INFECTION AND IMMUNITY, Dec. 1978, p. 908-918
0019-9567/78/0022-0908$02.00/0

Vol. 22, No. 3
Printed in U.S.A.

Isolation and Characterization of a High-Molecular-Weight Polysaccharide from the Slime of *Pseudomonas aeruginosa*

GERALD B. PIER,†* HAZEL F. SIDBERRY, SHANDOR ZOLYOMI, AND JERALD C. SADOFF
Department of Bacterial Diseases, Walter Reed Army Institute of Research, Washington, D.C. 20012

Received for publication 5 June 1978

A procedure is described for isolating a high-molecular-weight polysaccharide (PS) from the slime of *Pseudomonas aeruginosa* immunotype 1. The resultant material, obtained from the void volume of a Sephadex G-100 column, was composed of carbohydrate and water. No lipopolysaccharide (LPS), 2-keto-3-deoxyoctonoate, heptose, phosphate, or protein was detectable, and nucleic acid contamination was generally below 1%. The carbohydrate composition of the PS was glucose, rhamnose, galactose, arabinose, and mannose. PS had a molecular weight of between 100,000 and 350,000 and did not disaggregate when chromatographed in the presence of sodium deoxycholate. An antigen immunologically indistinguishable from PS could be obtained from LPS by either acetic acid hydrolysis and column chromatography or by allowing solutions of LPS to stand at room temperature for 3 days. Some of this LPS-associated polysaccharide eluted as the void volume of a G-100 column but differed from PS by its lack of galactose and arabinose. LPS also contained an immunodeterminant not shared with PS that was detected by its stability to dilute alkali treatment (0.1 N NaOH, 37°C, 2 h). PS was destroyed by alkali treatment. PS appeared to represent a form of LPS polysaccharide side chain that contains galactose and arabinose and is of a high molecular weight.

High-molecular-weight polysaccharide antigens isolated from outer bacterial surface layers have been shown to be effective, nontoxic vaccines for several gram-negative (2, 13) and gram-positive (3) bacterial infections. The production of an extracellular slime layer by *Pseudomonas aeruginosa* (16) suggested that this material might also yield a polysaccharide antigen with vaccine potential. Crude slime contains nucleic acids, proteins, lipopolysaccharide (LPS), a toxic glycolipoprotein (5), and polysaccharides (20, 24). Immunity induced with either crude slime, partially purified slime (1, 20), purified GLP (5), or partially purified LPS (15) has been shown to be effective in preventing death in mice challenged with live organisms. Cross-protection studies in mice led to the development of an immunotype scheme based on seven distinct LPS serotypes (11, 15). This immunotyping system is capable of classifying 90% of clinical *P. aeruginosa* isolates (4, 28).

The distribution of immunotypes among clinical isolates varies among medical centers, with no immunotype being predominant (28). We therefore began with *P. aeruginosa* immunotype 1 and sought to determine if a nontoxic, high-molecular-weight polysaccharide antigen could be isolated from its slime layer. In this report we describe the procedure developed to isolate a polysaccharide antigen from the slime layer of *P. aeruginosa,* its chemical characterization, and the serological relationship between polysaccharide and LPS.

† Present address: Channing Laboratory, Boston, MA 02115.

MATERIALS AND METHODS

Organism. *P. aeruginosa,* Fisher-Devlin immunotype 1 (no. 05139), was used throughout this study and was kindly provided by Mike Fisher of Parke-Davis Co., Detroit, Mich. The organism was maintained on Trypticase soy agar slants at 4°C, and transfers were made every two months.

Preparation of crude slime. Preparation of crude slime, based on the method of Alms and Bass (1), was as follows. (i) Solid culture: 500 ml of Trypticase soy agar with 3% glycerine in diphtheria toxin bottles was inoculated with 10 ml of a 4-h culture of *P. aeruginosa* grown in Trypticase soy broth, and incubated at 37°C for 48 to 72 h. After this growth period, 25 to 50 ml of saline was added to each bottle, and the growth was washed off with the aid of a glass scraper. The saline suspension was poured off and combined from each bottle, and the suspended cells were stirred with a magnetic bar for 3 to 24 h at 4°C. The cells were then removed by centrifugation, and the supernatant, after passage through a 0.45-μm filter, was slowly diluted with 95% ethanol with stirring at 4°C until the alcohol concentration was 80%. The resulting precipitate was collected by centrifugation, suspended in phosphate-buffered saline (PBS) of pH 7.2 (6.8 g of NaCl, 1.48 g

of Na_2HPO_4, and 0.43 g of KH_2PO_4 per liter), and designated crude slime from solid cultures. (ii) Liquid culture: material from liquid cultures was prepared by inoculating a 1-liter amount of Trypticase soy broth with 3% glycerine in 4-liter flasks with 10 ml of a 4-h Trypticase soy broth culture and incubating at 37°C for 48 to 72 h. Cells were removed by centrifugation, and the medium supernatant was concentrated to one-tenth the volume in a rotary evaporator. The pelleted cells were resuspended in PBS and dispersed by high speed in an omnimixer for 10 min. This slurry was centrifuged to remove cells, and the supernatant was added to the concentrated culture media. Ethanol (95%) was then added to a concentration of 30%, and the liquid was centrifuged to remove precipitated nucleic acids and cells. The alcohol concentration was then raised to 80%, and the resulting precipitate was collected by centrifugation. The pelleted material was redissolved in PBS and designated crude slime from liquid culture.

Purification of PS. Polysaccharide (PS) was isolated from crude slime as depicted in Fig. 1. Cetavalon (10%; cetyltrimethylammonium bromide) was added to dissolved crude slime to a final concentration of 1% and allowed to stand at room temperature for 30 min to precipitate nucleic acids. After centrifugation, 95% ethanol was added to the supernatant to a final concentration of 80%, and the resultant precipitate was collected by centrifugation and redissolved in PBS. The cetavalon and alcohol precipitation steps were then repeated one time.

After the second alcohol precipitation and centrifugation, the collected pellet was dissolved in 1% acetic acid at a concentration of approximately 5 mg/ml, and heated at 90°C for 18 h to cleave off and precipitate the lipid A portion of contaminating LPS. After cooling, the precipitated lipid was removed by centrifugation and the acetic acid supernatant was extracted 5 to 10 times with chloroform to remove residual lipid present. When there was no longer a precipitate at the interface of the aqueous and organic layers, the PS was precipitated from the aqueous layer by alcohol precipitation, collected by centrifugation, and redissolved in PBS (10 mg/ml).

