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EFFECT OF MULTIPLE PHENOL EXTRACTION ON THE COMPOSITION OF LIPOPOLYSACCHARIDE FROM S. marcescens 08 AND STRUCTURAL STUDIES ON ITS LIPID MOIETY

A DISSERTATION

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DOCTOR OF PHILOSOPHY

BY

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Oklahoma City, Oklahoma

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EFFECT OF MULTIPLE PHENOL EXTRACTION ON THE COMPOSITION OF LIPOPOLYSACCHARIDE FROM S. marcescens 08 AND STRUCTURAL STUDIES ON ITS LIPID MOIETY

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CHAPTER I

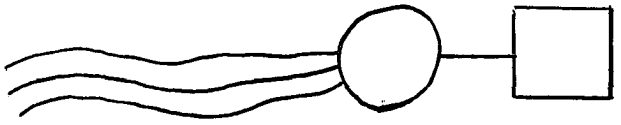
INTRODUCTION

Lipopolysaccharides (LPS) are the biologically active macromolecular complexes present in the cell wall of the Gram-negative bacteria. Because they are found in the cell wall, LPS are also known as Somatic O antigen. Many other terms such as endotoxin, Boivin O-antigen, tumor necrotizing agent, Shear's polysaccharide and Shwartzman material have been used to designate the substances responsible for the biological properties of these complexes.

Lipopolysaccharide preparations isolated from any of the Gram-negative bacteria elicit similar biological effects. They are antigenic, highly toxic and pyrogenic. A single dose of endotoxin will cause hemorrhage and regression of tumor tissues. In lethal doses they induce characteristic hemodynamic effects resulting in irreversible shock. On the other hand, the chronic administration of small amounts of the endotoxin to animals induces resistance to various infections, lethal doses of x-rays and traumatic and hemorrhagic shock.

Lipopolysaccharides are phosphorus-containing heteropolymers con-

sisting of a lipid moiety covalently linked to a core polysaccharide which is characterized by serologically specific terminal oligosaccharide units. The diagramatic presentation of this structural concept is shown below:



A protein is always found associated with these complexes. However, it is not known whether the protein is an integral part of the complex. Although the chemical structures and the linkages between the major components of the cell wall have not yet been elucidated, the localization of the lipopolysaccharides in the cell wall is generally known (1-7). The cell wall in Gram-negative bacteria is a three-layered structure consisting of a semi-rigid mucopeptide which in turn is enveloped by an interdigitated lipophilic complex of lipopolysaccharides and lipoproteins (Figure 1).

The polysaccharides of Serratia marcescens have been the subject of extensive investigation to determine their value in causing regression of malignant tumors. From culture filtrates of S. marcescens grown on glucose and inorganic salts, Shear and Turner isolated a polysaccharide which caused hemorrhage and necrosis in mouse sarcomas (8). Its components were an aldohexose, a methyl pentose, hexosamine and an unidentified phospholipid; hence, it was described as a lipopolysaccharide. Creech and his co-workers have investigated cells and culture filtrates from several strains of S. marcescens for polysaccharide components showing anti-tumor activity (9). The nitrogen, phosphorus and car-

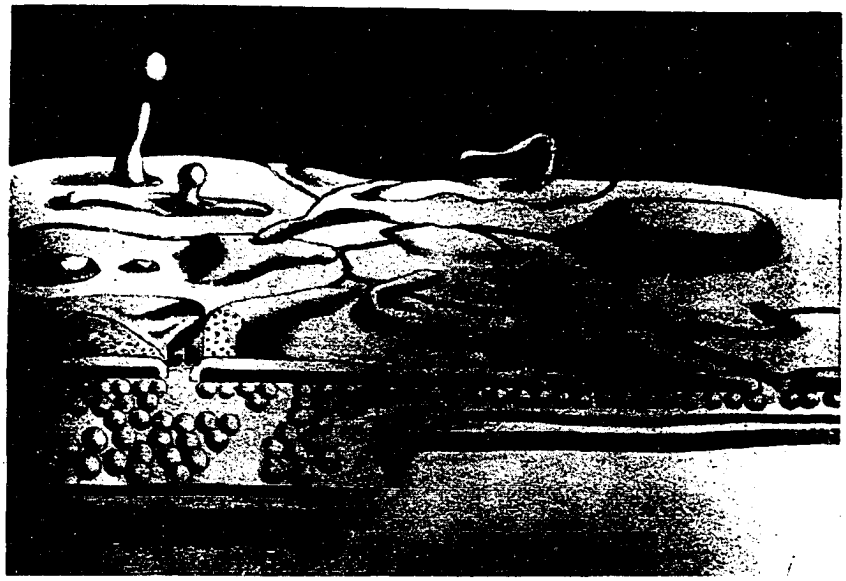


Figure 1. Proposed cell wall structure of Gram-negative bacteria (7).

Schematic view of the flattened wall of E. coli: LP = lipoprotein layer with protrusions and humps; it is dotted when seen in cross section. LS = lipopolysaccharide containing the channels Ch. P = protein elements covering the rigid glucamine-peptide layer (R). PM = the protoplasmic membrane.

bohydrate contents of the polysaccharide-lipid materials varied considerably depending on the strain of organism, composition of the medium and duration of the growth of culture. No analytical data were obtained on either the sugar or lipid components of the lipopolysaccharide. The first investigation of the chemistry of polysaccharide was reported by Rathgeb and Sylven (10). Fractionation of Shear's polysaccharide obtained from S. marcescens yielded a glucan and a polysaccharide containing N-acetyl hexosamine (10). However, the material showed no biological activity (11). Several polysaccharide-lipoprotein complexes of S. marcescens were isolated and their composition and properties were determined (12, 13). The "free" lipids associated with cellular polysaccharides were found to be phosphatides, free fatty acids and unsaponified material (14). The "bound" lipids of the lipopolysaccharides from the chromogenic strain S. marcescens 08 and non-chromogenic strain S. marcescens Bizio were isolated and their composition was determined (15). They contained fatty acids, glucosamine, phosphoric acid and a small amount of anthrone-positive carbohydrates.

The aim of the present investigation has been to study the effect of chemical modification of lipopolysaccharide from S. marcescens 08 on its chemical, physical and biological properties and to investigate the structural characteristics of its lipid moiety.

CHAPTER II

LITERATURE REVIEW

Although the biological properties of lipopolysaccharides are well known, the chemical characterization of these macromolecules has been less successful. Difficulties arise from the fact that culture conditions as well as isolation methods affect appreciably their physical and chemical characteristics. Extraction procedure and the chemical properties of the isolated and purified complexes will be reviewed briefly. A more complete survey of the published findings concerning the biological and chemical properties of these complexes has been covered fully in the recent reviews (16, 17, 18, 19).

Extraction Methods

The first extraction method was developed by Boivin and Mesrobeanu (20, 21). Wet or acetone-dried bacteria were extracted with 0.25 N trichloroacetic acid at 4°, and the extracts were precipitated by cold alcohol. The precipitate was redissolved in water, dialyzed and lyophilized (22). These preparations were antigenic and endotoxic.

