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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

FRAGMENTATION OF THE ENDOTOXIN COMPLEX FROM SERRATIA MARCESCENS BIZIO AND DETERMINATION OF THE STRUCTURE OF ITS POLYSACCHARIDE SIDE CHAIN

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CHI-SUN WANG

Oklahoma City, Oklahoma

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FRAGMENTATION OF THE ENDOTOXIN COMPLEX FROM SERRATIA MARCESCENS BIZIO AND DETERMINATION OF THE STRUCTURE OF ITS POLYSACCHARIDE SIDE CHAIN

APPROVED BY

Peter Alaupovic

Martin Griffin

Bernard Rabinovitz

Samuel Balmer

Jary G. Mayer

DISSERTATION COMMITTEE

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FRAGMENTATION OF THE ENDOTOXIN COMPLEX FROM SERRATIA MARCESCENS BIZIO AND DETERMINATION OF THE STRUCTURE OF ITS POLYSACCHARIDE SIDE CHAIN

CHAPTER I

INTRODUCTION

Many disease producing bacteria can be divided into two classes: those that secrete toxin into the surrounding medium and those that harbor the toxin within the bacterial cell. In the first class, the toxin is called exotoxin; in the second, endotoxin.

Endotoxin preparations isolated from different Gram-negative bacteria exhibit similar biological effects. They are antigenic, toxic and pyrogenic. In lethal doses they induce hemodynamic effects resulting in irreversible shock. The chronic administration of small amounts of endotoxin to animals induces resistance to various infections, x-ray irradiation and hemorrhagic shock.

A common feature of Gram-negative bacteria is the occurrence of endotoxins in the cell wall. This localization has been clearly shown by Bladen and Mergenhagen (1). Studying a human strain of the anaerobic coccus, Veillonella parvala, they observed impressive changes of the bacterial cells under electron microscope after phenol/water extraction (Fig. 1). The outer plastic layer or cell wall (OM, outer membrane) had

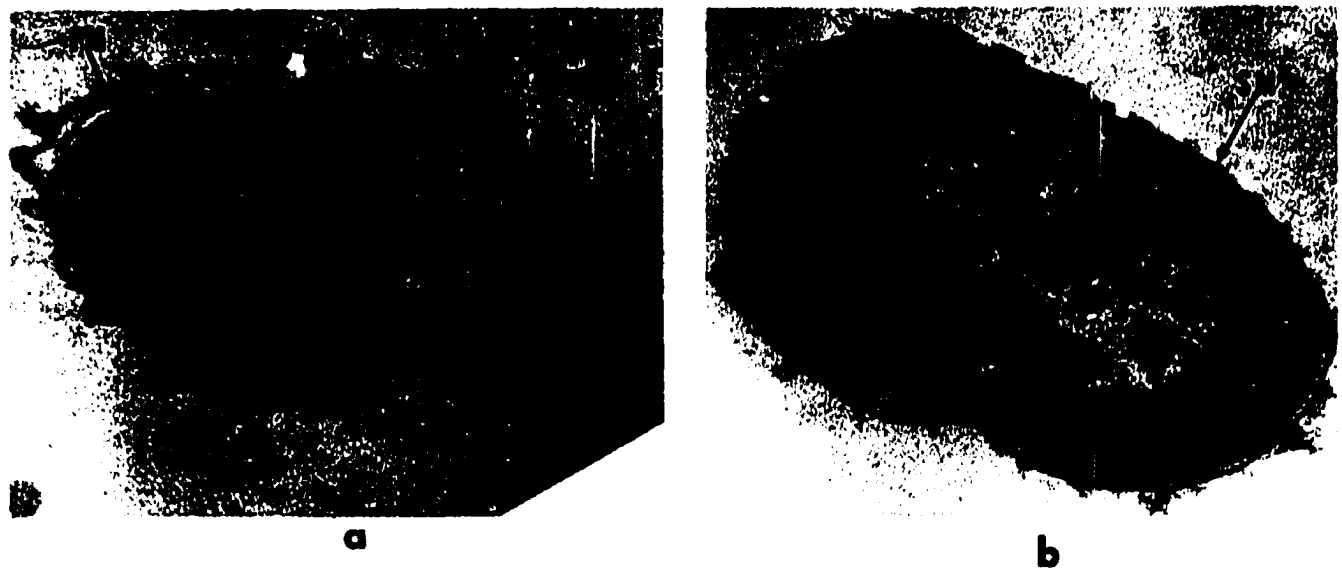


Figure 1. (a) Electron micrograph of a Veillonella cell: the outer membrane (OM), solid membrane (SM), and the plasma membrane (PM). (b) Electron micrograph of a Veillonella cell after phenol-water extraction (1). When compared with (a), it is evident that the outer membrane is absent. Magnification x 100,000.

been removed. But the cell retained its shape, owing to the still intact inner rigid layer (SM, solid membrane) and the plasma membrane (PM). The phenol/water extracts exhibited the biological and immunological activities characteristic of the somatic O-antigens or endotoxins.

In 1933, Boivin et al. (2, 3) first isolated endotoxin by trichloroacetic acid extraction of bacterial cells in the cold. Since then, this substance has been under extensive investigation. Terms such as Boivin O-antigen, lipopolysaccharide, tumor necrotizing agent, Shear's polysaccharide and Shwartzman material have been used to designate this biologically active substance. A complete survey of the findings related to these complexes may be found in several recent review articles (4, 5, 6, 7, 8, 9).

Endotoxins are not extracted from bacterial cells in the form of dissolved monomers. They form aggregates and tend to complex with a number of other natural products. This causes difficulties in obtaining these substances in a highly purified state (10). It also explains the very high molecular weight of endotoxin preparations as determined by light scattering photometry, analytical ultracentrifugation and gel-permeation chromatography techniques.

When Boivin investigated the isolated endotoxins, he considered the substance to be lipocarbohydrate (11), but failed to recognize a protein or protein-like constituent. It remained for Morgan and Partridge (12, 13) to demonstrate a protein moiety as a constituent of the endotoxin complex.

It is now generally agreed that undegraded preparations of endotoxin consist of lipid, protein and carbohydrate (7). A series of inves-

tigations from different laboratories using analytical and biochemical techniques have yielded similar results on the structure of endotoxins from various Gram-negative bacterial species. This has led to the conclusion that there is a common principle in the building-up of this macromolecule, as shown in Fig. 2:

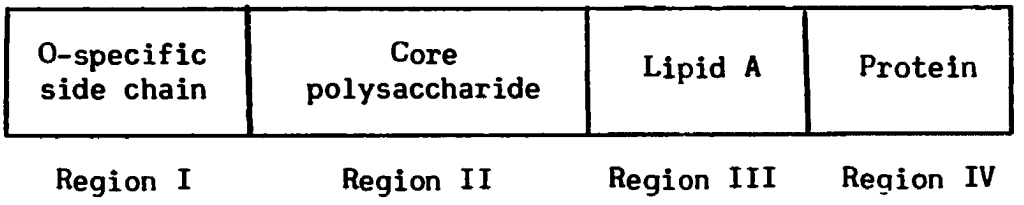


Figure 2. The four structural regions and their sequence in the endotoxin complex.

The side chain and the core are the two structural regions of the polysaccharide moiety. The side chain is a macromolecular entity consisting of repeating oligosaccharide units attached at a single point to the core polysaccharide. The core is in turn linked to the lipid and protein moieties. The lipid moiety is generally referred to as lipid A, following Westphal's terminology (17). Although lipopolysaccharide is only a fragment of the whole endotoxin complex, this name is frequently used as a synonym for endotoxin. The reasons for this are the popularity of the phenol/water extraction procedure which yields this product, and the fact that the lipopolysaccharide still retains the biological activity of whole endotoxin.

There are several labile linkages present in the endotoxin complex that make it possible to dissociate this macromolecule into its major components. Treatment of the substance with hot phenol/water (14) results in the separation of the lipopolysaccharide and the protein

moieties. The lipopolysaccharide can be further dissociated into the polysaccharide moiety and lipid A by heating with 1% acetic acid (15, 16). The same procedure may also lead to the dissociation of the endotoxin complex into degraded polysaccharide and conjugated protein (lipid A-protein). The degraded polysaccharide consists of a mixture of the O-specific side chain and the core-polysaccharide. These two structural components can be separated and purified by gel-permeation chromatography (18, 19, 20, 21). Due to the relative ease of isolation of highly purified polysaccharide fragments, this structural region represents the best known portion of the endotoxin molecule.

More than 20 sugars have been found to be constituents of the O-specific polysaccharide: hexoses (glucose, galactose, mannose); hexosamines (glucosamine, galactosamine, mannosamine); 6-deoxyhexoses (rhamnose, fucose); pentoses (ribose, xylose); and also rare sugars such as 3,6-dideoxyhexoses, 2-amino-2,6-dideoxyhexoses and 3-amino-3,6-dideoxyhexoses. Despite the wide compositional variations, the following sugars are present in all chemotypes (with rare exceptions): glucose, galactose, L-glycero-D-mannoheptose and 2-keto-3-deoxyoctonic acid (KDO) (5).

The sugar composition and the linkages of the constituent sugar units in the O-specific side chain are distinct and reflect the differences in serological specificity of bacterial species (6). The core is a complex oligosaccharide consisting of two distinguishable structural regions, namely, backbone and outer core. The inner backbone structure (through which the polysaccharide is linked to lipid A) is made up of heptose, KDO, phosphate and ethanolamine. To this short chain, the outer core which usually contains glucose, galactose and N-acetylglucosamine

provides the site for attachment of side chain.

The proposed structure of lipopolysaccharide of Salmonella typhimurium is shown in Figure 3.

The general architecture and composition of lipid A in O-antigens from various bacterial groups, including Salmonella, Shigella, Serratia and others seem to be alike (6). It is generally agreed that the main constituents of lipid A are glucosamine, fatty acids, and phosphate. Quantitative gas chromatographic analyses of fatty acid methyl esters of lipid A preparations from various bacteria have consistently revealed a relatively high concentration of β-hydroxymyristic acid. It appears to be a rather specific constituent and is frequently used as a marker for the presence of lipid A.

Westphal and Lüderitz (17) have suggested that the active group responsible for the toxicity and pyrogenicity of endotoxin is associated with lipid A. They coupled lipid A with an inert protein such as casein or serum albumin (17) and obtained an artificial lipoprotein which was very toxic and pyrogenic.

To what extent lipid A is responsible for toxicity is still a matter of controversy. It is questionable whether a quantitative relationship exists between lipid content and toxicity since Ribi et al. (22) have isolated a lipid A-poor lipopolysaccharide which was still a highly potent endotoxin.

The protein moiety of the endotoxin complex can be obtained in two forms. Treatment of the whole endotoxin complex with 1% acetic acid leads to the liberation of "conjugated protein" (12, 13) or "toxic protein" (23). Treatment of conjugated protein or of the whole endotoxin

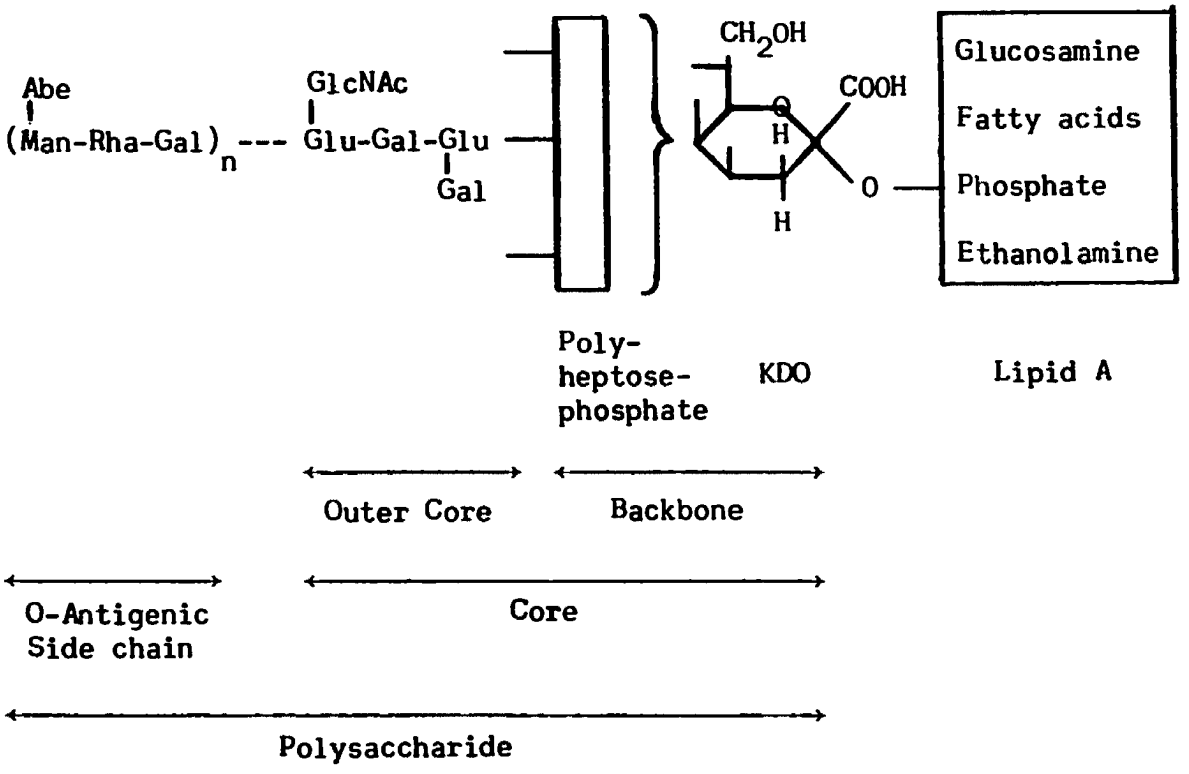


Figure 3. Postulated structure for LPS, modified according to Osborn (147), Heath et al. (148) and Nikaido (57).

complex with 90% phenol followed by ethanol precipitation produces "simple protein" (12). It has been suggested without proof that the protein moieties of endotoxin preparations from different bacteria are the same. Recent comparative studies on the protein moieties of the endotoxin complexes from Serratia marcescens and Escherichia coli by Wober and Alaupovic (24, 155) have indicated structural similarity on the basis of their immunological behavior and peptide patterns of tryptic digests. These authors also have presented evidence that the protein moiety is linked to the lipid moiety via covalent bonding rather than intermolecular association only. Two antigenic sites are present on the endotoxin molecule (24, 25, 156). One is located on the O-specific side chain, the other on the protein moiety.

The general goal of this study has been to isolate and characterize the endotoxin complex from S. marcescens Bizio, to degrade this macromolecule into some key fragments, and to determine their usefulness for studies on structural and biological properties of complete endotoxins. The specific aim has been to elucidate the chemical structure the O-specific side chain.

CHAPTER II

LITERATURE REVIEW

Isolation of the Intact Endotoxin Complex and Its Localization in the Cell Wall

The endotoxin complex contains several weak covalent linkages: phosphoglycosidic (26) and ketosidic bonds (27) are acid labile, while ester linkages are sensitive to alkali. Since some reagents designed to extract whole endotoxin may also attack chemically reactive functional groups within the endotoxin complex it is quite possible that too drastic isolation procedures may result in altered chemical and immunological properties of endotoxin preparations.

Boivin *et al.* (2, 3) first isolated an endotoxin complex from several strains of *Salmonella* and *Bacillus* by 0.25 N trichloroacetic acid extraction of cells at 4°C. This preparation later became known as Boivin antigen. The O-somatic antigen dissolves in the acid solution, whereas protein and nucleic acid remain with the cell debris. This is a general method of wide applicability to the "O" somatic antigens of smooth bacteria, but it generally fails to extract the more closely bound somatic antigens of rough bacteria and also polysaccharides from organisms which possess surface protein components (31).

Morgan (28) tested a number of glycols and utilized anhydrous diethylene glycol as a solvent for the extraction of the endotoxin from

Shigella dysenteriae. The product was a water-soluble undegraded material of high molecular weight. Chemically, it was a polysaccharide-lipid-protein complex (32) similar to that isolated by Boivin's method. Furthermore, the use of an anhydrous organic solvent was believed to reduce the possibility of extracting enzymes from the bacterial cells which might have been destructive to the antigen during later stages of purification in aqueous medium (3). Unfortunately, it was shown later that this method was not applicable to other bacteria.

Goebel et al. used 50% aqueous pyridine (29) or 50% aqueous glycol (30) for the extraction of the endotoxin complexes from S. flexeneri and S. sonnei, respectively.

Ribi et al. (33) showed that endotoxin preparations isolated by treatment of living cells with aqueous ether had biological properties similar to those resulting from the phenol extraction procedure.

Adams (34) used dimethylsulfoxide as an extraction agent for endotoxin complex; however, the simultaneous extraction of several other cell wall components complicated the subsequent purification procedures and limited greatly the applicability of this method.

Wilkinson (35), Leive et al. (36), Levy and Leive (37), Rogers et al. (38) reported that brief treatment with the chelating agent ethylenediaminetetraacetic acid (EDTA) in NaCl solution resulted in release of the lipopolysaccharide-protein complex from Escherichia coli and Pseudomonas aeruginosa.

The selective removal of the capsular, slim and other layers external to the rigid cell wall by extraction with organic solvents or enzymatic treatments offers a valuable means for investigating the anatomi-

cal interrelationships of the surface layers of the bacterial cell. Since most of the extraction procedures discussed are mild, it seems unlikely that the disruption of covalent linkages would occur during the isolation procedure. The extraction of the lipopolysaccharide-protein complex by solvent treatments of living cells indicates that the endotoxin is linked to the murein mainly, if not exclusively, through the physical forces; that is, they consist of either ionic or hydrophobic bonds, or both. The ability of EDTA to remove the endotoxin complex from the cell suggests that part of the complex is bound to the cell through a metal ligand.

Electron microscopic studies have shown the E. coli surface to consist of two layers separated by electron-dense material containing peptidoglycan (41, 42). Both electron-microscopic (42) and immunologic (43) evidence suggest that the lipopolysaccharide-protein complex is localized in the outer layer of the cell wall (43, 44).

Fragmentation of the Endotoxin Complex

The understanding of the chemical nature of the endotoxin complex has been derived mainly from degradation studies followed by proper characterization of each degradation product. A schematic summary of the degradation procedures is shown in Fig. 4.

Degradation of Endotoxin Complex with Phenol

In 1941, Morgan and Partridge (13) showed that the use of 90% phenol resulted in the dissociation of the endotoxin complex into the lipopolysaccharide and protein fragments. Palmer and Gerlough (157) treated whole bacteria with 95% aqueous phenol followed by water extraction. Phenol caused the endotoxin complex to dissociate in such a manner

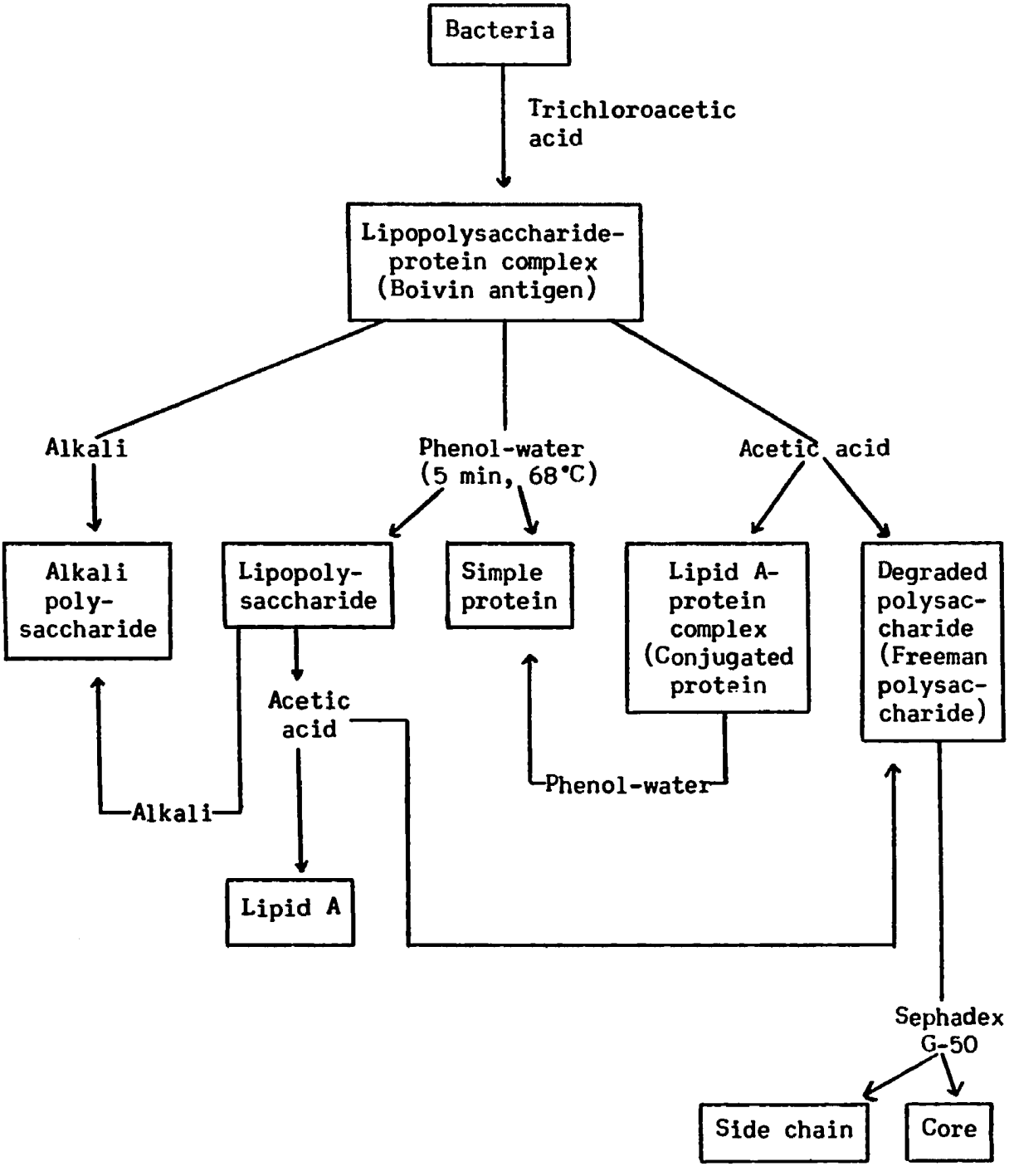


Figure 4. Preparation of fragments of O-antigen from bacteria.

that subsequent treatment with water led to the extraction of lipopolysaccharide of low protein content.

