol-insoluble fraction was extracted twice with 1% Na dodecyl sulfate solutions at 100° for 1–5 min each. The Na dodecyl sulfate-soluble extract contained more than 90% of the radioactivity and was used for further analysis.

RESULTS AND DISCUSSION

Treatment of the cell envelope of Gram-negative bacteria with Na dodecyl sulfate has been used for fractionation of the macromolecular constituents of this cell structure. Braun and Rehn (27) demonstrated that the insoluble residue remaining after treatment of the cell envelope of *E. coli* with boiling 4% Na dodecyl sulfate is composed primarily of the complex macromolecule, murein lipoprotein. Similarly, in the present study, the [^{3}H]glucosamine-labeled outer membrane of *E. coli* yields two fractions when extracted with 1% Na dodecyl sulfate at 100°; an insoluble fraction in which the radioactive hexosamine is about equally distributed between *N*-acetylmuramic acid and glucosamine (corresponding to the expected results from hydrolysis of murein lipoprotein) and a soluble fraction that contains all of the radioactive hexosamine in a complex consisting of lipopolysaccharide and protein. Fractionation of the crude Na dodecyl sulfate-soluble material on a phenol–urea–acetic acid polyacrylamide gel indicates the presence of 8–12 individual protein bands (Fig. 1). However, analysis of the radioactivity profile of this gel indicates that the bulk of the radioactive hexosamine present in the preparation resides in a single protein species. In order to purify the labeled protein, the crude Na dodecyl sulfate-soluble fraction was initially passed through a Bio-Gel P-100 column in 1% Na dodecyl sulfate solution. Initial attempts to further purify the radioactive material by conventional techniques (ion-exchange resins and gel filtration columns) were hampered by the fact that removal of Na dodecyl sulfate resulted in precipitation of the radioactive material. We observed, however, that treatment of the radioactive protein with 0.1 N NaOH at 70° for 30 min resulted in a preparation in which all the radioactivity was recovered in a water-soluble product after complete removal of Na dodecyl sulfate. Analysis (by gas–liquid chromatography) of the products, soluble in organic solvent, of alkaline treatment indicated the presence of several long-chain fatty acids, including β-hydroxymyristate. It has been established that this fatty acid is a unique constituent of the lipid-A moiety of lipopolysaccharide in the form of *N*- and *O*-acyl residues. Alkaline treatment would be expected to release the *O*-fatty acyl residues, which apparently is sufficient to permit solubilization of the lipopolysaccharide protein in aqueous solutions.

The water-soluble, radioactive material was applied to a column of DEAE–cellulose and fractionated with a linear gradient of KCl. Analysis of fractions of the eluate indicated that the bulk of the radioactive material is eluted in a relatively symmetrical peak at about 0.2 M KCl. In addition, this profile indicated that the radioactive material is coincident with both protein and KDO. Fractions containing the radioactive material were pooled and subjected to further fractionation on Sephadex G-150, ultimately yielding a protein that appeared to be homogeneous, as judged by electro-

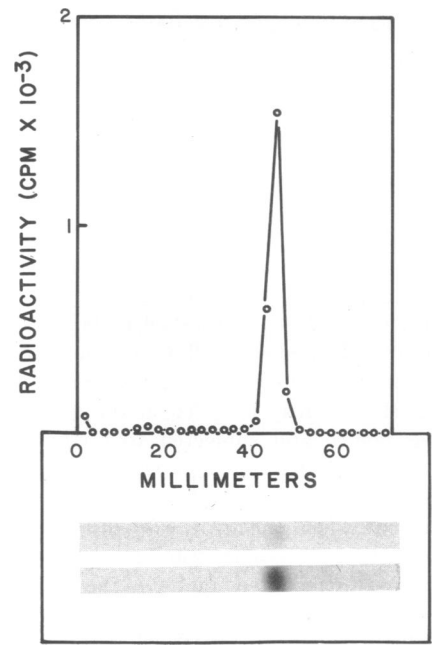

FIG. 2. Homogeneity of purified lipopolysaccharide protein. A 100-μl sample (50 μg of protein, 3000 cpm) of purified lipopolysaccharide protein was adjusted to 0.5% Na dodecyl sulfate and heated at 60° for 30 min, before it was applied to Na dodecyl sulfate–polyacrylamide gel. Electrophoresis was conducted for 6 hr at 8 mA per gel. Radioactivity (*profile*), protein (*lower gel*), and carbohydrate (*upper gel*) were detected.

phoresis on cellulose acetate strips in borate buffer at pH 9, by its elution profile from a DEAE–cellulose column, and by electrophoresis in Na dodecyl sulfate–polyacrylamide gels (Fig. 2). A single coincident band of protein and carbohydrate was evident in the gel; a symmetrical peak of radioactivity also coincided with this band.

