Degradative Effect of Phenol on Endotoxin and Lipopolysaccharide Preparations from *Serratia marcescens*

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It has been established that the well-known deproteinizing action of hot 45% aqueous phenol on whole cells or isolated and purified endotoxin of *Serratia marcescens* 08 is caused by the cleavage of a phenol-sensitive linkage within the lipid moiety. As a result of this degradation, both the lipopolysaccharide and simple protein fragments retained a part of the lipid moiety. Although not proceeding at the same fast rate as the cleavage of the lipid moiety, such phenol treatment also caused a partial hydrolysis of the O-specific side chain and ester-bound fatty acids. Hydrolysis of the O-specific side chain accounted for 5% of the lipopolysaccharide and that of ester-bound fatty acids accounted for 11% of the total fatty acid content after 60 min of treatment. It is suggested that the presence of these degradation products is one of the main causes of the heterogeneity of endotoxin and lipopolysaccharide preparations.

When extracted from bacterial cells with trichloroacetic acid, certain organic solvents, various salt solutions, or some chelating agents, endotoxins consist of polysaccharide, lipid, and protein moieties (14). Palmer and Gerlough (23) were the first to describe the use of 88 or 95% liquid phenol for the dissociation of the endotoxin complex in bacterial cells. By treating dried typhoid bacilli with liquid phenol and by extracting the phenol-insoluble residue with saline solution or water, they isolated an O-antigenic preparation which was characterized by high carbohydrate and low nitrogen content. Recognizing the significance of phenol extraction as a means for selective fragmentation of endotoxins, Morgan and Partridge (16) demonstrated that whole endotoxin from *Shigella dysenteriae* and *Salmonella typhosa* could be cleaved into an "undegraded" polysaccharide (lipopolysaccharide [LPS] consisting of the polysaccharide moiety and firmly bound lipid A) and simple protein. Westphal et al. (33) developed two alternative procedures for the isolation of the endotoxic LPS fragment by extracting aqueous suspensions of bacterial cells with an equal volume of 90% phenol at low or increased temperatures. Whereas LPS preparations isolated by aqueous phenol at 5 to 10 C still contained varying amounts of protein, those isolated at 65 to 68 C were practically free of protein moiety. Since the introduction of aqueous phenol treatment as a general method for the isolation of LPSs from bacterial cells (13, 14, 32) or whole endotoxins (2, 25, 29), it has been assumed that phenol treatment causes little, if any, cleavage of the covalently bound constituents of whole endotoxin other than the protein moiety. However, Nowotny et al. (19, 21) reported a marked heterogeneity of purified phenol-water-extracted endotoxins as revealed by ion-exchange column chromatography. Alaupovic et al. (2) observed chemical and immunological heterogeneity of phenol-water-extracted whole endotoxins and suggested that phenol treatment may cause considerable cleavage of certain chemical linkages. The role of phenol as a deproteinizing agent is well established. The need for a closer scrutiny of these degradative effects became more important when we determined that the deproteinizing action of phenol on endotoxins had been brought about by the cleavage of a sensitive linkage within the lipid moiety rather than at its point of attachment to the protein component (34). The results of our continuing studies of the effect of phenol treatment on the chemical and toxic properties of purified whole endotoxin isolated by trichloroacetic acid extraction of *Serratia marcescens* 08 form the basis of this report.

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MATERIALS AND METHODS

Bacteria. Cells of the chromogenic strain *S. marcescens* 08, cultivated as described by Alaupovic et al. (2), were supplied by General Biochemicals (Chagrin Falls, Ohio). The bacterial cells were harvested in late logarithmic phase by centrifugation in a Sharples continuous centrifuge and washed with distilled water.

Isolation of whole endotoxin. Approximately 1,000 g of wet cells was extracted twice with 2 liters of 5% trichloroacetic acid by a modified method of Boivin et al. (3). The combined extracts were dialyzed against tap and distilled water for 24 and 48 h, respectively. The dialyzed extracts were concentrated by vacuum distillation to approximately 500 ml and centrifuged in a Spinco model L ultracentrifuge at 105,000 x g for 1 h. The supernatant liquid which contained nucleic acids and acidic polysaccharides was decanted. The gel-like substance from the bottom of the centrifuge tube was lyophilized and extracted with chloroform-methanol (2:1, vol/vol) in a Soxhlet apparatus for 24 h to remove free lipids. The residue was suspended in distilled water and lyophilized. This preparation represented the purified whole endotoxin (PWE).