A milder extraction method was introduced by Morgan (23, 24, 25). This method avoided use of acids or alkali and was carried out at low temperature in order to reduce possible chemical modifications of endotoxins during the isolation procedure. Repeated extractions of the bac-

terial cells by diethylene glycol yielded a water soluble, strongly antigenic material from smooth strains of Shigella dysenteriae. The extracted material was purified by repeated ethanol and ammonium sulfate precipitations and dialyses. The final product was the whole lipopolysaccharide-protein antigenic complex. However, diethylene glycol was found to be ineffective when applied to other bacteria. Goebel et al. used 50 per cent aqueous pyridine (26) or 50 per cent aqueous glycol (27) for extraction of antigens from Shigella flexneri and Shigella sonnei, respectively. The product was contaminated with nucleic acids and inert polysaccharides. Goebel et al. later isolated the O-antigen and a colicine from the culture fluid of a strain of E. coli by ethanol and ammonium sulfate fractionation (28, 29, 30).

Westphal et al. developed an isolation procedure utilizing aqueous phenol for the extraction of the LPS (31). When E. coli cells were extracted with 45 per cent aqueous phenol in the cold, an active glycoprotein was obtained in the aqueous phase. When the same extraction was performed at 68° carbohydrates and nucleic acid were found in the aqueous phase and a protein in the phenol phase. It was found later that in both extraction procedures a lipid component, which Westphal termed "lipid A", remained attached to the polysaccharide (32).

A variation of the phenol method consists of pretreatment of the living or dried bacteria in formaldehyde before extraction with aqueous phenol, in which case the extracts do not contain nucleic acids (18).

An interesting extraction procedure was introduced by Ribi et al. (33). The endotoxic preparations obtained by aqueous ether extraction of living cells of Salmonella enteritidis and subsequent purifica-

tion by dialysis and ethanol precipitation were characterized by an exceptionally low lipid content. They exhibited a degree of toxicity similar to that of phenol extracted endotoxic preparations characterized by a relatively high lipid content.

Recently Adams used dimethyl sulfoxide for the extraction of LPS (34). After removal of large amounts of contaminating cell wall components, the yield of purified endotoxic preparations was still several times higher than that obtained by phenol-water extraction.

The simplest method for the isolation of LPS consists in their extraction by water. Roberts reported (35, 36) that endotoxic preparations could be released by suspending the cells of Gram-negative organisms in water for 1 hour at 80°.

To study the linkages between the major components, more vigorous extraction procedures were devised. For example, dried bacteria were treated with 0.25 N NaOH for 5 hours at 56° (37). The suspension was neutralized and extracted successively with trichloroacetic acid and 90 per cent phenol. The alkali-treated preparation contained a polysaccharide and part or all of the firmly bound O-deacylated lipid A. This indicated that linkage between lipid A and polysaccharide was alkali stable. On the other hand, treatment of bacteria with 0.1 N acetic acid at 90° (38) resulted in the isolation of a degraded polysaccharide low in nitrogen and phosphorus. Lipid A could not be detected in this degraded polysaccharide preparation.

Despite the fact that several procedures are available for the isolation of lipopolysaccharides from the cell wall, very little is known about their relation and mode of linkage to other cell wall components.

It has been suggested that LPS and K-antigens (acidic polysaccharide), are linked to other macromolecular complexes of cell wall primarily by physical bonds, i.e., either hydrophobic, ionic or both (39). It has been reported recently that short treatment of aqueous cell suspensions of at least some organisms with the chelating agent ethylenediamine tetraacetic acid or simply NaCl solution results in release of about half of the total LPS in soluble form (40-43). This suggests that part of the somatic LPS may be bound to the cell wall through a metal ligand such as magnesium or calcium. It has also been shown that a lysine requiring E. coli mutant excretes a lipopolysaccharide-protein complex into the culture medium when deprived of lysine (44-46). In this case, the protein might serve as a bridge between the LPS and other components of the cell wall.

Purification of LPS Complexes

The extraction procedures described above usually yield products contaminated with various substances such as nucleic acids, K-antigens (acidic polysaccharides), M-antigens (colanic acids), loosely bound proteins and free lipids. The extent of contamination by each of these substances depends on the individual extraction procedures. Since the integrity of the cell wall is more or less maintained by extraction with trichloroacetic acid or with aqueous ether, it is expected that the amount of nucleic acids in such extracts would be less than that resulting from phenol-water or dimethyl sulfoxide extraction as these reagents cause rupture of the cell wall, liberation of protoplasm and consequent admixture of internal and cell wall components.

Nucleic acids and acidic polysaccharides are usually removed by ultracentrifugation at 100,000 x g. Jones has shown that nucleic acids and acidic polysaccharide form insoluble complexes with 'Cetavlon' (cetyl trimethyl ammonium bromide) (47). These insoluble complexes can be separated by differential solubility in sodium chloride solution. Loosely bound lipids can be removed by refluxing the crude endotoxic extracts with chloroform or chloroform-methanol mixtures. Morgan and Partridge used anhydrous formamide to remove these lipids (24). Fractional precipitation with ethanol and salt removes a major portion of the proteins and some lipids (48). Phenol-water extraction is particularly effective in the removal of proteins and associated nitrogenous material (31). Zone electrophoresis has also been employed in the purification of a bacterial complex from Salmonella because of the anionic character of the LPS complex in neutral or slightly basic solution (49).

Column fractionation for the purification of crude LPS was also employed. Gardell used cellulose columns for the separation of a crude LPS suspension (50). Peterson and Sober obtained two fractions by the use of DEAE-cellulose column chromatography (51). Recently, the heterogeneity of LPS from S. marcescens was revealed by Nowotny using ion-exchange column chromatography (52, 53) and by Adams using precipitation and centrifugation techniques (54). So far, column fractionation has not been commonly used for the purification of endotoxic preparations because of the low recovery, limited amount of material which can be applied at one time and the prolonged time required for elution of the column. However, the unexplored gel filtration technique with its wide variety of newly developed gels may represent one of the most promising

tools for the purification of LPS.

Physical Properties of LPS Complexes

The physical properties of the purified complexes have been studied by several investigators. The general agreement is that the particle sizes and the water solubility of these complexes vary with the extraction method and the nature of the chemical treatment. The direct correlation of the molecular weight with the extraction method and major chemical components of different complexes is presented in Table 1 (18).

Boivin antigen (BA) and lipopolysaccharide (LPS) have molecular weights of the order of 1 to 20 million (55-58). These values depend on the state of aggregation of the complexes. It has been suggested that multivalent cations such as Mg^{++} and Ca^{++} cross-link the smaller units of the complexes to form the multi-structured organization of the bacterial cell wall. Hence, treatment of bacteria with a small amount of EDTA results in a rapid release of LPS (40-43). Similarly, the opalescent solutions of these complexes were clarified by the addition of a small amount of deoxycholate (58). Also, treatment of these complexes with alkali resulted in a rapid degradation (disaggregation) into subunits with a molecular weight of 200,000 (59).