Later, Westphal and his coworkers (14, 46) developed a one-step hot aqueous phenol procedure for the isolation of lipopolysaccharide from intact cells. Dried bacteria were heated at 68°C for a short time in a mixture of phenol and water (45:55, v/v), which caused dissociation of the protein-lipopolysaccharide complex and the partitioning of the corresponding fragments between the phenol and aqueous phases. After cooling, the extracted cell residues appeared at the bottom of the container; the phenol phase contained lipid and protein, and the aqueous phase contained the lipopolysaccharide. The hot phenol method can be also utilized for the degradation of trichloroacetic acid isolated endotoxin complex (45, 149).

The manner in which phenol causes the dissociation of the protein moiety from the lipopolysaccharide is not yet known. A recent investigation by Wober and Alaupovic (155, 156) indicated that dissociation of the protein moiety from the endotoxin complex after phenol treatment was the result of cleavage of a relatively weak covalent linkage within the lipid A moiety rather than a sharp dissociation of the protein moiety from the lipopolysaccharide part of the endotoxin complex. It has been generally assumed that, except for the fragmentation of endotoxin into the protein moiety and lipopolysaccharide, this procedure causes little, if any, cleavage of covalent linkages. Proteins do not seem to be altered or denatured by phenol treatment as shown by determination of the physical-chemical parameters and enzyme activity of the ribonuclease (47) as a model compound. However, multiple treatments of the lipopoly-

saccharide of Serratia marcescens 08 with phenol resulted in the release of O-specific side chain and the cleavage of ester linked fatty acids (45, 48).

Chemical and Biological Properties of LPS

The lipopolysaccharide fragment consists of lipid A, polysaccharide core and polysaccharide side chain. This substance still exhibits its endotoxic activities. Injection of 1 μg into man (and correspondingly smaller amounts in experimental animals) results, after a short latent period, in the development of fever, with body temperatures of up to 40°C. It also still retains other biological activities such as changes in blood pressure, fibrinolytic activity, complement activation, non-specific resistance to infection, Shwartzman activity, adjuvant activity and lethal toxicity (8, 9, 22).

There exists a close relationship between the serotype of a species and the chemotype of its lipopolysaccharide. This relationship was first demonstrated for each serological group of Salmonella species in the Kauffmann-White scheme (49). Each Salmonella species is characterized by the serological specificity of its heat stable O-antigen (LPS) and its heat labile H (flagellar) antigen. By analogy, Escherichia (50), Shigella (51), and other genera were classified later in a similar way.

Chemical and Biological Properties of Simple Protein

In 1941, when Morgan and Partridge (13) treated the endotoxin complex of S. dysenteriae (Shiga) with phenol, they observed a decrease in nitrogen content of the isolated product. These authors investigated the phenol soluble material and concluded that protein was an integral

component of the endotoxin complex. This proteinaceous substance was called "simple amphoteric protein." Goebel *et al.* also isolated a "simple protein" from the endotoxin complex of the Z type of *S. paradysenteriae* (Flexner) or its "conjugated protein" by ethanolic sodium hydroxide treatment followed by isoelectric precipitation (23, 29). It has been shown that the protein moieties of endotoxins isolated from various Gram-negative bacteria have similar chemical composition.

The most recent studies by Wober and Alaupovic (155) indicate clearly that the simple proteins isolated from endotoxins of *Serratia marcescens* 08 and *E. coli* O 141:K85(B) consist of the protein moiety and a small segment of lipid moiety. Contrary to the earlier views (12, 29) the protein moieties of endotoxins isolated by aqueous phenol cannot be classified as "simple" proteins if this term implies the absence of constituents other than amino acids. However, it has been suggested that for historic reasons the term "simple protein" be retained for protein fragments released by aqueous phenol treatment of intact endotoxin.

The function of the protein moiety as a cell wall component is not known. When injected into animals, it is immunogenic and represents one of the two antigenic sets of determinants of the endotoxin complex (24, 25, 155). The other biological role of the protein moiety is its bactericidal activity in colicinogenic bacteria. Several articles have dealt with this subject (52, 53).

Degradation of Endotoxin Complex with Acetic Acid

White (54) introduced the use of acetic acid for the degradation of endotoxin. His method involved repeated treatment of the bacteria with

0.1 N acetic acid at 90°C. Studies by several investigators (12, 13, 15) have demonstrated that the acetic acid treatment results in the fragmentation of endotoxin molecules into a polysaccharide and a protein component. Freeman and Anderson (15) purified the polysaccharide component from endotoxin of Bacterium typhosum Ty 2 by several precipitations with alcohol and acetic acid and established that it represents the O-specific hapten (59) of the endotoxin complex.

When applying this method to the whole endotoxin molecule, earlier investigators neglected the fact that the polysaccharide moiety was cleaved by the acetic acid hydrolysis into the polysaccharide core and O-specific side chain and that the former one was probably eliminated by dialysis of the reaction mixture. Polysaccharide core has been mainly isolated from mutants which produce an endotoxin complex deficient in O-specific side chain (56). The isolation of polysaccharide core from the degraded endotoxin complex has been achieved recently using a Sephadex G-50 column chromatography (18, 19, 20, 21) or by collection of the outer dialysate during dialysis of the degraded polysaccharide fraction (24).

The proteinaceous fragment released from the endotoxin complex by acetic acid treatment forms a precipitate during hydrolysis. Morgan and Partridge (13) established the protein nature of this precipitate and designated it as conjugated protein. When this hydrolytic method is applied to lipopolysaccharide, the expected products - side chain, core and lipid A - may also be isolated (20).

It is well known that acetic acid is capable of hydrolyzing only weak covalent bonds such as phosphoglycosidic or ketosidic linkages (26, 27). It has been suggested that the polysaccharide core is glycosidically

linked to the lipid moiety through keto-deoxyoctonate residues (55, 56). This suggestion is based on the fact that KDO is the only reducing end group of the polysaccharide core and that the lability of the polysaccharide core-lipid linkage is similar to that of a KDO-glycosidic bond.

Nikaido (57, 58) proposed that polysaccharide core and side chain of the endotoxin from Salmonella typhimurium are linked through a glycosidic bond (Figure 5). However, in view of the sensitivity to acetic acid, it seems quite unlikely that these two regions of polysaccharide moiety are bound glycosidically.

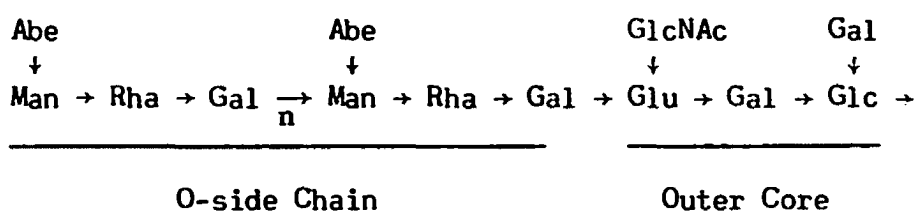


Figure 5. Proposed linkage of the O-side chain to the outer core of the polysaccharide moiety from Salmonella typhimurium.

The effects of acetic acid on several model compounds have been studied by Koeltzen and Conrad (79). There is no cleavage of either ester (19) or amide bonds during acetic acid hydrolysis. Ordinary glycosidic linkages are also stable to this treatment; the only monosaccharide which can be detected after such treatment is KDO. The glycosidic linkages of 3,6-dideoxyhexoses in polysaccharides are also acid labile; Tinelli and Staub (61) have reported that 1 N acetic acid treatment produces a degraded polysaccharide which is still not dialyzable but from which tyvelose has been lost.

Chemical Properties of O-specific Side Chains

Since a study of the structure of the O-specific side chain of

Serratia marcescens Bizio is one of the main aims of this investigation, our present knowledge of this portion of the endotoxin molecule will be discussed in detail. From studies on the polysaccharide moiety of the endotoxins, it has been established that the O-specific side chains are composed of repeating oligosaccharide units (5, 6, 7). The smallest repeating unit found in Salmonella groups, is a trisaccharide; in most cases the units are tetra- or pentasaccharides (6). From a comparative study of the wild type of E. coli K-12 with a mutant which had a deletion of the side chain in its lipopolysaccharide, Rapin and Mayer (62) suggested that the O-specific side chain of the wild type consisted of rhamnose-galactose disaccharide repeating units; however this suggestion has not been proved by chemical means. There are no reports indicating a single sugar as the only component of O-specific side chain.

The systematic investigations of the lipopolysaccharides from the genus Salmonella indicated a close relationship between the serotype and the chemotype (49). All 17 chemotypes of Salmonella O-antigens contain the basal sugars KDO, heptose, glucose, glucosamine, and galactose (7). This observation led to the concept that Salmonella polysaccharides might be composed of structurally identical or similar cores containing the basal sugars, and long side chains specific for each organism.

The constituent sugars of side chain are usually hexoses (glucose, galatose, mannose), hexosamines (glucosamine, galactosamine), 6-deoxyhexoses (rhamnose, fucose), and 3,6-dideoxyhexoses (tyvelose, abequose, colitose, ascarylose, paratose) (7). Other rare sugars, such as mannosamine (67), 4-amino-4,6-dideoxy glucose (68, 69), 2-amino-2,6-dideoxy-L-talose, 2-amino-2,6-dideoxy-L-galatose (7), 3-amino-3,6-dideoxy-

glucose (20), 3-amino-3,6-dideoxy-D-galactose (71) and D-rhamnose (89) have been reported to occur in lipopolysaccharides, but their exact location in the polysaccharide moiety has not been determined. Neuraminic acid is not a constituent of the lipopolysaccharide in at least the 80 bacterial species surveyed by Ellwood (72). KDO and heptose are constituents of the core polysaccharide and do not occur in the side chain.

D-ribose occurs in large amounts in preparations obtained from T1 forms (73, 74). Although its presence in other O-antigens has been reported (75), detection of D-ribose is most frequently an indication of the presence of nucleic acids as contaminants.

Most studies elucidating the structure of the O-specific side chains of various Salmonella species (Table 1) were done by Staub et al. (6, 7) and Robbins et al. (63, 116). Recently, Simmons studied systematically the structure of the O-specific side chains (Table 2) of the endotoxins from the genus Shigella (51, 64, 78). Hellerqvist et al. utilized methylation techniques with mass spectroscopic methods to confirm and supplement the structures of side chains proposed from earlier studies (65, 66).

Biological Properties of the O-specific Side Chain

The classical studies of Landsteiner and Scheer (59, 76) established the importance of stereochemical factors in determining the antigenic specificity of a synthetic antigen prepared by linking a simple organic substance to an azoprotein. However, knowledge about the importance of these factors in natural antigens is still rather limited. This is due to the fact that very few antigenic determinants of natural anti-

TABLE 1

REPEATING UNITS OF SIDE-CHAINS OF SALMONELLA O-SPECIFIC POLYSACCHARIDES*

O-groups	O-factors	Structure of Repeating Units
E_1	3,10	→α-Ac-Gal-(1→6)-α-Man-(1→4)-Rha-(1→3)→
E_2	3,15	→β-Gal-(1→6)-α-Man-(1→4)-Rha-(1→3)→ α-Glc ↓ 1 ↓ 4
E_3	(3),(15),34	→β-Gal-(1→6)-α-Man-(1→4)-Rha→ α-Glc ↓ 1 ↓ 6
E_4	1,3,19	→α-Gal-(1→6)-α-Man-(1→4)-Rha→ α-Tyv ↓
D_2	3,(9),46	→Gal-(1→6)-α-Man-(1→4)-Rha→ α-Abe ↓ 1 ↓ 3
B^-	4_1,12_1,27,27B	→α-Gal-(1→6)-β-Man-(1→4)-Rha→ α-Abe ↓ 1 ↓ 3
B	4_1,4_2,12_1	→α-Gal-(1→4)-β-Man-(1→4)-Rha→ α-Glc α-Abe ↓ 1 ↓ 1 ↓ 4 ↓ 3
B	4_1,4_2,5,12_1,12_2	→α-Ac-Gal-(1→4)-β-Man-(1→4)-Rha→ α-Tyv ↓
D	9,12_1,12_3	→Gal ⟶ Man-(1→x)-Rha→ α-Glc α-Tyv ↓ 1 ↓ ↓ 4
D_1	9,12_1,12_2,12_3	→α-Gal ⟶ Man-(1→x)-Rha→ → [Glc-(1→?)- β-Gal-(1→3)-GalNAc-(1→3)-GalNAc-Fuc→ Glc ↓ 1 ↓ 4
N	30	→β-Glc-(1→3)-GalNAc-Fuc→
U	43	→ [α-Gal ↓ 1 ↓ 3 β-Gal-(1→3)-GalNAc-(1→3)-GlcNAc-(1→4)-Fuc→
L	21	[α-Gal α-GlcNAc ↓ ↓ β-Gal-(1→3)-GalNAc-(1→3)-GalNAc→

*Ref. 6.

TABLE 2

REPEATING UNITS OF SIDE-CHAINS OF SHIGELLA O-SPECIFIC POLYSACCHARIDES*

Serotype	Structure
1a (I: 4)	α-Glc (1→4) →GlcNAc 1→2 Rha 1→4 Rha 1→
2a (II: 3, 4)	α-Glc (1→4) →GlcNAc 1→2 Rha 1→4 Rha 1→
Y_1 (-:3, 4)	→GlcNAc 1→2 Rha 1→4 Rha 1→
4a (IV:3, 4)	α-Glc (1→6) →GlcNAc 1→3 Rha 1→4 Rha 1→
3a (III:6, 7, 8)	α-AcGlc (1→2) →GlcNAc 1→3 Rha 1→4 Rha 1→
5 (V:7, 8)	α-Glc (1→2), α-Glc (1→3) →GlcNAc 1→3 Rha 1→4 Rha 1→
X (-:7, 8)	α-Glc (1→2) →GlcNAc 1→3 Rha 1→4 Rha 1→

*Ref. 51 with modifications.

gens have been adequately characterized. Secondary structural factors such as hydrogen bonding, conformation and carbon-carbon single bond rotation may occur in the antigenic determinants which have been defined and may effect antigenicity. Some aspects of immunological reactions of polysaccharides have been discussed by Allen (77) and Simmons (78).

Immunochemical investigations by Staub and Robbins resulted in the structural identification of the O-specific side chains and the localization of bacterial serological specificity. The monosaccharide that possesses the highest affinity for the anti-factor antibody is called the immunodominant sugar. Oligosaccharide fragments containing the immunodominant sugar are tested for precipitation inhibition (6).

These investigations were summarized by Lüderitz et al. (6) in the following manner: (a) in theory, each sugar unit can function as the immunodominant sugar; (b) the determinant structures overlap along the O-specific chain; (c) a family of antibodies is produced against a single immunodominant sugar; these antibodies are directed against larger or smaller areas of the determinant groups; (d) a determinant group commences with the immunodominant sugar and can extend to the right or to the left or in both directions to neighboring sugars, and (e) the Kauffmann-White scheme is an intentionally simplified one; many O factors can actually be differentiated into subfactors.

There appears to be a correlation between the relative pathogenicity of Salmonella strains and the composition of their O-specific side chains. The lipopolysaccharide which contains 3,6-dideoxyhexose is certainly more lipophilic in character. This may contribute to the special physical chemical properties of cell surfaces and represents the factor

responsible for its higher toxicity (50).

Nakano and Saito (80) and Medearis et al. (81) have suggested that the presence of O-specific side chains is an important factor in the virulence of a bacterium. This can be related to the ability of O-specific side chains to resist phagocytosis (80).

Bacterial infection by phage has also been correlated to the structure of the O-specific side chain (7). Losick and Robbins have concluded that the infection of a bacterial cell with one or more temperate phages may result in a change of specificity and consequently of the chemical structure of the O-antigen (82).

Salmonella Mutants with Atypical Specific Chains: T Forms

In 1956, two strains of Salmonella that could not be typed with any of the known sera were isolated in different hospitals in Europe (73). Fermentative studies indicated they were S. paratyphi and S. typhimurium. Kauffmann concluded that the new strains were actually mutants of these two species, in which the O-antigens had been replaced by new ones which he named T1 antigens. The T1 antigen contains a large amount of ribose in the specific side chain. Schlosshardt (84) suggested that many and perhaps all Salmonella species could in principle be converted into T1 forms. Berst et al. have reported the chemical structure of a T1 specific chain (74, 98).

Chemical and Biological Properties of Polysaccharide Cores

Due to neglect of the fact that polysaccharide core can be isolated from the outer dialysate after acetic acid degradation of the endo-

toxin complex, earlier studies of the core structure were carried out mainly with endotoxins isolated from mutant strains called the R forms. Since the R mutants occur spontaneously, they are frequently found in old cultures, in which they can be recognized by their specific morphological properties. Figure 6 shows the structure of the S. minnesota polysaccharide core.

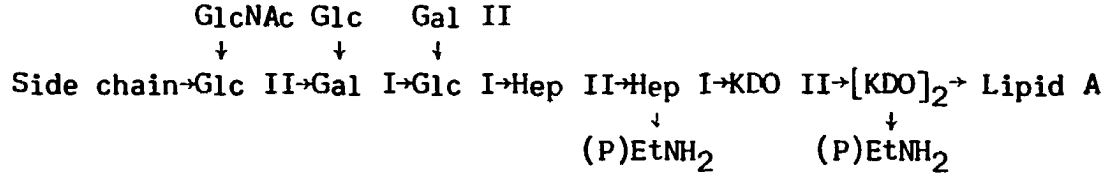


Figure 6. Structure of the polysaccharide core (4).

In contrast to structural specificity of O-specific side chains, the polysaccharide cores of all Salmonella species studied are structurally identical. This led to the idea that bacteria of other genera of Enterobacteriaceae may possess the same core structure as that found for Salmonella. Recently Johnston et al. (122) determined the structure of the terminal pentasaccharide of the polysaccharide core from Shigella flexneri (Figure 7).

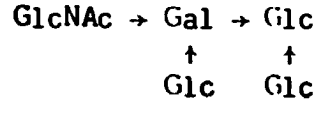


Figure 7. Structure of the terminal pentasaccharide of the polysaccharide core from Shigella flexneri.

A comparison of this structure with the corresponding portion of the Salmonella core shows that it is built up of the same sugar constituents but in a different way. Nevertheless, the general make-up of the two structures suggests a close evolutionary relationship between these two

groups.

In comparison to Salmonella, information about the core structure of E. coli R lipopolysaccharides is rather scanty. Results of genetic and chemical investigations (6, 89, 90, 91, 99, 100) are similar to those obtained with Salmonella. This would support the assumption that the core region of E. coli lipopolysaccharides might be very similar, if not identical to that of Salmonella. However, in 1948 Moller (92) isolated two R forms (R1 and R2) from representatives of E. coli O-group 8, which were serologically distinct. They did not cross react with each other. Schmidt et al. (153) studied the lipopolysaccharides from different E. coli R strains and demonstrated that their core regions had different quantitative and qualitative sugar compositions. Thus, it has become evident that there is more than one core type in E. coli lipopolysaccharides.

Schmidt et al. (95) compared polysaccharide cores from representatives of different enterobacterial genera, namely, Salmonella, Arizona, Escherichia and Citrobacter. From respective phage patterns and from the results of serological and chemical studies, the Arizona R-lipopolysaccharide corresponded in all respects to the Salmonella complete core type, thus indicating a close relationship between these two genera. The R lipopolysaccharides of Citrobacter and a mutant of E. coli differed from other R types by the very low content of galactose. The R structure of this E. coli mutant represents a new core type designated coli R3.

The mutants of all R classes are immunogenic and induce production of antibodies (4). The terminal nonreducing sugar residues of the defective lipopolysaccharides act as immunodominant sugars. S. typhi-

murium, S. paratyphi and S. enteritidis are some of the well known pathogens. Although R lipopolysaccharides are also toxic, the R forms are not pathogenic. This is due to the action of macrophages which phagocytize R mutants and render them harmless. The S forms, on the other hand, are protected from this fate because the long O-specific polysaccharide chains on their cell surfaces prevent the contact between the bacterial cell wall and macrophages (80, 81).