Isolation of LPS. To remove the protein moiety, PWE was extracted with 45% aqueous phenol for 30 min at 60 to 70 C (Fig. 1) by the method of Westphal and Jann (32). The extract was cooled, and the phenol phase was separated by low-speed centrifugation (2,000 rpm) at 4 C for 20 min. The phenol phase was washed three times with equal volumes of distilled water. The combined water phases were exhaustively dialyzed against distilled water and concentrated by vacuum distillation to a small volume (200 ml). This suspension was extracted twice in a separatory funnel with an equal volume of chloroform. After removal of

![Diagram](http://jb.asm.org/)
the chloroform phase, 3 volumes of acetone were added to the aqueous phase and the mixture was allowed to stand overnight at 4 C. The solvent was decanted from the precipitate, and the last traces of acetone were eliminated by evaporation. The precipitate was suspended in distilled water and lyophilized; the white, fluffy product was the LPS.

The LPS was extracted twice with 45% aqueous phenol in the same manner as described earlier (Fig. 1). The preparation obtained after chloroform extraction and acetone precipitation was designated as the multiple phenol-extracted LPS (LPS-M).

Isolation of the PS-SC. The isolation and purification of the O-specific side chain (PS-SC) of whole endotoxin was performed by a previously described procedure (28).

Isolation of compounds from phenol phase. The compounds present in the phenol phase (Fig. 1) were isolated according to the procedure developed by Ober and Alauuovic (34). The phenol phase I was washed several times with distilled water to remove any traces of LPS and other water-soluble compounds. Nine and one-half volumes of 95% ethanol were then added, and the mixture was allowed to stand for 7 to 10 days at 4 C. The precipitate was collected by centrifugation and washed several times with distilled water and with ethanol. To remove free lipids, the washed precipitate was extracted with chloroform-methanol (2:1, vol/vol) in a Soxhlet apparatus for 12 h. The washed and extracted precipitate represented the phenol fraction P-I. Phenol phases II and III were treated in the same manner (Fig. 1). However, since no precipitate was formed after addition of 95% ethanol, phenol phases II and III were submitted to steam distillation, concentrated to a small volume by vacuum distillation, and lyophilized. The residues were extracted with chloroform-methanol (2:1, vol/vol) to remove the free lipids; they were designated as phenol fractions II and III, respectively.

Paper chromatography and paper electrophoresis. The procedures for paper chromatography and electrophoresis were carried out by methods described previously (30).

Analytical methods. The presence of nucleic acids was monitored by ultraviolet absorption of a 1% aqueous solution of whole endotoxin or LPS at 260 nm. A lack of inflection at this wavelength indicated that the endotoxic preparation was free from nucleic acids. The anthrone-positive carbohydrate content was determined by the method of Kohler (10). The reducing sugar content was determined by the method of Somogyi and Nelson as described by Marais et al. (15). Samples were hydrolyzed with 2 N HCl for 4 h at 100 C. Glucose was used as a standard for both the anthrone-positive and reducing sugars determinations. Hexosamine content was determined by the method of Rondle and Morgan (26). Samples were hydrolyzed with 4 N HCl for 6 h. For the determination of muramic acid, samples were hydrolyzed with 4 N HCl under reflux for 4 h. After neutralization with NaOH, the hydrolysate was applied to a column packed with a mixture of Norit A and Celite (1:1, wt/wt), and the hexosamine fraction was eluted with water (24). The presence of muramic acid was tested. If present, it would have been eluted with 5% ethanol and detected by its absorption peak at 505 nm in the Elson-Morgan reaction (27). Heptose content was estimated by the cysteine-sulfuric acid reaction (4) with d-glycerol-L-talo heptose as a standard. The 2-keto-3-deoxyoctulosonic acid (KDO) content was determined by the thiobarbituric acid method (31). Amino acids and fatty acids were determined as described previously (34).

Infrared spectroscopy. The infrared spectra were recorded by Sadler Research Laboratories, Philadelphia, Pa., using potassium bromide pellets (1.5 to 2.0 mg of substance and 250 mg of KBr).

Analytical ultracentrifugation. Sedimentation velocities of endotoxin and LPS preparations were carried out in a Spinco model E ultracentrifuge as described previously (30).