Short incubation of LPS with serum (60) and treatment with sodium dodecyl sulfate (61) resulted in enhancement of diffusion in agar gel indicating disaggregation of the molecules. Recently Ribi *et al.* reported that sodium deoxycholate could dissociate toxic endotoxin into nontoxic subunits with molecular weight of about 20,000 (58). Dialysis of this solution resulted in reaggregation of subunits into an active endotoxin

TABLE 1

CHEMICAL COMPONENTS AND MOLECULAR WEIGHTS OF DIFFERENT LIPOPOLYSACCHARIDE PREPARATIONS

Extracts	Method of Preparation	Chemical Components	Molecular Weights
Boivin antigen (BA)	Trichloroacetic acid	PS[a]-lipid A-protein	Several millions
Lipopolysaccharide (LPS)	Phenol-water	PS-lipid A	Several millions
Alkali-polysaccharide (AP)	Alkali treatment on LPS	PS-O-deacylated lipid A	200,000
Degraded polysaccharide (DP)	Acetic acid treatment on LPS	PS	20,000 to 30,000

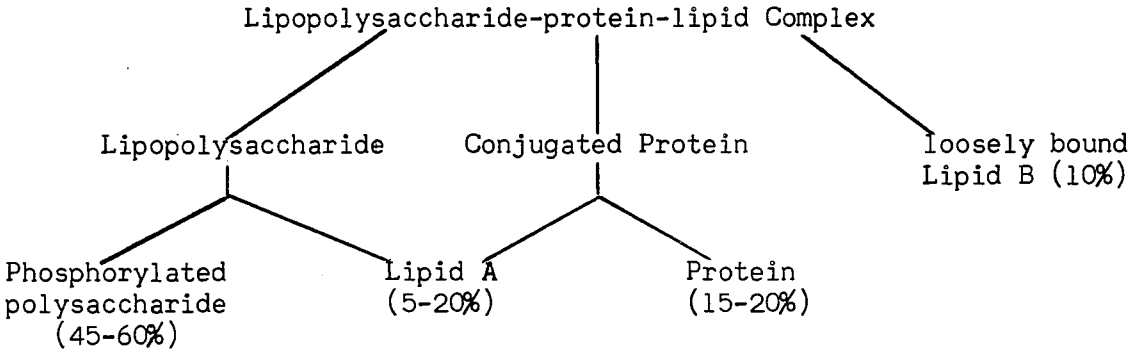
[a]PS = polysaccharide

of molecular weight of 500,000 to 1 million. The data on the correlation of biological activity with molecular size (62, 63, 64) have indicated that the active endotoxic preparations consist of micellar aggregates of linear lipopolysaccharide subunits held together by the Van der Waals attractions of the non-polar groups of long-chain fatty acid esters in the complexes. A lipopolysaccharide was disaggregated to a unit of a molecular weight of 400,000 by three procedures: (1) removal of esterified fatty acids by alkaline hydroxylaminolysis, (2) succinylation, and (3) dissolution with sodium dodecyl sulfate (65). Disaggregation of endotoxic preparations by sodium dodecyl sulfate did not decrease pyrogenicity in the rabbit, but the removal of lipid resulted in loss of pyrogenicity and toxicity.

The high particle-weight and low water-solubility have always represented the main obstacles for more refined studies on the homogeneity of LPS. It is, therefore, still questionable whether the many interesting biological properties of LPS belong to a single or to several different molecules in the same preparation. In most previous investigations a reduction in particle size of LPS preparations has been achieved only by procedures which would cleave the covalent bonds. Under the circumstances it is impossible to conclude whether changes in physical state and biological properties resulted from disaggregation (intermolecular) or degradation (intramolecular). The dissociation with either sodium dodecyl sulfate or sodium deoxycholate indicates clearly the importance of hydrophobic bonding in the aggregation of LPS molecules, but does not exclude the possible involvement of other types of intermolecular forces.

Chemistry of the Lipopolysaccharide Complex

The endotoxins or somatic O-antigens of Gram-negative bacteria are complex macromolecules composed of polysaccharides, lipids and proteins. With the aid of dissociating agents such as acetic acid, phenol, alkaline ethanol or formamide the complex can be dissociated into its component parts. Westphal has suggested (66) that endotoxin complexes could be broken down according to the following scheme:



Following treatment of the complex with mild acetic acid (0.1 N), a loosely bound phospholipid (Lipid B), a polysaccharide and a conjugated protein (presumably lipid A-protein conjugate) could be isolated. The loosely bound phospholipid could also be removed selectively from the complex by formamide extraction. The extraction of endotoxic complex with phenol-water results in the separation of lipopolysaccharide and protein. This separation can be achieved also by a selective ethanol precipitation of the protein moiety from an alkali treated LPS preparation. The lipopolysaccharide can be dissociated into the phosphorylated polysaccharide moiety and lipid A by mild acid hydrolysis.

Although the phosphorylated polysaccharide is serologically active, the protein moiety is required for eliciting the specific antigen-

icity of the complex. The investigations of Westphal's group on the correlation of sugar composition and serological specificities of LPS isolated from various strains of Salmonella have clearly shown the importance of and enhanced the interest in the chemistry of polysaccharide moiety. The first classification of bacteria according to their serological specificity was devised by Kaufmann and White with Salmonella. Similarly, other entero bacterial genera have been classified.

The chemical constituents of each major component of the lipopolysaccharide-protein-lipid complexes will be discussed separately.

The Constituents of the Polysaccharide Moiety of Somatic O-antigen

More than 20 constituent sugars have been found in O-antigens of Gram-negative bacteria and this number is increasing. Several of these monosaccharides have been reviewed including 3,6-dideoxyhexoses (67), heptoses (68), deoxyhexoses (69) and a variety of amino sugars (70, 71).

Hexoses. The most commonly found hexoses are galactose and glucose. They are primarily the constituents of the common core structure, but they may also occur in the side chains of the polysaccharide moiety. Mannose is frequently found in the side chain.

6-Deoxyhexoses. L-Fucose, L-rhamnose and D-rhamnose have been found as the constituents of the side chains. Recently, 6-deoxy-L-talose has been identified in the lipopolysaccharide of E. coli 45 (72).

3,6-Dideoxy hexoses. The 3,6-dideoxy hexoses are possibly the most important sugar constituents of LPS. It has been suggested that these hexoses are antigenic determinants directly responsible for the serological specificity (73). The 3,6-dideoxy sugars were detected and

identified by the groups of Lederer, Staub and Westphal (74). The nomenclature and occurrence of these sugars are presented in Table 2.

Heptoses. Jesaitis and Goebel first reported a heptose as a constituent of a bacterial lipopolysaccharide in 1952 (75). Since then, the presence of heptoses has been demonstrated in almost all O-antigens analyzed, with the exception of the genus Xanthomonas (76). A heptose identified as L-glycero-D-mannoheptose has been found in the polysaccharides of the following organisms: Shigella soonei, S. flexneri, S. dysenteriae, E. coli B, Salmonella minnesota, S. ruiru, S. typhimurium, Proteus mirabilis and Serratia marcescens. The LPS of the latter two organisms contained an additional heptose, namely, D-glycero-D-mannoheptose. During hydrolysis, the heptose was released in the form of a phosphate ester which could be isolated as barium salt. Heptoses were detected in the core region of the polysaccharide.