Chemical and Biological Properties of Lipid A

In 1954, Westphal and Lüderitz (17) first isolated a phospholipid from lipopolysaccharide. They referred to this compound released from endotoxin by mild acid hydrolysis as lipid A. It is now generally accepted that lipid A contains fatty acid, glucosamine and phosphate (6). The hydrochloric acid hydrolysis of lipid A and chloroform extraction results in the isolation of the fatty acid fraction. Ikawa and coworkers (158) were the first to identify lauric acid, myristic acid, palmitic acid, and β-hydroxymyristic acid as the major fatty acids of lipid A from E. coli. Burton and Carter (121), Kasai and Yamano (110), Taylor et al. (159) and Alaupovic et al. (149) performed quantitative gas-liquid chromatographic analysis of fatty acids from different lipid A preparations and obtained similar results. These studies have shown that β-hydroxymyristic acid is a rather specific constituent of lipid A. However, β-hydroxylauric acid was shown to be the main hydroxy acid of lipid A from Pseudomonas aeruginosa (96). Hydroxylaminolysis of lipid A results in an approximately 60% release of fatty acids as hydroxamate derivatives (6). The remainder of fatty acids resistant to hydroxylamino-

lysis were shown to be almost exclusively β-hydroxymyristic acid (6). It was, therefore, concluded that in lipid A β-hydroxymyristic acid was bound through an amide linkage while the other fatty acids and the remaining part of the β-hydroxymyristic acid formed ester linkages.

Despite considerable work on the structural analysis of lipid A, its exact structure is still unknown. Most studies were performed with preparations that were not submitted to rigorous purification procedures. As a matter of fact, there have been no claims or proofs for a homogeneous lipid A preparation. Despite this limitation, some worthwhile information was obtained by several investigators. Nowotny (160) suggested that the basic structure of lipid A from various organisms consists of a chain of acylated D-glucosamines linked through phosphodiester bonds. On the basis of sodium borohydride reduction and alkaline degradation of lipid A, Burton and Carter (121) challenged this structural proposal and considered two glycosidically bound acylated glucosamine molecules as the basic unit of lipid A. It has also been suggested by Alaupovic et al. (149) and Lüderitz (4) that in intact lipid A, glycosidically-bound diglucosamine units may be linked together by phosphodiester bridges.

Tsang (45) fractionated the lipid A preparation from Serratia marcescens 08 by silicic acid column chromatography into five major fractions. He characterized the fractions by sodium borohydride reduction followed by hydrolysis. On the basis of the glucosamine/glucosaminol ratio (which served as indicators for the size of the molecule), he concluded that lipid A does not represent the intact lipid moiety, but a mixture of degraded polyglucosamine fragments of various sizes. Recently, Adams and Singh (162) used a similar fractionation scheme and with the

aid of methylation confirmed this observation.

It was suggested by Tsang (45), and by Wober and Alaupovic (155) that phenol causes disruption of the lipid moiety. It seems that this random cleavage is one of the major reasons for heterogeneity of lipid A preparations. A further degradation of lipid moiety occurs during the hydrolytic isolation procedure of lipid A from lipopolysaccharide. It is, therefore, not surprising that heterogeneous lipid A preparations were obtained by all investigators who utilized this isolation procedure. It is obvious that any meaningful structural studies will depend exclusively upon the isolation of an intact lipid moiety.

The biological function of lipid A was investigated by several groups. The main difficulty in investigating the function of lipid A is the poor solubility of it in aqueous solutions. Binkley et al. (23) were the first to postulate a toxic factor T as the component of endotoxin. This factor T was later shown by Westphal and Lüderitz to be lipid A. Westphal and Lüderitz (17) were able to couple lipid A to an inert protein such as casein or serum albumin to obtain an artificial lipoprotein which was shown to be pyrogenic and toxic. In 1971, Wober and Alaupovic (156) using a dispersion of lipid A in Tris-SDS buffer demonstrated that this preparation exhibited toxicity similar to that of lipopolysaccharide. It has also been shown that the glycolipid of R mutant, which contains only KDO and lipid A, is just as toxic as the lipopolysaccharide of the corresponding wild type (8). The conjugated protein (lipid A-protein) after pronase digestion reveals an even higher level of toxicity (156). It can be concluded from these studies that lipid A is most probably responsible for the toxic property of endotoxins.

Chemical and Biological Properties of Conjugated Protein

During acetic acid degradation of the endotoxin, a precipitate appears in the reaction mixture. Morgan and Partridge (12, 13) first recognized this precipitate as "conjugated" protein. It has long been assumed, without any proof, that the "simple" protein and the "conjugated" protein are distinguished by the absence or presence of lipid A, respectively. A recent study on the protein moiety of S. marcescens and E. coli by Wober and Alaupovic (156) indicated that the protein portions of simple and conjugated protein are the same. The difference is that conjugated protein contains the intact lipid moiety, whereas the simple protein contains only a small fragment of the lipid moiety. The identity of the "simple" and "conjugated" protein moiety is based on the amino acid compostion and identical tryptic peptide patterns (155, 156). After acetic acid degradation, there was no detectable free lipid extracted by chloroform from the reaction mixture. The lipid was exclusively present in the precipitate. Treatment of the precipitated conjugated protein with 0.1 N HCl at 100° caused the release of a lipid which was similar to that isolated from lipopolysaccharide. It was concluded by Wober and Alaupovic (156) that conjugated protein contained the intact lipid moiety of the endotoxin complex.

The conjugated protein is biologically active. It has been shown that the lipid portion of conjugated protein is the toxophore of the endotoxin complex. Both "conjugated" and "simple" proteins were able to induce the formation of antibodies which cross-reacted with the intact endotoxin complex (156). Colicine activity of O-antigen was destroyed by trypsin and formaldehyde, suggesting that the protein moiety of the endo-

toxin complex is involved in the bactericidal activity (161).

Biological studies have revealed that both the "conjugated" protein and the "simple" protein give rise to the formation of antibodies which react with the intact O-antigen complex (23, 25). Both "toxic carbohydrate" and "conjugated" protein with lipid A as their common constituent were toxic to about the same extent. On the other hand, "simple" protein and polysaccharide hapten exhibited no toxicity.

So far, the nature of the linkage between the protein moiety and lipid A is not known yet. Morgan (13) suggested from his results that the protein binds to other components of the antigenic complex through intermolecular binding rather than covalent type forces. A different view was proposed by Wober (24). He suggested, with some evidence, that lipid A is linked to the protein moiety through β-aspartyl-N-acetyl-glucosaminylamine, which occurs frequently in glycoproteins as a carbohydrate protein linkage group.

Alkaline Degradation

Alkaline extraction of Gram-negative bacteria was first performed by Krumwiede and Cooper (101) using alkaline hypochlorite solution. The method was refined by Furth and Landsteiner (102) who obtained a purer polysaccharide. The principal action of alkali is deacylation. Archibald reported that treatment with either 0.5 N NaOH or $NaBH_4$ at room temperature had no effect on the phosphodiester linkage (113).

"Alkali" polysaccharides are obtained when dried bacteria (103) or lipopolysaccharides (104) are treated with 0.25 N NaOH (56). The alkali treated preparation consisted of the polysaccharide moiety and a part or all of the firmly bound O-deacylated lipid moiety. This indicated

that the linkage between lipid A and polysaccharide moiety was alkali stable. Quantitative periodate oxidation of the "alkali" polysaccharide indicated the presence of additional vicinal hydroxyl groups which had become available during the mild hydrolysis. Alkali treated polysaccharides had a molecular weight of about 200,000 and exhibited O-specificity (104), but were non-antigenic and non-toxic. However, they reacted as antigens when fixed to erythrocytes (106, 107). The loss of serological activity of some lipopolysaccharides after alkaline was attributed to the hydrolytic cleavage of O-acetyl groups and 3,6-dideoxyhexoses (146).

Hydroxylaminolysis (45, 108, 109, 110) cleaves the ester bound fatty acids in lipid A while hydrazinolysis removes the amide bound fatty acids (86). Treatment of lipid A with hydrazine resulted, among others, in the isolation of an oligosaccharide containing glucosamine and KDO but with β-hydroxymyristic acid absent. This observation excluded the possibility that the linkage of the polysaccharide core to lipid A was through the hydroxyl group of this fatty acid (86).

Enzymatic Degradation

Although quite a few bacteriolytic enzymes are known (111), it is quite surprising that no endoglycosidase which can degrade the lipopolysaccharide moiety, is yet available (5). Early studies by Freeman and Anderson (15) indicated that "degraded" polysaccharide was stable to treatment with amylase and glucosidase. However, some glycosidases, such as glucosidase, galactosidase, N-acetyl-glucosaminidase are powerful tools for the elucidation of the structure of oligosaccharides obtained from partial hydrolysis of lipopolysaccharides.

Studies utilizing proteolytic enzyme treatment have been carried

out quite successfully. Weber and Alaupovic (155, 156) employed trypsin and pronase digestion of "conjugated" protein and "simple" protein to obtain lipid A-rich tryptic and pronase cores. So far, proteolytic digestion has been utilized primarily for the removal of the protein moiety rather than for the elucidation of the primary structure of the protein moiety.

Kim and Watson (114) utilized papain to inactivate the endotoxic activity of lipopolysaccharide. They suggested that this was probably due to the splitting of ester and amide linkages by which fatty acids were bound to the glucosamine backbone of lipid A. Papain has been reported to be capable of splitting linkages of both types (116).

Recently, Nigam et al. (117) reported the presence of an enzyme from amoeba which was also capable of hydrolyzing the ester linkages in lipid A, with a maximum release of 50% of the long chain fatty acids.

Degradation by Periodate

O-antigens may or may not contain a 3,6-dideoxyhexose, but never has more than one member of this class been found in any one antigen. The glycosidically linked 3,6-dideoxyhexoses do not contain vicinal hydroxyl groups and therefore are resistant to periodate oxidation.

Staub and Tinelli (112) isolated a polysaccharide in which only the branched nonreducing terminal tyvelose was not destroyed. O-specificity is retained in periodate treated polysaccharides. This is important for the evaluation of the role played by 3,6-dideoxy sugars in immunological specificity.

CHAPTER III

METHODS AND EXPERIMENTAL PROCEDURES

Preparative Method

Bacterium

Cells of Serratia marcescens Bizio were obtained from General Biochemicals, Chagrin Falls, Ohio. Cells were grown in a medium containing NH_4Cl 0.1%, $MgSO_4 \cdot 7\ H_2O$ 0.02%, KH_2PO_4 0.5% and glucose 0.8%, at pH 7.2. Cells were harvested in late log phase by centrifugation in a Sharples continuous-flow centrifuge, washed in water and extracted in the wet state.

Isolation Procedures

Isolation of the endotoxin complex. Wet cells were extracted (Fig. 8) twice with trichloroacetic acid (2 liter TCA/kg cells) according to a modification of the method of Boivin and Mesrobeanu (3). The extracts were dialyzed against running water for 48 hours and then against distilled water for 24 hours, concentrated in vacuo to a small volume (approximately 300 ml) and centrifuged in a Spinco Model L ultracentrifuge at 40,000 rpm (105,000 x g). The nucleic acid-free sediment was lyophilized and free lipid was removed by chloroform/methanol (2:1, v/v) extraction in a Soxhlet apparatus for 24 hours. The residue represented

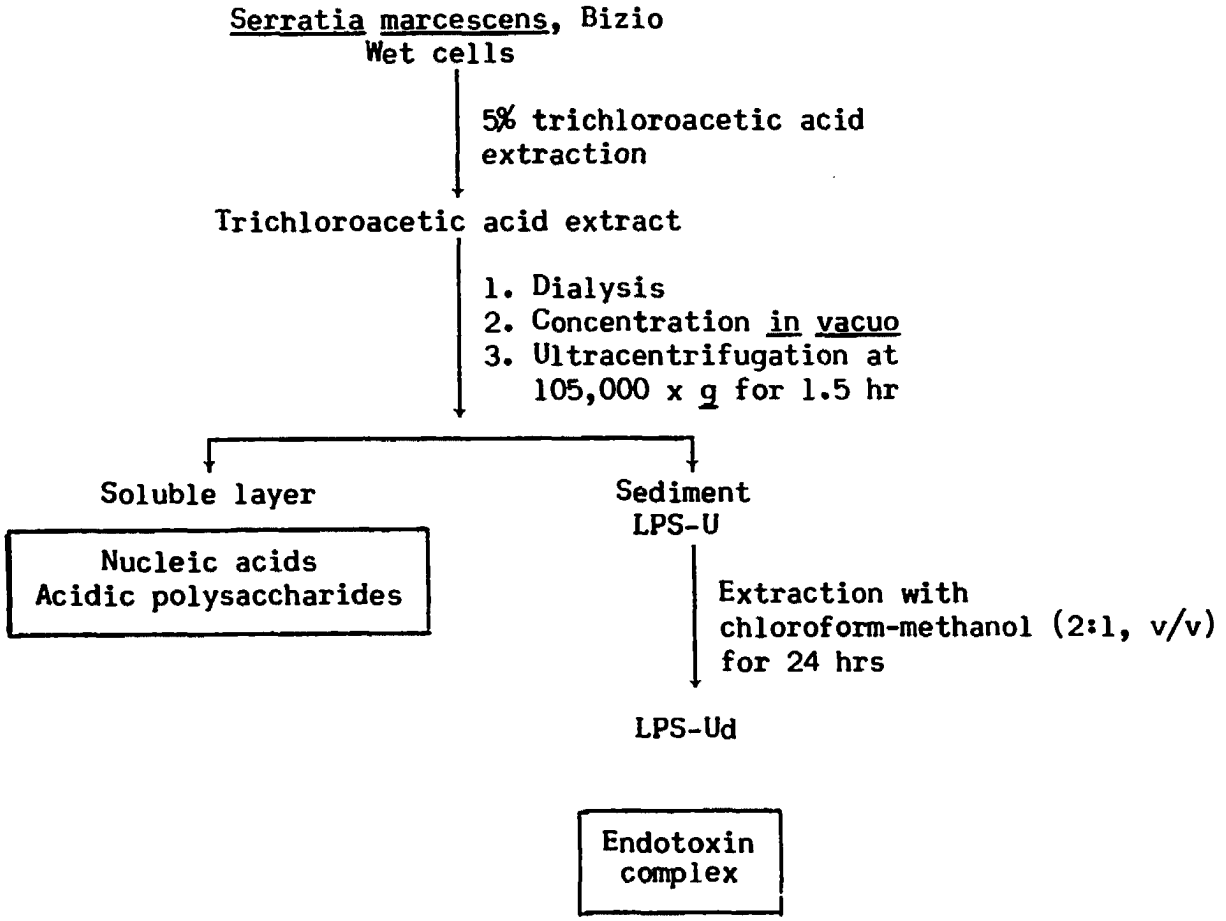


Figure 8. Isolation of endotoxin complex.

the purified parent endotoxin complex coded LPS-Ud (0.5 1% yield).

Isolation of lipopolysaccharide (LPS-A) and "simple" protein (PX-B) by degradation of the endotoxin complex with phenol. The lyophilized LPS-Ud was treated with 45% aqueous phenol solution for 30 minutes at 68°C according to the method of Westphal et al. (14) (Fig. 9).

The reaction mixture was cooled, and the phenol and aqueous phases were separated by low-speed centrifugation (2,000 rpm) at 4°C. The phenol phase was washed 3 times with equal volumes of water. The combined aqueous phases were dialyzed and concentrated in vacuo to a small volume. The solution was extracted twice with equal volumes of chloroform. Acetone was added to the aqueous phase to 75% saturation and the mixture was allowed to stand overnight in the cold. The overlayering solvent was removed from the precipitated material and the last traces of acetone were eliminated by evaporation and lyophilization. The isolated white dry substance was coded LPS-A (40% yield). The phenol phase was dialyzed against running tap water and then distilled water. The precipitate which formed after dialysis was collected by filtration through a sintered glass funnel. After washing with water, the precipitate was extracted with chloroform-methanol (2:1, v/v) in a Soxhlet extractor for 24 hours. This residue represents the so-called "simple" protein (PX-B) (30% yield).

Isolation of the conjugated protein and polysaccharide side chain and core after degradation of the endotoxin complex with acetic acid. Approximately 1 g of LPS-Ud was dissolved in 500 ml preheated 1% acetic acid (Fig. 10) and hydrolyzed for 4 hours with stirring at 90°C according to the method of Morgan and Partridge (13). After cooling, the

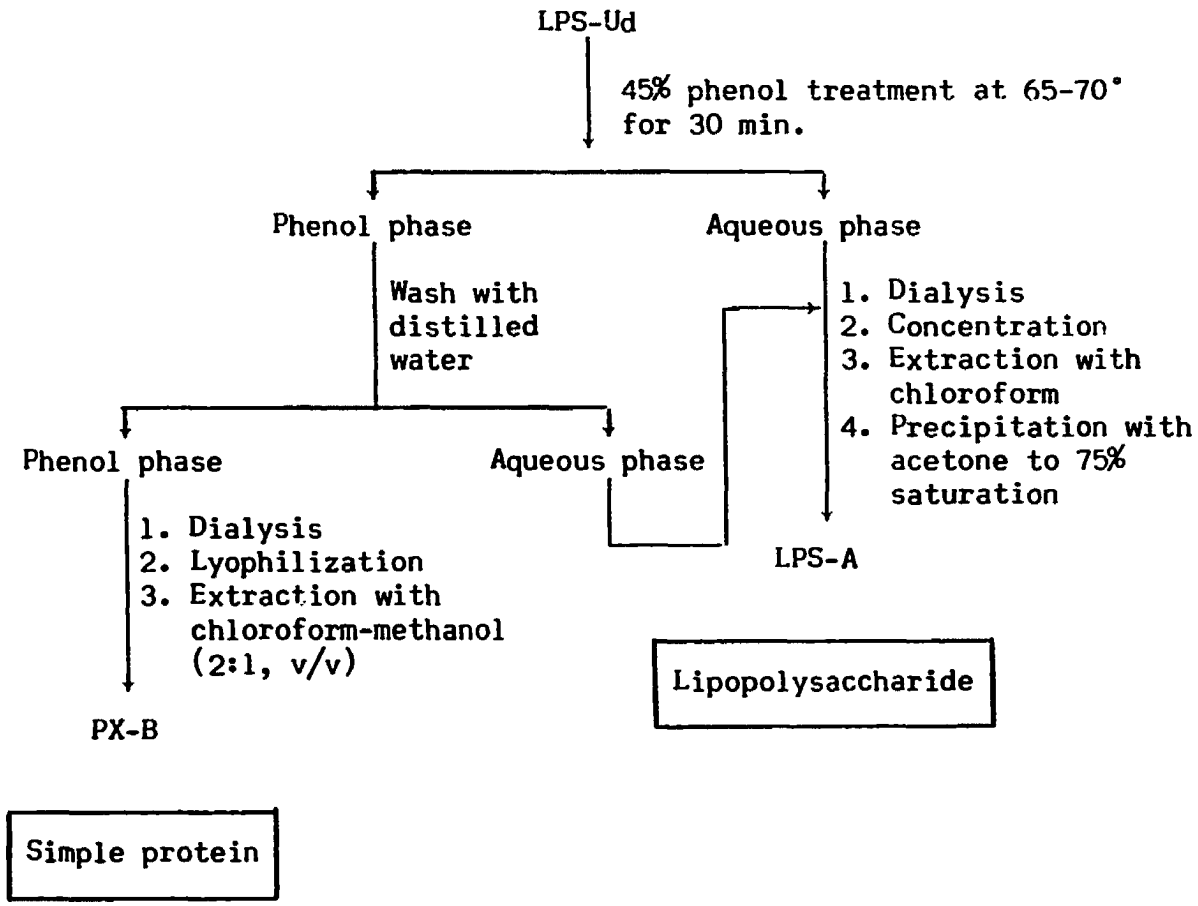


Figure 9. Degradation of the endotoxin complex with phenol.