Residual nucleic acid was removed by digestion with nucleases. Ribonuclease (0.05 mg/ml) was added, and the mixture was incubated at 37°C for 18 h. Deoxyribonuclease was then added to the solution at a final concentration of 0.05 mg/ml. $MgSO_4$ at a 0.1 M final concentration was also added, and incubation continued for an additional 4 h at 37°C. Remaining protein was then removed by treatment with an equal volume of 90% phenol. After vigorous mixing, the solution was cooled and maintained at 4°C for a minimum of 12 h. The aqueous and phenol layers were then separated by centrifugation and, after removal of the water layer, the PS was precipitated from it by the addition of alcohol to 80% (vol/vol) and collected by centrifugation. The PS was redissolved in PBS (5 to 7 ml) and applied to a column of Sephadex G-100 (2.6 by 100 cm) equilibrated in PBS (flow rate of 30 ml/h). The eluate was monitored at wavelengths of 206 and 254 nm. The void volume fractions were combined, and the PS was precipitated by the addition of alcohol to 80% (vol/vol). The collected pellet was redissolved in water and desalted on a column of Sephadex G-50 (1.5 by 60 cm) equilibrated in distilled water. The void volume from this column was collected and lyophilized.

Nucleic Acids Removed	Dissolve crude slime in PBS; add cetavalon to 1%, 22°C, 30 min., spin out precipitated nucleic acids; precipitate supernate with alcohol to 80%, collect and repeat cetavalon step one time.
LPS Removed	Dissolve alcohol precipitate in 1% acetic acid, pH 2.8; heat 90°C, 18 h. Cool. Spin out precipitated lipid A.
Lipids Extracted	Extract acetic acid layer 5-10 times with chloroform; precipitate aqueous layer with alcohol to 80%, collect precipitate.
Nucleic Acids Digested and Protein Removed	Redissolve in PBS, add RNase, 0.05 mg/ml, 37°C, 18 h; add DNase, 0.05 mg/ml, $MgSO_4$, 0.01M, 37 C, 4 h. Add equal volume of 90% phenol, 4 C, 2-12 h.
Purification by Gel Filtration	Separate phenol and aqueous layers by centrifugation, precipitate aqueous layer with alcohol to 80%, collect by centrifugation, redissolve in PBS, apply to G-100 column, collect and precipitate void volume, desalt, lyophilize.

FIG. 1. *Summary of PS antigen purification procedure.*

Purification of LPS. LPS was purified either by phenol-water extraction of whole cells by the method of Westphal et al. (27) or from crude slime as follows: crude slime or concentrated liquid culture supernatants were ultracentrifuged at 105,000 × *g* for 3 h, and the pellet was resuspended in PBS and ultracentrifuged again under the same conditions. The pellet was then resuspended in PBS and digested with nucleases under the same conditions as those for the PS. After deoxyribonuclease treatment, the mixture was digested with Pronase B (1 mg/ml) at 37°C for 24 h. This material was then applied to a column of Sepharose 4B (2.6 by 100 cm; flow rate, 30 ml/h), and the fractions eluting as the void volume were combined, precipitated with alcohol, collected by centrifugation, and dialyzed and lyophilized.

Isolation of lipid A and polysaccharide side chains from LPS. LPS was treated with 1% acetic acid (2.5 mg/ml) at 90°C for 18 h to cleave off the lipid A moiety (26). The lipid A precipitate was removed by centrifugation, washed three times with distilled water, and lyophilized. The acetic acid supernatant was extracted three times with chloroform and then applied to a column of Sephadex G-100 (2.6 by 100 cm) equilibrated in PBS (flow rate, 30 ml/h). The eluate was monitored at 206 and 256 nm, and the peak fractions were combined, precipitated with alcohol (80% vol/vol), resuspended in water, and then dialyzed and lyophilized.

Preparation of antiserum. Antiserum was prepared in New Zealand white rabbits as follows.

(i) Whole organisms. Organisms grown in the defined medium of Weiss and Long (25) were pelleted by centrifugation and resuspended in saline containing 0.4% Formalin to an optical density at 650 nm (OD_{650}) of 1.0. After ascertaining sterility, 0.5 ml of this suspension was given intravenously (i.v.) on days 1 and 4.

A 1.0-ml dose was given on days 7 and 10. On days 17 and 20, 0.5 ml of a live culture grown for 24 h in Trypticase soy broth-glycerine at 37°C and adjusted to an OD_{650} of 1.0 was given i.v., followed by 1.0 ml of live organisms on days 23 and 26. On day 33 the rabbits were bled by cardiac puncture and the serum was collected.

(ii) Crude slime. Antiserum to crude slime was produced by an initial intramuscular (i.m.) injection of 10 mg of crude slime in complete Freund adjuvant followed on days 7 and 10 with 5.0-mg i.v. injections. On day 15, 7.5 mg of crude slime was given i.v. in 1.0 ml of saline. The rabbit was bled by cardiac puncture on day 24, and the serum was collected.

(iii) PS. Antiserum to purified PS was prepared by giving an initial i.m. injection of 1.0 mg of PS in complete Freund adjuvant, followed on days 7, 10, 13, 20, 23, and 26 with 1.0 mg i.v. in 1.0 ml of saline. On day 33, 50 ml of blood was removed by cardiac puncture and the serum was collected.

(iv) LPS. Antisera to LPS was prepared by injecting subcutaneously 1.0 mg of LPS in complete Freund adjuvant. The rabbit was bled on day 7 of 50 ml of blood.

Gel diffusion and immunoelectrophoresis. Gels for immunodiffusion were prepared by dissolving agarose to 1% in a previously described barbital buffer (23). Microscope slides were coated with 3 ml of heated gel, 10 μl of antiserum was then added to the appropriate well and allowed to drain into the gel, and a second 10-μl amount of serum was then added. At this time the antigen solution in 10-μl amounts was added to the appropriate well. Gels were incubated overnight at room temperature in a humid atmosphere.

Electrophoresis of antigens was performed in 1.5% Special Noble agar (Difco, Detroit, Mich.) dissolved in 0.025 M sodium barbital (pH 8.6). Slides were electrophoresed for 40 min at 10 mA and 90 V, using 0.05 M sodium barbital as conducting buffer. Antiserum was then added to the trough, and the slides were developed by overnight incubation at room temperature in a humid atmosphere.

Serological analysis. Titration of antisera and calculations of concentrations of antibody were performed by using the indirect solid-phase radioimmunoassay (SPRIA) of Zollinger et al. (29). To sensitize the polyvinyl microtiter plates with the PS antigen, it had to be derivatized with stearoyl chloride by a modification of the method of Hammerling and Westphal (14). A 5-mg portion of PS was suspended in 1 ml of *N',N'*-dimethyl formamide in a glass ampoule. A 0.5-ml amount of pyridine was added, and then 2 mg of stearoyl chloride dissolved in 0.1 ml of *N',N'*-dimethyl formamide was added. The glass ampoule was sealed and put at 56°C for 48 h with occasional shaking. After this incubation period, the contents were removed to a centrifuge tube and 1.0 ml of saline was added. The PS was precipitated by the addition of 25 volumes of ethanol, and the precipitate was collected by centrifugation, washed 3 times with ethanol, resuspended in 2.5 ml of distilled water, and desalted over a prepacked column of Sephadex G-25 (Pharmacia PD-10, Uppsala, Sweden) equilibrated in distilled water. The void volume was collected and lyophilized.