Immunological methods. The immunological properties of endotoxin and LPS preparations were studied by double-diffusion and immunoelectrophoresis (34). Antiserum to whole cells was prepared by injecting white rabbits with a saline suspension (1 mg/ml) of heat-killed cells of S. marcescens 08. The total dose of 7 mg of cells was administered intraperitoneally in six equal fractions at regular intervals for 2 days. The last injection consisted of 2 mg of heat-killed cells and an equal volume of complete Freund adjuvant. Antibodies to PWE were prepared by intraperitoneal administration of a mixture consisting of 0.5 mg of PWE suspended in 1 ml of saline solution and 1 ml of complete Freund adjuvant at four weekly intervals. Animals were bled by cardiac puncture. The presence of antibodies was tested at appropriate intervals of time by double diffusion in agar gels.

Lethality for mice. White BALB/c inbred mice (Texas Inbred Mouse Co., Houston, Texas) weighing 15 to 20 g were divided into groups of 10 animals each. The animals were injected intraperitoneally with 1 ml of saline solution containing graded dosages of endotoxin or LPS preparations. Deaths occurring within 6 days were recorded. When dosage was determined, an additional group of 10 mice was injected at this dose level to verify the estimated mean lethal dose (LD₅₀) value.

RESULTS

Characterization of whole endotoxin. The crude endotoxin extracted from the bacterial cells by trichloroacetic acid contained varying amounts of extraneous non-endotoxic compounds. Nucleic acids and acid polysaccharides were removed by ultracentrifugation, and free lipids were removed by exhaustive extraction with a mixture of chloroform and methanol. However, even after such treatments, the immunoelectrophoretic pattern of endotoxin preparations (CWE) revealed three precipitin arcs with antibodies to whole cells or PWE (Fig. 2,
Fig. 2. Immunoelectrophoretic patterns of crude (CWE) and purified (PWE) whole endotoxins in 1% agar gel. The trough contains antibodies to purified whole endotoxin. The incubation period was 24 h.

lower pattern). We have demonstrated (28, 34) that the precipitin line formed in the anodal compartment was that of the free protein moiety of endotoxin. The immunoprecipitin arc of a band migrating rapidly towards the cathode was identified as that of the PS-SC (28), whereas the line closer to the antigen well represented the intact endotoxin (34). These findings clearly showed that trichloroacetic acid extraction caused a partial cleavage of the protein moiety and PS-SC. To evaluate the extent of this hydrolytic cleavage, the whole endotoxin (CWE) freed from all extraneous compounds was chromatographed on Sepharose 4B. Results of this experiment showed an almost total recovery of intact endotoxin in the void volume: the amount of PS-SC was too small to be detected in the appropriate fraction. This elution volume had been determined with a sample of PS-SC in a separate experiment. It was established later that the trace amounts of PS-SC could be eliminated from whole endotoxin by repeated ultracentrifugations of trichloroacetic acid extracts. Trace amounts of protein moiety were removed by low-speed centrifugation (12,000 × g for 30 min). Under these conditions, the protein moiety and a small portion of endotoxin sedimented, while the main portion of endotoxin remained in the supernatant liquid. After purification by Sepharose 4B column chromatography or repeated low- and high-speed centrifugations, PWE exhibited a single precipitin arc close to the antigen well (Fig. 2, upper pattern). This highly purified whole endotoxin was used as the starting material for all other experiments in this study.

The PWE was poorly soluble in water or common aqueous buffers. It was soluble, however, in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.6, containing 0.1 to 0.2% sodium dodecyl sulfate. In agar electrophoresis, it migrated slowly as a single band into the cathodal compartment (Fig. 2, upper pattern). On immunoelectrophoresis, it displayed a single precipitin line with antibodies to PWE as well as with antibodies to whole bacterial cells. The infrared spectrum (Fig. 3) was characterized by pronounced ester absorption at 1,740 cm⁻¹ and by amide I and amide II bands at 1,650 cm⁻¹ and 1,520 cm⁻¹, respectively. The absorption band at 1,230 cm⁻¹ was attributed to δP=O, and that at 1,060, cm⁻¹ was attributed to the carbohydrate. The PWE had a high content of reducing sugars and D-glucosamine and almost an equal percentage of fatty acids and amino acids (Table 1). Heptose accounted for 5.8% of the whole endotoxin. Hexoses, identified by paper chromatography, included D-glucose and D-galactose. KDO was also detected by paper electrophoresis and the thiobarbituric acid procedure. The absence of muramic acid in the whole endotoxin indicated clearly that the mucoprotein layer of the cell wall was not extracted by trichloroacetic acid. PWE was highly toxic for mice (Table 2).