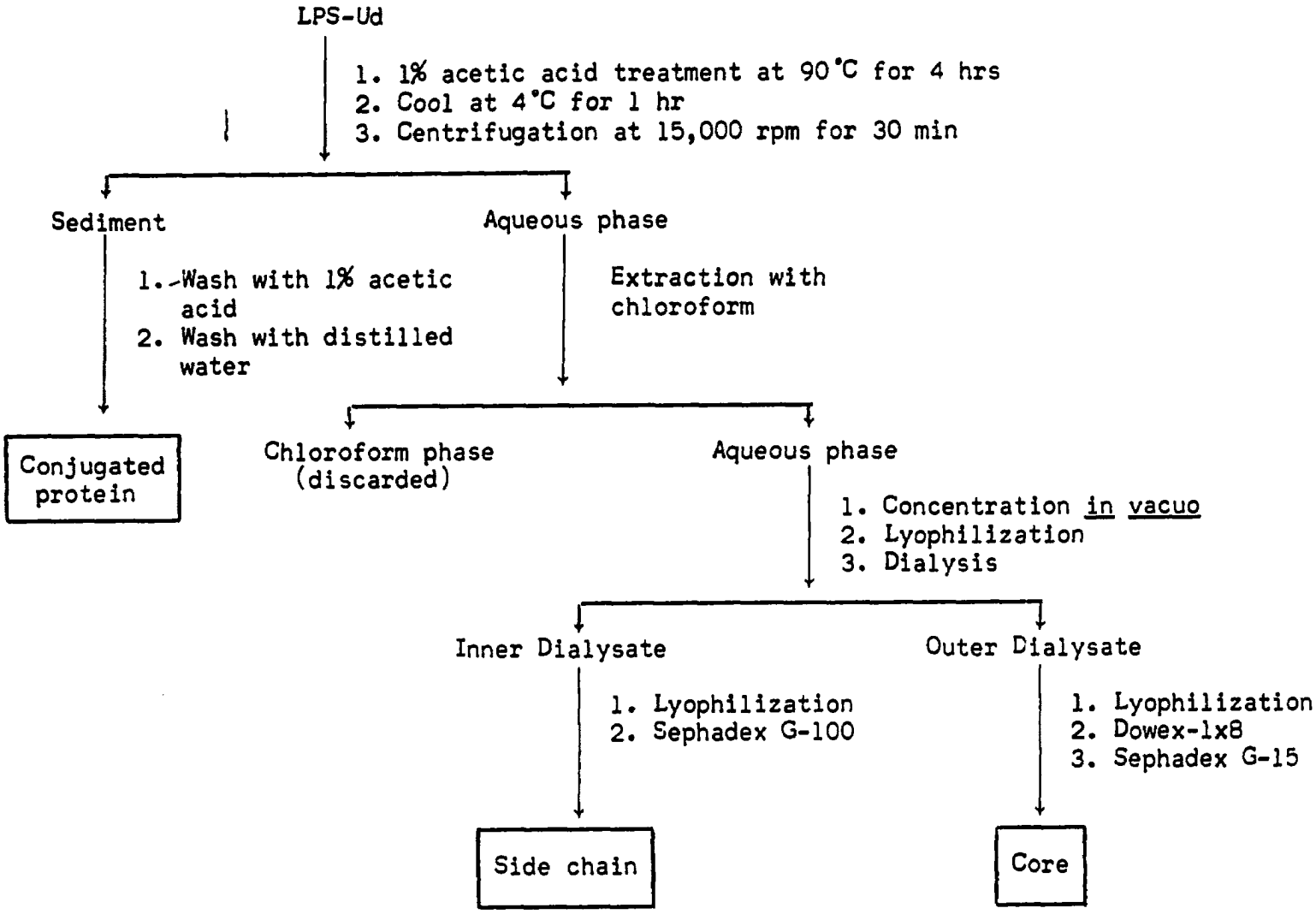


Figure 10. Degradation of the endotoxin complex with acetic acid.

precipitate (conjugated protein) was separated from the clear supernate by centrifugation at 15,000 rpm (27,000 x g) for 30 minutes at 10°C. The protein (50% yield) was washed with 1% acetic acid and water three times and tested each time for impurities by high voltage electrophoresis.

After removal of the conjugated protein, the clear supernate was extracted three times with chloroform. The lyophilized aqueous phase containing the "degraded polysaccharide" was dissolved in distilled water and then dialyzed against distilled water for 7 days. The inner dialysate contained the O-specific side chain, while the outer dialysate consisted mainly of polysaccharide core.

The inner dialysate was further purified by Sephadex G-100 column chromatography. About 200 mg of inner dialysate was dissolved in 3 ml distilled water and applied to a column (100 x 2.5 cm). The flow rate was about 30 ml/hour. Three ml fractions were collected and a 0.1 ml aliquot of each fraction was tested for carbohydrate by the anthrone-sulfuric acid method of Koehler (118).

The outer dialysate was purified by ion exchange chromatography on Dowex 1x8 (bicarbonate form). Two hundred mg of outer dialysate was applied onto the column (30 x 1 cm) and after an initial passage of 100 ml of distilled water the core was eluted with 100 ml 0.2 M ammonium bicarbonate. The eluant was lyophilized. In order to remove salt from this preparation, the substance was passed through a Sephadex G-15 column (100 x 2.5 cm). It was necessary to carry out this step twice in order to remove the salt completely. The purified polysaccharide core eluted at the void volume was utilized for further chemical studies.

Isolation of the lipid moiety from lipopolysaccharide. One gm

of LPS-A was heated with 100 ml of 0.1 N HCl at 100°C (Fig. 11). The mixture was refluxed for 30 minutes, cooled in an ice bath and extracted with three portions of chloroform. The combined chloroform extracts were washed with water to remove traces of acid and concentrated in vacuo. The free fatty acids were removed by repeated treatment of residue with acetone. After centrifugation, acetone was removed by decantation and the precipitate (lipid A) was designated A-AI (20% yield).

Isolation of the pronase cores of "simple" protein (PX-B) and conjugated protein (CP-B). The isolation of pronase cores was performed according to the method of Wober and Alaupovic (156). Fifty mg of PX-B or CP-B were suspended in 0.02 M $(NH_4)_2CO_3$ buffer, pH 8.6, heated for 30 seconds in a boiling water bath and cooled in an ice bath to 37°C (Fig. 12). Pronase (B grade, Calbiochem, Los Angeles, California), dissolved in a small volume of the same buffer, was added (protein/pronase, 100:1, w/w) to the protein solution and the reaction mixture was stirred for 24 hours at 37°C. The same amount of enzyme was added again and the reaction continued for another 24 hours. Addition of concentrated acetic acid (pH 3.5-3.8) to the reaction mixture resulted in the formation of a precipitate which was separated from the clear supernate by centrifugation at 15,000 rpm for 30 minutes at 10°C. After washing three times with 1% acetic acid and three times with distilled water, the lyophilized precipitate was designated pronase core of PX-B (10% yield) or pronase core of CP-B (40% yield).

Partial acid hydrolysis of the polysaccharide side chain. Two hundred mg of side chain were hydrolyzed with 0.5 N HCl for 2 hours at 100°C. The hydrolysate was dried by rotary evaporation in vacuo. The

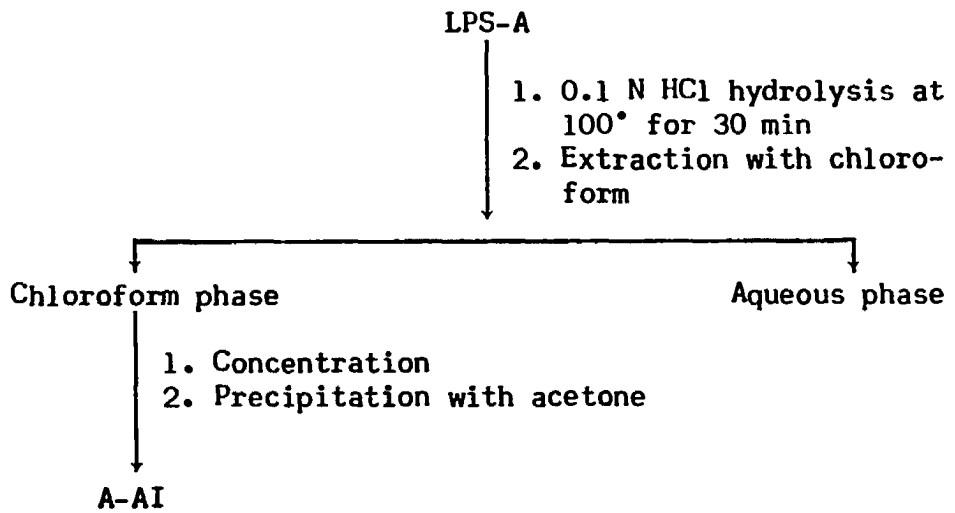


Figure 11. Isolation of the lipid moiety from LPS-A.

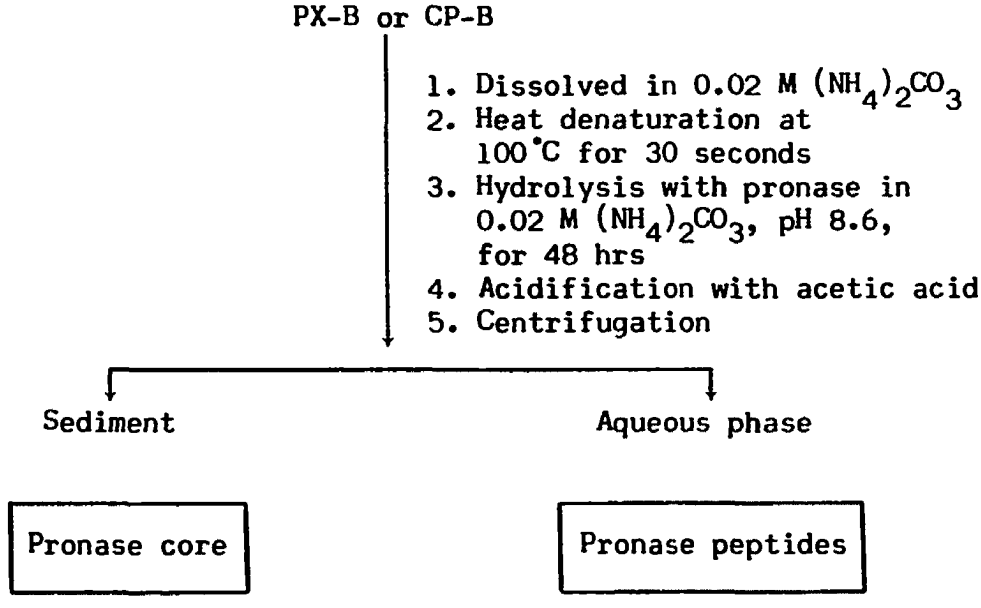


Figure 12. Preparation of pronase cores from PX-B and CP-B.

whole sample was then dissolved in 3 ml water and applied on a Sephadex G-15 column (100 x 2.5 cm). The disaccharide fraction was pooled and purified by paper chromatography in solvent system (a), as described under the methods of paper chromatography.

Analytical Methods

Paper Chromatography and High Voltage Paper Electrophoresis

Separation of carbohydrates was performed by descending chromatography on Whatman No. 1 paper. The solvent systems used were: (a) ethyl acetate/pyridine/water (3.6:1:1.15, v/v) (123), and (b) n-butanol/acetic acid/water (4:1:5, v/v).

The preparative paper chromatography was performed exclusively with the solvent system (a).

High voltage paper electrophoresis was performed at 3,000 volts for 30 minutes in a buffer composed of pyridine/acetic acid/water (1:10:189, v/v), pH 3.65. Neutral sugars were detected by alkaline silver nitrate, amino acids and amino sugars by ninhydrin (0.2% ninhydrin in 1-butanol), and sugar phosphates by the staining procedure of Hanes and Isherwood (124) or by methyl violet (125).

Thin Layer Chromatography

Lipids were separated by thin layer chromatography according to the method of Mangold *et al.* (126) on glass plates coated with silica gel G. Chromatograms were developed with petroleum ether/diethyl ether/acetic acid (80:20:1, v/v) as the solvent system for neutral lipids, and chloroform/methanol/water (85:19:2, v/v) or diisobutyl ketone/formic

acid/water (40:15:2, v/v) (127) as the solvent system for phospholipids. Glycerides, fatty acids, and phospholipids were detected by spraying with 50% sulfuric acid and charring, or with bromothymol blue (128), and the phospholipids with molybdenum blue (129) and ninhydrin.

Agarose Electrophoresis

Electrophoresis was performed in 1% agarose gel by the method of Grabar and Williams (130) employing the barbital buffer, pH of 8.6, ionic strength 0.05. The plates were fixed for 60 minutes in a solution of acetic acid-ethanol-water (5:70:25, v/v), washed for 8 hours in distilled water and dried at room temperature. Amido Black 10B was used for protein staining.

Gel Permeation Column Chromatography

Gel filtration on Sephadex columns was performed according to the standard procedures. Sephadex G-100 was utilized for the purification of O-specific side chain. Sephadex G-15 was used for desalting and also purification of the polysaccharide core.

Ultracentrifugal Analysis

Ultracentrifugal analyses were performed in a Spinco Model E ultracentrifuge equipped with a phase plate schlieren diaphragm and an automatic temperature control unit. Plate measurements were made with a Nikon microcomparator (Nikon Co., Japan) having a sensitivity of 0.001 mm. Sedimentation rates were determined at constant temperature (25°-26°C) with a rotor speed of 56,100 rpm. Samples were dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.1% sodium dodecyl sulfate. The observed sedimentation coefficient was calculated by the usual method (132).

Partial Specific Volume

Partial specific volume was determined by a pycnometric method (138). The values obtained from three separate determinations were averaged.

Infrared Spectroscopy

Infrared spectra were obtained with a Beckman Infrared Spectrophotometer JR10 using potassium bromide pellets (1.5-1.8 mg of substance and 250 mg KBr).

NMR Spectroscopy

NMR spectroscopy was performed by Sadtler Research Laboratory, Inc., Philadelphia, Pa. The samples were examined on a Varian A-60 A MHZ, proton magnetic resonance spectrometer. Hydrogen deuterium oxide was used as solvent and internal standard. The determinations were made at a probe temperature of 36°.

Amino Acid Analysis

Approximately 2 mg of each sample (in duplicate) were hydrolyzed with 6 N HCl in evacuated, sealed tubes at 110°C for 24 hours. To each sample, 0.05 μmole of norleucine and 100 μg of heptadecanoic acid were added as internal standards. The hydrolysates were extracted three times with chloroform to remove fatty acids. The aqueous phases were evaporated to dryness *in vacuo*, redissolved in 2 ml of 0.2 N sodium citrate buffer, pH 2.2, filtered through a sintered glass filter and aliquots were used for the determination of amino acids. Analysis was performed on a Beckman Model 120C amino acid analyzer with the long (36 cm) and short (6 cm) columns packed with Beckman PA28 and PA35 resins, respectively. Neutral

and acidic amino acids were eluted at 37°C on the long column with 0.2 N citrate buffer, pH 4.25, for 130 minutes. Basic amino acids were separated on the short column. The amino acid analyzer was calibrated with the Type 1 amino acid calibration mixture.

Fatty Acid Analysis

Chloroform extracts containing heptadecanoic acid as an internal standard were evaporated to dryness, redissolved in 0.5 N methanolic KOH and extracted with n-heptane. The methanol phase was then acidified and evaporated to a small volume and the fatty acids were extracted with chloroform and esterified with boron trifluoride-methanol reagent (Applied Science Laboratories, Inc., State College, Pa.). To a fatty acid sample dissolved in 1 ml benzene, 3 ml 10% boron trifluoride in methanol was added. The mixture was heated in a boiling water bath for 15 minutes, water was then added, and methyl esters of fatty acids were extracted with 3x6 ml hexane. After evaporation of hexane, the fatty acid esters were analyzed on a Barber-Colman gas chromatograph, Series 5000. The glass column (6 ft. in length and 5 mm in diameter) was packed with 15% diethyl glycol succinate on chromsorb W AW, 80/100 mesh (Superlco, Inc., Bellefont, Pa.). The flow rate of air was 306 ml/min, carrier gas (argon) was 54 ml/min and hydrogen gas was 30-42 ml/min. The injector temperature was 220°C while detector temperature was 245°C. The chromatograph was calibrated with NHI mix D (Supelco, Inc.).

Qualitative and Quantitative Determination of Neutral Sugars by Gas-liquid Chromatography

Polysaccharides were hydrolyzed with 1 N HCl at 100°C for 4

hours. Sugars were determined by gas-liquid chromatography of their reduced and acetylated derivatives (133). The glass column (6 ft.) was packed with 3% ECNSS-M on Gaschrom Q, 80/100 mesh (Supelco., Inc.).

Quantitative Determination of Formaldehyde and Acetaldehyde

The sample in aqueous solution was analyzed by gas-solid chromatography. The glass column was packed with porapak QS, 80/100 mesh (Waters Assoc., Inc., Framingham, Mass.). The column temperature was 150°C, while the injector and detector temperatures were 200°C and 235°C, respectively.

Elementary Analyses

Elemental nitrogen, carbon and hydrogen analyses were performed by Galbraith Co., Knoxville, Tennessee. Organic and inorganic phosphate were determined by the method of Gerlach and Penticke (134) and Chen et al. (135).

Colorimetric Analysis for Carbohydrate

Anthrone positive carbohydrate. The anthrone positive carbohydrates were estimated according to the method of Koehler (118).

Reducing sugars. The reducing sugars were determined by the method of Somogyi and Nelson described by Navais et al. (136). Polysaccharides were hydrolyzed with 1 N HCl in sealed tube for 4 hours at 100°C.

D-Glucose. D-Glucose content was determined by the glucose oxidase method (145).

D-Galactose. D-Galactose content was determined by the galactose oxidase method (144).

Hesoxamine. Hexosamine content was determined by the method of Rondle and Morgan (143). Samples were hydrolyzed in sealed tube with 4 N HCl at 100°C for 6 hours.

2-Keto-3-deoxy-octonic acid. The semi-quantitative determination was performed colorimetrically with the thiobarbituric acid method (137).

L-Rhamnose. L-Rhamnose was determined by the method of Dische and Shettles (138).

Periodate Oxidation

The consumption of periodate was followed by measuring the decrease in absorption of periodate ion at 222.5 mμ according to the method of Aspinall and Ferrier (139).

Methylation Analysis

Polysaccharide was methylated by Hakomori's method described by Sanford and Conrad (140). The methylated polysaccharide was hydrolyzed with 1 N HCl in aqueous methanol at 100°C. The hydrolyzed and methylated sugars were reduced and acetylated. The sugar derivatives were then analyzed on a gas chromatograph (Barber-Colman, Series 5000) employing a glass column packed with 3% ECNSS-M on Gaschrom Q, 80/100 mesh (141). Xylitol pentaacetate was utilized as an internal standard.

Enzymatic Degradation of Oligosaccharides

Degradation by α-Glucosidase

Three-tenths ml of α-glucosidase (Sigma Chemical Company, St. Louis, Mo., 1 mg/ml in water) was added to 0.3 ml of substrate solution

(1 mg/ml). Five-tenths ml of 0.1 M phosphate buffer, pH 7, was added and the reaction mixture was incubated at 37°C for 12 hours. Five-tenths ml of this solution was assayed for free glucose by the glucose oxidase method.

Degradation by β-Glucosidase

A 0.45 ml aliquot of β-glucosidase (Sigma, 1 mg/ml in 0.1 M sodium acetate buffer, pH 5) was added to 0.3 ml of substrate solution (1 mg/ml) and incubated for 6 hours at 37°C. The release of glucose was determined by the glucose oxidase method.

Degradation by Hesperidinase

A 0.5 ml aliquot of hesperidinase (Miles Laboratory, Inc., Elkhart, Ind., 1 mg/ml in 0.1 M sodium acetate buffer, pH 3.5) was added to 0.5 ml of substrate solution (1 mg/ml) and incubated at 37°C for 12 hours. After incubation, the reaction mixture was desalted by ion exchange chromatography on Dowex 50(H^+) and the released monosaccharide was identified by paper chromatography. When the substrate was rhamnosyl-glucose, the assay was based also on the glucose release determined by glucose oxidase.

Preparation of Antisera

White rabbits were injected twice a week intraperitoneally with 2 ml of a saline suspension of antigen (1 mg/ml), emulsified with 2 ml of complete Freund's adjuvant. Subcutaneous injections were avoided to prevent local necrosis (Schwartzman phenomenon). Animals were bled by cardiac puncture at the end of four weeks. The presence of antibodies was tested by double diffusion in agar gel.

Immunodiffusion and Immunoelectrophoresis

The immunological properties of antigenic substances were tested by double diffusion (131) and immunoelectrophoresis (164) in 1% agar gels employing Veronal buffer, pH 8.6, ionic strength 0.1. The agar plates were prepared by heating the mixture in a boiling water bath for 20 minutes, followed by cooling to 56°C. The solution was then transferred at 56°C to leveled glass slides supported by a plastic frame. After cooling, the slides were transferred to a closed chamber saturated with moisture and equilibrated for at least 2 hours prior to use. Wells of 3 mm diameter were made with a gel punch. The plates were allowed to develop for 24-30 hours after filling the wells with antigen and antibodies. Developed plates were washed several times with 0.15 M NaCl and distilled water, and dried at room temperature.

Lethalility for Mice

White male mice (Balb-C inbred mice, Texas Inbred Mouse Co., Houston, Texas) weighing 17-22 g were used for toxicity studies. The endotoxin preparations were dissolved in 0.05 M Tris buffer containing 0.5% SDS, pH 7.6, and injected interperitoneally. Control groups were injected with the buffer. Each group contained 6 mice. Deaths occurring within 7 days were recorded. The LD_{50} was calculated according to Cornfield and Mantel (142).

$$M = h - d\left(\frac{A}{n} - 0.5\right)$$

Where M = log max. dose,

d = log interval equals 0.3,

A = number of dead animals,

n = number of animals in each group.

CHAPTER IV

RESULTS

Fragmentation of the Endotoxin Complex

Preparation of the Endotoxin Complex and Its Degradation Products

The endotoxin complex was prepared by the trichloroacetic acid extraction procedure. The absence of nucleic acid contamination was indicated by the lack of an inflection at 260 mμ of the UV absorption spectrum. The absence of uronic acid in the preparation indicated that the acidic polysaccharides had been completely removed by ultracentrifugation. The lack of muramic acid showed that the murein layer was not extracted along with the lipopolysaccharide-protein complex.

The crude endotoxin complex (LPS-U) contained a significant amount of non-covalently bound lipid (about 20% of its dry weight). Thin layer chromatography of the free lipid indicated the presence of triglycerides, free fatty acids and phosphatidylethanolamine as the principal phospholipid. There was also indication of the presence of glycolipids. Further investigation of this non-covalently bound lipid was not attempted.