The plates were then sensitized with 25 μl of a 0.1-mg/ml solution of one of the following: stearoyl chloride-derivatized PS; intact LPS; stearoyl chloride-derivatized PS treated with 0.1 N NaOH at 37°C for 2 h and neutralized with HCl and PBS; or LPS treated with 0.1 N NaOH in an identical manner and subsequently neutralized.

Inhibition of the SPRIA was by the method of Zollinger and Mandrell (30). The amount of antibody to be inhibited was that amount of antibody contained in a dilution of antiserum twice as concentrated as the dilution of antiserum giving 25% of the maximal counts bound (approximately 0.1 μg of specific antibody).

Phage depolymerase enzyme treatment. The phage 2 and phage 8 PS depolymerase enzymes (6) were kindly provided by P. F. Bartell, Newark, N.J. PS antigen and LPS (2 mg/ml in PBS) were mixed with an equal volume of the enzyme preparations, incubated at 37°C for 24 h with shaking, and then tested for serological activity in immunodiffusion gels.

Chemical analysis. Heptose and methyl pentoses were determined by the cysteine-sulfuric acid method (22). 2-Keto-3-deoxyoctonate (KDO) was determined by the thiobarbituric acid assay (22). Nucleic acids were determined by OD_{260} and OD_{280} in a Beckman 25 scanning spectrophotometer. Protein was measured by the method of Lowry et al. (21). Water content was determined by a Karl-Fischer titration performed by Gailbraith Laboratories, Knoxville, Tenn. LPS content was measured by the carbocyanin dye method of Janda and Work (18). Phosphate was determined by the method of Gerlach and Deuticke (12).

Quantitative and qualitative assay of carbohydrates and lipids were performed by gas-liquid chromatography (GLC), and the resultant peaks were identified on the basis of co-chromatography with known standards and mass spectroscopy. GLC of the fatty acid methyl esters of the lipids was performed on material that had been hydrolyzed for 24 h with 3% methanolic HCl at 100°C. Separation was done on a column of 3% OV-17 on Gas Chrom Q (100/120 mesh, ¼ inch by 6 feet [ca. 0.6 cm by 1.8 m]) and analyzed by dual flame ionization detectors in a Hewlett-Packard 5830A gas chromatograph. The temperature was programmed from 160 to 220°C at a rate of 5°C/min, and the carrier gas was nitrogen at a flow rate of 20 ml/min. The injector temperature was 250°C and the detector temperature was 275°C. GLC of carbohydrates was performed on material hydrolyzed with 3% methanolic HCl for 6 h at 100°C and then derivatized to the trimethyl silyl esters as described by Chambers and Clamp (7). The derivatized sugars were injected onto a column of 3% SE-30 on Gas Chrom Q (see above). Conditions for analysis were the same as those for the lipids, except that programming of the temperature of the column oven was as follows: the initial column temperature of 140°C was held for 1.5 min, and the column temperature was then raised to 155°C at a rate of 10°C/min. The temperature rise was then slowed to 5°C/min until a temperature of 190°C was reached. This temperature was held for 6 min and the column temperature was then raised to 225°C at a rate of 5°C/min. This temperature was held for 2 min, and a final rise of temperature to 250°C at 5°C/min was performed and held for 10 min.

Mass spectroscopy of the trimethylsilyl-derivatized

sugars and fatty acid methyl esters was done in a Finnegan 3100B GLC mass spectrometer utilizing methane and isobutane chemical ionization. Samples were separated on a column of 3% OV-17 on Gas Chrom Q (see above). The column temperature was held at 140°C for 3 min and then raised to 220°C at a rate of 4°C/min. Source pressure was 1,500 μm and source temperature was 120°C. Mass spectra were compared to those of known standards, except for the spectra of 2-acetamido-2,6-dideoxygalactose, which was unavailable as a pure standard.

Physical determination. Ion-exchange chromatography of purified PS was performed on diethylaminoethyl-Sephadex A-50 and QAE-Sephadex A-50 gels equilibrated in either distilled water, 0.01 M tris(hydroxymethyl)aminomethane of pH 4.0 or pH 8.0, or 0.01 M NaCl of pH 5.2, and on CM-Sephadex A-50 and SP-Sephadex A-50 gels equilibrated in either distilled water, 0.01 M phosphate buffer (pH 8.6) or 0.1 M phosphate buffer (pH 8.6). Column size was 1.6 by 60 cm in each case, and the flow rate was 30 ml/h. Eluting fractions were monitored and collected as described for the PS.

Sizing of the PS was done on Sepharose CL-6B gels equilibrated in PBS on a column (2.6 by 100 cm) with a flow rate of 40 ml/h. Calibration of the column was performed with dextran polymers (Pharmacia Co., Uppsala, Sweden).

To determine if the PS was aggregated, a sample was chromatographed on columns of Sephadex G-100 and Sepharose CL-6B in 0.05 M glycine–0.005 M ethylenediaminetetraacetate (pH 8.8) containing 3% sodium deoxycholate. PS was first dissolved in this buffer containing 10% sodium deoxycholate at 56°C for 30 min prior to application to the column.

The UV absorption spectrum of the PS was determined in a Beckman 25 scanning spectrophotometer.

Rabbit pyrogenicity test. The rabbit pyrogenicity test was kindly performed by Patricia Altieri, Department of Biologics Research, Walter Reed Army Institute of Research. New Zealand white rabbits weighing 2.5 to 3.1 kg were given graded doses of the PS and monitered for a rise in temperature greater than 0.3°C in a 3-h period.

Mouse toxicity test. ICR mice, obtained from the Walter Reed Army Institute of Research animal colony, were injected intraperitoneally with doses of PS and LPS in saline and observed for signs of endotoxin shock.

RESULTS

Characterization of crude slime. The chemical composition of crude slime prepared from *P. aeruginosa* immunotype 1 is shown in Table 1. Nucleic acids account for over two-thirds of the material present, with protein, LPS, and PS responsible for the majority of the remaining material. Serologically, slime was shown to be composed of numerous antigens (Fig. 2). After electrophoresis, five distinct lines could be visualized with antiserum to whole organisms. Treatment of crude slime with Pronase B reduced the number of visible antigens to three. Antiserum to crude slime (Fig. 2b) revealed the presence of three antigens in untreated and two antigens in Pronase B-treated slime.

