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# A Surface Polysaccharide of *Escherichia coli* O111 Contains O-Antigen and Inhibits Agglutination of Cells by O-Antiserum

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The repeating pentasaccharide of O-antigen from *Escherichia coli* O111 contains galactose, glucose, *N*-acetylglucosamine, and colitose, the latter representing the major antigenic determinant. Phenol extraction of this strain was previously shown to release two fractions (I and II) containing O-antigen carbohydrate, and both fractions were believed to be lipopolysaccharide. We have now characterized fractions I and II and conclude that only fraction II represents lipopolysaccharide. Fraction II contains phosphate, 2-keto-3-deoxyoctonate, β-hydroxymyristic acid, and potent endotoxin activity, whereas fraction I was deficient in all of these properties of the lipid A and core oligosaccharide regions of lipopolysaccharide. Fractions I and II each represented 50% of the total cellular O-antigen, and both were present on the cell surface. Both fractions were metabolically stable, and no precursor-product relationship existed between them. Fraction II had a number-average molecular weight of 15,800, corresponding to an average of 12 O-antigen repeats per molecule. In contrast, fraction I had a number-average molecular weight of 354,000, corresponding to an average of 404 O-antigen repeats per molecule. Before heat treatment, cells of *E. coli* O111 are poorly agglutinated by O-serum; although this indicates the presence of a capsule, the corresponding K-antigen was never detected. We conclude that fraction I, when present on the cell surface, inhibits agglutination of unheated cultures of *E. coli* O111 by O-serum because: (i) a variant strain which lacks fraction I was agglutinated by O-serum without prior heating; (ii) erythrocytes coated with purified fraction I behaved like bacteria containing fraction I in showing inhibition of O-serum agglutination; and (iii) heat treatment released fraction I and rendered bacterial cells agglutinable in O-serum.

Polysaccharide and lipopolysaccharide (LPS) are often present together as antigenic determinants on the surface of gram-negative bacteria (15, 32, 33). K-antigens (now defined as capsular polysaccharides; 32) and O-antigens (polysaccharide usually covalently linked to lipid A-core oligosaccharide, thus forming LPS) were originally defined by Kauffmann and Vahlne (19) by using bacterial agglutination reactions. Most polysaccharide K-antigens are extracellular components loosely bound to the bacterial surface by noncovalent interactions, although in some cases they are found covalently linked to lipid A (15, 33). O-antigen covalently linked to lipid A-core oligosaccharide (i.e., LPS) is firmly bound to the cell surface because lipid A is itself an integral component of the outer membrane.

O-antigens that are not linked to lipid A have sometimes been called haptens. Some are found linked to the isoprenoid carrier lipid as an intermediate in LPS biosynthesis (21, 22) and have been shown to accumulate in mutants which are unable to transfer O-antigen to lipid A-core oligosaccharide (20). In strains without such mutations, haptens can also accumulate as pure O-antigen lacking covalently attached lipid. Thus, one half of the O-antigen of *Escherichia coli* O100 was described as a hapten which was believed to represent a byproduct of overproduced O-antigen (13). Similarly, one half of the O-antigen of *E. coli* O113 was described as a

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hapten, but it was believed to represent a cytoplasmic precursor of LPS (2).

*E. coli* serotypes O100, O111, and O113 are characterized as inagglutinable in O-antiserum unless first heated at 100°C. This is usually the criterion for the presence of a capsule, but no K-antigens have been found in these strains (18, 32, 33; G. T. Barry and T. Tsai, Fed. Proc. 22:206, 1963). In view of the importance of bacterial surface structures both in bacterial physiology and in the host-pathogen relationship, we felt it necessary to thoroughly characterize the polysaccharide surface antigens of such bacterial strains. We have examined the surface polysaccharides of four independent clinical isolates of *E. coli* group O111. We now report that 50% of the O-antigen from three of these strains is not covalently linked to the lipid A-core oligosaccharide characteristic of LPS. This fraction of O-antigen is not a cytoplasmic component, nor is it a precursor to LPS as was previously believed (2). When present on the cell surface, it inhibits O-serum agglutination, as shown both by immunochemical analysis of purified fractions I and II and by the fact that the single strain lacking this fraction of O-antigen is agglutinated by O-serum without prior heating.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Four independent clinical isolates of *E. coli* group O111 were studied. Strain CL99, which lacks UDP-glucose epimerase (8) and has been cured of a lysogenic phage (5), was derived from *E. coli* O111 strain J5 originally obtained from E. Heath (Department of Biochemistry, University of Iowa College of Medicine, Iowa City). When grown in the presence of galactose, this strain synthesizes O-antigen identical to its parent clinical isolate (8). *E. coli* group O111 strains 12015 and 29552 were obtained from the American Type Culture Collection, Rockville, Md. The test strain for group O111, designated Stoke W, was obtained from the Centers for Disease Control, Atlanta, Ga. Cells were grown in defined medium at 37°C as previously described (9), except for the substitution of phosphate buffer (7.08 g of $Na_2HPO_4$ and 13.6 g of $KH_2PO_4$ per liter, adjusted to pH 7.4 with 5 M KOH) for Tris-hydrochloride buffer.

**Preparation of total membranes and purification of outer membranes.** The total membrane fraction was prepared by three passages of cells through a French press at 16,000 lb/in$^2$, followed by centrifugation as described (23). Outer membranes were purified from the French press lysate by density gradient centrifugation in sucrose (9) or by the previously described method using lysozyme and EDTA (34).

**Extraction of cells and subcellular fractions.** Cells or subcellular fractions were extracted with aqueous phenol (40), EDTA (27), phosphate-buffered saline (PBS) (pH 7.4) at 100°C, or aqueous *n*-butyl alcohol (29). Phenol, EDTA, PBS, and *n*-butyl alcohol were removed from extracts by chromatography on Sephadex G-25 (PD-10 columns, Pharmacia) previously equilibrated with distilled water or buffers as indicated. Material recovered in the column void volume was retained for further study.

**Fractionation of cell lysates and extracts.** The lysate generated by passage of cells through a French press was centrifuged at 113,000 × *g* for 2 h, yielding a membrane pellet and supernatant; both were extracted with phenol. Phenol extracts of cells or subcellular components were fractionated by isopycnic density gradient centrifugation in cesium chloride (29), chromatography on Sepharose 4B in the presence of 1% (wt/vol) sodium dodecyl sulfate (SDS) (29), polyacrylamide gel electrophoresis in SDS (9), or isoelectric focusing in polyacrylamide gels (9).