Qualitative and quantitative analysis of the endotoxin complex, LPS-Ud, indicated the presence of carbohydrate, lipid and protein. LPS-Ud

was used as the parent compound for fragmentation studies. The "conjugated" protein (CP-B), O-specific side chain and polysaccharide core were obtained by acetic acid hydrolysis and the lipopolysaccharide (LPS-A) and simple protein (PX-B) by phenol degradation of the endotoxin complex. Degraded lipid A was isolated by chloroform extraction of the mild acid-treated lipopolysaccharide. Lipid A-rich fragments were obtained by pronase digestion of the "simple" protein and "conjugated" protein (CP-B pronase core and PX-B pronase core).

The Physical-Chemical Properties of the Endotoxin Complex and Its Fragments

The solubility of endotoxin fragments correlated well with their carbohydrate content. The side chain and polysaccharide core were readily soluble in distilled water. LPS-Ud and LPS-A gave opalescent solutions, while PX-B and CP-B were insoluble. Degraded lipid A was soluble in chloroform or chloroform/methanol mixtures but insoluble in acetone. Due to these solubility difficulties, the ultracentrifugal analyses were performed in Tris-Cl buffer in the presence of SDS. The ultracentrifugal sedimentation patterns and the observed sedimentation coefficients of endotoxin fragments are shown in Fig. 13 and Table 3.

LPS-Ud, PX-B and CP-B migrated in agarose electrophoresis as single bands stainable with Amido Black (Fig. 14). LPS-A showed no positively stained band indicating the absence of protein in the preparation.

The infrared spectra of endotoxin complex and its fragments (Fig. 15, 16) were characterized primarily by the absorption peaks at 1735-1725 cm^{-1}, 1660-1650 cm^{-1} and 1550-1540 cm^{-1}, attributed to ester, amide I and amide II absorptions, respectively. The broad peak at

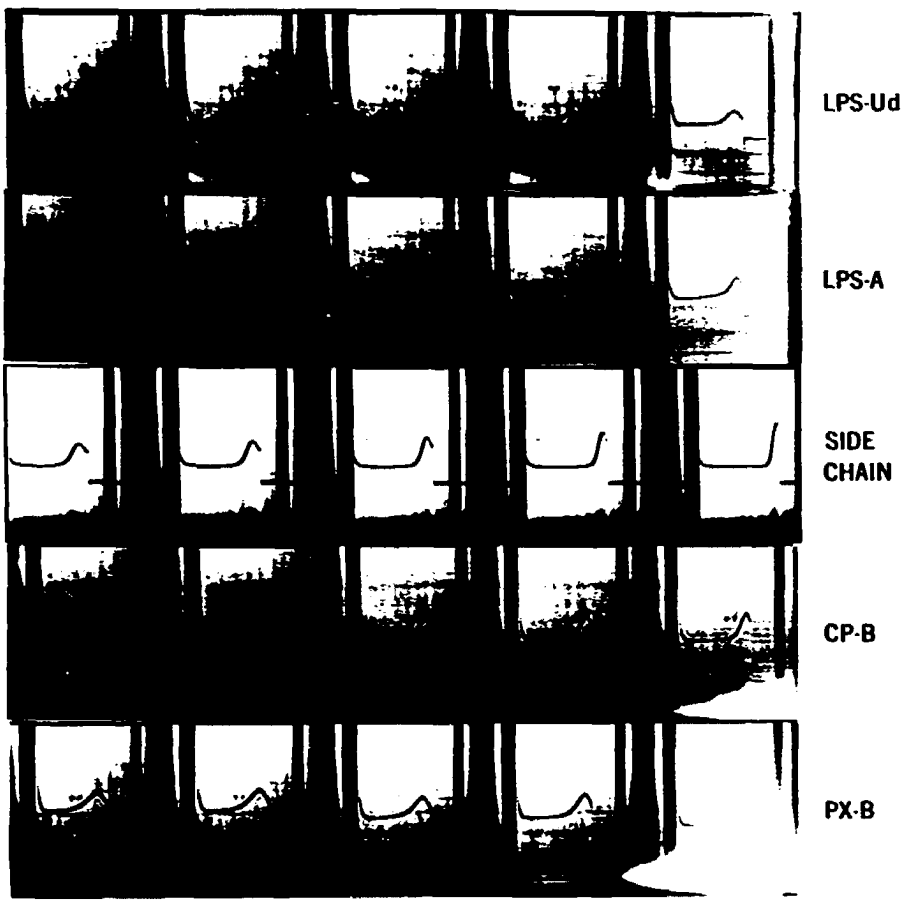


Figure 13. The ultracentrifugal schlieren patterns of endotoxin complex and its fragments. (a) Intact endotoxin (LPS-Ud), (b) lipopolysaccharide (LPS-A), (c) side chain, (d) conjugated protein (CP-B) and (e) single protein (PX-B).

Samples (5 mg/ml) dissolved in 0.05 M Tris-Cl buffer containing 0.1-0.2% SDS, pH 7.6, were centrifuged at 52,640 rpm at 25°C. Exposures were taken from right to left at 16 minute intervals.

TABLE 3

OBSERVED SEDIMENTATION COEFFICIENTS OF ENDOTOXIN COMPLEX AND ITS FRAGMENTS

	S_{obs}
LPS-Ud	1.82
LPS-A	2.15
CP-B	1.38
PX-B	1.43
Side chain	1.95

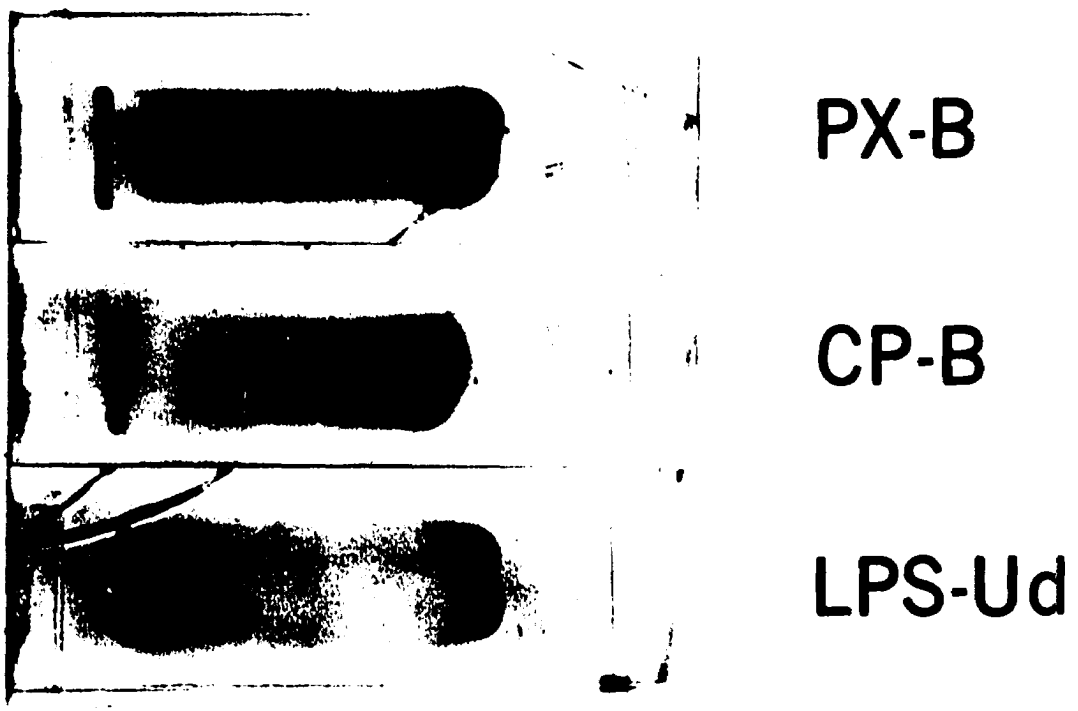


Figure 14. Agarose electrophoresis of intact endotoxin (LPS-Ud), conjugated protein (CP-B) and simple protein (PX-B).

Samples (20 mg/ml) were dissolved in 0.05 M Tris-Cl buffer containing 0.5% SDS, pH 7.6, and diluted to a final concentration of 10 mg/ml with 1% agarose in barbital buffer. 30 µl of the solution was applied on the origin. 1% agarose gel; barbital buffer, pH 8.6; ionic strength 0.05; 40 minutes.

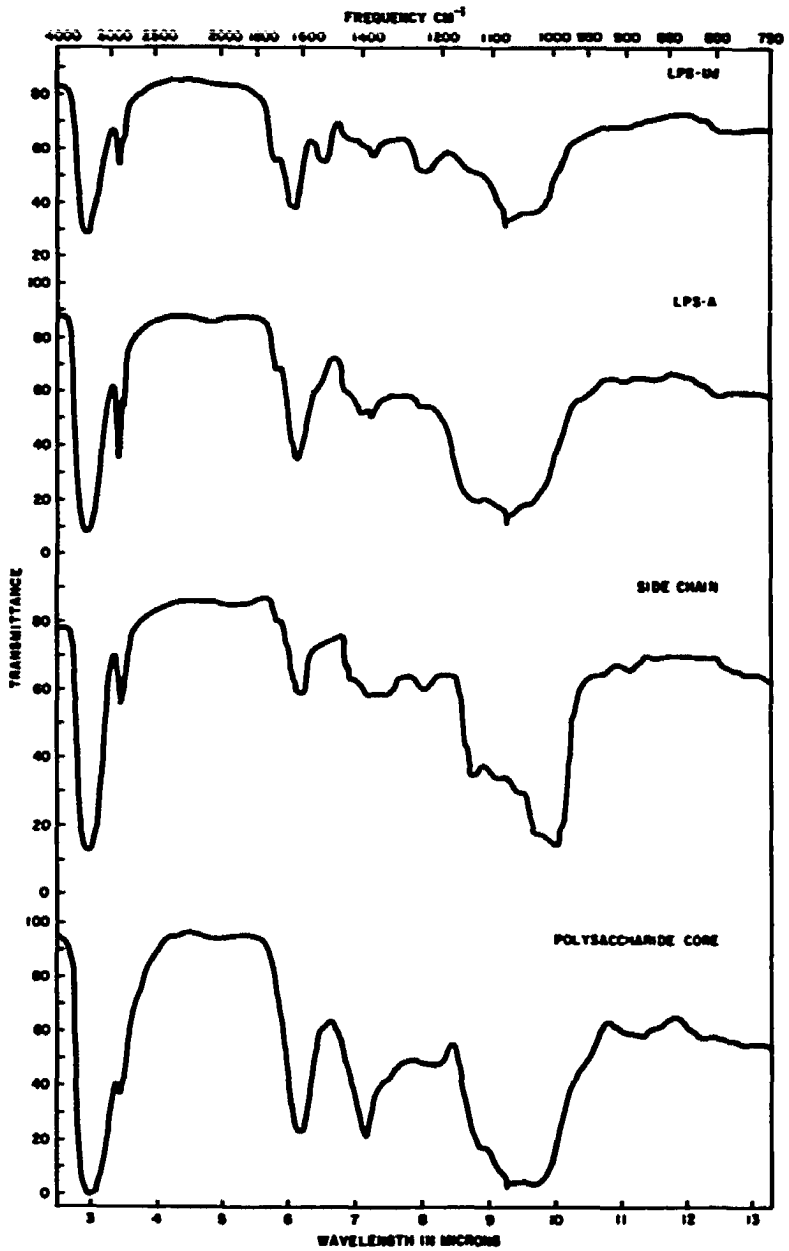


Figure 15. Solid infrared spectra of the intact endotoxin LPS-Ud and its fragments. Intact endotoxin (LPS-Ud), lipopolysaccharide (LPS-A), side chain and polysaccharide core (1.5-1.8 mg substance/250 mg KBr).

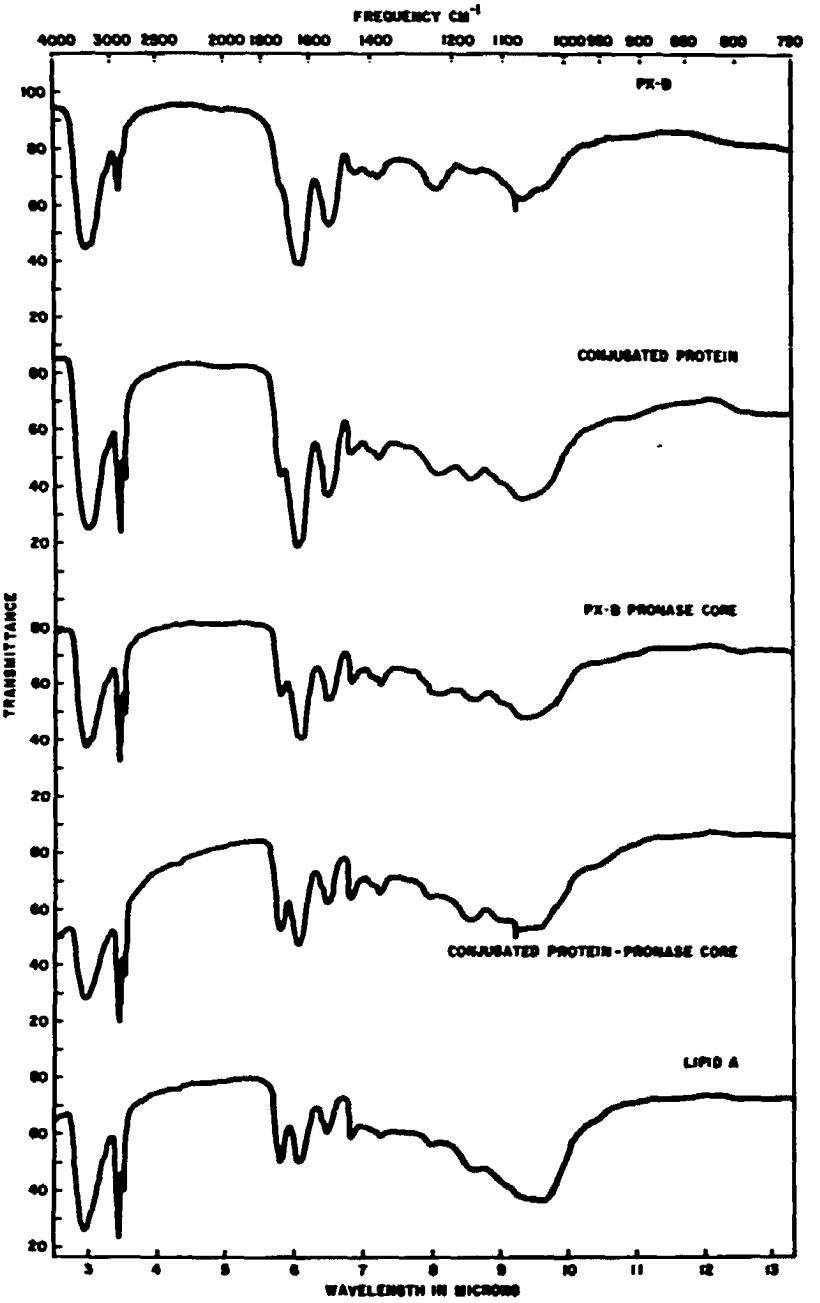


Figure 16. Solid infrared spectra of the endotoxin complex fragments. Simple protein (PX-B), conjugated protein (CP-B), pronase core (PX-B), pronase core (CP-B) and lipid A.

3400-3280 cm^{-1} is due to the -OH or $-NH_2$ group absorption. The absence of ester and amide absorptions in the spectrum of the side chain indicated that this endotoxin fragment is a neutral carbohydrate. The fact that both side chain and polysaccharide core were devoid of an ester absorption peak clearly showed that the lipid moiety is an entity separable from the polysaccharide moiety. The increase in lipid content of the pronase cores of both CP-B and PX-B was demonstrated by an increase in their ester/amide I absorption ratios (Table 4). The increased lipid content of these fragments correlated also with their toxic effect.

Immunological and Electrophoretic Properties of the Endotoxin Complex and Its Fragments

The endotoxin complex, LPS-Ud, reacted with antibodies to LPS-Ud (Fig. 17a, b) and antibodies to LPS-A (Fig. 17c). The lipopolysaccharide fragment, LPS-A, also reacted with both antibodies to LPS-Ud and LPS-A. Since conjugated protein, CP-B, gave a negative reaction with antibodies to LPS-Ud (Fig. 17a, b) it was concluded that this antiserum was specific for the antigenic determinants located in the polysaccharide moiety. The intact endotoxin complex showed no migration in the electric field (Fig. 17c). The migrating precipitin line formed in LPS-Ud pattern (Fig. 17a) was due most probably to the presence of a contaminating lipopolysaccharide fragment which showed a characteristic anodic mobility.

PX-B had a slightly greater mobility toward the anode than CP-B. This was due most probably to its smaller molecular size (Fig. 18). Since pronase treatment decreased the solubility of cores from both "simple" and "conjugated" protein, their diffusion in agar gel to form precipitin

TABLE 4

ESTER/AMIDE I RATIOS OF SOME ENDOTOXIN FRAGMENTS

	Length of Amide I Band in cm	Length of Ester Band in cm	Ester/Amide I Ratio
CP-B	13.0	7.9	0.61
CP-B Pronase Core	7.3	6.3	0.86
PX-B	11.3	5.6	0.49
PX-B Pronase Core	8.2	5.2	0.63
Lipid A	5.9	5.9	1.0

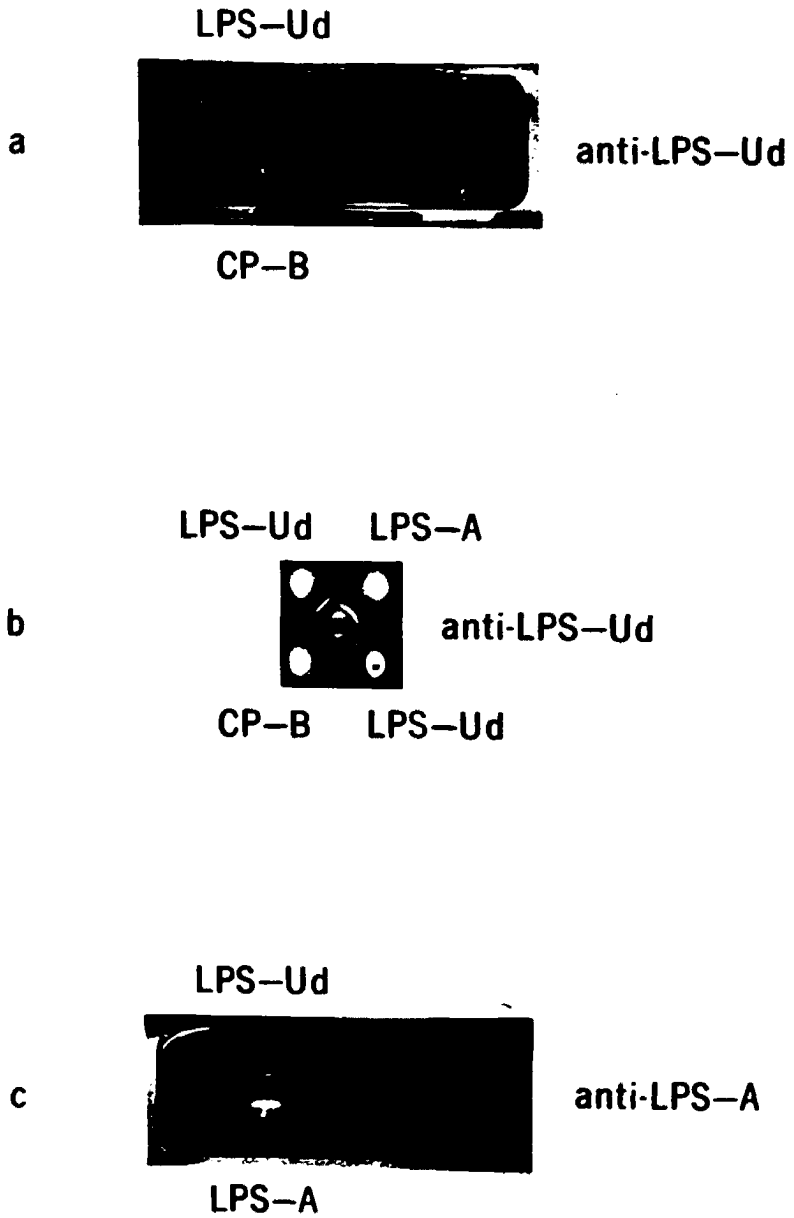


Figure 17. (a) Immunoelectrophoretic pattern of intact endotoxin (LPS-Ud) and conjugated protein (CP-B); (b) immunodiffusion pattern of intact endotoxin (LPS-Ud), lipopolysaccharide (LPS-A) and conjugated protein (CP-B); (c) immunoelectrophoretic pattern of intact endotoxin (LPS-Ud) and lipopolysaccharide (LPS-A). Samples (10 mg/ml) were dissolved in distilled water.