Purification of PS antigen. The purification scheme detailed in the section above is outlined in Fig. 1. The two cetavalon precipitations removed 90 to 95% of the nucleic acid from the crude slime. LPS was present in crude slime after the cetavalon treatment as detected by the presence of KDO and lipid A (thiobarbituric acid assay and carbocyanin dye test, respectively). LPS was not completely removed from the crude slime by either ultracentrifugation, chromatography in LPS disaggregating buffers such as deoxycholate, or by ion-exchange or gel-filtration chromatography. Hydrolysis of LPS with acetic acid and heat, and subsequent removal of the lipid and low-molecular-weight polysaccharide components, was utilized to remove LPS from the slime. Treatment for 18 h at 90°C was necessary to insure removal of all intact LPS; later studies with purified PS showed that it readily withstood up to 72 h of this treatment.

TABLE 1. *Chemical composition of crude slime*[a]

Component	Assay	Wt %
Nucleic acids	OD_{260} and OD_{280}	67.8
Protein	Lowry	9.2
LPS	Thiobarbituric acid for KDO[b]	3.0
PS	Recoverable from slime	5–20.0

[a] Crude slime was dialyzed and lyophilized for these determinations.

[b] KDO = 5% by weight of the LPS.

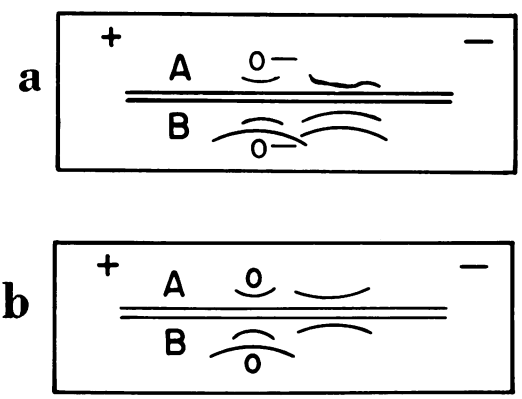

FIG. 2. *(a) Immunoelectrophoresis of crude P. aeruginosa immunotype 1 slime reacted with antisera to whole organisms. A, Pronase B-treated crude slime. B, Untreated crude slime. (b) Immunoelectrophoresis of crude slime versus antisera to crude slime. A, Pronase B-treated crude slime. B, Untreated crude slime.*

The precipitated lipid A was removed by centrifugation, and chloroform extraction was performed to remove any material soluble in organic solvents.

Chloroform extraction was repeated until there was little to no precipitate present at the interface of the aqueous and organic layers. Nucleases were used to bring down the level of nucleic acid contamination to 1 to 2%. Finally, a phenol extraction was performed to remove remaining proteins, which constituted about 5% of the material before this step. Chromatography on Sephadex G-100 gels separated high-molecular-weight PS in the preparation from smaller polysaccharides and LPS side chains. The pattern of elution from the G-100 column, as followed by the absorbance of the eluate at 206 and 254 nm, is shown in Fig. 3. The UV absorbance pattern of PS showed it to have two peaks at wavelengths of 206 and 163 nm (Fig. 4) and is compared to that of nucleic acids.

Chemical composition of PS and LPS. The PS antigen was composed primarily of carbohydrate and water (Table 2). No KDO, lipid, phosphorous, heptose, or protein could be detected in the final product, and nucleic acid contamination was generally at a level of 1 to 2% (Table 2). LPS contained protein, lipid, carbohydrate, phosphorus, and a higher amount of contaminating nucleic acid. GLC analysis of three lots of PS prepared from solid cultures, one lot from liquid culture, intact LPS, and the acetic acid-hydrolyzed polysaccharide portion of LPS are shown in Table 3. Intact LPS contained rhamnose, glucose, mannose, heptose, KDO, 2-acetamido-2-deoxygalactose, 2-acetamido-2-deoxyglucose, and 2-acetamido-2,6-dideoxygalactose. 2-Acetamido-2,6-dideoxygalactose was identified on the basis of its mass spectrum, showing a molecular ion of the methylated and trimethylsilyl-derivatized compound at 363 u (molecular ion plus 1 proton from ionization), and a strong peak at 347 u, a loss of the methyl group, which is typical for these derivatized carbohydrates. The rest of the fragmentation pattern was consistent with the structure of 2-acetamido-2,6-dideoxygalactose. Galactose was not found in the intact LPS.

The polysaccharide portion of the LPS was purified from acetic acid-hydrolyzed LPS by chromatography on Sephadex G-100. Four carbohydrate peaks were obtained (Fig. 5). The first three peaks contained the sugars rhamnose, glucose, and mannose. The first two peaks, when tested in gel diffusion (not shown), gave a reaction of identity with PS. The third peak gave no precipitin line and accounted for only 5% of the starting weight. The first two peaks accounted for 72% of the starting weight of LPS used (average of three determinations), with the second of these two peaks accounting for 50% of the LPS starting weight. The fourth peak (smallest molecular weight) contained detectable KDO and heptose.

Analysis of the lipid content of the LPS gave the following molar percentages: 2-hydroxydecanoic acid, 12%; 3-hydroxydodecanoic acid, 33%; 2-hydroxydodecanoic acid, 36%; dodecanoic acid, 6%; hexadecanoic acid, 7%; and octadecanoic acid, 7%. Six major fatty acids were found, with 2-hydroxydodecanoic acid and 3-hydroxydodecanoic acid accounting for 69% on a molar basis. Furthermore, the C_{18} fatty acid was found to contain a single double bond on the basis of

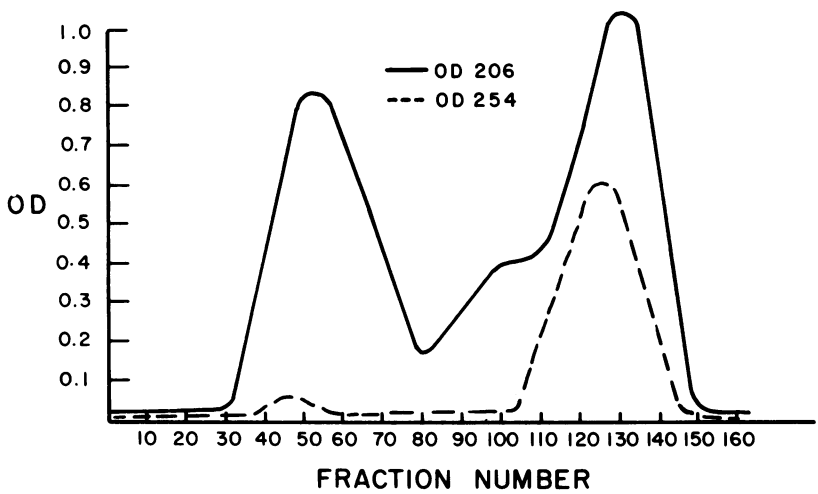

FIG. 3. *Elution profile of PS antigen from Sephadex G-100 column (2.6 by 100 cm). Fractions of 4 ml were collected.*