**Analytical procedures.** Quantitative assays for phosphate (1) and colitose (25) were previously described. 2-Keto-3-deoxyoctonate was quantitated by using a modification of the thiobarbituric acid assay (6). This involved hydrolysis of polysaccharide for 5 min at 95°C in $10^{-3}$ N $H_2SO_4$ and periodate oxidation at 2°C for 90 min. Under these conditions 1 nmol of a 2-keto-3-deoxyoctonate standard could be detected in the presence of 2,200 nmol of colitose linked to O-antigen in fraction I. RNA was estimated by absorption at 258 nm with *E. coli* RNA as a standard. The *Limulus* and rabbit pyrogen assays for endotoxin were performed by the Division of Control Activities, Bureau of Biologics, Food and Drug Administration, Rockville, Md., as previously described (12).

**Analysis of β-hydroxymyristic acid.** Each sample (+100 nmol of 18:0 internal standard) was incubated with 4 N KOH (0.5 ml) at 100°C for 5 h in a sealed ampoule. After cooling, the samples were acidified with 1 ml of 3 N HCl and extracted three times with 2.0 ml of diethyl ether. The ether extracts were evaporated to dryness. Each sample was dissolved in 1.0 ml of $BF_3$/MeOH and incubated at 70°C for 20 min to form methyl esters of the fatty acids. The samples were subsequently handled as previously described (36) and analyzed on 6-ft (ca. 1.83-m) glass columns packed with 15% HI-EFF1BP on 100/120 Gas Chrom Q (Applied Science Laboratories), employing a Hewlett-Packard 5840A gas chromatograph.

**Radiochemical methods.** Cells were grown in the presence of 0.1 mM galactose plus [$^3$H]galactose (NET-201; New England Nuclear Corp.) at a final specific activity of 0.17 Ci/mmol. Since *E. coli* CL99 contains a mutation in UDP-glucose epimerase, galactose is only incorporated into polysaccharides containing galactose (26). Protein was labeled by growing cells in 0.5 mM leucine plus [$^{14}$C]leucine (NET-279) at a specific activity of 0.44 Ci/mmol.

**Acetylation of fraction I.** Fraction I (16 mg) was dissolved in 2 ml of formamide-pyridine (1:2). Acetic anhydride (0.6 ml) was added dropwise, and the solution was stirred for 6 h at room temperature. The acetylated material was precipitated with 3 to 4 ml of cold water with cooling at 4°C and dialyzed against water (2 liters, changed two times) over 48 h. Acetylated fraction I was then lyophilized, yielding 22.5 mg. The acetyl content was 40.32% (wt/wt), determined as benzyl acetate by gas chromatography (4) after drying at 80°C in a vacuum oven.

**Molecular weight determination of fraction I.** Molecular weights were determined by equilibrium experiments in a Beckman model E analytical ultracentrifuge

by using a multiplexed He-Ne laser with the Rayleigh interference optical system. The interference patterns were recorded on Kodak 3414 high-resolution aerial photography film and were measured with a Nikon 6-C comparator equipped with digital lead screws and an Equalite fringe center detector. The chemical nature of the material made simple halogenated organic compounds the solvents of choice, thus requiring the use of aluminum double-sector centerpieces. It was determined that dichloromethane ($\rho_{20}$ = 1.3266 g/cm$^3$) and trichloromethane ($\rho_{20}$ = 1.4832 g/cm$^3$) were optimal, since sedimentation equilibrium occurred in the former and floatation equilibrium occurred in the latter. The fact that single component solvents were used precluded any difficulties arising from preferential solvent component interactions and made it possible to obtain both molecular weights and the partial specific volume from the concentration distributions in the two solvents (28).

The experiments were conducted at a temperature of 20°C and at rotor speeds from 12,000 to 20,000 rpm. These speeds were selected so that, depending on the solvent, either the meniscus or the bottom was depleted, permitting ready calculation of the apparent reduced number-average molecular weight as a function of position in the cell (41). The reduced molecular weight, $M' = M(1 - \bar{\nu}\rho)$, was used since the value of $\bar{\nu}$ has to be determined from the values of $M'$ in the two solvents. Apparent reduced cell number-average molecular weights were calculated using the equation (39):

$$M'_{n,\text{cell}} = \int_{r_m}^{r_b} r\,c\,dr \Big/ \int_{r_m}^{r_b} (r\,c/M'_{n,r})dr \qquad (1)$$

where

$$M'_{n,r} = c_r/(\omega^2/RT) \int_{r_m}^{r} r\,c\,dr \qquad (2)$$

It should be noted that for floatation equilibrium, the lower limit of integration in the last equation will be $r_b$ rather than $r_m$ and that the values of $M'$ will be negative. The values of the apparent reduced cell weight-average molecular weights are given by the apparent reduced number-average molecular weight at the bottom for sedimentation equilibrium and at the meniscus for sedimentation floatation (41). It is essential that the meniscus or bottom be at essentially zero concentration for equation 2 to be valid and to have this relationship be correct.

Pseudo-ideality was assumed because of the very low solute concentrations used. The reduced apparent molecular weights were plotted as functions of the square of rotor speed and extrapolated to zero speed to eliminate rotor speed effects on distribution. The reduced molecular weights obtained for each solvent were then used to calculate the weight-average and number-average molecular weights and the partial specific volume.

**Antiserum and agglutination of bacterial cells.** O- and OK-antisera were produced in rabbits, and bacteria were tested for serum agglutination according to standard procedures (32).

**Binding of O-antigen to sheep erythrocytes.** Sheep erythrocytes were collected by centrifugation at 2,000 × *g* for 6 min, washed three times in PBS, and finally suspended in 10 volumes of PBS. Erythrocytes (1.5 ml of the 10% erythrocyte suspension) were mixed with 1.5 ml of O-antigen solution (1,000, 100, or 10 μg/ml, heated at 100°C for 1 h or unheated). The mixture was incubated in a water bath at 37°C for 1 h and then centrifuged for 6 min at 2,000 × *g*, followed by two washings (6 min, 2,000 × *g*) in PBS. The final pellet was suspended in 15 ml of PBS, as a 1% erythrocyte suspension.