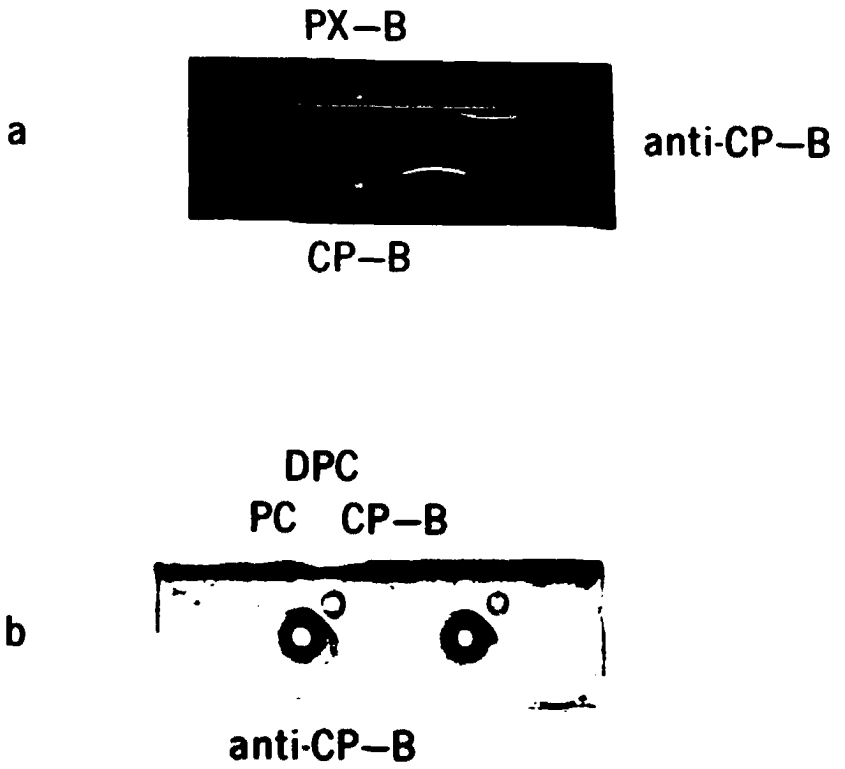


Figure 18. (a) Immunoelectrophoretic pattern of simple protein (PX-B) and conjugated protein (CP-B); (b) immunodiffusion pattern of conjugated protein (CP-B), pronase core (PC) and deacylated pronase core (DPC) of CP-B. Samples (10 mg/ml) were dissolved in distilled water.

bands against the antibody was prevented. The hydroxylamine treated pronase core of CP-B gave a reaction of partial identity with anti-CP-B serum, indicating that pronase treatment did not remove the antigenic determinant of CP-B completely (Fig. 18). The reaction of non-identity between LPS-A and CP-B run against a mixture of their antibodies clearly excluded the possible role of lipid A as an antigenic determinant (Fig. 19). The O-specific side chain did not give a precipitin line with antibodies to LPS-A, due, possibly, to the non-branching structure of this polysaccharide.

Chemical Characterization

The results of the elementary analysis and chemical composition of endotoxin and its fragments are shown in Table 5. The composition of the O-specific side chain was quite simple; it contained only D-glucose and L-rhamnose. The polysaccharide core was composed of D-glucose, D-glucosamine, heptose, KDO and ethanolamine. Heptose and KDO were the two specific components of polysaccharide core. A rather unique feature of this polysaccharide core was the absence of galactose, which otherwise occurs in most polysaccharide cores of Gram-negative bacteria. The absence of fatty acids in both side chain and polysaccharide core confirmed the observation from infrared spectroscopy. The presence of only trace amounts of amino acids in the hydrolysate of LPS-A showed that the phenol treatment of endotoxin resulted in a complete removal of protein from lipopolysaccharide.

Degraded lipid A contained no neutral sugars. It consisted of large amounts of glucosamine and fatty acids, similar to preparations from other Gram-negative bacteria (6).



Figure 19. Immunodiffusion pattern of conjugated protein (CP-B) and lipopolysaccharide (LPS-A). Samples (10 mg/ml) were dissolved in distilled water.

TABLE 5

ELEMENTARY ANALYSIS AND CHEMICAL COMPOSITION OF THE ENDOTOXIN COMPLEX

	LPS-Ud %	LPS-A %	Side Chain %	Poly-saccharide Core %	Lipid A %	CP-B %	PX-B %	CP-B Pronase Core %	PX-B Pronase Core %
P	1.09	1.52	Neg[a]	0.98	1.56	1.35	0.70	1.61	1.97
C	45.23	37.79	41.50	37.72	56.19	55.06	48.09	56.33	54.14
H	7.10	6.70	5.93	6.99	9.58	8.96	7.01	9.42	8.73
N	5.75	1.53	Neg[a]	1.96	1.53	6.47	10.46	3.50	4.53
Fatty Acids	10.2	15.5	Neg[a]	Neg[a]	47.2	34.2	10.96	49.0	37.0
D-Glucosamine	4.2	5.5	Neg[a]	6.5	13.2	6.3	2.2	8.4	6.3
Amino Acids	5.8	0.8	Neg[a]	Neg[a]	Neg[a]	32.5	48.2	16.3	17.3
D-Glucose	19.7	30.6	55.0	13.0	Neg[a]	Neg[a]	Neg[a]	Neg[a]	Neg[a]
L-Rhamnose	12.0	18.0	55.0	Neg[a]	Neg[a]	Neg[a]	Neg[a]	Neg[a]	Neg[a]
Heptose	9.9	14.6	Neg[a]	20.3	Neg[a]	Neg[a]	Neg[a]	Neg[a]	Neg[a]
KDO	Pos[b]	Pos[b]	Neg[a]	Pos[b]	Neg[a]	Neg[a]	Neg[a]	Neg[a]	Neg[a]
Ethanolamine	Pos[b]	Pos[b]	Neg[a]	Pos[b]	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]	N.D.[c]

[a]Neg = Negative; [b]Pos = Positive; [c]N.D. = Not Determined.

The results of amino acid composition of LPS-Ud, CP-B, PX-B, and pronase cores of CP-B and PX-B are shown in Table 6. Amino acid content increased in the direction LPS-Ud CP-B PX-B. Of course, the non-protein part of each substance showed a reverse trend.

It has been shown that the only difference between CP-B and PX-B is in their content of lipid A resulting from different degradation procedures used in their isolation (155, 156). The "conjugated" protein formed by the acetic acid hydrolysis of LPS-Ud contained the intact lipid moiety as evidenced by the absence of chloroform-extractable free lipid in the hydrolysate. Phenol, however, cleaved the lipid moiety, leaving only a small fragment of lipid A still covalently bound to the protein moiety in the "simple" protein. Pronase digestion caused degradation of the protein moiety, as shown by the decrease in amino acid content of the pronase cores. Neither fatty acids nor glucosamine were found in the pronase peptides.

Since PX-B and CP-B are both derived from LPS-Ud, their amino acid ratios should be similar (Table 7) if no degradation of the protein moiety occurred during isolation. The amino acid composition of these protein moieties were statistically evaluated by the one way analysis of variance method and were found not to be significantly different (165). The observed similarity in the ratios based on a lysine content of 1 mole suggests that neither phenol nor acetic acid altered the protein moiety.

An interesting feature of the amino acid compositions was the absence of cystine. It has also been observed in Serratia marcescens 08 and E. coli that the protein moiety of the endotoxin complex does not contain this amino acid (155).

TABLE 6

AMINO ACID COMPOSITION OF LPS-Ud, CP-B, CP-B PRONASE CORE, PX-B AND PX-B PRONASE CORE

	LPS-Ud		CP-B		CP-B Pronase Core		PX-B		PX-B Pronase Core	
	μMoles/g	mg/100	μMoles/g	mg/100	μMoles/g	mg/100	μMoles/g	mg/100	μMoles/g	mg/100
Lysine	22	0.32	126	1.85	65	0.95	159	2.32	90	1.31
Histidine	6	0.10	48	0.74	10	0.16	42	0.65	24	0.37
Arginine	40	0.69	103	1.79	51	0.89	131	2.29	60	1.04
Aspartic acid	63	0.84	347	4.62	80	1.07	603	8.02	156	2.07
Threonine	29	0.34	148	1.76	55	0.65	242	2.88	70	0.63
Serine	31	0.33	151	1.59	77	0.84	228	2.40	108	1.13
Glutamic acid	53	0.78	294	4.33	82	1.21	401	5.90	86	1.27
Proline	17	0.20	104	1.20	41	0.47	223	2.57	122	1.41
Glycine	52	0.39	260	1.95	93	0.70	380	2.86	161	1.21
Alanine	59	0.53	333	2.97	131	1.17	455	4.05	161	1.43
1/2 Cystine	-	-	-	-	-	-	-	-	-	-
Valine	25	0.29	195	2.28	100	1.17	224	2.62	83	0.97
Methionine	-	-	24	0.36	25	0.37	39	0.58	-	-
Isoleucine	16	0.21	113	1.48	71	0.93	140	1.84	72	0.95
Leucine	34	0.45	227	2.98	265	3.48	306	4.02	139	1.82
Tyrosine	4	0.08	50	0.91	25	0.45	176	3.18	29	0.52
Phenylalanine	13	0.21	103	1.70	108	1.78	124	2.05	71	1.17
Total		5.76		32.51		16.29		48.23		17.30

TABLE 7

COMPARISON OF MOLAR RATIOS OF AMINO ACIDS OF
LPS-Ud, CP-B AND PX-B

	LPS-Ud	CP-B	PX-B
Lysine	1	1	1
Histidine	0.27	0.38	0.26
Arginine	1.82	0.82	0.82
Aspartic acid	2.86	2.75	3.79
Threonine	1.32	1.17	1.52
Serine	1.41	1.20	1.43
Glutamic acid	2.41	2.33	2.52
Proline	0.77	0.82	1.40
Glycine	2.36	2.06	2.40
Alanine	2.68	2.64	2.86
1/2 Cystine	-	-	-
Valine	1.14	1.54	1.40
Methionine	-	0.19	0.24
Isoleucine	0.73	0.89	0.88
Leucine	1.55	1.80	1.92
Tyrosine	0.18	0.39	1.10
Phenylalanine	0.59	0.82	0.78

Gas-liquid chromatography of the methyl esters of fatty acids from LPS-Ud (Fig. 20) and its fragments revealed that β-hydroxymyristic acid was present in relatively high concentration, along with saturated and unsaturated fatty acids of chain length C_{12}-C_{16} (Table 8). "Simple" protein contained a significant amount of firmly bound fatty acids and glucosamine. The main fatty acid of this compound was also β-hydroxymyristic acid. As expected, the fatty acid content of the pronase cores was higher than that of their parent compounds CP-B and PX-B.

The molar ratios of glucosamine/phosphate/fatty acids in the pronase core of "conjugated" protein were found to be approximately 1:1:3.6. Since in lipid A glucosamine contains only two available hydroxyl groups and one amino group for ester and amide bond formation, a maximum of 3 moles of fatty acid can be expected to bind directly with 1 mole of glucosamine. It is, therefore, concluded that the hydroxyl group of β-hydroxymyristic acid is also involved in ester bond formation.

Toxicity of the Endotoxin Complex and Its Fragments

LD_{50} determinations were performed with samples suspended in Tris-Cl buffer containing SDS. The buffer itself was not toxic. The results of this study (Table 9) showed that all fragments which contained lipid A were toxic. Pronase cores of both protein preparations exhibited higher toxicity than their parent compounds. The core-polysaccharide and side chain were not toxic even at high doses (100 mg/kg).

Structural Studies on the O-specific Side Chain

Homogeneity Criteria

The homogeneity of side chain was based on some of its physical

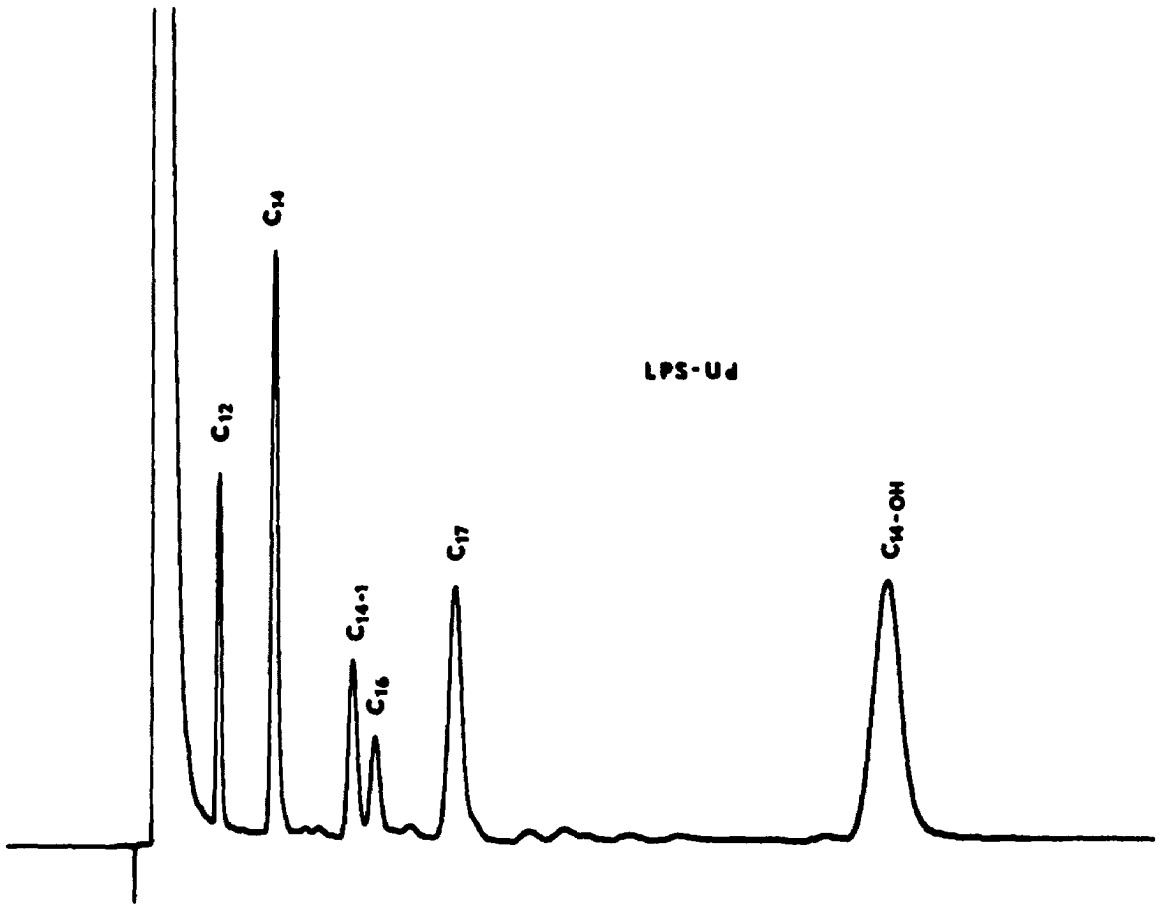


Figure 20. Gas-liquid chromatogram of fatty acid methyl esters of LPS-Ud.

TABLE 8

FATTY ACID COMPOSITION OF ENDOTOXIN FRAGMENTS

	LPS-Ud %	LPS-A %	Lipid A %	CP-B %	CP-B Pronase Core %	PX-B	PX-B Pronase Core %
C_{12}	1.23	2.23	4.31	4.89	4.65	0.95	4.01
C_{14}	2.05	3.46	9.27	6.95	9.17	1.77	7.30
Unknown	1.66	3.77	6.79	7.17	12.71	2.27	7.20
C_{16}	0.63	1.76	2.13	1.96	4.19	1.33	3.65
C_{16-1}	0.10	0.60	2.18	0.45	1.44	0.51	1.04
β-OH-C_{14}	4.06	5.70	21.01	12.01	16.83	3.33	13.92
Total	10.15	19.05	45.68	34.20	48.99	10.16	37.12

TABLE 9

LD_{50} VALUES OF THE ENDOTOXIN COMPLEX AND ITS FRAGMENTS ON INBRED BALB-C MICE

	LD_{50} mg/kg of mouse
LPS-Ud	6.5
LPS-A	3.5
Side chain	non-toxic
Core-polysaccharide	non-toxic
Lipid A	25.0
CP-B	6.0
CP-B pronase core	3.5
PX-B	15.0
PX-B pronase core	10.0

properties and chemical composition as major criteria. Sephadex G-100 column chromatography (Fig. 21) and ultracentrifugal schlieren patterns of the polysaccharide side chain (Fig. 13) exhibited single symmetrical peaks. Chemical studies on the side chain provided the most definite criterion of the homogeneity of this macromolecular compound. D-Glucose and L-rhamnose as the only component sugars present accounted for the total dry weight of the side chain. Elementary analysis indicated the absence of phosphorus and nitrogen. Gas-liquid chromatographic analysis of sugars and lipids and the amino acid analysis showed a complete absence of contamination from other components of endotoxin.

Physical-Chemical Properties

Some physical-chemical constants for the side chain are presented in Table 10. The agreement in molecular weights determined by sedimentation equilibrium and reducing end analysis indicated that the polysaccharide had one mole of terminal reducing sugar per mole of the molecule. The molecular weight obtained by the ultracentrifugal analysis probably represents the closer value to the true molecular weight.

Chemical Composition

Infrared spectroscopy of the side chain (Fig. 15) resulted in a spectrum typical of neutral polysaccharide. The anthrone test showed that the side chain contained 97% carbohydrate when glucose was used as standard. Results of gas chromatographic (Fig. 22) and colorimetric sugar analyses showed that side chain contained 55% glucose and 55% rhamnose. The molar ratio of D-glucose to L-rhamnose was 1/1.1 which indicated the polysaccharide consisted of a glucose-rhamnose disaccharide re-

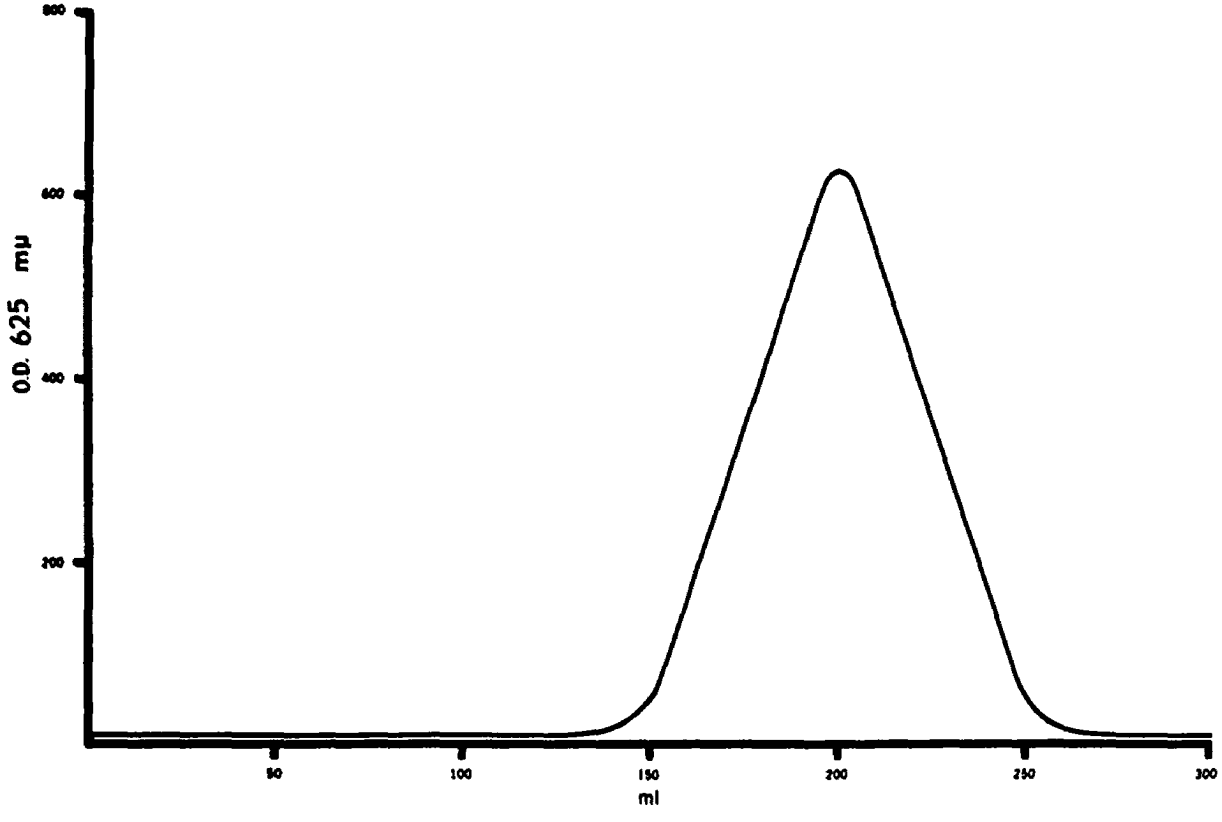


Figure 21. Gel-filtration chromatography of side chain on Sephadex G-100.

Experimental conditions: Column (2.5 x 100 cm) was eluted with distilled water. Volume of collected fractions was 3 ml per tube. Flow rate was 30 ml per hour. Column was monitored by determing the carbohydrate content with the anthrone method. Void volume: 120 ml.