In order to confirm that the radioactive hexosamine-containing material is covalently bound to protein in the isolated material, the homogeneous preparation was subjected to proteolytic digestion. The homogeneous material and its product of digestion were subjected to electrophoresis on cellulose acetate strips in borate buffer at pH 9 (Fig. 3). The product of digestion exhibited a mobility somewhat greater than that of the undigested protein, moving as a single component as judged by the carbohydrate stain, Alcian Blue; the material could not be made visible by the Ponceau Red protein stain. In contrast, the undigested protein migrated at a slower rate and could be made visible by staining for either carbohydrate (Alcian Blue) or protein (Ponceau Red). Preparative amounts of both the undigested lipopolysaccharide protein and the lipopolysaccharide peptide, obtained by proteolytic digestion, were eluted from cellulose acetate strips and subjected to chemical analysis. The results of these analyses, expressed as molar ratios, are as follows: Intact lipopolysaccharide protein—KDO, 1.0; glucosamine, 1.3; neutral sugar (glucose + heptose), 1.0; organic phosphate, 2.3; amino acid, 21.0. Lipopolysaccharide peptide—KDO, 1.0; glucosamine, 1.3; neutral sugar (glucose + heptose), 1.0; organic phosphate, 2.0; amino acid, 4.7. Thus, the intact lipopolysaccharide protein and the lipopolysaccharide peptide exhibit essentially identical molar ratios of lipopolysaccharide constituents, whereas the molar ratio of amino acids in the peptides has been reduced to about one-fifth of that of the protein. Qualitatively, the amino-acid analysis of the protein is not remarkable, although analysis of the lipopolysaccharide peptide indicates significant enrichment in the acidic amino acids, aspartate and glutamate. From Na dodecyl sulfate–acrylamide gels and chemical analyses, the estimated minimum molecular weight of lipopolysaccharide protein is 14,000, about 70% protein and 30% lipopolysaccharide.

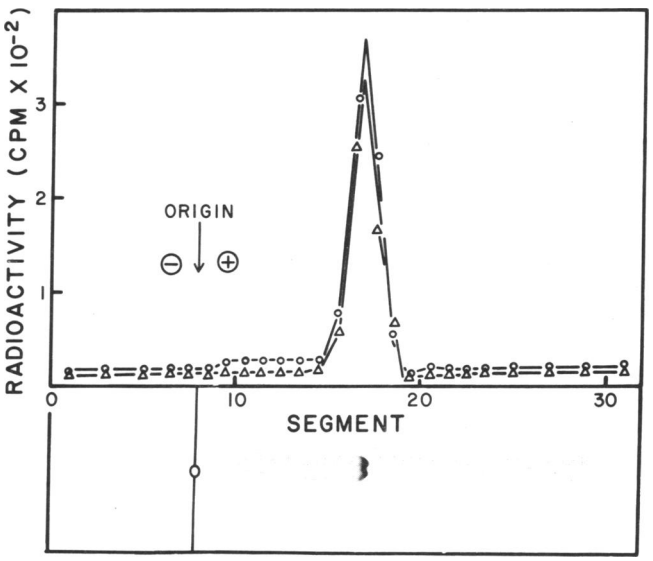

FIG. 4. Electrophoretic behavior of [^{14}C]galactose- and [^{3}H]glucosamine-labeled lipopolysaccharide protein. A 10-μl sample of labeled lipopolysaccharide protein isolated from the *in vitro* galactosyl transferase assay mixture was applied to a 1-inch strip of cellulose acetate in a band about 1 cm in length. Electrophoresis was conducted in 1% sodium borate buffer (pH 9) for 80 min at 250 V. After it was dried, the strip was cut longitudinally to divide the sample channel into two equal parts; one was stained for protein and the other was cut into segments for determination of ^{14}C (△——△) and ^{3}H (○——○).