**Hemagglutination test.** Twofold dilutions of antisera in saline were prepared, and 25 μl of each dilution was added to 25 μl of coated erythrocytes. After mixing, samples were incubated at 37°C for 30 min, followed by 3 h at room temperature, and then examined for agglutination.

**Immunoelectrophoresis.** Immunoelectrophoresis was carried out by the method of Scheidegger (37). Agarose HSA ("Litex"; Glostrup, Denmark) and Veronal buffer (pH 8.6) were used. In some experiments 1% (wt/vol) Cetavlon (hexadecyl trimethylammonium bromide) was included in the agarose.

## RESULTS

**Fractionation and purification of two polysaccharide components.** Previous work from this laboratory (29) had shown that phenol extraction of *E. coli* O111 strain 12015 yields two polysaccharide fractions separable by density gradient centrifugation. Both fractions contained colitose, glucose, *N*-acetylglucosamine, and galactose, sugars characteristic of the O-antigen of this strain (29). Fraction I, with a density of 1.36 g/cm$^3$, and fraction II, with a density of 1.44 g/cm$^3$, both contained lipid A and core oligosaccharide, although at very low levels in fraction I. Thus both fractions were believed to represent LPS. Similar results were obtained with *E. coli* O111 strain CL99 when grown in the presence of galactose (D. C. Morrison and L. Leive, unpublished data).

We have been studying surface components of strain CL99 grown in the presence of galactose (9, 10). In the present work, we found that fraction I prepared from this strain by phenol extraction and density gradient centrifugation in CsCl (Fig. 1A) was contaminated 5 to 10% with fraction II. Thus, if fraction I obtained from the gradient shown in Fig. 1A was reextracted with phenol, 5 to 10% of the [$^3$H]galactose banded with a density of 1.44 g/cm$^3$, as expected for fraction II (Fig. 1B). In contrast, fraction II did not yield any fraction I when reextracted with phenol (data not shown).

The presence of fraction II in fraction I was confirmed by polyacrylamide gel electrophoresis in SDS (Fig. 2). Fraction II from the CsCl gradient shown in Fig. 1A separated as multiple components, which we have previously shown

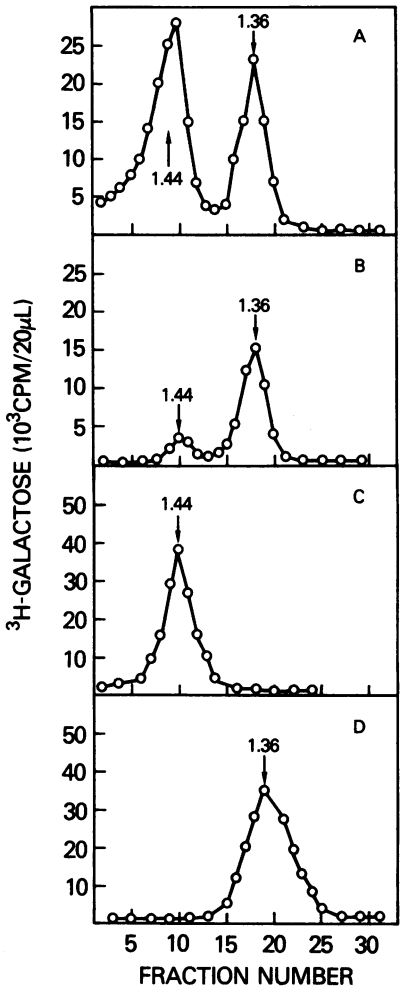


FIG. 1. Isopycnic density gradient centrifugation of fractions I and II. Samples were extracted with phenol and passed through a column of Sephadex G-25 equilibrated with 0.12 M Tris-hydrochloride (pH 8.1). Fractions I and II were recovered in the column void volume free from phenol. Cesium chloride was added to 1.40 g/cm$^3$, and centrifugation at 4°C was for 48 h in an SW50.1 rotor at 42,000 rpm. (A) Phenol extract of cells. (B) Phenol extract of fraction I (from [A] above, fractions 17 to 21). (C) Phenol extract of fraction II from the outer membranes prepared as shown in Fig. 3. (D) Phenol extract of fraction I from the top of the sucrose gradient shown in Fig. 3.

(9) to differ in the number of O-antigen repeating units per molecule of lipid A (Fig. 2, lanes A and B). Equal amounts of [$^3$H]galactose from fractions I and II were applied to the SDS gels (Fig. 2), but only 5 to 10% of the radioactivity from fraction I was recovered in the gel (Fig. 2, lanes C and D; determined by densitometry), and this material separated into multiple components similar to fraction II. Approximately 90% of the [$^3$H]galactose in fraction I did not enter the gel, but could be recovered in the gel sample well after electrophoresis. Thus, unlike true LPS, fraction I behaved as a neutral polysaccharide and did not migrate during electrophoresis.

**Separation and purification of fractions I and II from cell lysates.** Disruption of bacteria by passage through a French press resulted in soluble fraction I and membrane-associated fraction II. Thus, fractionation of the lysate by density gradient centrifugation in sucrose yielded an outer membrane fraction containing 50% of the

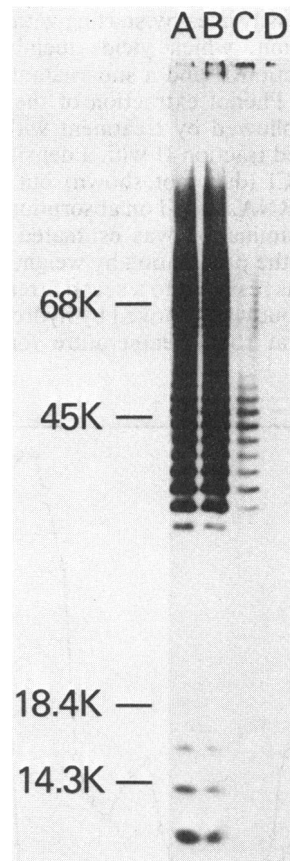


FIG. 2. Polyacrylamide gel electrophoresis of fractions I and II after density gradient centrifugation in cesium chloride. Samples from the cesium chloride gradient shown in Fig. 1A were desalted on Sephadex G-25 equilibrated with distilled water and lyophilized. Samples were run on a 7.5 to 20% gradient gel of polyacrylamide containing SDS. [$^3$H]galactose was detected by fluorographic exposure to X-ray film. Samples from Fig. 1A, fractions: (A) 5 to 9; (B) 10 to 14; (C) 15 to 18; (D) 19 to 22. Equal amounts of tritium were applied to each lane of the gel.