TABLE 10

PHYSICAL-CHEMICAL PROPERTIES OF THE SIDE CHAIN

Sedimentation coefficient, $S_{obs.}$	1.95×10^{-13} sec.
Partial specific volume, $\bar{v}$	0.619 ml/g
Molecular weight (Sedimentation equilibrium)	13,200
Molecular weight (Reducing end analysis)	11,300

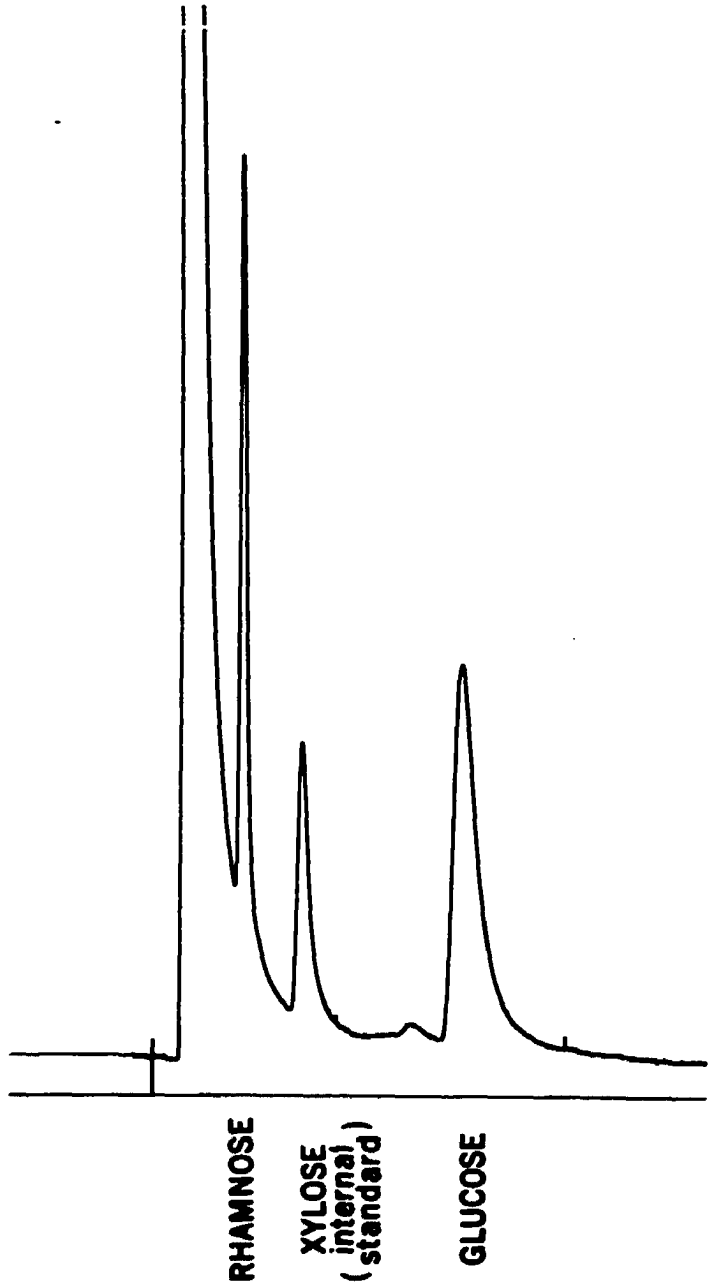


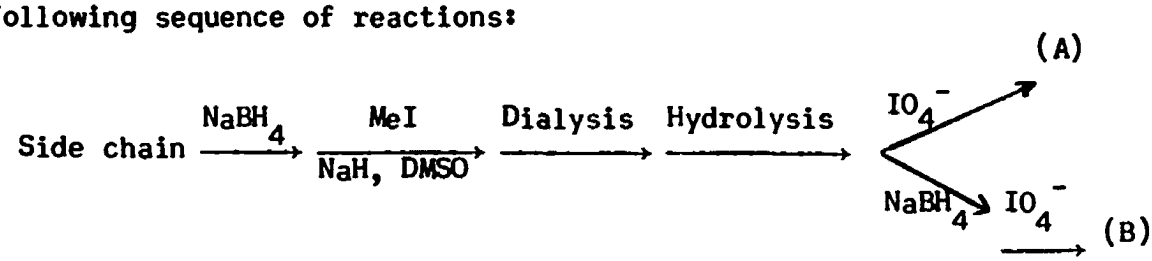
Figure 22. Separation of alditol acetates of side chain by gas-liquid chromatography.

peating unit. The absence of ester carboxyl absorption in the IR-spectrum indicated that side chain did not contain O-acetyl groups which occur very frequently in the O-specific side chains of Gram-negative bacteria.

Linkage Studies

The absence of 1→3 linkages. The complete degradation of side chain by periodate oxidation established by the failure to detect D-glucose and L-rhamnose in the reaction mixture by either paper chromatography or gas-liquid chromatography, indicated clearly that the 1→3 linkage was not present in the polysaccharide.

Linkages between D-glucose and L-rhamnose. Since D-glucose and L-rhamnose are present in equimolar amounts, the theoretical periodate consumption for each of the four possible glycosidic linkages is shown in Table 11. Since one mole of the D-glucose-L-rhamnose disaccharide consumed 2.8 moles of periodate, the D-glucose must be linked 1→6, whereas L-rhamnose can be linked either 1→2 or 1→4. To distinguish between the 1→2 and 1→4 linkages of L-rhamnose, the side chain was submitted to the following sequence of reactions:



The side chain polysaccharide was first reduced with sodium borohydride and the reduced product was methylated with methyl iodide according to the Hakomori method, dialyzed and hydrolyzed with 1 N hydrochloric acid. One aliquot of hydrolysate was oxidized with periodate (reaction sequence

TABLE 11

THE THEORETICAL PERIODATE CONSUMPTION FOR LINKAGES BETWEEN D-GLUCOSE AND L-RHAMNOSE

Linkage	D-Glucose	L-Rhamnose
	moles periodate/mole monosaccharide	
1→2	1	1
1→3	0	0
1→4	1	1
1→6	2	-

A), while the other aliquot was first reduced with sodium borohydride and then oxidized with periodate (reaction sequence B). The theoretical formation of formaldehyde and acetaldehyde from the methylated D-glucose and L-rhamnose in these two reaction sequences is shown in Table 12. It was already established from the results of periodate oxidation of side chain that D-glucose was linked 1→6. In this case, one mole of formaldehyde should be released from one mole of methylated D-glucose during both reaction sequences A and B. However, as shown in Table 12, if methylated L-rhamnose is linked 1→2, reaction sequence A would yield from each repeating unit one mole of formaldehyde and reaction sequence B two moles of formaldehyde. Neither reaction sequence would result in liberation of acetaldehyde. On the other hand, if methylated L-rhamnose is linked 1→4, both reaction sequences would yield one mole of formaldehyde and one mole of acetaldehyde.

The results of gas-liquid chromatographic analysis of reaction products (Fig. 23) showed that the formaldehyde ratio of reaction sequences B/A was 1.6:1 and that no acetaldehyde was formed. This finding indicated clearly that the L-rhamnose was bound through a 1→2 linkage. The absence of OH absorption in the IR-spectrum of methylated side chain (Fig. 24) confirmed the completeness of methylation reaction and, therefore, the validity of formaldehyde ratios.

Studies on the mode of linkages in side chain by methylation analysis. The side chain was methylated by Hakomori's method and then hydrolyzed. The methylated monosaccharides were reduced with sodium borohydride and acetylated by acetic anhydride in pyridine. The methylated acetyl alditols were analyzed by gas-liquid chromatography

TABLE 12

THEORETICAL FORMATION OF FORMALDEHYDE AND ACETALDEHYDE FROM THE METHYLATED D-GLUCOSE AND L-RHAMNOSE IN REACTION SEQUENCES A AND B*

L-Rhamnose Linkages	D-Glucose		L-Rhamnose	
	Formal-dehyde	Acetal-dehyde	Formal-dehyde	Acetal-dehyde
	moles		moles	
Reaction sequence A				
1→2	1	0	0	0
1→4	1	0	0	1
Reaction sequence B				
1→2	1	0	1	0
1→4	1	0	0	1

*It is assumed that D-glucose is linked 1→6.

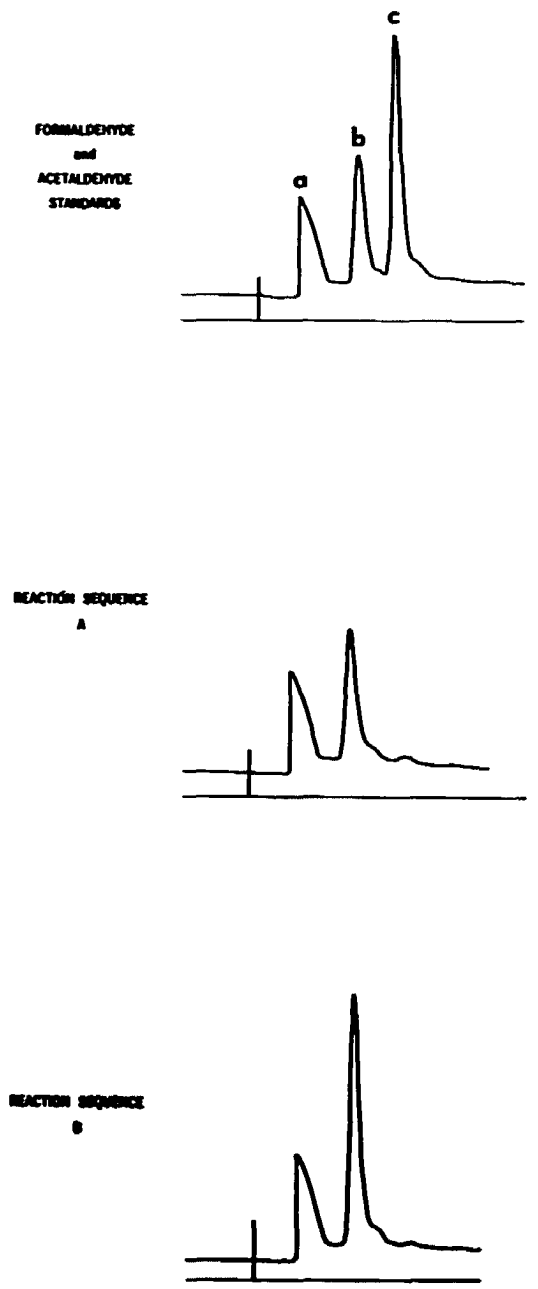


Figure 23. Gas-solid chromatography of formaldehyde and acetaldehyde formed by periodate oxidation of the methylated monosaccharides from side chain. Peak (a) H_2O, peak (b) formaldehyde, and peak (c) acetaldehyde.

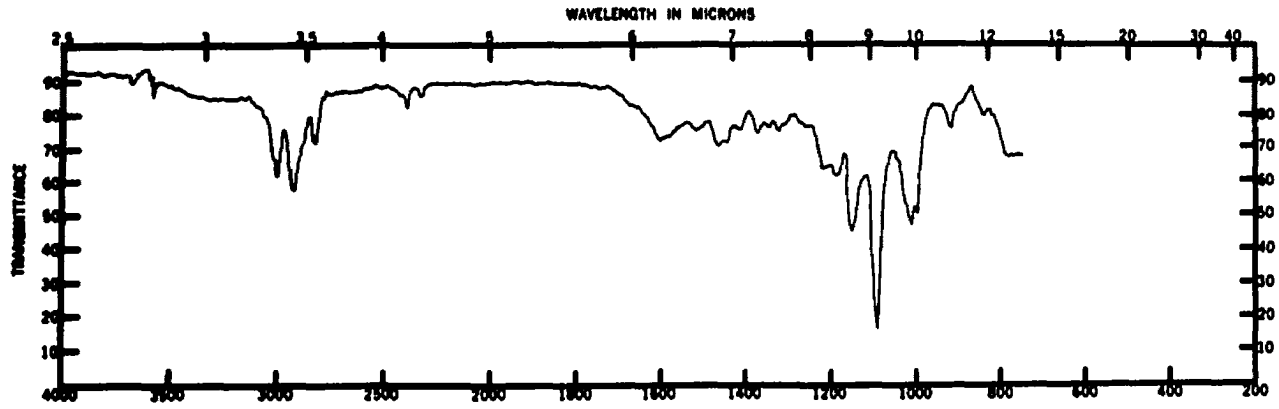


Figure 24. Infrared spectrum of methylated side chain in chloroform.

(Fig. 25). The identification of alditol acetate of 2,3,4-tri-O-methyl-D-glucose confirmed the linkage of D-glucose to be 1→6. The first peak from the chromatogram is probably the alditol acetate of 3,4,-di-O-methyl-L-rhamnose as suggested by the periodate experiment described above, however, authentic sample was not available. The absence of the alditol acetates of di-O-methyl-D-glucose and mono-O-methyl-L-rhamnose showed that there was no branching in the polysaccharide structure.

Partial Hydrolysis of the Side Chain

To demonstrate that side chain consists of repeating units of glucose-rhamnose disaccharide, the polysaccharide was hydrolyzed with 0.5 N HCl for 2 hours at 100°C. The hydrolysate was then concentrated to a smaller volume and applied on a Sephadex G-15 column. The elution pattern is shown in Fig. 26. Eluates I, II and III represented the monosaccharide, disaccharide and trisaccharide fractions, respectively. The preparative paper chromatography of the disaccharide fraction resulted in the separation of two silver nitrate positive bands with mobilities between those of glucose and lactose. These two bands were designated as D-1 ($R_{f\ glu}$ 0.66) and D-2 ($R_{f\ glu}$ 0.42).

The structures of D-1 and D-2 were studied by enzymatic degradation with α- and β-glucosidases and with hesperidinase. The α-glucosidase cleaves the non-reducing end of α-D-glucosides, the β-glucosidase that of β-glucosides and hesperidinase that of β-L-rhamnosides. The release of monosaccharide was assayed by the glucose oxidase method or by paper chromatography. Results shown in Table 13 suggested that fragments D-1 and D-2 had following structures:

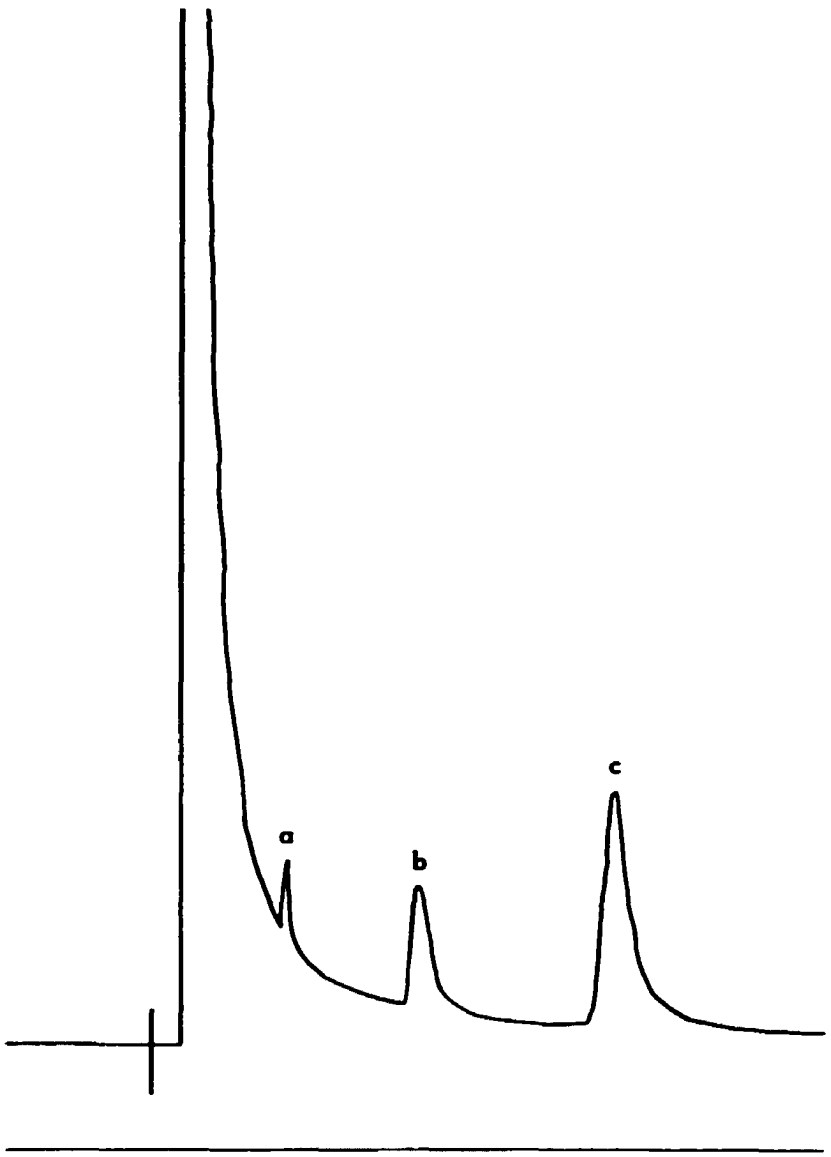


Figure 25. Gas-liquid chromatogram of the methylated alditol acetates from side chain.

Peak (a) 3,4-Di-O-Me-L-rhamnose, peak (b) 2,3,4-Tri-O-Me-D-glucose, and peak (c) xylose, internal standard.

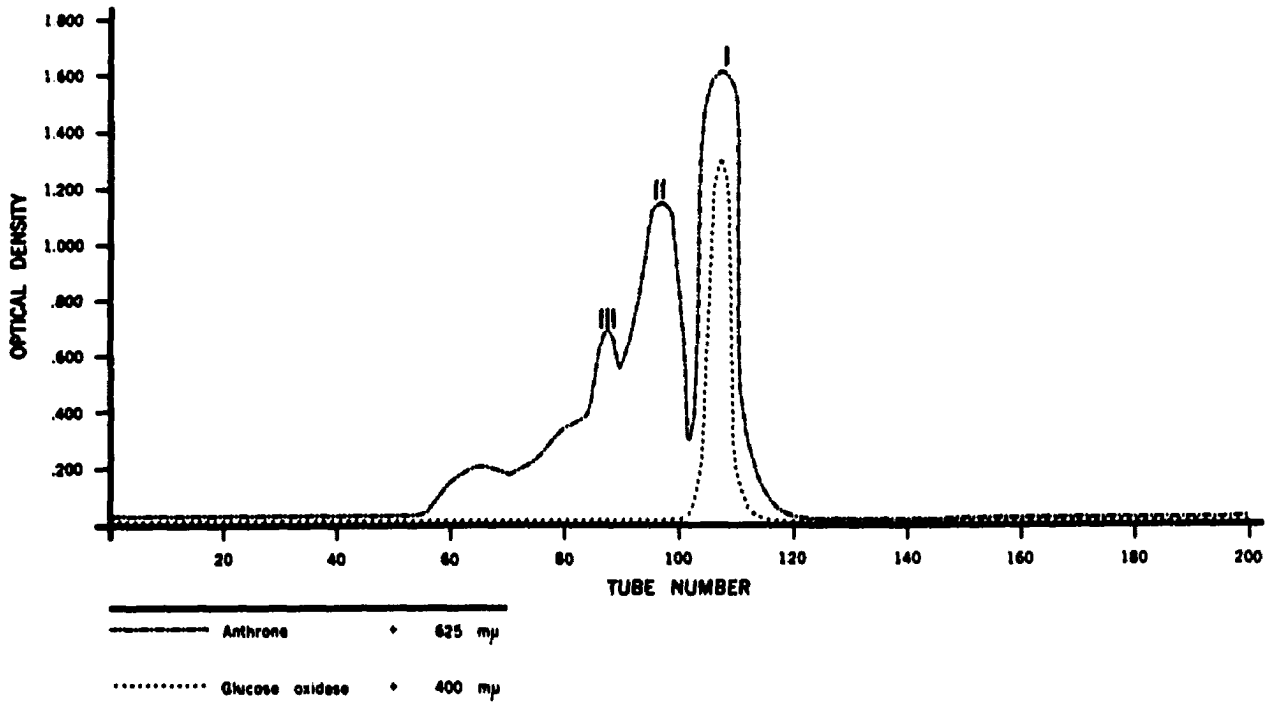


Figure 26. The gel-filtration chromatography of partially hydrolyzed side chain.

Experimental conditions: Column (2.5 x 100 cm) was eluted with distilled water. Volume of collected fractions was 3 ml per tube. Flow rate was 30 ml per hour. Column was monitored by determing the mixture of sugars with anthrone method and glucose by glucose oxidase.

TABLE 13

DEGRADATION OF D-1 AND D-2 BY GLYCOSIDASES

	D-1	D-2
α-Glucosidase	Neg[a]	Neg[a]
β-Glucosidase	Pos[b]	Neg[a]
Hesperidinase	Neg[a]	Pos[b]

[a]Neg = Negative reaction; [b]Pos = Positive reaction.

D-1: D-Glu $\xrightarrow{1\ \beta\ 2}$ L-Rha

D-2: L-Rha $\xrightarrow{1\ \beta\ 6}$ D-Glu

The slower moving silver nitrate positive bands were probably trisaccharides and tetrasaccharides. All fractions contained both glucose and rhamnose. Since neither Rha→Rha nor Glu→Glu disaccharides were observed, these results suggested that the side chain consisted of a D-glucose-L-rhamnose disaccharide repeating unit rather than of a random distribution of these two sugars.

When a mixture of β-glucosidase and hesperidinase was used for the digestion of the whole polysaccharide, neither glucose nor rhamnose was released. However, the enzymatic treatment of the oligosaccharide eluted from Sephadex G-15 resulted in the release of both D-glucose and L-rhamnose. These results indicated that the glycosidases possessed some specificity for the size of their substrates. The results of enzymatic digestion also demonstrated that both D-glucose and L-rhamnose had the β-anomeric configuration.

Anomeric Configuration of the Side Chain

Results of the enzymatic degradation already indicated that both D-glucose and L-rhamnose had the β-anomeric configuration. This result was confirmed by the NMR-spectrum of side chain (Fig. 27) which exhibited only a single β-anomeric proton peak at 5.0 τ.