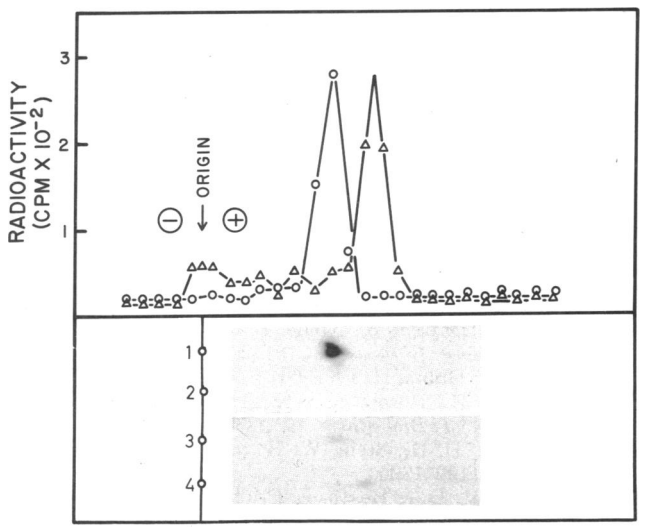

FIG. 3. Electrophoretic behavior of lipopolysaccharide protein and lipopolysaccharide peptide on cellulose acetate. A 5-μl sample of lipopolysaccharide protein (20 μg of protein, 600 cpm) and 5 μl of proteolytic digest (600 cpm) were applied to cellulose acetate strips in bands about 0.5 cm in length; three duplicate strips, each 1-inch wide, were prepared in this manner. Electrophoresis was conducted in sodium borate buffer (pH 9) for 80 min at 300 V. After drying, one strip was cut longitudinally between the two sample channels, and then cut into segments for determination of radioactivity (shown in *profile*); a second strip was stained for protein (*1* and *2*); and the third was stained for carbohydrate (*3* and *4*). Intact lipopolysaccharide protein was applied in channels *1* and *3;* lipopolysaccharide peptide was applied in channels *2* and *4*. ○, Before digestion; △, after digestion.

We conclude, therefore, that both of the major complex carbohydrate-containing macromolecules of the cell envelope of *E. coli*, murein (27) and lipopolysaccharide, are actually constituents of complex proteins in the outer membrane of the organism. Furthermore, essentially all of the lipopolysaccharide of the cell envelope occurs as lipopolysaccharide protein in its native state. Thus, extraction of cell envelope of this organism with 1% Na dodecyl sulfate results in essentially total solubilization of the KDO present in the envelope. With the isolation procedure described, essentially all of the Na dodecyl sulfate-soluble KDO may be recovered in the homogeneous lipopolysaccharide protein. It appears, therefore, that isolation of lipopolysaccharide from *E. coli* by the hot aqueous phenol extraction procedure does result in partial degradation of this complex macromolecule. This conclusion was corroborated by extraction of pure [^{3}H]glucosamine-labeled lipopolysaccharide protein by the hot aqueous phenol procedure; about 75% of the radioactivity was recovered in the aqueous phase (free of protein) and 25% of the radioactivity was retained in the phenol layer. These results sug-

gest that degradation of lipopolysaccharide most likely occurs in the lipid A–protein linkage region of the polymer.

Further confirmation that the lipopolysaccharide protein is the primary native form of the polymer was established by isolation of the product of an *in vitro* incubation mixture designed to determine the galactosyl transferase reaction (26). Thus, incubation of the cell-envelope particulate (from [^{3}H]glucosamine-grown cells) with UDP[^{14}C]Gal followed by the isolation procedure outlined above resulted in quantitative recovery of the [^{14}C]galactose that was incorporated into macromolecules in the lipopolysaccharide protein. These results are summarized in Fig. 4, which illustrates that the [^{3}H]glucosamine (incorporated during growth) and the [^{14}C]-galactose (incorporated *in vitro* from UDP[^{14}C]Gal) were coincident in their electrophoretic mobility, corresponding to the properties of the lipopolysaccharide protein. Paper chromatographic analysis of mild acid hydrolysates of this product indicated that the [^{14}C]galactose is present in the inner-core oligosaccharides that possess the specific acceptor sites for galactosyl transferase.

We have examined several strains of this organism, all of which were derived from *E. coli* 0111-B$_4$, for occurrence of the lipopolysaccharide protein. In addition to the mutant described in these studies, the mutant lacking UDP-galactose 4-epimerase, *E. coli* J-5, and a heptose-less mutant, *E. coli* RC59, also contained their entire complement of lipopolysaccharide in a complex protein that appears identical to the one described above by analysis on Na dodecyl sulfate–polyacrylamide gels. Analysis of either the wild-type organism, *E. coli* 0111-B$_4$, or mutants grown under conditions that permit them to produce complete lipopolysaccharide, indicated the presence of a portion of the lipopolysaccharide in the form of the lipopolysaccharide protein described above, but in addition, by Na dodecyl sulfate–polyacrylamide gel fractionation, additional lipopolysaccharide-containing proteins of apparent higher molecular weight were observed.