[$^3$H]galactose incorporated into polysaccharide; the remaining 50% was found at the top of the gradient (Fig. 3). Inner membranes could be recovered from fractions 22 to 32 of the sucrose gradient (data not shown) and contained very little [$^3$H]galactose under these conditions. The polysaccharide associated with the outer membrane had a density of 1.44 g/cm$^3$ in CsCl (Fig. 1C), showing it to be fraction II, whereas the polysaccharide from the top of the sucrose gradient had a density of 1.36 g/cm$^3$ in CsCl (Fig. 1D), showing it to be fraction I. These two fractions thus correspond to the two fractions extracted directly from cells with phenol (reference 29 and Fig. 1A).

Fractions I and II can also be separated from French press lysates by starting with differential centrifugation, which yields membranes containing fraction II and a supernatant containing fraction I. Phenol extraction of the membrane fraction, followed by treatment with RNase A (25), yielded fraction II with a density of 1.44 g/cm$^3$ in CsCl (data not shown) but which still contained RNA. Based on absorption at 258 nm, RNA contamination was estimated to account for 20% of the preparation by weight. This RNA fraction was resistant to a second treatment with RNase A, but was removed by hydrolysis with 1 N NaOH at room temperature for 17 h and dialysis (no absorption peak remaining at 258 nm). Since treatment with alkali also removes fatty acids, this step was only used in purification of material for phosphate analysis. Phenol extraction of the supernatant fraction, followed by treatment with RNase A, yielded fraction I with a density of 1.36 g/cm$^3$ in CsCl (data not shown). Nucleic acid contamination was 11% by weight (based on absorbance at 258 nm), and only 56% of this was removed by base hydrolysis. Most of the remaining nucleic acid was removed by chromatography on Sepharose 4B in the presence of SDS. Fraction I eluted in the void volume, and nucleic acid was included in the column matrix (data not shown).

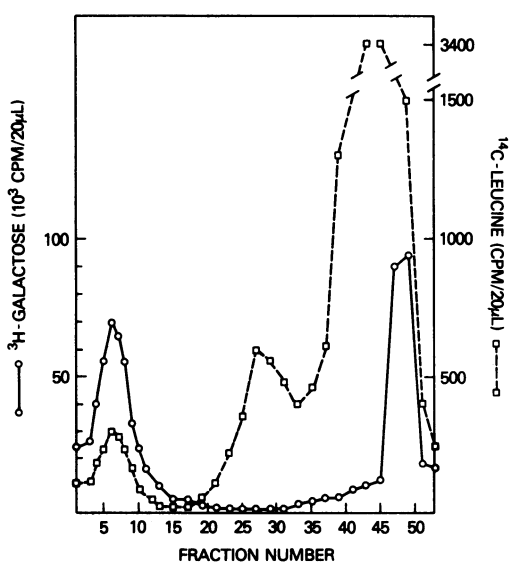


FIG. 3. Fractionation of the French press lysate by density gradient centrifugation. Cells, labeled with both [$^3$H]galactose and [$^{14}$C]leucine, were lysed by three passages through a French press. The lysate was applied to a step gradient of sucrose (6 ml each of 55, 50, 45, 40, 35, and 30% [wt/vol] sucrose), and centrifugation was for 24 h at 4°C in an SW27 rotor at 25,000 rpm. Fractions 3 to 13 contain the outer membranes.

**Molecular weight of fraction I.** A previous report from this laboratory gave an approximate value of $1.5 \times 10^6$ as the molecular weight of native fraction I, and this number was not decreased by SDS or Triton X-100 (29). In an attempt to derive a more accurate molecular weight with minimal effect of inter- and intramolecular interactions, fraction I was acetylated and analyzed by ultracentrifugation in organic solvents. A weight-average molecular weight of 718,000, a number-average molecular weight of 528,000, and a partial specific volume of 0.728 cm$^3$/g were obtained by using the reduced weight-average and number-average molecular weights obtained in dichloromethane and in trichloromethane. The value of 1.36 for the ratio of the weight-average molecular weight to the number-average molecular weight indicates significant polydispersity. When corrected for the weight percent O-acetyl content (40.32%), native fraction I was calculated to have a weight-average molecular weight of 481,000 and a number-average molecular weight of 354,000.

**Endotoxin activity and chemical characterization of fractions I and II.** Fractions I and II were both previously shown to contain carbohydrates characteristic of the O-antigen of serotype O111; however, fraction I was much less toxic to mice and contained less lipid A than fraction II (29). We have now used the *Limulus* and rabbit pyrogen assays, as well as chemical techniques, to show that fraction I does not represent normal LPS as does fraction II.

Endotoxin activity was monitored during preparation of fractions I and II (Table 1). The initial endotoxin activity of fraction I, prepared by fractionation of the French press lysate of cells, is 1% of fraction II on a weight basis, and this value declined to 0.1% after further purification on Sepharose 4B. Fraction I contained 4% of the activity of fraction II on a weight basis when assayed after direct phenol extraction of cells and fractionation by density gradient centrifugation. This value decreased to 0.1% after reextraction of the fraction I peak with phenol

TABLE 1. Endotoxin activity of fractions I and II during purification[a]

| Fraction | Stage of purification | Endotoxin activity[b] (μg/mg) |
|---|---|---|
| II | Phenol extract of pellet from French press lysate | 900 |
| I | Phenol extract of supernatant from French press lysate | 10 |
| I | Fraction I above after chromatography on Sepharose 4B | 1 |
| II | CsCl gradient of phenol extract of cells | 1,000 |
| II | CsCl gradient of phenol extract of fraction II above | 500 |
| I | CsCl gradient of phenol extract of cells | 40 |
| I | CsCl gradient of phenol extract of fraction I above | 1 |

[a] Endotoxin activity was quantitated by using serial 10-fold dilutions in the *Limulus* assay.

[b] Values are given as equivalents of the endotoxin standard EC-2.