Hexosamines. D-glucosamine is a common constituent of LPS. It occurs in the "backbone" of lipid A. It is also a regular constituent of the core structure of LPS of Salmonella species and E. coli and it may function occasionally as a component sugar of the serologically specific side chains. Furthermore, D-glucosamine has long been known as a constituent of the rigid mucopeptide layer. D-galactosamine may also be present. Both D-mannosamine and L-fucosamine have recently been found in Salmonella and other enterobacteriaceae (77, 78). D-Fucosamine and D-viosamine (4-amino-4,6-dideoxy-D-glucose) were detected in the degraded polysaccharide of the LPS of Chromobacterium violaceum (79, 80). Viosamine and its galacto epimer thomosamine(4-amino-4,6-dideoxy-D-galactose) have been isolated from E. coli (81-84). The list of amino sugars pres-

TABLE 2

ENTEROBACTERIAL 3,6-DIDEOXY-HEXOSES

Trivial Name	Chemical nomenclature	Occurrence
Abequose	3,6-Dideoxy-D-xylo-hexose 3,6-Dideoxy-D-galactose 3-deoxy-D-fucose	Salmonella Citrobacter Pasteurella
Colitose	3,6-Dideoxy-L-xylo-hexose 3,6-Dideoxy-L-galactose 3-deoxy-L-fucose	Salmonella E. coli
Tyvelose	3,6-Dideoxy-D-arabino-hexose 3,6-Dideoxy-D-mannose 3-deoxy-D-rhamnose	Salmonella Pasteurella
Ascarylose	3,6-Dideoxy-L-arabino-hexose 3,6-Dideoxy-L-mannose 3-deoxy-L-rhamnose	Pasteurella Ascaris
Paratose	3,6-Dideoxy-D-ribo-hexose 3,6-Dideoxy-D-glucose 3-deoxy-D-quinovose	Salmonella Pasteurella

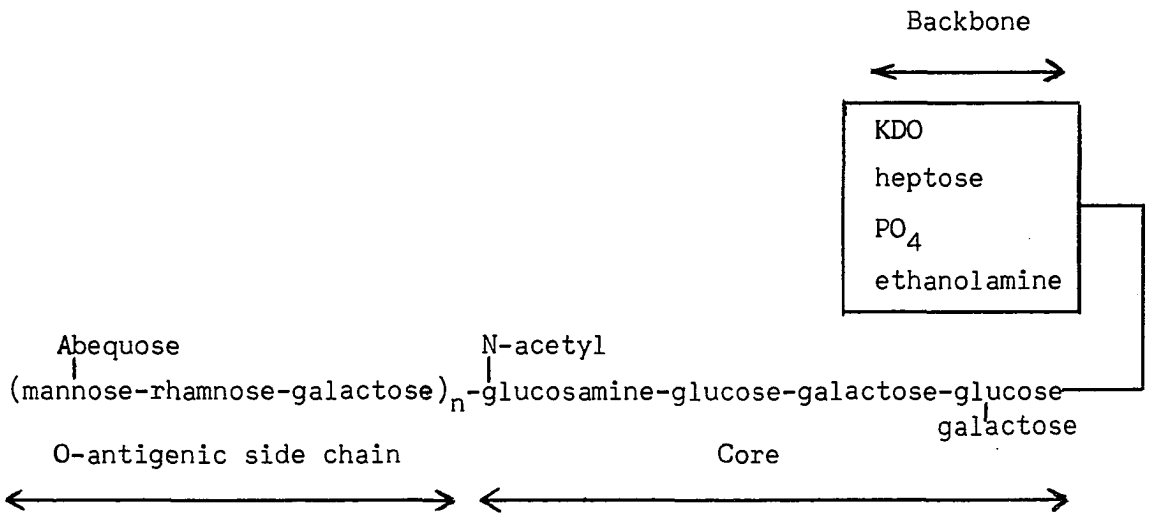
ent in lipopolysaccharides is rapidly increasing. It is probable that the presence of unusual amino sugars has been overlooked in the past.

2-Keto-3-deoxy-octonic acid (KDO). KDO was isolated recently from E. coli 0111 lipopolysaccharide by Heath and Ghalambor (85), who also studied its structure and biosynthesis. Since then KDO detectable by the periodate-thiobarbituric acid reaction, has been found as a common constituent of all O-antigens analyzed. Osborn suggested that KDO might serve as a link between the polysaccharide and lipid A (86). This concept was supported by Edstrom and Heath in their studies on the incorporation of KDO into LPS (87). Similar to N-acetylneuraminic acid, KDO is activated through its monophosphate nucleoside derivative, cytidine monophosphate (CMP)-KDO, rather than through the diphosphate nucleoside. The transfer of KDO from CMP-KDO to a partially degraded Lipid A preparation was catalyzed by a particulate enzyme fraction from E. coli 0111 (87). The LPS itself was not an acceptor. In the synthetic product KDO was linked as a glycoside; this conclusion was based on its alkali stability and acid lability, and on the fact that the carbonyl group could be reduced with sodium borohydride only when the product was first treated with acid.

O-Phosphorylethanolamine. Recently, Grollmann and Osborn isolated and identified O-phosphorylethanolamine in hydrolysates of LPS and their polysaccharide moieties from S. typhimurium and E. coli (88). It is believed to be an integral constituent of O-antigens linked to heptose phosphate through phosphodiester linkages. Previously, the occurrence of ethanolamine in an E. coli lipopolysaccharide had been reported by Ikawa et al. (89).

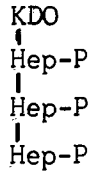
Structure and Biosynthesis of Polysaccharide Moiety

Structural analyses and biosynthetic studies of LPS have been performed almost exclusively on the lipopolysaccharides of Salmonella species. According to the current structural concepts, the polysaccharide moieties of LPS consist of an inner core linked to specific side chains carrying the determinants of O-antigen activities. The structure postulated for the polysaccharide of S. typhimurium may be represented schematically as follows (90):

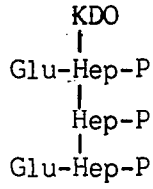


Although the composition and structure of the O-antigenic side chains vary widely among Salmonellae, the structure of the core region appears to be similar in all species (18). Nikaido (91) initiated studies on the biosynthesis of the polysaccharide core by utilizing mutant organisms of Salmonella and E. coli which were unable to synthesize required polysaccharide precursors such as UDP-galactose or UDP-glucose. Such mutants formed incomplete LPS lacking both the O-antigenic side chains and that portion of the inner core distal to the point of the bio-

synthetic lesion. Thus, mutants unable to form UDP-glucose (92-94) produced a polysaccharide containing only the backbone components:



The polysaccharide moiety of LPS from mutants deficient in UDP-galactose consisted of backbone components and glucose (95):



There are four nucleotide-sugar:LPS transferase systems which carry out the successive additions of monosaccharides to the incomplete lipopolysaccharide of these mutants (90, 96):