Determination of Reducing End Sugar

When the polysaccharide was treated with periodate, the release of acetaldehyde was observed. Since only a terminal L-rhamnose is capable of releasing the aldehyde, this result suggested that the reducing

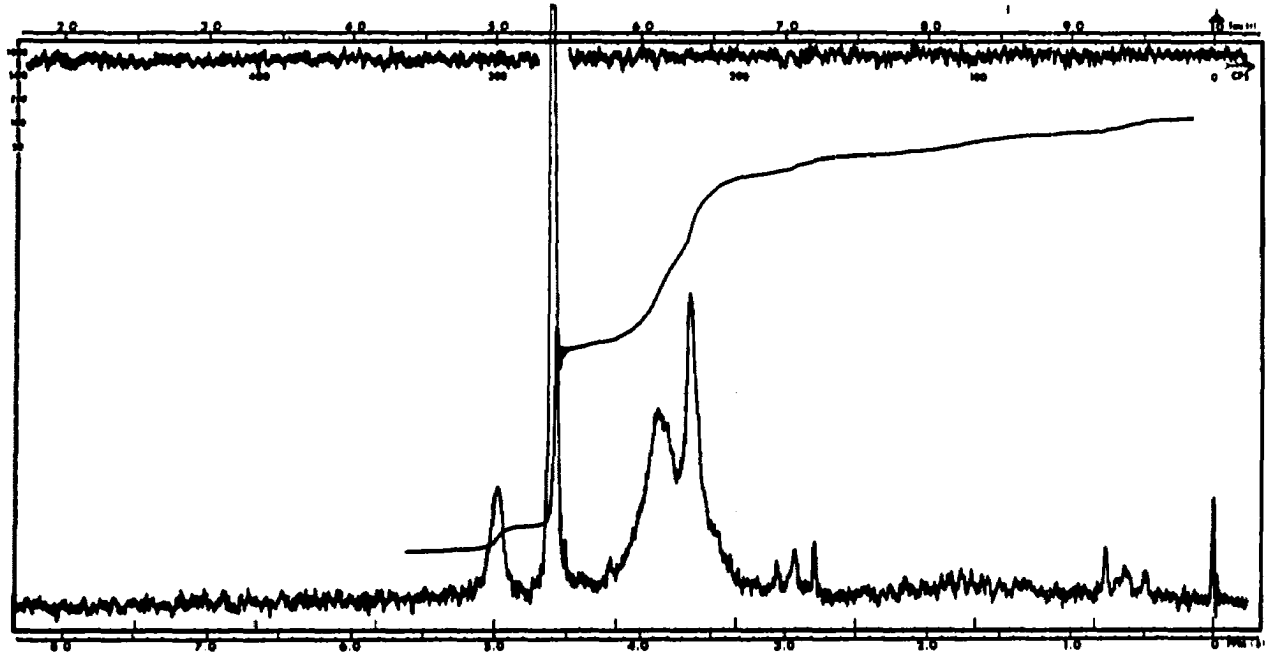


Figure 27. NMR spectrum of side chain dissolved in HDO.

end sugar was L-rhamnose.

Structure of O-Specific Side Chain

The results presented indicate that the O-specific side chain from Serratia marcescens Bizio has the following structure:

$$\xrightarrow{6} \text{D-Glu} \xrightarrow{1\ \beta\ 2} \text{L-Rha} \xrightarrow{1\ \beta}_{n}$$

with n equal to 43 by the calculation from molecular weight determination (Fig. 28).

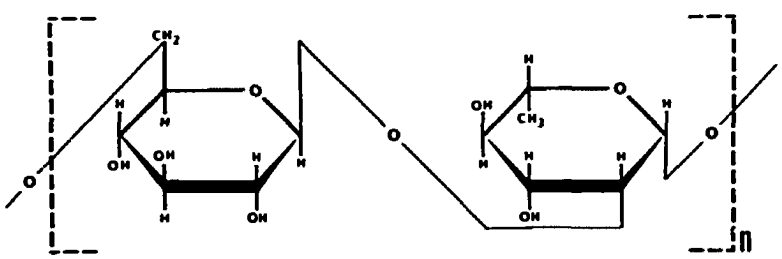


Figure 28. Structure of O-specific side chain from Serratia marcescens Bizio.

CHAPTER V

DISCUSSION

Treatment of the non-chromogenic strain of Serratia marcescens Bizio with trichloroacetic acid results in the isolation of the lipopolysaccharide-protein complex. The acidic polysaccharide and nucleic acids are also extracted by this agent, but can be removed by fractional ultracentrifugation. It has been shown that acidic polysaccharides posses relatively low toxicity (149). This leaves the group of lipopolysaccharide-protein complexes as the sole carriers of endotoxic characteristics. Results of present study showed that the endotoxin complex LPS-Ud contained small amounts of its own degradation products as detected by immunochemical analysis. However, gel-permeation chromatography of LPS-Ud on Sepharose 4B resulted in quantitative recovery of the substance at the void volume with no detectable polysaccharide side chain present in later appropriate fractions. This suggested that degradation of the endotoxin complex during the extraction process was negligible.

Fragmentation Studies

Several fragmentation techniques have been discussed from the point of their specificity and applicability. The treatment of the endotoxin complex from S. marcescens Bizio by acetic acid resulted in the degradation of the polysaccharide moiety into water-soluble polysaccharide

side chain and core. A precipitate also formed during hydrolysis which was identified as "conjugated" protein (a fragment consisting of lipid A and protein moiety). Analysis of the chloroform extracts from either the aqueous phase or the precipitate after hydrolysis showed that lipid moiety was not liberated in an extractable form. The polysaccharide side chain and core were separated from one another by dialysis. The inner dialysate contained the side chain of the endotoxin complex (judged by the absence of KDO and heptose), while the outer dialysate contained mainly the polysaccharide core. The action of acetic acid also resulted in some degradation of the polysaccharide side chain since small amounts of rhamnose-containing oligosaccharides were detected in the outer dialysate. The only free monosaccharide present in the outer dialysate was KDO.

Studies on the characterization of these fragments showed that acetic acid treatment resulted in fragmentation of endotoxin into the polysaccharide side chain and core and "conjugated" protein. "Conjugated" protein contained a non-degraded lipid moiety as well as the protein portion of the endotoxin complex. Although some degradation of the polysaccharide side chain and core occurs (especially in the KDO region of the core), this degradation procedure still represents the best procedure for the fragmentation of the polysaccharide moiety, as judged by its high specificity and high yields.

Phenol treatment of the endotoxin complex resulted in the cleavage of complex into a lipopolysaccharide fragment and "simple" protein. The lipopolysaccharide, in turn, was degraded by acetic acid hydrolysis into polysaccharide side chain and core, and lipid A. The protein isolated from the phenol phase contained not only amino acids but also glu-

cosamine and fatty acids. β-Hydroxymyristic acid was the major fatty acid constituent. This indicated that the so-called "simple" protein still contained a portion of the lipid A moiety, if detection of β-hydroxymyristic acid is accepted as evidence for its presence. Since the lipid portion of "simple" protein could not be removed by organic solvents and "simple" protein exhibited homogeneity in the analytical ultracentrifuge and on immunoelectrophoresis, it is concluded that the lipid moiety is covalently bound to the protein. This conclusion is contrary to that of earlier investigators who claimed the isolation of a lipid-free "simple" protein by similar methods (12, 13, 14, 23). "Simple" protein was judged to be lipid-free because of its low content of phosphorus. This is, however, an inadequate criterion. A lack of knowledge of the components of lipid A and a lack of sensitive analytical methods may also explain why lipid A was not recognized as an integral part of "simple" protein. Since both lipopolysaccharide and "simple" protein contained lipid A, it was concluded that phenol caused internal cleavage of the lipid moiety and resulted in the formation of the polysaccharide and protein moieties with lipid fragments attached to both. It is difficult to explain why acetic acid did not result in a similar cleavage. One possible explanation is that phenol as an excellent solvent for protein unfolded the endotoxin molecule and exposed a weak linkage which became then susceptible to the hydrolytic action of phenol. In addition phenol also caused some side reactions such as cleavage of phosphoglycosidic linkages and the release of ester bound fatty acids from lipid A (48). However, these reactions are slow, and treatment of endotoxin with phenol for only short periods of time eliminates these side reactions.

The standard method for the isolation of lipid A is the mild acid hydrolysis of lipopolysaccharide (0.1 N HCl, 30 min, 100°C). The resulting chloroform extractable and acetone precipitable substance is a typical preparation of lipid A. However, since lipid preparations isolated by this procedure are mixtures consisting of acylated polyglucosamines of different length, they do not represent substances suitable for the structural study of lipid A. On the other hand, as these preparations do not contain any portions of the polysaccharide or protein moieties they are useful for studies of the role of lipid A in the biological characteristics of endotoxin.

Present study has established unequivocally that "conjugated" protein contains the intact lipid moiety. If protein could be removed by proteolytic digestion, the undigestible residue should represent the intact lipid preparation. Results showed that a prolonged digestion of "conjugated" protein with pronase resulted in the removal of large amounts of the protein moiety. However, the undigestible residue still contained about 16% of amino acids. It is probable that the lipid moiety inhibited the action of the proteolytic enzyme. After deacylation of the pronase core by hydroxyaminolysis, a further removal of protein was achieved (150). This represents a way to isolate the intact polyglucosamine backbone of lipid moiety. Pronase digestion of "simple" protein also resulted in a product which contained a higher percentage of lipid moiety than the starting material.

Linkages Between the Structural Moieties

Some insights on linkages between the structural moieties can be obtained from studies on their stabilities to various hydrolytic agents.

The acid treatment of the endotoxin complex causes the cleavage of polysaccharide side chain, core, and "conjugated" protein. It is, therefore, concluded that the linkages between the side chain and core, and between the polysaccharide core and lipid A are acid labile. Since the action of acetic acid on the endotoxin complexes from other bacteria is similar (7, 18, 19, 20) it seems that the linkages between structural moieties of endotoxin complexes from different Gram-negative bacteria are the same. The lability of the linkage between the side chain and core to both acetic acid and phenol (48) excludes the possibility that these two portions of the polysaccharide moiety are bound glycosidically via galactose and glucose residues as suggested by Nikaido for lipopolysaccharide of Salmonella (57, 58). The glycosidic linkage between galactose and glucose can not be split by either acetic acid or phenol under the specified conditions. Therefore, the known acid-labile linkages such as phosphoglycosidic, ketosidic, and glycosidic linkages of dideoxyhexose should be considered as the possible candidates for the bond between side chain and core. The absence of keto sugars and dideoxyhexoses in side chains of a number of Gram-negative bacteria including Serratia marcescens make the latter two possibilities unlikely. This leaves the phosphoglycosidic bond as the most probable linkage between the side chain and core. The presence of a reducing end on the side chain and a phosphomonoester group in the core supports this point of view. From biosynthetic studies, it is known that the hexose region of the polysaccharide core is the site for the attachment of the side chain (151). Under these circumstances the isolation of a hexose monophosphate from the partially hydrolyzed core would represent the evidence for this suggestion.

It was established that in lipopolysaccharide from Salmonella typhimurium (86) the polysaccharide core was linked to the lipid A through a glycosidic bond between KDO and glucosamine. It is very probable that a similar linkage also exists in the endotoxin complex of Serratia marcescens Bizio. The presence of free KDO in the acetic acid hydrolysate and the absence of any KDO-containing oligosaccharide suggest that the KDO residues are located at the reducing end of the polysaccharide core. Otherwise, a cleavage of the polysaccharide core during acetic acid degradation would be expected.

It is known that the linkage between lipid A and the protein moiety of endotoxin from S. marcescens 08 is alkali stable (24). The present investigation also demonstrates this stability. Failure to split an O-glycosidic linkage with serine or threonine by β-elimination according to the method of Carubelli et al. (166) indicated the possibility that protein and lipid moieties may be linked through an N-glycosidic bond. Neither glutamic acid nor glutamine has ever been reported to be involved in carbohydrate-protein linkages (152). On the other hand, it is well known that asparagine is linked to glucosamine in glycoproteins in the form of 2-acetamido-1-β-(L-β-aspartamido)-1,2-dideoxy-D-glucose or β-aspartylacetylglucosaminylamine (BAGA) (152). Theoretically, both glutamine and asparagine may represent the attachment sites for lipid A.

Composition and Properties of the Endotoxin Complex and Its Fragments

Degradation of the endotoxin complex results in the formation of several fragments, as shown in Fig. 29.

Immunological studies indicated that LPS-Ud, LPS-A and CP-B were

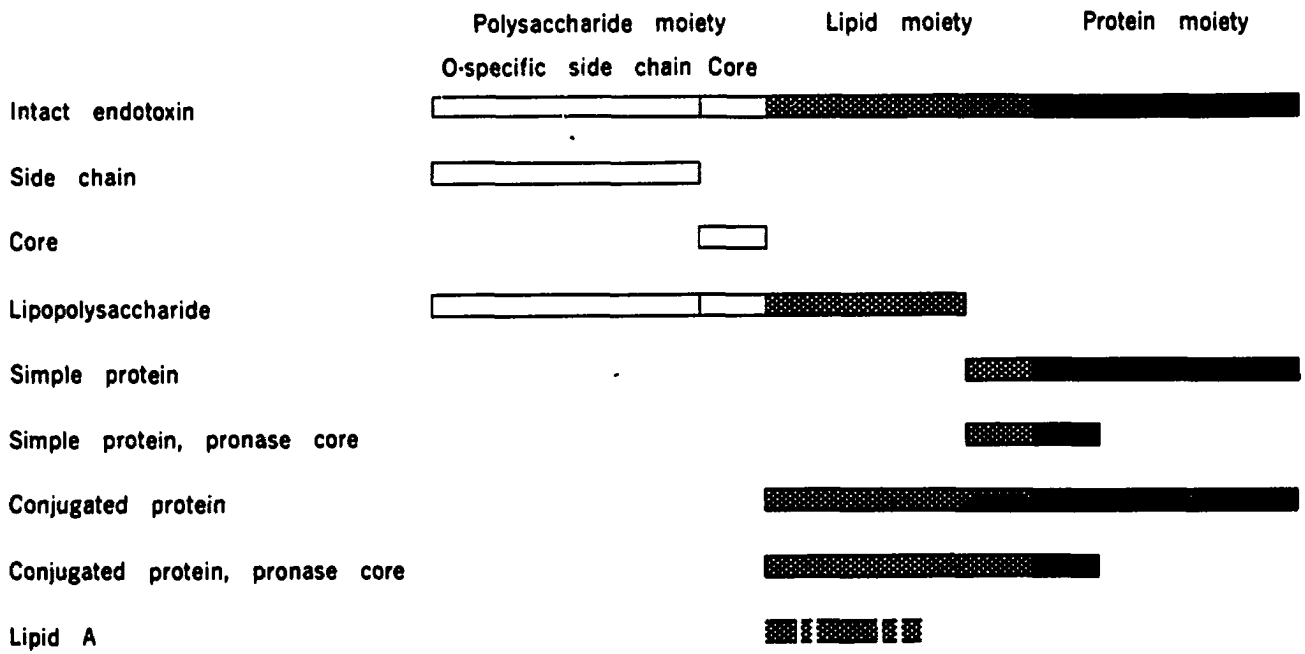


Figure 29. Fragments of endotoxin from Serratia marcescens Bizio

antigenic and immunogenic. The non-identical reactions of CP-B and LPS-A against a mixture of their antibodies suggested that the polysaccharide and protein represented two different antigenic components. If lipid A were involved in antibody formation, a reaction of partial or total identity between CP-B and LPS-A would be obtained; however, this was not the case. Polysaccharide core was not immunogenic, a finding expected from its low molecular weight. The side chain, when reacted against anti-LPS-A, formed no precipitin line. It seems that the absence of a branched structure in the side chain may be responsible for the failure to form a precipitin line. PX-B cross-reacted within antibodies to CP-B. Since the protein portions of PX-B and CP-B are the same, this reactivity was expected. The pronase cores of CP-B and PX-B gave no precipitin lines with antibodies to CP-B. This was due to their poor solubility in saline, since hydroxyamine-treated pronase core did form a precipitin line when reacted with antibodies to CP-B. The antigenic and immunogenic activities of deacylated pronase cores suggest that the antigenic components of the protein moiety are located very close to the lipid A moiety. There was no cross-reactivity between LPS-Ud from the Bizio strain and the antibody to S. marcescens O8 and vice versa. It was thus concluded that the antigenic components of the endotoxin complexes from the two strains of Serratia were different.

Ratios of fatty acids, glucosamine and phosphate indicated a close compositional similarity between the "conjugated" and "simple" proteins. An equally significant similarity in the molar ratios of amino acids showed the identity of the protein moieties of both preparations. A lower fatty acids to glucosamine ratio of PX-B indicated that phenol

treatment of endotoxin did cause the release of ester-bound fatty acids. Pronase treatment of CP-B and PX-B caused an increase in the relative content of lipid moiety in the undigested cores. The molar ratio of glucosamine to aspartic acid in the pronase core of CP-B was 7:1; after deacylation and further pronase digestion, the ratio increased to 20:1 (150). There were no glucosamine or fatty acids found in the pronase peptides. This result suggested that lipid moiety consisted of a polyglucosamine backbone with a minimum of 20 glucosamine residues.

The polysaccharide core of S. marcescens Bizio isolated from acetic acid hydrolysis contains glucose, glucosamine, heptose and KDO in a molar ratio of 2:1:3:1. The absence of galactose in the polysaccharide core showed that the structure and composition of the core from S. marcescens is different from those of Salmonella, Shigella and Escherichia coli (95, 122, 153). A similar qualitative composition of the polysaccharide core from Serratia marcescens 08 suggests that the cores of all Serratia species are similar, if not identical, in structure. The toxicity of the endotoxin complex represents one of its most characteristic biological properties. All fragments (LPS-Ud, LPS-A and CP-B) which contain a portion or the entire lipid moiety exhibited toxicity in mice. Pronase digestion of CP-B and PX-B did not result in the lowering of toxicity; rather an increase was observed. This suggested that the protein moiety was not responsible for the toxicity of the endotoxin complex. Lipid A preparations isolated by mild acid hydrolysis (LD_{50} 25 mg/kg) were shown to be highly toxic. The lower toxicity of this preparation compared to that of LPS-Ud (LD_{50} 6.5 mg/kg) could be explained by the structural alteration of lipid moiety during the isolation procedure. The side chain

and polysaccharide core did not show any toxicity even at high dosages (2 mg/mouse). These data clearly demonstrated the importance of lipid moiety for toxicity and supported the suggestion of Westphal and Lüderitz that lipid A is the toxic factor of endotoxin (17).

Structure of the Polysaccharide Side Chain

The structure of the polysaccharide side chain of the endotoxin complex from S. marcescens Bizio was determined to be:

$$\xrightarrow{6} \text{D-Glu} \xrightarrow{1\ \beta\ 2} \text{L-Rha} \xrightarrow{1\ \beta}_{43}$$

Recent studies in this laboratory have shown that the composition of side chain from S. marcescens 08 differs markedly from that of the Bizio strain. It contains glucosamine, glucose and galactose in a molar ratio of 2:1:1. The lack of cross-reactivity of the lipopolysaccharide from the Bizio strain with the antibody prepared to the lipopolysaccharide from 08 strain can therefore be explained by structural differences of these compounds. The disaccharide unit of the side chain of Bizio is the same as the glycon portion of hesperidine (Fig. 30). Hesperidine was thought to be vitamin P due to its ability to regulate capillary permeability and fragility (154). The ability of hesperidinase to degrade the disaccharide rhamnosyl-glucose (isolated from partially hydrolyzed side chain) suggests that the rhamnose has L-configuration and confirms that the anomeric configuration is β as predicted by NMR spectroscopy.

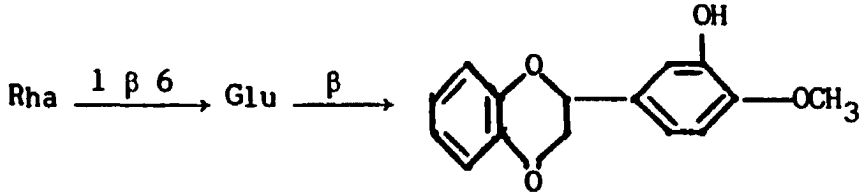


Figure 30. Structure of hesperidine.

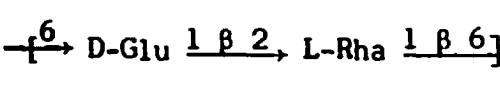
CHAPTER VI

SUMMARY

A nucleic acid-free endotoxin was isolated from Serratia marcescens Bizio by trichloroacetic acid extraction. Treatment of the endotoxin complex with various hydrolytic agents such as acetic acid, phenol, HCl or pronase resulted in the isolation of the following fragments: polysaccharide side chain, polysaccharide core, lipopolysaccharide, "conjugated" protein and its pronase core, "simple" protein and its pronase core and degraded lipid moiety. They were characterized by elementary analysis, by determination of amino acids, carbohydrates and fatty acids, electrophoresis, analytical ultracentrifugation, IR-spectroscopy and by immunological properties.

Toxicity studies showed that the toxic site of the endotoxin complex was located in the lipid moiety. Immunochemical studies indicated that the two sets of antigenic determinants of endotoxin resided in the polysaccharide and protein moieties.

Studies on the side chain demonstrated that it consists of a glucose-rhamnose disaccharide repeating unit of following structure:



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