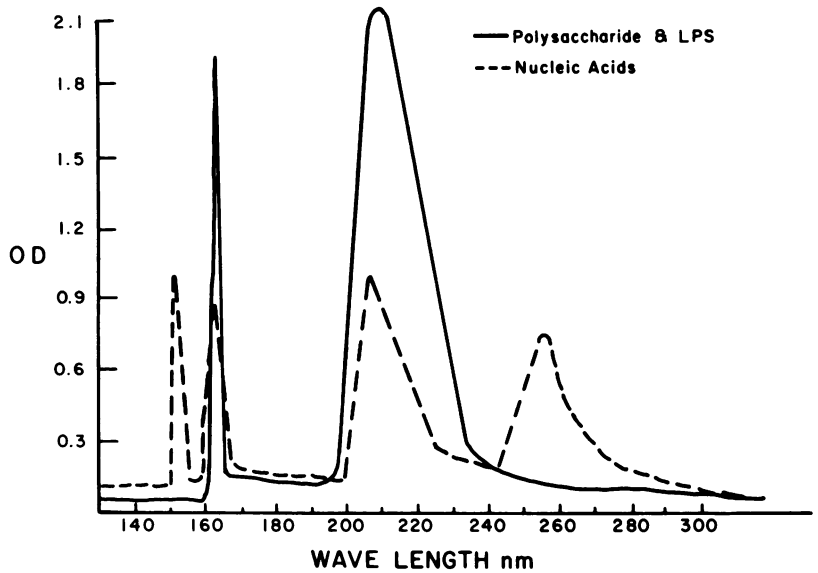

FIG. 4. *Absorbance of UV light by PS, LPS, and nucleic acids from 140 to 300 nm.*

TABLE 2. *Chemical composition of the PS and LPS from P. aeruginosa immunotype 1*[a]

Component	PS	LPS
Carbohydrate	75[b]	80
Lipid	0.0	10.5
Nucleic acid	1.0	1.6
Protein	0.0	4.3
Phosphate[c]	0.0	2.8
Water	21.7	ND[d]

[a] Average of three lots.
[b] Weight percent.
[c] Phosphate values determined after material passed over a diethylaminoethyl-Sephadex A-50 column in 0.1 M NaCl to remove last traces of nucleic acids.
[d] ND, Not determined.

its eluting from the GLC column ahead of octadecanoic acid and having a molecular ion of 297 by mass spectroscopy (molecular ion of octadecanoic acid = 299).

Physical properties of PS. Sizing of the PS on Sepharose CL-6B gels showed the PS to elute as a broad peak between molecular weight markers of 350,000 and 100,000. Dextran polymers used to calibrate the column eluted as sharp peaks when run under identical conditions, or along with the PS, indicating that PS is composed of polymers of a heterogeneous size ranging from 350,000 to 100,000 daltons.

Even though PS migrates towards the anode when electrophoresed, attempts to bind PS to either anion or cation exchange resins under a variety of conditions were unsuccessful. This property did allow for removal of the last traces of nucleic acid on a diethylaminoethyl-Sephadex A-50 column in 0.01 M NaCl.

Chromatography of the PS on either a G-100 gel or Sepharose CL-6B gel with 3% sodium deoxycholate did not change the elution pattern of the PS. Thus under these disaggregating conditions the PS eluted in the void volume of the G-100 column and as a broad peak on the CL-6B gel. Under similar conditions, a majority of the LPS eluted considerably after the void volume.

Toxicity of PS. Toxicity of the PS was tested in rabbits by the standard pyrogenicity test. No change in temperature greater than 0.1°C was observed in any rabbit when doses up to 25 μg/kg of body weight were given. This represents a dose 100 times greater than the currently allowed pyrogenic level of the polysaccharide vaccine to group C *Neisseria meningitidis*. Further tests for LPS contamination using the sensitive carbocyanin dye test were unable to detect any LPS in up to 5 mg of PS, indicating less than one part in 10,000 of LPS present. When mice were injected with doses of PS at levels of 400 mg/kg of body weight, no signs or symptoms of endotoxin shock were observed. This contrasts with a dose of 4 mg of LPS per kg of body weight needed to induce typical signs of endotoxin poisoning in mice and a 50% lethal dose of 20 mg of LPS per kg of body weight.

Immunology of the PS and LPS antigens. When PS and LPS were present together in the same antigen well in immunodiffusion gels, two separate precipitin lines developed (Fig. 6). Adsorption of antisera with PS removed the reac-

TABLE 3. *Molar ratios of sugars composing the PS and LPS of P. aeruginosa immunotype 1*[a]

Prepn	Ara	Rham	Man	Gal	Glu	KDO	Gluam	Galam	Hep	Dideoxy-gal
Lots 1-5 combined	0.07	0.65	0.16	0.32	1.0	0	0	0	0	0
Lot 8	0.02	0.66	0.05	0.33	1.0	0	0	0	0	0
Lot 10	0.03	0.66	0.10	1.08	1.0	0	0	0	0	0
Liquid culture	0.10	0.23	0.30	0.01	1.0	0	0	0	0	0
Intact LPS	0	0.95	0.04	0	1.0	0.06	0.20	0.20	0.10	++[b]
PS from LPS[c]	0	0.04	0.04	0	1.0	0	0	0	0	0

[a] Ara, Arabinose; Rham, rhamnose; Man, mannose; Gal, galactose; Glu, glucose; KDO, 2-keto-3-deoxyoctonoate; Gluam, 2-acetamido-2-deoxyglucose; Galam, 2-acetamido-2-deoxygalactose; Hep, L-glycero-D-manno-heptose; dideoxy-gal, 2-acetamido-2,6-dideoxygalactose.

[b] Lack of a standard prohibits assignment of a molar value.

[c] PS obtained from LPS hydrolyzed for 18 h at 90°C with 1% acetic acid and then subjected to column chromatography. Void volume peak, second peak, and third peak are analyzed here.

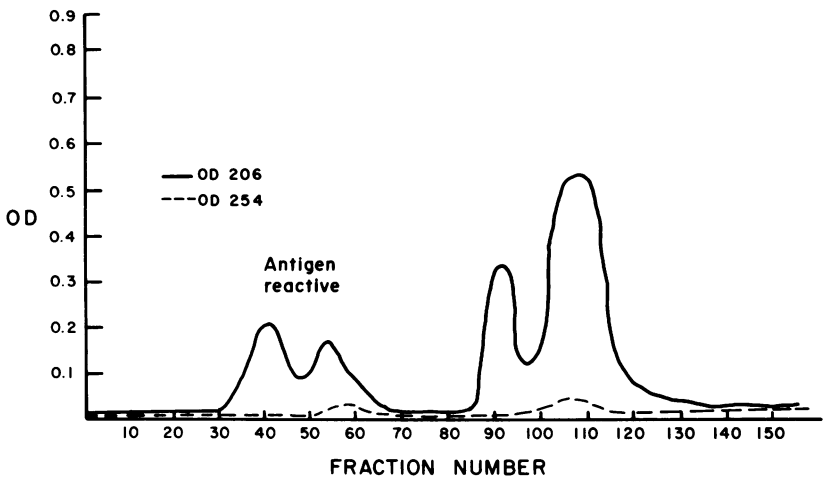

FIG. 5. *Elution profile of polysaccharide released from LPS following hydrolysis with 1% acetic acid at 90°C for 18 h. Peaks 1 and 2 (from left) gave a reaction of identity with PS.*

tivity to PS but not to LPS. However, as stated above, acetic acid hydrolysis of LPS released an antigen giving a reaction of identity with PS. This antigen could also be released from LPS simply by allowing a solution of LPS in PBS to sit at room temperature for 3 days or at 37°C for 24 h (Fig. 7). Adsorption of antisera with LPS removed its activity to both PS and LPS.