Glucosyl Transferase I

UDP-Glucose + LPS ⟶ Glu-LPS (1)

Galactosyl Transferase I

UDP-Galactose + Glu-LPS ⟶ Gal-glu-LPS (2)

Glucosyl Transferase II

UDP-Glucose+Gal-glu-LPS ⟶ Glu-gal-glu-LPS (3)

N-acetyl glucosamine Transferase

UDP-N-acetylglucosamine+ glu-gal-glu-LPS ⟶ N-acetyl-glucosamine-glu-gal-glu-LPS (4)

The enzyme activities are primarily localized in the cell-envelope fractions which also contain the incomplete lipopolysaccharide acceptor. The transfer of glucose onto the heptose phosphate backbone can be catalyzed by enzyme systems derived from mutants deficient in UDP-glucose. The inability to synthesize UDP-glucose is due to the deficiency in enzyme phosphoglucose isomerase. In the absence of exogenous glucose this mutant is unable to form glucose-6-phosphate and hence is unable to synthesize UDP-glucose. In this case the polysaccharide contains only heptose, phosphate, ethanolamine and KDO. Reactions 2 to 4 have been studied mainly with preparations obtained from a mutant which lacks UDP-galactose-4-epimerase and is therefore deficient in UDP-galactose. The mechanism of biosynthesis of the tetrasaccharide sequence of the polysaccharide core appears to involve sequential transfer of the monosaccharide residues from the nucleotide sugars directly to the growing polysaccharide. The incomplete lipopolysaccharide, however, is not active by itself as an acceptor for sugar residues, but is transformed into an active acceptor by mixing with a phospholipid fraction obtained from cell envelope of sonicated cells by extraction with alcohol-chloroform (97). The most active component of this lipid fraction has been identified as phosphatidyl ethanolamine. The specificity of phospholipids in the monosaccharide transferase reactions resides in both the polar and the non-polar portions of the lipid molecule (98-100). The role of phospholipid in the biosynthesis of the polysaccharide core will be further discussed under the section on lipid B.

In the biosynthesis of the O-specific side chains, which consist of repeating units of oligosaccharides, different mechanisms seem to be

involved. Recently, Osborn and Robbins have independently found (101, 102) that the initial reaction in the biosynthesis of the O-specific side chain consists in the transfer of a sugar to a membrane-bound lipid, the so-called antigen carrier lipid (ACL). The reaction sequence is as follows:

$$\text{UDP-Gal} + \text{ACL-P} \rightleftharpoons \text{Gal-P-P-ACL} + \text{UMP}$$

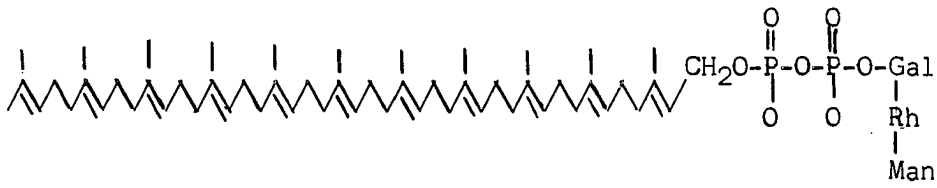
$$\text{Gal-P-P-ACL} + \text{TDP-Rh} \rightarrow \text{Rh-Gal-P-P-ACL} + \text{TDP}$$

$$\text{Rh-Gal-P-P-ACL} + \text{GDP-Man} \rightarrow \text{Man-Rh-Gal-P-P-ACL} + \text{GDP}$$

$${}_n\text{Man-Rh-Gal-P-P-ACL} \rightarrow (\text{Man-Rh-Gal})_n\text{-P-P-ACL} + (n\text{-}1)\text{Pi} + (n\text{-}1)\text{ACL-P}$$

$$(\text{Man-Rh-Gal})_n\text{-P-P-ACL} + \text{"Core"} \rightarrow (\text{Man-Rh-Gal})_n\text{-Core} + \text{Pi} + \text{ACL-P}$$

Galactose-1-phosphate is first transferred from uridine diphosphate-galactose (UDP-Gal) to ACL-phosphate to form a galactose pyrophosphate-ACL. Rhamnose is then transferred from thymidine diphosphate-rhamnose (TDP-Rh) to the galactose unit to form rhamnosyl galactose pyrophosphate-ACL. The sequence is completed by the transfer of mannose from guanosine diphosphate-mannose (GDP-Man) to the disaccharide pyrophosphate-ACL. The trisaccharide units are polymerized forming O-antigen chains which are then transferred to the "core" to form the complete lipopolysaccharide. It is not certain at what step the cytidine diphosphate-abequose (CDP-Abe) participates in the reaction sequence. The chemical nature of the ACL and its linkages to the sugars has been elucidated recently (103). The ACL has been identified as a polyisoprenoid compound with eleven isoprene units. The lipid is linked to the O-antigen repeating sequence through a pyrophosphate bridge (103):



The Lipid Components of the Somatic O-antigen

According to their chemical nature and biological activity, at least two distinct lipid components related to the O-antigen are present in the cell envelope of Gram-negative bacteria. These are lipid A and lipid B.

Lipid B. Lipid B is a loosely linked portion of the whole antigen complex from which it can be obtained in a yield of about 10% by treatment with formamide or alkaline ethanol (24, 25, 27). Detailed analyses indicate that it belongs to a cephalin type of phospholipid (25, 27). Recently, it has been found that a lipid B or lipid B-like material acts as a cofactor in certain enzymic steps in the biosynthesis of the polysaccharide core. The active component was identified as phosphatidyl ethanolamine. It has been suggested that this lipid provides either an essential site for enzyme binding or that it alters the physical state of the lipopolysaccharide which alone is not active as an acceptor of nucleotide-sugars. In the presence of the lipopolysaccharide-lipid B complex the heat stability of the transferases is markedly increased (99, 100).

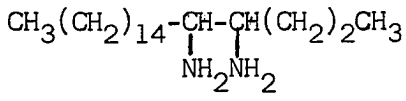
Lipid A. Lipid A is a covalently linked component of LPS from which it may be obtained by mild acid hydrolysis. Ikawa and coworkers contributed considerably to the present knowledge of the chemistry of lipid A. In their studies the lipid moiety was isolated from a hemorrhagic LPS of E. coli (104). Lipid A obtained from the acid hydrolysate

as chloroform soluble and acetone insoluble material was shown to be a phosphorus-containing lipid. Since then, lipid A preparations isolated from LPS of various bacterial groups including Salmonella, E. coli, Shigella, Serratia marcescens and others seem to exhibit great similarity in their physical and chemical properties (19). The yield of lipid A depends appreciably upon the methods utilized for the isolation of LPS and their subsequent hydrolysis. Usually, the yield is 5-20%. However, in the case of R mutants the yield may be as high as 65% (105).