TABLE 2. Chemical analysis of fractions I and II

| Fraction | Content (nmol/mg) | | | |
|---|---|---|---|---|
| | Colitose | Phosphate | 2-Keto-3-deoxyoctonate | β-Hydroxymyristic acid |
| II[a] | 1,400 | 470 | 40 | 180 |
| I[b] | 2,200 | 20 | <1 | 3 |

[a] Fraction II was prepared by phenol extraction of total membranes prepared after lysis of cells with the French press. Colitose, phosphate, and 2-keto-3-deoxyoctonate were assayed after removal of residual RNA by base hydrolysis. β-Hydroxymyristic acid was assayed before base hydrolysis.

[b] Fraction I was prepared by phenol extraction of the supernatant prepared after lysis of cells with the French press. RNA was removed by chromatography on Sepharose 4B.

and isolation by a second density gradient centrifugation. There was no significant decrease in the activity of fraction II after identical treatment. The loss of endotoxin activity during purification was confirmed by the rabbit pyrogen assay (data not shown).

We assayed our purest preparations of fractions I and II for colitose, phosphate, 2-keto-3-deoxyoctonate, and β-hydroxymyristic acid (Table 2). Fractions I and II contained 32 and 20% colitose by weight, respectively. Fraction II contained phosphate, 2-keto-3-deoxyoctonate, and β-hydroxymyristic acid, which are markers for lipid A and core oligosaccharide; fraction I was deficient in these components (Table 2).

**Isoelectric focusing of fractions I and II.** Fraction II migrated during polyacrylamide gel electrophoresis in SDS, separating into multiple components; fraction I, as shown above, appeared neutral under these conditions. In contrast, we found that fraction II appeared homogeneous when analyzed by isoelectric focusing in polyacrylamide gels, giving a component of approximate pI 6.6 (Fig. 4, lane A). Fraction I entered the gel during isoelectric focusing, but ceased to migrate at a pH below 10 (Fig. 4, lane B). This fraction I preparation was contaminated with 5 to 10% of fraction II, which appeared as a band at pH 6.6 (Fig. 4, lane B).

**Release of fractions I and II from cells and their cellular location.** Release of fractions I and II from cells labeled with [$^3$H]galactose was quantitated under various conditions. When cells were disrupted with a French press, fraction II remained attached to the outer membrane, whereas fraction I was released as a soluble component (Fig. 3); however, when cells were disrupted by lysozyme-EDTA treatment followed by osmotic lysis (34), 50 to 75% of fraction I and >95% of fraction II purified with outer membranes (data not shown). These results indicate that both fractions are associated with the outer membrane, but that fraction I is more

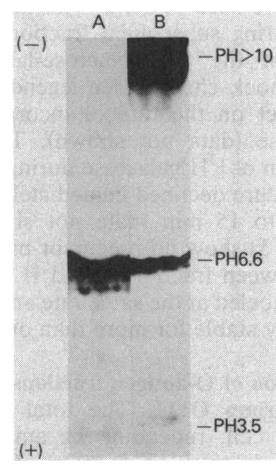


FIG. 4. Isoelectric focusing of fractions I and II. Cells were labeled with [$^3$H]galactose and passed three times through a French press, and the lysate was applied to a sucrose gradient (see Fig. 1). Fractions I and II were recovered from the top of the sucrose gradient and outer membrane fraction, respectively, and, after removal of sucrose by column chromatography on Sephadex G-25, both fractions were extracted with phenol. Phenol was removed before lyophilization and subsequent electrophoresis. (A) Fraction I; (B) fraction II. The isoelectric focusing gel contained 9 M urea, 2% (wt/vol) Nonidet P-40, and 2% ampholines (pH 3.5 to 10). Electrophoresis was at 400 V for 18 h, followed by 500 V for 1.5 h. [$^3$H]galactose was detected by fluorographic exposure to X-ray film.

loosely attached. Treatment of cells with EDTA released 50% of both fractions I and II and only 4 to 8% of cellular β-galactosidase. Since cytoplasmic components are not usually released by EDTA treatment (30), these data confirm that both fractions are located on the cell surface.

Heating cells in PBS at 100°C for 1 h released greater than 95% of fraction I, whereas less than 15% of fraction II was released; release was virtually complete within 2 min. However, neither fraction was released by 1 M NaCl, repeated water washing, or 25 passages of cells through a syringe fitted with a 25 gauge needle. Since these last three procedures usually release capsular material (15), fraction I, though more loosely bound to the outer membrane than LPS, is not as easily removed as a true capsule.

**Stability of O-antigen fractions I and II.** We performed the following experiments to determine whether fraction I was a precursor to fraction II. The extent of labeling of fractions I and II after a 10-min exposure of cells to [$^3$H]galactose was measured, and the labeled cells were then chased with an excess of unlabeled galactose for both 15 and 60 min to determine the stability of these two fractions. Cells labeled continuously with [$^{14}$C]galactose, but otherwise treated identically to the [$^3$H]galactose-labeled cultures, were used to control for any losses or variation during subsequent fractionation. Manipulation of the [$^{14}$C]galactose-labeled cells during a "mock chase" (see legend to Fig. 4) had no effect on the rate of incorporation of [$^{14}$C]galactose (data not shown). The rate of incorporation of [$^3$H]galactose during the actual chase procedure declined immediately, reaching zero at 10 to 15 min (data not shown). The results (Fig. 5) show no precursor-product relationship between fractions I and II. Both fractions were labeled at the same rate and appeared metabolically stable for more than one cell doubling time.

**Quantitation of O-antigen fractions I and II in strains of group O111.** The total amount of colitose per cell, reflecting the amount of O-antigen, was determined. Samples of cells ($10^{10}$) of each strain contained 6.8 nmol of colitose (estimated standard deviation, $\bar{\sigma}$, of ±0.6 nmol of colitose). Cells were also extracted with phenol, and the extract was subjected to isopycnic density gradient centrifugation in CsCl to separate O-antigen fractions I and II (Table 3). Strains Stoke W, CL99, and 12015 all yielded the same amount of colitose, and thus O-antigen, in fractions I and II. In contrast, strain 29552 lacked detectable fraction I by this method and contained twice the amount of O-antigen in fraction II as the other three strains.