When examined in the SPRIA, antisera to whole organisms, crude slime, purified PS, and purified LPS were all capable of binding to PS- and LPS-sensitized plates (Table 4). Antisera to whole organisms, crude slime, and LPS were able to bind to alkali-treated LPS (0.1 N NaOH, 37°C, 2 h), whereas none of the four sera demonstrated binding to alkali-treated PS.

Inhibition of the PS-anti-PS antigen-antibody reaction was obtained with PS, LPS, and the acetic acid-hydrolyzed polysaccharide portion of LPS (peaks 1 and 2). Treatment of these three inhibitors with alkali destroyed their ability to inhibit this antigen-antibody reaction. PS, LPS, and the acetic acid-hydrolyzed polysaccharide portion of LPS were also able to inhibit the LPS-anti-LPS reaction, and this inhibition was also destroyed by treating the inhibitors with alkali. However, alkali-treated LPS and intact LPS were able to inhibit the alkali-treated LPS-anti-LPS system, whereas PS and the acetic acid-hydrolyzed polysaccharide from LPS were not. Table 5 summarizes these results. These data suggested that LPS had a set of antigenic determinants that were shared with PS and labile to alkali as well as a set of determinants not shared with PS and stable to alkali treatment. The alkali-labile shared set of determinants was expressed on the portion of the LPS molecule obtained by acetic acid hydrolysis and column chromatography, whereas the alkali-stable determinants were associated with another part of the molecule.

Further evidence that LPS contained a set of determinants distinct from PS was obtained from experiments utilizing phage 2 and phage 8

polysaccharide depolymerase enzymes. Figure 8 shows that treatment of the immunotype 1 PS with phage 2, but not phage 8, enzyme destroyed the precipitin activity of the PS in gels, whereas LPS still gave a precipitin line after treatment. The untreated control LPS (LPS + PBS) and the LPS treated with phage 8 enzyme released into the supernatant during the incubation period the antigen on LPS giving a line of identity with PS. LPS treated with phage 2 enzyme gave only a single precipitin line corresponding to intact LPS. This confirmed that both the PS isolated from slime that contains galactose and arabinose and the galactose- and arabinose-deficient antigen bound to LPS lost their gel precipitating activity after phage 2 polysaccharide depolymerase enzyme treatment. LPS remained unaffected by this treatment, and must therefore contain a separate set of immunodeterminants.

DISCUSSION

We have demonstrated that a high-molecular-weight nontoxic PS can be isolated from the slime of *P. aeruginosa* immunotype 1. Earlier work has demonstrated the potential importance of high molecular weight in purified bacterial polysaccharides that are used as human vaccines (13, 19). The PS we have isolated is composed of polymers of a heterogeneous size between 350,000 and 100,000 daltons. It is free of detectable lipid, protein, and KDO, has a low level of nucleic acid contamination, and is composed of rhamnose, glucose, galactose, arabinose, and mannose. The antigenic determinants of PS which react with rabbit antisera in immunodiffusion gels and radioimmunoassay are relatively acid stable (1% acetic acid for up to 72 h at 90°C), but very alkali labile (0.1 N NaOH for 1 h at 37°C). The PS gave a single precipitin line by gel diffusion and immunoelectrophoresis analysis.

Initially PS was isolated from 18-h cultures. PS purified from 48- to 72-h cultures was found to be chemically and immunologically identical to PS from 18-h cultures (unpublished data). Yields of PS were maximal when 48- to 72-h cultures were used, and these conditions were used in subsequent purifications.

Purification of PS free from LPS was possible only by hydrolysis of contaminating LPS into its lipid and polysaccharide portions, with subsequent removal of these products. Since acetic acid hydrolysis of purified LPS yields a product

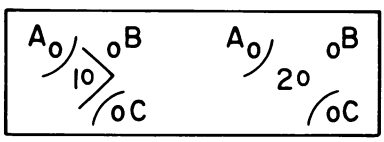

FIG. 6. *Precipitin lines in immunodiffusion gels of PS, LPS, and a mixture of PS and LPS versus antisera to whole organisms. (1) Unadsorbed antisera. (2) Antisera adsorbed with PS. (A) LPS antigen; (B) PS antigen; (C) mixture of PS and LPS antigens.*

TABLE 4. *Binding of antisera to P. aeruginosa immunotype antigens in the SPRIA*

Antiserum to:	Antigens[a]			
	PS	NaOH PS[b]	LPS	NaOH LPS
Whole organisms	415.4	0	381.3	3.37
Crude slime	19.1	0	26.8	1.98
PS	26.7	0	31.7	0
LPS	74.8	0	276.6	28.2

[a] In micrograms of antibody bound per milliliter.
[b] Alkali treatment of antigens was done in 0.1 N NaOH at 37°C for 2 h.

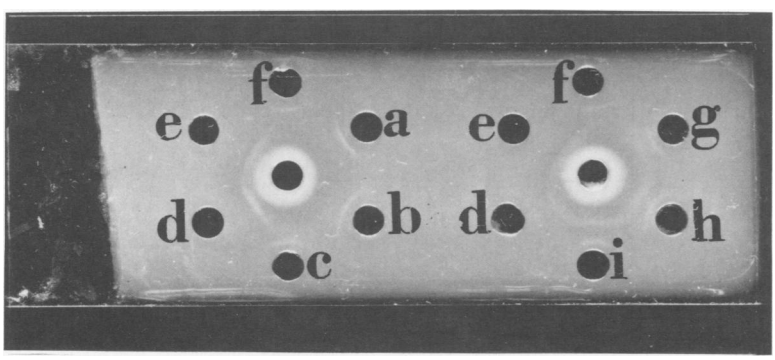

FIG. 7. *Precipitin lines in immunodiffusion gels of LPS solutions taken at days 0 through 6 posthydration, PS, and the acetic acid-hydrolyzed portion of LPS versus antisera to whole organisms. (a) LPS at day 1 posthydration. (b) LPS at day 2 posthydration. (c) LPS at day 3 posthydration. (d) PS antigen. (e) acetic acid-hydrolyzed PS portion of LPS. (f) Freshly dissolved LPS. (g) LPS at day 4 posthydration. (h) LPS at day 5 posthydration. (i) LPS at day 6 posthydration. Note development of a faint line on day 3 posthydration (c) that gives a line of identity with PS and acetic acid-hydrolyzed LPS. Line is very clear on day 4 (g).*

TABLE 5. *Summary of inhibition reactions in the SPRIA*[a]

Inhibitor:	Antigen bound to plate[b]		
	PS	LPS	NaOH LPS
PS	100	80	0
LPS	100	100	100
NaOH PS	0	0	0
NaOH LPS	0	0[c]	100

[a] The amount of antibody inhibited was that amount of antibody contained in a dilution of serum giving twice the number of counts of labeled secondary immunoglobulin equal to 25% of the maximum counts bound.