Effect of phenol extraction. After a single treatment of PWE with 45% aqueous phenol for 30 min at 60 to 70 C, the separated aqueous and phenol phases were characterized by determination of some of their chemical, physical, and immunochernical properties. The aqueous phase was dialyzed exhaustively against distilled water. The crude LPS preparation was precipitated by the addition of 3 volumes of acetone to the aqueous phase. The LPS was only slightly soluble in water. Its infrared spectrum was very similar to that of PWE (Fig. 3). However, in comparison with PWE, LPS showed a marked decrease in the amino acid content with a concomitant increase in reducing sugars and glucosamine (Table 1). All sugars detected in PWE were also present in LPS. In the analytical ultracentrifuge, the water-soluble part of LPS displayed a single, symmetrical peak with a sedimentation coefficient (s₂₅,₅₀) of 1.4S. On immunoelectrophoresis (Fig. 4), the LPS exhibited two precipitin lines with antiserum to PWE. The fast-moving precipitin arc had the same mobility as the PS-SC which was isolated by 1% acetic acid hydrolysis of PWE (28). The precipitin line closer to the antigen well was that of intact LPS. This relative immobility of endotoxin and LPS preparations in agar or agarose electrophoresis seems to be a characteristic property of these compounds. Holmgren et al. (7) have already shown that each of the LPSs isolated from 29 strains of E. coli gave immunoprecipitin lines close to the application well. The LPS preparation was only slightly less toxic for mice than was PWE (Table 2).

To characterize further the electrophoreti-
Fig. 3. Infrared spectra of purified whole endotoxin (PWE) and lipopolysaccharide fragments obtained by single (LPS) and multiple (LPS-M) extractions of endotoxin with hot 45% aqueous phenol.

| Table 1. Chemical composition of whole endotoxin and lipopolysaccharide fractions |
|---------------------------------|-------------|-------------|---------------|------------|------------|-------------|-------------|
| Fraction           | Nitrogen (%) | Phosphorus (%) | Reducing sugars (%) | Anthrone-positive carbohydrates (%) | Glucosamine (%) | Heptose (%) | Fatty acids (%) | Amino acids (%) |
| PWE                | 5.75        | 1.11        | 38.2               | 18.7          | 17.4        | 5.8         | 7.9         | 7.3         |
| LPS                | 5.43        | 1.10        | 55.8               | 24.6          | 21.2        | 8.6         | 8.9         | 0.5         |
| LPS-M              | 2.45        | 0.35        | 41.8               | 20.8          | 12.3        | 4.9         | 2.0         | 0.1         |

cally fast-moving compound and to determine the extent of its formation by the phenol treatment of PWE, LPS was chromatographed on Sepharose 4B. The elution pattern of LPS was characterized by two carbohydrate-positive peaks (Fig. 5). The first fraction (LPS-I) at the void volume was identified chemically as LPS. It displayed, on immunoelectrophoresis, a single precipitin arc close to the antigen well and exhibited high toxicity for mice (Table 2). The second fraction (LPS-II) showed a single, fast-migrating cathodal precipitin arc with antibodies to PWE. It was highly soluble in water. The ultracentrifugal pattern was characterized by a single, symmetrical peak with a sedimentation coefficient ($s_{20,w}$) of 1.2S. It contained no phosphorus, heptose, KDO, amino acids, or long-chain fatty acids. Its qualitative and quantitative hexosamine and hexose contents were the same as those of the recently described PS-SC (28). When applied to the Sepharose 4B column, a sample of PS-SC was eluted at the
The incubation period was 24 h.

The column fractions were eluted from a column (2.5 by 40 cm) with 0.15 M NaCl in 0.05 M phosphate buffer, pH 7.0. The volume collected was 3 ml per tube. The flow rate was 16 ml/h. The column was monitored by determining carbohydrates with the anthrone reaction.

FIG. 5. Gel filtration on Sepharose 4B of lipopolysaccharide preparations obtained by single (broken line) and multiple (solid line) extractions of endotoxin with hot 45% aqueous phenol. Fractions were eluted from a column (2.5 by 40 cm) with 0.15 M NaCl in 0.05 M phosphate buffer, pH 7.0. The volume collected was 3 ml per tube. The flow rate was 16 ml/h. The column was monitored by determining carbohydrates with the anthrone reaction.