**Agglutination of cells by O- and OK-antisera.** *E. coli* O111, strain Stoke W, is the test strain for group O111. As expected, unheated cells of this strain were poorly agglutinated by O-serum but became agglutinable after heat treatment; in addition, OK-serum agglutinated unheated cells

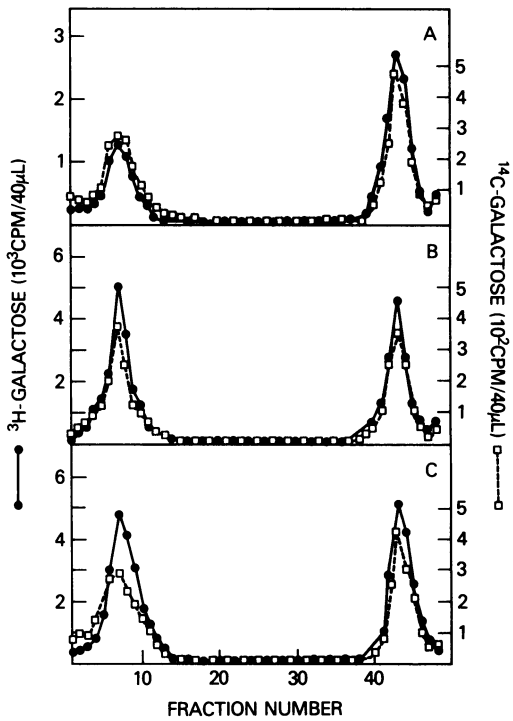


FIG. 5. Pulse-labeling of fractions I and II and their stability during a chase. One culture of *E. coli* O111 (100 ml) was continuously labeled for several generations in medium containing [$^{14}$C]galactose (0.65 μCi/ml, representing 0.1 mM galactose). At time zero, 25 ml was harvested by centrifugation, washed with 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-NaOH buffer, pH 7.4 (buffer A), and frozen. At the same time, the remaining 75 ml was pelleted and resuspended in the supernatant medium to effect a "mock chase." At 15 and 60 min after resuspension, 25-ml portions were harvested by centrifugation, washed in buffer A, and frozen. A second culture (150 ml) was labeled at time zero with [$^3$H]galactose (10 μCi/ml plus carrier galactose in the medium to equal 0.1 mM). At 10 min, 50 ml of culture was harvested, washed, and frozen. The remaining 100 ml was harvested by centrifugation, suspended in prewarmed medium containing 0.1 mM unlabeled galactose, and returned to the water bath shaker. At 15 and 60 min after resuspension, 50-ml samples were harvested, washed, and frozen. The cells labeled with [$^3$H]galactose (pulse-labeled and 15- and 60-min pulse-chased) were mixed with the analogous [$^{14}$C]galactose cells (identical manipulation but no chase) at a $^3$H$^{14}$C ratio of 10:1. Each sample was lysed with the French press, applied to a sucrose step gradient, and centrifuged for 48 h at 25,000 rpm in an SW27 rotor. Gradient fractions were collected and counted. (A) 10-min pulse; (B) 15-min chase; (C) 60-min chase.

TABLE 3. Quantitation of O-antigen fractions I and II in strains of *E. coli* group O111[a]

| Strain | % Total O-antigen as: | |
|---|---|---|
| | Fraction I | Fraction II |
| Stoke W | 54 | 46 |
| CL99 | 56 | 44 |
| 12015 | 54 | 46 |
| 29552 | <0.5 | >99.5 |

[a] Values were determined by assaying colitose in fractions I and II after their extraction from cells with phenol and separation in CsCl.

of this strain better than O-serum (Table 4). Identical results were obtained for strains CL99 and 12015 (Table 4).

In contrast, unheated cells of strain 29552 were agglutinated by O-serum; indeed, either heated or unheated cells gave identical high titers with O- or OK-sera (Table 4). These titers were the same as observed when O-serum was used to agglutinate heated cells of the other three strains (Table 4). Since only strain 29552 lacked chemically detectable O-antigen fraction I, these data indicate that fraction I inhibits O-serum from agglutinating unheated cells of strains 12015, CL99, and Stoke W.

**Agglutination of erythrocytes coated with O-antigen fractions I and II.** The role of fraction I in inhibiting agglutination of cells by O-serum was further tested by coating erythrocytes with purified fraction I or II and measuring O- and OK-serum titers in subsequent agglutination reactions. O-serum titers were significantly lower than OK-serum titers when erythrocytes coated with suboptimal concentrations of fraction I and, to a lesser extent, those coated with suboptimal amounts of fraction II were tested in agglutination reactions (Table 5). This result was most dramatic when unheated fraction I was used for coating (Table 5) and mimicked the results of O- and OK-serum agglutination of unheated bacterial cells which contained fraction I (Table 4).

TABLE 4. O- and OK-serum agglutination of unheated and heated cells

| Strain | Treatment[a] | Titer[b] | |
|---|---|---|---|
| | | O-serum | OK-serum |
| Stoke W | Unheated | 8 | 160 |
| | Heated | 1,280 | ND |
| CL99 | Unheated | 8 | 160 |
| | Heated | 1,280 | ND |
| 12015 | Unheated | 8 | 80 |
| | Heated | 1,280 | ND |
| 29552 | Unheated | 1,024 | 1,280 |
| | Heated | 1,280 | ND |

[a] Cells were heated at 100°C in PBS for 30 min.
[b] Reciprocal value of highest serum dilution causing agglutination. ND, Not determined.

TABLE 5. Agglutination of erythrocytes coated with fractions I and II

| Antigen | Amt used to coat erythrocytes (mg/ml) | Titer[a] | |
|---|---|---|---|
| | | O-serum | OK-serum |
| Fraction I, unheated | 1.0 | 5,120 | 20,480 |
| | 0.1 | 40 | 1,280 |
| | 0.01 | 0 | 0 |
| Fraction I, heated, 100°C | 1.0 | 10,240 | 10,240 |
| | 0.1 | 10,240 | 10,240 |
| | 0.01 | 640 | 10,240 |
| Fraction II, unheated | 1.0 | 5,120 | 10,240 |
| | 0.1 | 5,120 | 10,240 |
| | 0.01 | 160 | 2,560 |
| Fraction II, heated, 100°C | 1.0 | 5,120 | 5,120 |
| | 0.1 | 10,240 | 5,120 |
| | 0.01 | 5,120 | 5,120 |

[a] Reciprocal value of highest serum dilution giving hemagglutination.