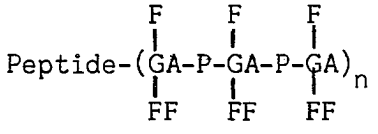
The hydrochloric acid hydrolysis of lipid A preparations followed by ether extraction results in the separation of a fatty acid fraction. Ikawa and coworkers were the first to identify lauric acid, myristic acid, palmitic acid and β-hydroxy myristic acid in such an extract from E. coli (106). Burton and Carter (107), Kasai and Yamano (108, 109), Taylor et al. (44) and Alaupovic et al. (15) have performed quantitative gas-liquid chromatographic analyses of the methyl esters of fatty acids from different lipid A preparations and have obtained very similar results. In addition to these long-chain fatty acids, Burton and Carter (107) also found acetyl groups in the lipid A from E. coli O111B4. These studies have shown that β-hydroxy myristic acid as a rather specific constituent of lipid A represents a valuable marker for the detection of this lipid.

In addition to the fatty acid fraction, the acid hydrolysis of lipid A results in a water-soluble and a water-insoluble fraction. The former contains D-glucosamine, D-glucosamine-4-phosphate and glucosamine-6-phosphate (110). Kasai has postulated a second amino sugar of unknown structure in a lipid A preparation derived from E. coli (113). The water soluble fraction may also contain a small amount of free amino acids and

ethanolamine (89). A water and ether insoluble substance found in lipid A preparation from a special strain of E. coli was identified by Ikawa as a long chain diamine, the structure of which was established as 4,5-diamino-n-eicosane (89, 111). However, this diamine, called necrosamine, has not been found in lipid A preparations from other bacteria. The structure is shown below:



Although much work has been devoted to structural analysis of lipid A, its exact structure is still unknown. Nowotny (110) investigated the lipid A moieties of the phenol extracted LPS from several strains of Salmonella. Nowotny claimed the isolation of three D-glucosamine phosphate derivatives, one of which contained a peptide. It was suggested that the amino group and the C3 and C6 hydroxyl groups of glucosamine were acylated by fatty acids. It seems on the basis of the available analytical data that lipid A consists of an acylated poly-D-glucosamine phosphate chain. The proposed structure is shown below (110):



GA = glucosamine

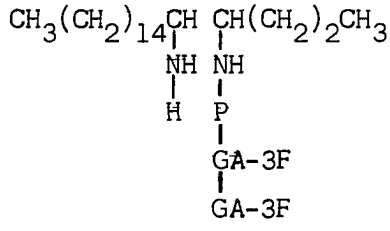
F = fatty acid

P = phosphate

Little is known at present about the nature of the linkages of

fatty acids to glucosamine units and the linkages between the glucosamine residues. Equally possible are a glycosidic bond and a phosphodiester bond as illustrated in Figure 2.

Ikawa recently proposed a sphingomyelin-like structure for lipid A of LPS from E. coli with necrosamine as a central component (112):

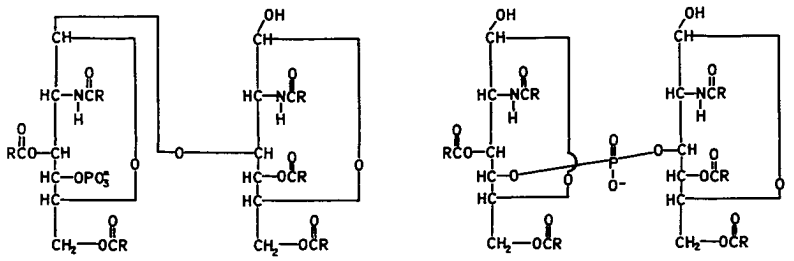


GA = glucosamine

F = fatty acid

P = phosphate

Several approaches have been taken to obtain more information about the precise structure of lipid A. Burton and Carter (107) studied the action of sodium borohydride on lipid A from E. coli 0111B4. From the nitrogen and glucosamine content of the reduced substance, it was concluded that about half of the original glucosamine was reduced. These findings and the stability of the reduced product to drastic alkali treatment suggested the occurrence of glycosidic linkages in lipid A. However, the action of sodium borohydride in ethanol on lipid A involves more than a simple reduction of the aldehydic groups available in the carbohydrate moiety. Trans-esterification of the fatty acids with the solvent may yield ethyl esters. Reduction of the ester linkages may result in deacylation. Therefore, it is not valid to compare the content of gluco-



I II

Figure 2. Proposed structure of lipid A (107).

samine in the deacylated, reduced products with that of the originally fully acylated material. A direct determination of glucosaminol content in the reduced product would provide more conclusive information for the calculation of glucosaminol produced.

The preparation of a homogeneous starting material for the structural studies has yet to be achieved. In general, lipid A preparations represent a mixture of fractions which may be separable by using extraction and precipitation methods (114) or by the aid of silicic acid column chromatography (107, 108, 113). Using the latter procedure a major fraction of purified lipid A may be obtained together with variable amounts of minor fractions. The latter represents the gradually degraded, more polar lipid A fractions containing less esterified fatty acids than the major fraction (108). There are basically three variables which affect polarity of the lipid A fractions; the amount of fatty acids, the number of glucosamine residues, and the nature as well as quantity of peptides or amino acids. So far, the insolubility of lipid A and its structural complexities still defy efforts for the isolation of a demonstrably homogeneous product.

The role that lipid A plays in the biological activities of endotoxin remains controversial. Many groups have worked on the problem. Goebel et al. were the first to postulate a toxic factor, T, as the component of endotoxins which endows protein or polysaccharide with toxicity (27, 115). The question is still under discussion as to what extent lipid A is responsible for toxicity. It is generally accepted that a quantitative relationship does not exist between toxicity and lipid content since lipid A-poor lipopolysaccharides, isolated by Ribi et al.

(116), are the most potent endotoxins. Preparations devoid of lipid A, however, like degraded polysaccharide are not toxic. On the other hand, mutant strains of Salmonella yielded endotoxic lipopolysaccharides which contained mainly lipid A and were devoid of polysaccharide moiety (105). Unfortunately, lipid A preparations obtained from lipopolysaccharides by acid hydrolysis have often been found to be unsuitable for studies on toxicity. Contradictory results have been obtained with different preparations in different laboratories. Biologically active preparations have been described as being pyrogenic (117, 118), as exhibiting tumor inhibitory (113) and necrotizing effects (119), as enhancing dermal reactivity to epinephrine (120), as displaying non-specific protection (117), and as giving adjuvant effects (117). On the other hand, the ineffectiveness of lipid A has also been described in some of these biological activities. This discrepancy may derive partly from the fact that lipid A obtained by acid hydrolysis may have undergone a partial degradation. Also, the low toxicity may be due to the poor solubility of lipid A preparations. When an artificial complex, composed of lipopolysaccharide and casein (non-pyrogenic) was dissociated with acetic acid, a lipocasein was obtained which exhibited strong pyrogenicity (113, 121). However, the possibility cannot be excluded that these preparations still contained small amount of lipopolysaccharide.

It has been shown that, in analogy to the detoxification of lipopolysaccharides, the pyrogenic activity of lipid A is destroyed by incubation with serum (122). It is not known whether this is due to the formation of complexes of serum components with lipopolysaccharide or lipid A (123, 124), or whether detoxification is the result of an attack on

lipopolysaccharide or lipid A by an enzyme such as an esterase (125).