The role of the protein moiety of the lipopolysaccharide protein in biosynthesis of the lipopolysaccharide remains to be established. Whether or not lipopolysaccharide protein is biosynthesized initially as an intact molecule on the inner-envelope membrane and subsequently translocated to its ultimate localization in the outer membrane, as indicated by the results of Osborn *et al.* (28), or if the protein moiety of the lipopolysaccharide may serve as a "carrier" protein to which lipopolysaccharide may be alternately attached and detached, is not known. It would seem likely, however, that establishment of the lipopolysaccharide protein as the major form of lipopolysaccharide in its native state may permit the elucidation of some of these important physiological questions. Many of the precise structural details of the lipopolysaccharide protein have not been determined. It will be of particular significance to evaluate the structural and physical characteristics of the protein component itself and, specifically, to determine the structural details of the linkage site between the protein and lipopolysaccharide moieties of this complex macromolecule.

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1. Wu, M.-C. & Heath, E. C. (1973) *Fed. Proc.* **32,** 1460.
2. Osborn, M. J. (1969) *Annu. Rev. Biochem.* **38,** 501-538.
3. Heath, E. C. (1971) *Annu. Rev. Biochem.* **40,** 29-56.
4. Fuller, N. A., Wu, M.-C., Wilkinson, R. G. & Heath, E. C. (1973) *J. Biol. Chem.*, in press.
5. Westphal, O. & Jann, K. (1965) in *Methods in Carbohydrate Chemistry*, ed. Whistler, R. L. (Academic Press, New York), Vol. 5, pp. 83-91.
6. Osborn, M. J., Gander, J. E., Parisi, E. & Carson, J. (1972) *J. Biol. Chem.* **247,** 3962-3972.
7. Schnaitman, C. A. (1971) *J. Bacteriol.* **108,** 553-563.
8. Okuda, S. & Weinbaum, G. (1968) *Biochemistry* **7,** 2819-2825.
9. Wober, W. & Alaupović, P. (1971) *Eur. J. Biochem.* **19,** 340-356.
10. Elbein, A. D. & Heath, E. C. (1965) *J. Biol. Chem.* **240,** 1919-1925.
11. Adelberg, E. A., Mandel, M. & Chen, G. C. C. (1965) *Biochem. Biophys. Res. Commun.* **18,** 788-795.
12. Ghosh, S. & Roseman, S. (1962) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), Vol. 5, pp. 414-417.
13. Maxwell, E. S., Kurahashi, K. & Kalckar, H. M. (1962) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), Vol. 5, pp. 185-186.
14. Takayama, K., MacLennan, D. H., Tzagoloff, A. & Stoner, C. D. (1966) *Arch. Biochem. Biophys.* **114,** 223-230.
15. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406-4412.
16. Segrest, J. & Jackson, R. L. (1972) in *Methods in Enzymology*, ed. Ginsburg, V. (Academic Press, New York), Vol. 28, p. 57.
17. Ptashne, M. (1967) *Proc. Nat. Acad. Sci. USA* **57,** 306-313.
18. Graham, J. L. & Grunbaum, B. W. (1963) *Amer. J. Clin. Pathol.* **39,** 567-578.
19. Wardi, A. H. & Allen, W. S. (1972) *Anal. Biochem.* **48,** 621-623.
20. Ghalambor, M. A., Levine, E. M. & Heath, E. C. (1966) *J. Biol. Chem.* **241,** 3207-3215.
21. Elson, L. A. & Morgan, W. T. J. (1933) *Biochem. J.* **27,** 1824-1828.
22. Seifter, S., Dayton, S., Novic, B. & Muntwyler, E. (1950) *Arch. Biochem. Biophys.* **25,** 191-200.
23. Ames, B. & Dubin, D. T. (1960) *J. Biol. Chem.* **235,** 769-775.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265-275.
25. Spackman, D. H., Stein, W. H. & Moore, S. (1958) *Anal. Chem.* **30,** 1190-1206.
26. Edstrom, R. D. & Heath, E. C. (1967) *J. Biol. Chem.* **242,** 3581-3588.
27. Braun, V. & Rehn, K. (1969) *Eur. J. Biochem.* **10,** 426-438.
28. Osborn, M. J., Gander, J. E. & Parisi, E. (1972) *J. Biol. Chem.* **247,** 3973-3986.