There is one published report of reproducing the inability of bacterial cells to be agglutinated by O-serum by using successive application of O-antigen, and then capsule polysaccharide, on the surface of erythrocytes (24). Our attempts to show O-serum inagglutination of erythrocytes by successive application of O-antigen fraction II followed by fraction I have been unsuccessful (data not shown).

Fractions I and II were tested as inhibitors of hemagglutination, using erythrocytes coated with homologous or heterologous fractions (Table 6). The only remarkable result was the small amount of fraction I required to inhibit agglutination of fraction I-coated erythrocytes with OK-serum. This indicates that fraction I binds antibody molecules in the OK-serum which are important for the agglutination of fraction I-coated erythrocytes; these antibodies do not significantly take part in agglutination in O-serum.

**Immunoelectrophoresis.** Fractions I and II were further characterized by immunoelectrophoresis against OK-serum. In the absence of detergent, both fractions I and II gave single precipitin lines located slightly towards the cathode (data not shown). In the presence of the detergent Cetavlon, fraction II moved further towards the cathode, while fraction I moved slightly towards the anode, except for a minor component migrating like fraction II (Fig. 6). Identity between this minor component and fraction II was shown by cutting the antiserum

TABLE 6. Inhibition of hemagglutination with fractions I and II[a]

| Serum, dilution | Fraction used as inhibitor | Concn[b] (μg/ml) of inhibitor preventing agglutination of erythrocytes coated with 1 mg/ml of: | |
|---|---|---|---|
| | | Fraction I[b] | Fraction II[b] |
| OK-serum, 1:1,280 | I | 0.03 | 0.16 |
| OK-serum, 1:1,280 | II | 0.31 | 0.63 |
| O-serum, 1:1,280 | I | 0.16 | 0.31 |
| O-serum, 1:1,280 | II | 0.16 | 0.31 |

[a] Serum (25 μl of a 1:1,280 dilution in PBS) was mixed with twofold dilutions of fractions I and II in round-bottomed, plastic microtiter plates (Nunc, Denmark) and incubated at 37°C for 30 min on a Thomas rotator. Erythrocytes (50 μl of a 1% suspension coated with antigen) were added, and agglutination was scored after incubation at 37°C for 30 min.

[b] Least concentration necessary to give inhibition of hemagglutination.

dish short at the cathode and observing fusion of the precipitin lines (data not shown). The fraction I preparation used was contaminated by 1% with fraction II, based on the *Limulus* and rabbit pyrogen assays (see above), and the immunochemical detection of a minor fraction II contaminant in this fraction I preparation is consistent with these data.

Although fractions I and II reacted with OK-serum as described above, O-serum was deficient in antibodies capable of precipitating fraction I. Thus, both in double diffusion and in immunoelectrophoresis, only weak precipitin reactions were observed when fraction I was tested with O-serum, compared to the strong reaction observed with fraction II (data not shown).

The standard method of extracting O- and OK-antigens for immunoelectrophoresis consists of heating cells at 60°C in PBS (32, 33). Such an extract of *E. coli* O111, strain Stoke W, yielded precipitin lines identical to those obtained with fractions I and II after immunoelectrophoresis against OK-serum (Fig. 6A and B, respectively), showing that these molecules are the antigens responsible for the OK-serum reactivities of this organism. Identical results were obtained by using extracts of strains CL99 and 12015 (data not shown).

In contrast, the 60°C extract of strain 29552 lacked a precipitin line corresponding to fraction I when tested in immunoelectrophoresis (Fig. 6C). Thus three strains (Stoke W, CL99, and 12015), which are inagglutinable in O-serum before heating, contain chemically and immunochemically detectable O-antigen fraction I, whereas strain 29552, which is agglutinable in O-serum before heating, does not.

## DISCUSSION

Fraction II described in this report has the structure of a classical LPS, the O-antigen carbohydrate being covalently linked to lipid A via core oligosaccharide. This fraction separates, during gel electrophoresis in SDS, into multiple components based on the number of repeats of O-antigen units per molecule of lipid A (9), and we have calculated an average number of 12 O-antigen repeats per molecule of lipid A based on these data. The chemical composition of the core (16, 17) and antigenic side chain (K. Eklind, P. J. Garegg, L. Kenne, A. A. Lindberg, and B. Lindberg, Abstr. IXth Int. Symp. Carbohydr. Chem., London, 1978, p. 493) of LPS from *E. coli* O111 is known, and based on these data and a value of 2,800 daltons for lipid A (14), we estimate an average molecular weight of 15,800 for this LPS. Since there are two colitose residues in each O-antigen repeat, the theoretical weight percent colitose would be 22%. Chemical analysis of fraction II gave a value of 20% colitose by weight. Fraction II also contained potent endotoxin activity in the *Limulus* and

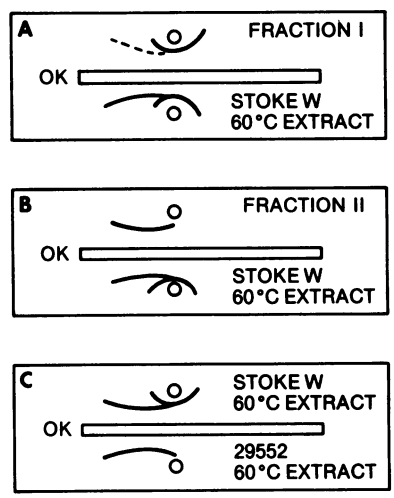


FIG. 6. Immunoelectrophoresis of fractions I and II and the 60°C extract of cells. Cell extracts were prepared by heating suspensions ($10^{10}$ to $10^{11}$ cells per ml) at 60°C for 20 min and removing cells by centrifugation at 20,000 × *g* for 20 min. Samples (25 μl) of fraction I or II (1 mg/ml) or of the 60°C extract were placed in the wells, and OK-serum was placed in the antibody trough. Agarose contained 1% Cetavlon (wt/vol), and electrophoresis was at 150 V for 45 min. Designations above and below the antibody trough indicate which sample was in the corresponding well. (A) Extract of Stoke W compared to O-antigen fraction I; (B) extract of Stoke W compared to O-antigen fraction II; (C) extract of Stoke W compared to the extract of strain 29552.

rabbit pyrogen assays, and chemical analysis showed it to contain phosphate, 2-keto-3-deoxyoctonate, and β-hydroxymyristic acid, which are chemical markers for lipid A and core oligosaccharide. The actual molar amount of β-hydroxymyristic acid assayed was 2.8 nmol/nmol of LPS, based on our average molecular weight estimate of 15,800 for fraction II.