The Protein Component of the Somatic O-antigen

Morgan, and later Goebel have shown that the protein components of both Shigella dysenteria and Shigella flexneri (24-26), can be obtained in two different forms. Splitting of the whole antigen complex with 1% acetic acid at 100° leads to a "conjugated protein", degraded polysaccharide and lipid B. This "conjugated protein" represents 15-20% of the antigen complex. It contains 12% nitrogen and 1% phosphorus. Treatment of the conjugated protein with 90% phenol, followed by ethanol precipitation results in the isolation of a so-called "simple protein" (24-26), which also can be obtained by dissociation of the antigen complex with phenol or alkali. This partially degraded protein contains up to 14% nitrogen and no phosphorus. Although it has been assumed that the presence of a lipid in conjugated protein represents the major difference between these two protein preparations, this has never been proven experimentally.

The conjugated protein is biologically active. It is toxic (26) and antigenic (25), but the simple protein is devoid of toxic activity. Morgan and Partridge (25) showed that conjugated protein and lipopolysaccharide could be recombined to form a complex similar to the native O-antigen complex. Polysaccharides of nonbacterial origin could be coupled with conjugated protein and thus transformed into antigenic complexes (126, 127). Likewise, simple protein or proteins of nonbacterial origin, such as serum proteins, egg albumin or casein form complexes upon coupling with lipopolysaccharide (25). Artificial complexes, consisting of casein

and lipopolysaccharide from S. paratyphi were found to be antigenic (128).

Goebel and coworkers demonstrated that colicines K, V, and A were intimately associated with the O-antigenic complexes of the respective colicinogenic bacteria (129-131). The O-antigens, therefore, were not only toxic for mammals but for bacteria, as well. When these O-antigens were dissociated into their component parts, the bactericidal activity remained associated with the conjugated protein component while the lipopolysaccharide was the carrier of the endotoxic activity (29). Recently Mesrobeanu et al. (132) have described a thermolabile antigenic glycolipid-polypeptide with neurotoxic activity. This polypeptide was considered to be the polypeptide fraction of Boivin's antigen. A similar glycolipoprotein has been isolated by Wober and Alaupovic from the phenol phase of the trichloroacetic acid extract of S. marcescens (133). The colored material m.p. 281-290, was completely soluble in dimethyl sulfoxide and slightly soluble in phenol. Characterization of the preparation included determination of nitrogen (11.5%), protein (70-75%) and fatty acid (2-3%) content as well as qualitative fatty acid analysis and quantitative amino acid composition.

CHAPTER III

METHODS AND EXPERIMENTAL PROCEDURES

Analytical Methods

Paper Chromatography

Paper chromatography of carbohydrates. All chromatograms for sugars, amino sugars and sugar phosphates were run on Whatman No. 1 filter paper by the descending method with the following solvent systems:

(1) ethyl acetate:pyridine:water (3.6:1:1.5)

(2) propan-1-ol:ammonia (sp. gr. 0.88):water (6:3:1)

(3) t-butylalcohol:picric acid:water (80 ml:1 g:20 ml)

The neutral sugars were detected by alkaline silver nitrate and p-anisidine hydrochloride, the amino sugars by ninhydrin (0.2% ninhydrin in 1-butanol), and the sugar phosphates by the staining procedure according to Hanes and Isherwood (134) or by methyl violet (135). Heptoses were detected by acetylacetone following periodate oxidation (136).

Paper chromatography of peptides. Peptides were run on Whatman No. 3 paper with the following solvent system:

(4) 1-butanol:acetic acid:water (4:1:5)

Peptides were stained with 1% ninhydrin solution in ethanol.

Thin Layer Chromatography

Thin layer chromatography of phospholipid and phosphomucolipid. Thin layers of silica gel G or Adsorbosil 2 (Applied Science Lab., College Station, Pa.) were applied to glass plates, 20 x 20 cm. Various combinations of chloroform, methanol and water or chloroform, methanol and ammonium hydroxide were used as solvent systems. The chromatograms were developed for 40 to 50 min. and the spots were detected by spraying with a sulfuric acid-water solution (1:1 v/v) followed by heating on an electric hot plate for 10 min. at 180°. Phosphorus compounds were detected by spraying with molybdenum blue reagent (137).

Sephadex thin layer chromatography. Sephadex TLC was carried out on glass plates 50 x 20 cm. covered with 0.5 mm thick layers of G-150 or G-200 superfine Sephadex. The plates were run by descending chromatography in a closed chamber. The eluant solvent consisted of 0.15 M sodium chloride in 0.05 M phosphate buffer (pH 7). After completion of a run, the separated substances were transferred from the gel layer by adsorption onto a superimposed sheet of Whatman No. 1 filter paper for 10 minutes. The papers were dried and stained in the usual way with o-toluidine blue for phosphomucolipid and lissamine green for protein.

Gel Filtration Column Chromatography

Gel filtration column chromatography was performed on Sephadex columns (Pharmacia, Inc., Piscataway, N. J.) according to the standard procedure. The gel permeation chromatography of LPS-AM was performed on an SR-1 column (3 x 25 cm) packed with Sepharose 4B. The eluents and required volumes for each fractionation are indicated in Results.

Gas-Liquid Partition Chromatography of Methyl Esters of Fatty Acids

Nonvolatile methyl esters were prepared by esterification of fatty acids with the absolute methanol-BF_3 reagent (Applied Science Lab., College Station, Pa.) in a glass-stoppered test tube at 63° according to the method of Metcalfe and Schmitz (138). The methanol solution was cooled and extracted with four volumes of ether. The combined ether extracts were evaporated to dryness and the residue was redissolved in hexane. One to two µl were injected into a Barber-Colman gas chromatograph (Series 5000), equipped with an argon ionization detector and a 6 foot U-shape glass column packed with 15 per cent diethylene glycol on Chromosorb W (80-100 mesh). The column was maintained at 172-175°.

Quantitative Analyses

Nitrogen and phosphorus. Elemental nitrogen and phosphorus analyses were performed by Galbraith Laboratories, Knoxville, Tennessee.

Organic and inorganic phosphorus. Organic and inorganic phosphorus were determined according to the method of Gerlach and Deuticke (139).

Anthrone positive carbohydrates. The anthrone positive carbohydrates were estimated according to the method of Koehler (140).

Reducing sugars. The reducing sugars were determined by the method of Somogyi and Nelson (141), following hydrolysis of fractions by 2 N HCl under reflux for 4 hours and neutralization of the hydrolysates by evaporation over NaOH *in vacuo*.

Hexosamine. Hexosamine content was determined by the method of Rondle and Morgan (142). The fractions were hydrolyzed with 2 N HCl for

16 hours and neutralized under NaOH in vacuo.

Muramic acid. The samples were hydrolyzed with 4 N HCl for 4 hours under reflux. Following neutralization, the hydrolysate was applied on a column packed with a mixture of Norit A and Celite (1:1, w/w) and the hexosamine was eluted with water (143). Muramic acid, if present, would have been eluted with 5% ethanol. This compound can be detected by its absorption peak at 505 mμ in the Elson-Morgan reaction. Quantitative values can be obtained by multiplying the values obtained from the Elson-Morgan method by a factor of 3, according to the suggestion of Strange (144).