The same volume as LPS-II. The PS-SC and LPS-II showed identical reactions with antibodies to PWE (Fig. 6). Neither of these two compounds was toxic for mice (Table 2). We concluded, on the basis of these results, that LPS-II was identical to the PS-SC.

Two additional treatments of LPS-I with 45% aqueous phenol at 60 to 70°C for 30 min each resulted in further cleavage of PS-SC. The infrared spectrum of this LPS-M was similar to that of PWE and LPS, except for a diminished ester carbonyl absorption (Fig. 3). Its chemical composition (Table 1) was characterized by a relatively small content of fatty acids and a negligible amount of amino acids. The ratio between anthrone-positive carbohydrates and glucosamine was higher for LPS-M than for LPS, indicating a loss of glucosamine by repeated phenol extractions of LPS. The LPS-M displayed two immunoprecipitin lines with antibodies to PWE (Fig. 7). The precipitin line closer to the antibody well gave an identical reaction with the analogous precipitin line of LPS. They both corresponded to PS-SC. Precipitin lines closer to the antigen well were those of intact LPS. The Sepharose 4B column chromatography of LPS-M also resulted in the separation of two carbohydrate-positive peaks with elution volumes identical to those of LPS (Fig. 5). The fraction eluted at the void volume (LPS-M-I) was identified as LPS, whereas the fraction LPS-M-II was shown immunochromically to be identical to PS-SC (Fig. 6). As expected, the multiple phenol extraction of LPS resulted in a higher yield of PS-SC. The low toxicity of LPS-M preparation (Table 2) was

FIG. 6. Immunodiffusion patterns of O-specific side chain (PS-SC) and fractions (LPS-II and LPS-M-II) retained on Sepharose 4B column. Antibody well contains antiserum to whole purified endotoxin. The incubation period was 24 h.

FIG. 7. Immunodiffusion patterns of lipopolysaccharides isolated by single (LPS) and multiple (LPS-M) extractions of endotoxin by hot 45% aqueous phenol. The antibody well contains antiserum to whole purified endotoxin. The incubation period was 24 h.

### Table 2. Toxicity of whole endotoxin and lipopolysaccharide fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Lethality (LD₅₀) for mice (µg/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PWE</td>
<td>270</td>
</tr>
<tr>
<td>LPS</td>
<td>550</td>
</tr>
<tr>
<td>LPS-I</td>
<td>450</td>
</tr>
<tr>
<td>LPS-II</td>
<td>&gt;2,000</td>
</tr>
<tr>
<td>LPS-M</td>
<td>&gt;2,000</td>
</tr>
<tr>
<td>LPS-M-I</td>
<td>750</td>
</tr>
<tr>
<td>LPS-M-II</td>
<td>&gt;2,000</td>
</tr>
<tr>
<td>PS-SC</td>
<td>&gt;2,000</td>
</tr>
</tbody>
</table>

The trough contains antibodies to purified whole endotoxin. The incubation period was 24 h.

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*Note: The text is a continued excerpt from a scientific article.*
due most probably to its relatively larger proportion of free PS-SC and a small proportion of intact LPS. After column fractionation, the LPS-M-I exhibited an LD₅₀ value characteristic of endotoxic compounds, whereas LPS-M was nontoxic.

Whereas the addition of ethanol resulted in precipitation of a dark purple substance from the first phenol phase (Fig. 1), it failed to precipitate any compounds from the second and third phenol phases. However, after removal of phenol by steam distillation, the subsequent lyophilization of aqueous suspensions yielded small amounts of yellow-colored substances. The precipitate from the first phenol phase and the residues of the second and third phenol phases were extracted with chloroform-methanol. The yield of extracted residues which were designated as P-I, P-II, and P-III was 7.1, 2.5, and 1.7%, respectively. All three fractions were insoluble in water, dilute acids, and common organic solvents. They contained amino acids, fatty acids, glucosamine, and phosphorus as the most characteristic constituents (Table 3). Fraction P-I had a very high content of amino acids and only small amounts of glucosamine, fatty acids, and phosphorus. It corresponded chemically and immunochemically to the so-called simple protein. It has been established recently (34) that simple protein isolated from the endotoxin of S. marcescens 08 consists of the entire protein moiety and a small segment of the lipid moiety. In contrast to fraction P-I, fractions P-II and P-III had a higher percentage of glucosamine and fatty acids and a lower percentage of amino acids. Qualitative sugar analysis indicated the presence of heptose and KDO. These results indicate that fractions P-II and P-III consisted of a small amount of simple protein and varying amounts of the polysaccharide core-lipid fragment and other unidentified degradation products of the lipid moiety. Results of qualitative gas-liquid chromatography analyses showed that P-I, P-II, and P-III fractions and their corresponding chloroform extracts contained lauric, myristic, palmitic, and β-hydroxy-myristic acids as the major fatty acid constituents.