[b] Maximum percent inhibition obtained with a concentration of 1 mg of inhibitor per ml or less.

[c] Alkali-treated LPS did not inhibit the LPS–anti-LPS reaction because the dilution of serum employed for this reaction contained only antibody directed at the alkali-labile portion of the LPS.

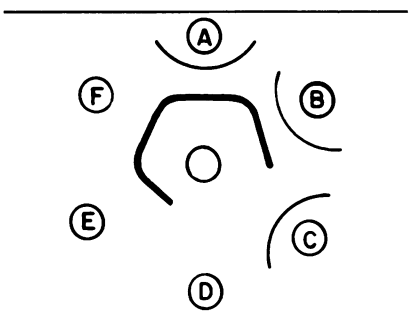

FIG. 8. *Reactivity of PS and LPS antigens after treatment with phage 2 and phage 8 PS depolymerases versus antisera to whole organisms. (A) LPS + PBS (control). (B) LPS + phage 8 depolymerase. (C) LPS + phage 2 depolymerase. (D) PS + phage 2 depolymerase. (E) PS + phage 8 depolymerase. (F) PS + PBS (control).*

immunologically indistinguishable from PS that also eluted as the void volume of a G-100 column, PS preparations must contain this material to a variable degree. This may somewhat account for the varying amounts of galactose, mannose, and arabinose in different PS preparations. This product of the acetic acid hydrolysis of LPS may represent high-molecular-weight O-side chain of *P. aeruginosa* LPS if such an entity exists.

The size of O-side chains in *P. aeruginosa* LPS has not been conclusively established. Fensom and Meadow (10) established the existence of two polysaccharide regions in the LPS of *P. aeruginosa* strain 8602, presumably corresponding to a low-molecular-weight core and a high-molecular-weight side chain region. Horton et al. (17) found that the polysaccharide from acetic acid-hydrolyzed LPS of the seven Fisher-Devlin immunotypes were heterogeneous on Sephadex G-75 and G-50 gels. Chester and Meadow (8) subjected *P. aeruginosa* PAC1 LPS to acetic acid hydrolysis and, after removal of the lipid A, recovered three polysaccharide peaks from Sephadex G-75 molecular sieve gels. The lowest molecular weight peak was interpreted to represent a common core structure. Alkaline ethanol treatment of this LPS, which removes *o*-acetylated fatty acids and thus reduces nonpolar interactions, followed by Sephadex G-200 chromatography, confirmed that the polysaccharide portion of this LPS was composed of various sized polymers, some of which had a molecular weight of greater than 100,000. These findings therefore suggest the existence of some high-molecular-weight polysaccharide side chains in *P. aeruginosa* LPS, which could also be part of our PS preparation.

Extraction of LPS from *P. aeruginosa* immunotype 1 by means of the phenol-water method yielded very poor results. This could be explained by the fact that we found most of the LPS partitioned in the phenol phase of the extract. Better yields were obtained simply by ultracentrifuging crude slime and treating it with nucleases and Pronase B and then chromatographing the material over a Sepharose 4B column. Ultracentrifugation alone did not very effectively purify LPS, and, even after six ultracentrifugations, considerable amounts of nucleic acid (15%), protein (10%), and free PS (25%) were present in the pellet. Therefore, the enzyme digestions were needed to reduce the levels of nucleic acid and protein contamination, and the chromatography step was needed to separate LPS from free PS.

The results on the analyses of the carbohydrate and lipid composition of *P. aeruginosa* immunotype 1 LPS (Table 3) contrasts with those reported by Horton et al. (17). They did not report any mannose in the LPS, perhaps because it occurs in very small amounts, and large amounts of LPS need to be injected onto the GLC column to see the mannose peak. Furthermore, they did not identify the presence of hydroxy fatty acids as the major constituents of the lipid A, nor did they identify the C_{18} fatty acid as unsaturated. Drewry et al. (9) reported that the lipid A of *P. aeruginosa* contains large amounts of hydroxy fatty acids but did not identify octadecanoic acid.

We were unable to purify LPS free of polysaccharide immunologically related to PS, though the LPS gave a single precipitin line in immunodiffusion gels if freshly dissolved or stored frozen. If the LPS was allowed to stand at room temperature for 3 days or incubated at 37°C for 24 h, an antigen that gave a reaction of identity

with PS in gels was released free into the supernatant. This antigen could also be recovered from LPS by acetic acid hydrolysis and column chromatography. The resultant antigens eluted as three peaks from a Sephadex G-100 column: a void volume peak and a peak of molecular weight 60,000, both of which gave lines of identity in gels with PS; and a peak of molecular weight 25,000, which was chemically identical to the material in the larger peaks but nonreactive in gels. The larger sized antigens completely inhibited the PS-anti-PS reaction in the SPRIA. Likewise, both PS and the polysaccharide portion of LPS were susceptible to the action of phage 2 polysaccharide depolymerase enzyme. Galactose and arabinose were consistently found in all PS preparations, whereas the LPS never contained galactose or arabinose (Table 3). Although the ratio of galactose and arabinose to glucose found in the liquid culture-prepared antigen was low, PS prepared from 11-liter fermentor cultures and isolated by a slightly different method contained a ratio of galactose to glucose of 1.27 to 1.0 and arabinose to glucose of 0.53 to 1.0 (unpublished data). Horton et al. (17) also did not detect galactose or arabinose in *P. aeruginosa* immunotype 1 LPS.

Although we cannot detect any serological differences between PS and the polysaccharide isolated from LPS, these chemical differences suggest that PS is a different molecule. Serologically, intact LPS differs from PS in that besides the presence of the alkali-labile determinants shared with PS, LPS contains an alkali-stable determinant not found in PS as demonstrated by immunodiffusion, SPRIA, and phage 2 polysaccharide depolymerase treatment (Tables 4 and 5). This alkali-stable determinant may represent the immunotype determinant.