The only similarities between fractions I and II are the sugars characteristic of the O-side chain of *E. coli* O111 and the presence of O-antigen determinants. We show herein that other physical, chemical, and biological properties of fraction I are drastically different from those of fraction II and are inconsistent with the previous conclusion (29) that fraction I represented LPS. Endotoxin activity associated with fraction I, quantitated by the *Limulus* and rabbit pyrogen assays, declines during the purification procedure to a level of 0.1% of fraction II on a weight basis. This residual activity probably represents minor contamination with fraction II, so these data suggest the absence of biologically active lipid A. Fraction I contains less than 2.5% of the 2-keto-3-deoxyoctonate found in fraction II, suggesting the lack of core oligosaccharide. Although phosphate is present in fraction I preparations at a level of about 4% that of fraction II, we are uncertain whether it represents phosphate covalently linked to fraction I polysaccharide or phosphate derived from minor contamination (0.5% by weight) with nucleic acid. However, results of gel electrophoresis in SDS, isoelectric focusing, and immunoelectrophoresis indicate that fraction I is a neutral polysaccharide, lacking substituents (e.g., $PO_4$) capable of ionization at near physiological pH.

Fraction I has a weight-average molecular weight of 481,000 and a number-average molecular weight of 354,000. These values were determined by methods which minimize inter- and intramolecular interactions, and they suggest a marked degree of size heterogeneity in fraction I. Molecular weight values of 481,000 and 354,000 correspond to 550 and 404 repeating units of O-antigen, respectively, using a molecular weight of 878 calculated from the chemical composition of a repeating unit (7). In addition, fraction I contains 32% colitose by weight, which approaches the theoretical value of 34% for pure O-antigen units.

On the basis of the number-average molecular weight of 354,000, the amount of β-hydroxymyristic acid and 2-keto-3-deoxyoctonate in fraction I on a molar basis was 33 and <40%, respectively, of the values determined for fraction II; thus fraction I cannot represent normal LPS as does fraction II. At present we are left with two possibilities: (i) after chromatography on Sepharose 4B, fraction I is still contaminated with a small amount of fraction II, or (ii) fraction I represents extremely long polymers of O-antigen linked to incomplete lipid A core. We are continuing to investigate the precise structure of fraction I, including the possibility that it contains diacylglycerol as was recently shown for surface polysaccharides of *E. coli* K92 and meningococcal groups A, B, and C (11).

Other differences between fractions I and II also support the conclusion that fraction I is not LPS. Fraction II will separate into multiple components during polyacrylamide gel electrophoresis in SDS, whereas fraction I appears electrically neutral under these conditions (no native charge and no bound SDS). Since lipid A and core oligosaccharide represent the negatively charged portion of LPS (31), and lipid A most likely represents the SDS binding region (14), these data also suggest that fraction I lacks lipid A and core oligosaccharide. The conclusion that fraction I is not LPS is also supported by our recent finding that although both fractions I and II are released from the cell surface by extraction with aqueous *n*-butyl alcohol, only fraction II is released as a complex with outer membrane protein (10). Interactions between LPS and outer membrane protein are known to involve the lipid A and core oligosaccharide regions of LPS (3, 38).

There is no precursor-product relationship between fractions I and II, and both are metabolically stable during at least one cell doubling time. Thus, fraction I is not related to precursor, which is linked to acyl carrier lipid as an intermediate in LPS biosynthesis (21, 22). In addition, we conclude that both fractions are located on the surface of the cell because they are releasable by EDTA and associated with outer membranes which are purified after lysozyme-EDTA lysis of cells; O-antigen precursors are not located on the cell surface (20).

Several *E. coli* serotypes [O111:H2, O113:H4, O141:H4, and O100:H2, previously designed O111:K58:H2, O113:K75:H4, O141:K85:H4, and O100:K?(B):H2, respectively] are inagglutinable in O-serum unless heated; however, it was never possible to produce a pure K58, K75, or K85 antiserum by absorption, nor was it possible to detect a K-specific antigen by chemical analysis or crossed immunoelectrophoresis (18, 32, 33; Barry and Tsai, Fed. Proc. 22:206, 1963). Deletion of these and several similar K-antigen numbers was therefore proposed (32). However, all of these results can be explained if the presence of an O-antigen polysaccharide, analogous to fraction I, prevents agglutination of these strains by O-serum.

We conclude that in *E. coli* group O111 the

presence of O-antigen fraction I on the bacterial surface does inhibit agglutination by O-serum because: (i) strains which contain fraction I are poorly agglutinated by O-serum before heating; (ii) a strain which lacks fraction I is agglutinated by O-serum before heating; and (iii) O-serum titers are significantly lower than OK-serum titers when erythrocytes appropriately coated with fraction I, or bacteria containing fraction I, are tested in agglutination reactions.

The mechanism by which fraction I adheres to the cell surface is unknown. Fraction I may interact with fraction II on the surface of the bacterial cell in a manner which masks the antigenic determinants reacting with O-serum, either by steric hindrance or some other physicochemical interaction. Agglutination by O-serum would therefore be inhibited. Since fraction I is almost completely released by heating at 100°C, masking would no longer occur in heated cultures. Release of fraction I from cells by heating would also explain the differences between O- and OK-sera; release of fraction I would alter its immunogenicity, as is known to occur with K antigens (33). Strains from two other groups, O100 and O113, also contain O-antigen which is not linked to lipid A. They, like serotype O111, contain no K-antigen but are inagglutinable by O-serum before heating. The presence of these apparently analogous O-antigen fractions in serotypes O100, O111, and O113 suggests that a common mechanism may be responsible for the inability of unheated cultures to be agglutinated by O-serum.

There is little known about the conformation of O-antigen carbohydrate, but precedence for secondary and tertiary structure in other carbohydrate polymers (35) indicates that polymer structure and interactions could be involved in the adherence of fraction I to the cell surface and its ability to mask antigenic determinants.

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