The aqueous-phenol treatment of whole bac-

![Diagram](http://jb.asm.org/)

**Table 3. Partial chemical characterization of phenol fractions**

<table>
<thead>
<tr>
<th>Phenol fraction</th>
<th>Phosphorus (%)</th>
<th>Glucosamine (%)</th>
<th>Fatty acid (%)</th>
<th>Amino acid (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-I</td>
<td>1.30</td>
<td>2.5</td>
<td>1.00</td>
<td>76.3</td>
</tr>
<tr>
<td>P-II</td>
<td>1.83</td>
<td>10.4</td>
<td>4.17</td>
<td>7.4</td>
</tr>
<tr>
<td>P-III</td>
<td>1.28</td>
<td>4.1</td>
<td>2.72</td>
<td>2.3</td>
</tr>
</tbody>
</table>

**Fig. 8. Fractionation of degradation products resulting from hot 45% aqueous phenol treatment of purified lipopolysaccharide.**
tential cells also resulted in the isolation of simple protein, PS-SC, and free fatty acids as the main degradation products.

A time study of the effect of aqueous phenol treatment on the fragmentation of purified LPS was designed according to a scheme presented in Fig. 8. An LPS preparation (LPS-I) which contained no free PS-SC or fatty acids was treated with 45% aqueous phenol at three different time intervals (10, 30, and 60 min). The extent of fragmentation increased with time. Although negligible (1%) after 10 min, the hydrolysis of ester-bound fatty acids amounted to 11% of the total fatty acid content after 60 min of treatment. The cleavage of the PS-SC accounted for 2 to 3% of the LPS-I after 30 min and for 5% after 60 min.

**DISCUSSION**

Results of this study have confirmed our previous finding (34) that the well-established deproteinizing action of hot 45% aqueous phenol is caused by the cleavage of a phenol-sensitive linkage within the lipid moiety rather than at the point of its attachment to the protein moiety. Under such experimental conditions, phenol treatment of either native or isolated whole endotoxin results in the formation of two very characteristic fragments recognizable as LPS (33) and simple protein (16, 34). Since both fragments contain various proportions of covalently linked structural elements of the lipid moiety, neither the LPS nor the simple protein can be utilized as starting materials for the isolation of the intact lipid moiety. The degradative action of hot aqueous phenol is not confined solely to the cleavage of the lipid moiety. It also affects the polysaccharide moiety by causing a partial hydrolytic cleavage of the PS-SC and the already fragmented lipid moiety by partial hydrolysis of the ester-bound fatty acids. Removal of PS-SC from some of the LPS molecules leads to the formation of an additional fragment which consists of the polysaccharide core and a part of the lipid moiety. This fragment is similar, if not identical, to the LPS preparations that can be isolated from R mutants deficient in PS-SC (13, 14, 22).

The major fragments and the degradation products resulting from the phenol treatment of native or isolated endotoxins can be separated from one another by a combination of procedures such as partition, extraction, and column chromatography (Fig. 1 and 8). The LPS and PS-SC were partitioned into the water phase, and the simple protein and polysaccharide core-lipid fragment were partitioned into the phenol phase. Most of the free fatty acids were dissolved in phenol, but some were dispersed in water. If LPS were isolated directly from whole cells, the phenol phase would also contain lipid constituents of cytoplasmic membrane such as phospholipids, glycerides, and fatty acids (34). Fatty acids were removed from both the aqueous and phenol phases by exhaustive extraction with chloroform or n-heptane. The LPS can be separated from the PS-SC by gel filtration on Sepharose 4B. The isolation of polysaccharide core-lipid fragment was only attempted when LPS was used as the starting material. The fragment was isolated by exhaustive dialysis of phenol phase and subsequent lyophilization of phenol-free dialysate.