The relative lack of toxicity and pyrogenicity shown by the PS in mouse and rabbit tests is in contrast to that of LPS. Attempts to immunize humans with a heptavalent LPS vaccine have been hampered by the toxicity of this preparation. The isolation of a relatively nontoxic, high-molecular-weight PS from the slime of *P. aeruginosa* is an encouraging first step toward development of a safe and effective vaccine.

ACKNOWLEDGMENTS

We thank J. McLeod Griffiss for his review of the manuscript and Elizabeth A. Sadoff for her invaluable help in preparation of the manuscript.

G. B. Pier is the recipient of a National Research Council Postdoctoral Research Fellowship Award.

LITERATURE CITED

1. **Alms, T. H., and J. A. Bass.** 1967. Immunization against *Pseudomonas aeruginosa*. 1. Induction of protection by an alcohol precipitated fraction from the slime layer. J. Infect. Dis. **117**:249–256.
2. **Anderson, P., G. Peter, R. B. Johnston, Jr., L. H. Wetterlow, and D. H. Smith.** 1973. Responses of children immunized with polyribophosphate, the capsular antigen of *Haemophilus influenzae* type b. Pediatrics **52**:637–644.
3. **Austrian, R.** 1977. Prevention of pneumococcal infection by immunization with capsular polysaccharides of *Streptococcus pneumoniae*: current status of polyvalent vaccines. J. Infect. Dis. Suppl. **136**:S38–S42.
4. **Baltimore, R. S., A. S. Dobek, F. R. Stark, and M. S. Artenstein.** 1974. Clinical and epidemiological correlates of *Pseudomonas* typing. J. Infect. Dis. Suppl. **130**: S53–S59.
5. **Bartell, P. F., T. E. Orr, and B. Chudio.** 1970. Purification and chemical composition of the protective slime antigen of *Pseudomonas aeruginosa*. Infect. Immun. **2**: 543–548.
6. **Bartell, P. F., T. E. Orr, and G. K. H. Lam.** 1966. Polysaccharide depolymerase associated with bacteriophage infection. J. Bacteriol. **92**:56–62.
7. **Chambers, R. C., and J. R. Clamp.** 1971. An assessment of methanolysis and other factors used in the analysis of carbohydrate containing materials. Biochem. J. **125**: 1009–1018.
8. **Chester, I. R., and P. M. Meadow.** 1975. Heterogeneity of the lipopolysaccharide from *Pseudomonas aeruginosa*. Eur. J. Biochem. **58**:273–282.
9. **Drewry, D. T., J. A. Lomax, G. W. Gray, and S. G. Wilkinson.** 1973. Studies of lipid A fractions from the lipopolysaccharide of *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes*. Biochem. J. **133**:563–572.
10. **Fensom, A. H., and P. M. Meadow.** 1970. Evidence for two regions in the polysaccharide moiety of the lipopolysaccharide of *Pseudomonas aeruginosa* 8602. FEBS Lett. **9**:81–84.
11. **Fisher, M. W., H. B. Devlin, and F. Gnabski.** 1969. New immunotype schema for *Pseudomonas aeruginosa* based on protective antigens. J. Bacteriol. **98**: 835–836.
12. **Gerlach, E., and B. Deuticke.** 1963. Eine einfach Methode zur Mikrobestimmung von Phosphat in der Papierchromatographie. Biochem. Z. **337**:477–479.
13. **Gotschlich, E. C., I. Goldschneider, and M. S. Artenstein.** 1969. Human immunity to the meningococcus. V. The effect of immunization with meningococcal group C polysaccharide on the carrier state. J. Exp. Med. **128**:1385–1395.
14. **Hammerling, U., and O. Westphal.** 1967. Synthesis and use of O-stearoyl polysaccharides in passive hemagglutination and hemolysis. Eur. J. Biochem. **1**:46–50.
15. **Hanessian, S., W. Regan, D. Watson, and T. H. Haskell.** 1971. Isolation and characterization of the antigenic components of a new heptavalent *Pseudomonas* vaccine. Nature (London) New Biol. **229**:209–210.
16. **Haynes, W. C.** 1954. *Pseudomonas aeruginosa*—its characterization and identification. J. Gen. Microbiol. **5**: 939–950.
17. **Horton, D., G. Rodemeyer, and T. H. Haskell.** 1977. Analytical characterization of lipopolysaccharide antigens from seven strains of *Pseudomonas aeruginosa*. Carbohydr. Res. **55**:35–47.
18. **Janda, J., and E. Work.** 1971. A colorimetric estimation of lipopolysaccharides. FEBS Lett. **10**:343–345.
19. **Kabat, E. A., and A. E. Berzer.** 1958. The effect of variation in molecular weight on the antigenicity of dextran in man. Arch. Biochem. Biophys. **78**:306–318.
20. **Liu, P. V., Y. Abe, and J. C. Bates.** 1961. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. J. Infect. Dis. **108**:218–228.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. **Osborn, M. J.** 1963. Studies on the Gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonoate

in the lipopolysaccharide of *Salmonella typhimurium*. Proc. Natl. Acad. Sci. U.S.A. **50**:499–506.

23. **Pier, G. B., and S. H. Madin.** 1976. *Streptococcus iniae* sp. nov., a beta-hemolytic streptococcus isolated from an Amazon freshwater dolphin, *Inia geoffrensis*. Int. J. Syst. Bacteriol. **26**:545–553.
24. **Sadoff, J. C.** 1974. Cell-wall structures of *Pseudomonas aeruginosa* with immunologic significance: a brief review. J. Infect. Dis. Suppl. **130**:S61–S64.
25. **Weiss, E., and J. L. Long.** 1969. Simplified method for the production of carbohydrate antigen from *Neisseria meningitidis*. Appl. Microbiol. **18**:843–847.
26. **Westphal, O., and O. Luderitz.** 1954. Chemisch Erforschung von Lipopolysacchariden gramnegativen Bakterien. Angew. Chem. **66**:407–417.
27. **Westphal, O., O. Luderitz, and F. Bister.** 1952. Uber die Extraktion von Bakterien mit Phenol/Wasser. Z. Naturforsch. **79**:148–155.
28. **Young, V. M., and M. R. Moody.** 1974. Serotyping of *Pseudomonas aeruginosa*. J. Infect. Dis. Suppl. **130**: S53–S59.
29. **Zollinger, W. D., J. M. Dalrymple, and M. S. Artenstein.** 1976. Analysis of the parameters affecting the solid phase radioimmunoassay quantitation of antibody to meningococcal antigens. J. Immunol. **117**:1788–1798.
30. **Zollinger, W. D., and R. E. Mandrell.** 1977. Outer-membrane protein and lipopolysaccharide serotyping of *Neisseria meningitidis* by inhibition of a solid-phase radioimmunoassay. Infect. Immun. **18**:424–433.