Although the trichloroacetic acid (pKₐ = 0.08) is a much stronger acid than phenol (pKₐ = 10.0), the isolation of a relatively intact endotoxin complex by trichloroacetic acid is due most probably to its extraction at low temperature (4°C). The importance of reaction temperature is best illustrated by the finding that cold aqueous phenol extraction does not dissociate the protein moiety from the endotoxin complex (33). Like phenol, trichloroacetic acid can also affect an acid-sensitive linkage(s) within the lipid moiety and the linkage between the LP-SC and the core portion of the polysaccharide moiety. The extent of this hydrolytic degradation is so negligible, however, that the reaction products could only be detected by sensitive immunological techniques. No free fatty acids were released when an intact endotoxin preparation was treated with trichloroacetic acid for 30 min at 4°C. The effectiveness of phenol as a hydrolytic reagent at higher reaction temperatures is probably enhanced by its property as an excellent solvent for proteins (9). Such a dual function of phenol may be a reasonable explanation for the fast rate of hydrolytic cleavage of the protein moiety. In contrast, the cleavage of the PS-SC is a relatively slow process depending mainly, if not exclusively, on the hydrolytic activity of phenol as a weak acid. It has already been established that the linkage between the PS-SC and the core portion of the polysaccharide moiety is very sensitive to mild acetic acid hydrolysis (5, 17, 28, 30). The present study shows that this linkage is also susceptible to the hydrolytic action of phenol. Recently, Lindberg and Holme (12) have detected, in LPS preparations from smooth and rough strains of *Salmonella typhimurium*, the presence of free PS-SC considered to represent free haptenic precursors rather than the products of hydrolytic degradation. Results of our experiments with a highly puri-
fied LPS preparation (LPS-I) now demonstrate that the free PS-SC could also be derived, especially in smooth strains, from the hydrolytic action of phenol. An increasing yield of PS-SC after repeated phenol extractions of LPS is an obvious consequence of this hydrolytic cleavage.

The usual acid sensitivity of some of the glucosamine bonds within the lipid moiety is demonstrated not only by the fast cleavage of the protein moiety but also by further degradative removal of glucosamine, phosphorus, and fatty acids from LPS by repeated extraction with phenol. The nature of these degradation products has not been established. It has been demonstrated, however, that certain proportions of fatty acids in phenol phases are products of the hydrolytic cleavage of their ester bonds. Although the initial hydrolysis (10 min) of ester-bound fatty acids was negligible (1%), after 60 min it amounted to 11% of the total fatty acids. It seems that this hydrolytic cleavage can also be the result of a simultaneous role of phenol as a solvent and as a weak acid. In contrast, trichloroacetic acid—a strong acid, but a poor solvent for long-chain acids—caused a negligible cleavage of esterified fatty acids at low temperature.

These results raise a number of questions regarding the structural chemistry of endotoxins. First, they provide some clues regarding the heterogeneity of various LPS preparations (2, 11, 19, 21) isolated by hot 45% aqueous phenol extraction. Unless submitted to meticulous purification procedures, such preparations may contain, in addition to intact LPS, capsular polysaccharides, nucleic acids, the free PS-SC, the core-lipid fragment, some unidentified lipid fragments, and fatty acids. Second, these results show that our present views about the structure of the lipid moiety are inadequate and incomplete. One of the major problems concerning the structural studies of the lipid moiety is the fact that an intact lipid moiety cannot be isolated from LPS preparations obtained by phenol extraction procedures. The lipid A preparations consist of a mixture of various degradation products (20); if prepared from LPS, the lipid A includes degradation products of only one segment of the lipid moiety (34). Although the accumulated evidence (1, 6, 8) indicates the existence of glycosidically bound diglucosamine fragments as the basic structural elements of the lipid moiety, their number and mode of attachment is not known. A final question concerns the nature of the linkage between the PS-SC and the polysaccharide core. It has been suggested (18) that in *S. typhimurium* LT2 the PS-SC is linked glycosidically (1 → 4) to the subterminal α-glucose residue of the core. However, the relative acid stability of this type of glycosidic linkage (11) seems to be incompatible with the unusual susceptibility of the PS-SC of *S. marcescens* (28, 30), *E. coli* (17), and *Pseudomonas aeruginosa* (5) to mild acetic acid and/or phenol hydrolysis.

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