Uronic acids. Uronic acids were determined by a modified carbazole method of Dische (145).

Heptoses. The determination of heptoses was carried out by the cysteine-sulfuric acid reaction (146) using D-glycero-L-talo heptose as standard.

2-Keto-3-deoxy-octonic acid (KDO). Since a pure sample of KDO was not available as standard, its semi-quantitative determination was performed colorimetrically with thiobarbituric acid (147).

Amino nitrogen. Determination of amino nitrogen was performed according to the method of Rosen (148) using glutamic acid as standard. The sample was hydrolyzed with 6 N HCl for 24 hours in a sealed tube at 107° and the hydrolysate was neutralized in the usual manner prior to analysis.

Fatty acids. Fatty acid esters (FAE) were estimated by the method of Snyder and Stephens (149) and the percentage of esterified fatty acids was calculated from a reference curve which had been obtained

with tripalmitin as standard. The results were expressed as per cent palmitic acid. To calculate the total fatty acid content, all lipid fractions were first hydrolyzed with 6 N HCl for 20 hours under reflux, and the resulting hydrolysates were extracted with chloroform. The combined chloroform extracts were evaporated, and the residual fatty acids were converted to methyl esters and quantitatively determined according to the procedure described for FAE.

Amino Acid Analysis

Samples (3-4 mg) were hydrolyzed with 1 ml of 5.7 N distilled HCl in evacuated, sealed tubes for 24 hours at 107°. The hydrolysates were dried in a rotatory evaporator at 37°, and repeatedly redissolved in distilled water and evaporated to dryness to assure removal of HCl. The residues were analyzed in duplicate on a Beckman Model 120 C amino acid analyzer. The long and short columns were packed with Beckman PA 18 and PH 35 resins, respectively. The neutral and acidic acids were eluted with 0.2 N citrate buffer, pH 3.28 for 85 minutes, followed with the same buffer, pH 4.25, for 125 minutes. The basic amino acids were eluted with 0.2 N citrate buffer, pH 5.25, for 57 minutes.

The analyzer was calibrated with the type 1 amino acid calibration mixture. In studies of lipid A fractions it was also calibrated with glucosamine, galactosamine, glucosaminol and galactosaminol. The hexosaminols were prepared from the corresponding hexosamines by the method of Bragg and Hough (150). Other amino compounds, such as diaminopimelic acid, ethanolamine, O-phosphoethanolamine and O-phosphoserine were also used for calibration.

Detection of Nucleic Acids

The ultraviolet absorption spectrum of 1% aqueous solution of LPS fractions was recorded. The lack of inflection at 260 mμ was interpreted as an indication of absence of nucleic acids in the LPS preparations.

Enzymatic Hydrolysis

Enzymatic dephosphorylation was performed whenever possible according to the method of Baddiley (151). Soluble or insoluble substrates (mg/ml) in 0.15% ammonium carbonate were incubated with intestinal phosphomonoesterase (Sigma Chemical Co., St. Louis, Mo.) for 20 hours at 37°.

Snake venom phosphodiesterase (Sigma Chemical Co., St. Louis, Mo.) was used to hydrolyze phosphodiester linkages. The incubation mixture contained 0.5 ml of substrate (1-2 mg), 0.1 ml of 1.0 M glycine buffer, pH 9.3, 0.2 ml of 0.1 M $MgCl_2$, and 1 mg of snake venom diesterase (152). The incubation was performed at 37° for 4 hours.

Infrared Spectroscopy

The infrared spectra were recorded by Sadtler Research Laboratories, Philadelphia, Pa. The samples were run as potassium bromide wafers on a Perkin-Elmer Model 521 Grating Spectrophotometer.

Analytical Ultracentrifugation

Ultracentrifugal analyses were carried out in a Spinco Model E ultracentrifuge equipped with a phase plate schlieren diaphragm. Plate measurements were made with a Nikon micro-comparator having a sensitivity of 0.001 mm. Sedimentation coefficients were determined at constant

temperature (25-26°), employing rotor speed of 52,640 rpm. Solutions of the samples in 0.15 M NaCl were spun in single sector or synthetic boundary cells. The observed sedimentation coefficients were corrected to values in water at 20° by the usual methods (153).

Immunodiffusion

The presence of antibodies was tested by Ouchterlony's method of double diffusion in agar gel (154) as follows: a 1% agar solution in veronal buffer, pH 8.6 μ 0.1, was prepared by heating the mixture in a boiling water bath for 20 minutes, followed by cooling to 56°. The solution was then transferred at 56° to precleaned, leveled, glass slides supported by a plastic frame. The solution was allowed to cool and gel on the slides at room temperature for four hours. The slides were then transferred to a closed chamber saturated with moisture and equilibrated for at least 2 hours prior to use. Wells of constant diameter (3 mm) were made with a gel punch (LKB, Stockholm, Sweden). They were correspondingly filled with antibodies and antigen. The plates were allowed to develop for 24 hours in a closed chamber at room temperature.

Preparation of Antisera

White rabbits were injected successively with a saline suspension (1 mg/ml) of heat-killed cells of chromogenic strain S. marcescens 08. The total dose of 7 mg of cells was administered in six equal aliquots at intervals of two days. The last injection consisted of 2 mg of heat-killed bacteria and an equal amount of Freund's adjuvant. Animals were bled by cardiac puncture at the end of four weeks. The presence of antibodies was tested by double diffusion in agar gels.

Lethality for Mice (LD_{50})

White male mice weighing 15-20 g, obtained from the National Animal Company, were divided into groups of 10 each. Animals were injected intraperitoneally with 1 ml of a saline solution containing graded dosages of LPS fractions and deaths occurring within 6 days after injection were recorded. When range was determined, an additional group of 10 mice was injected at this dose to verify the estimated LD_{50} values.

Experimental Procedures

Isolation and Fractionation of Lipopolysaccharides

The chromogenic strain S. marcescens 08 grown on an enriched medium (N-Z-amino type E, 2%; glycerine, 0.1%; NaCl, 0.5%; meat extract, 0.3%; pH 7.3) was supplied as frozen cells by General Biochemicals, Chagrin Falls, Ohio. Cells were harvested at the late log phase. The cells were washed in water and collected by centrifugation in a Sharples continuous flow centrifuge. They were extracted from the wet state.

In a typical experiment (Figure 3), approximately 200 g of wet cells were extracted three times with 300 ml of 5% trichloroacetic acid (TCA), according to a modification of the method of Boivin et al. (20). The combined extracts were dialyzed against tap and distilled water for 24 hours and 48 hours, respectively, concentrated by vacuum distillation to approximately 100 ml and centrifuged at 40,000 rpm (105,000 x g). The soluble layer contained the nucleic acids and acidic polysaccharides. Lyophilization of the gel-like bottom portion yielded the partially purified nucleic acid-free parent lipopolysaccharides (LPS-U) as an amorphous powder. To remove the protein moiety the LPS-U fraction was treated with

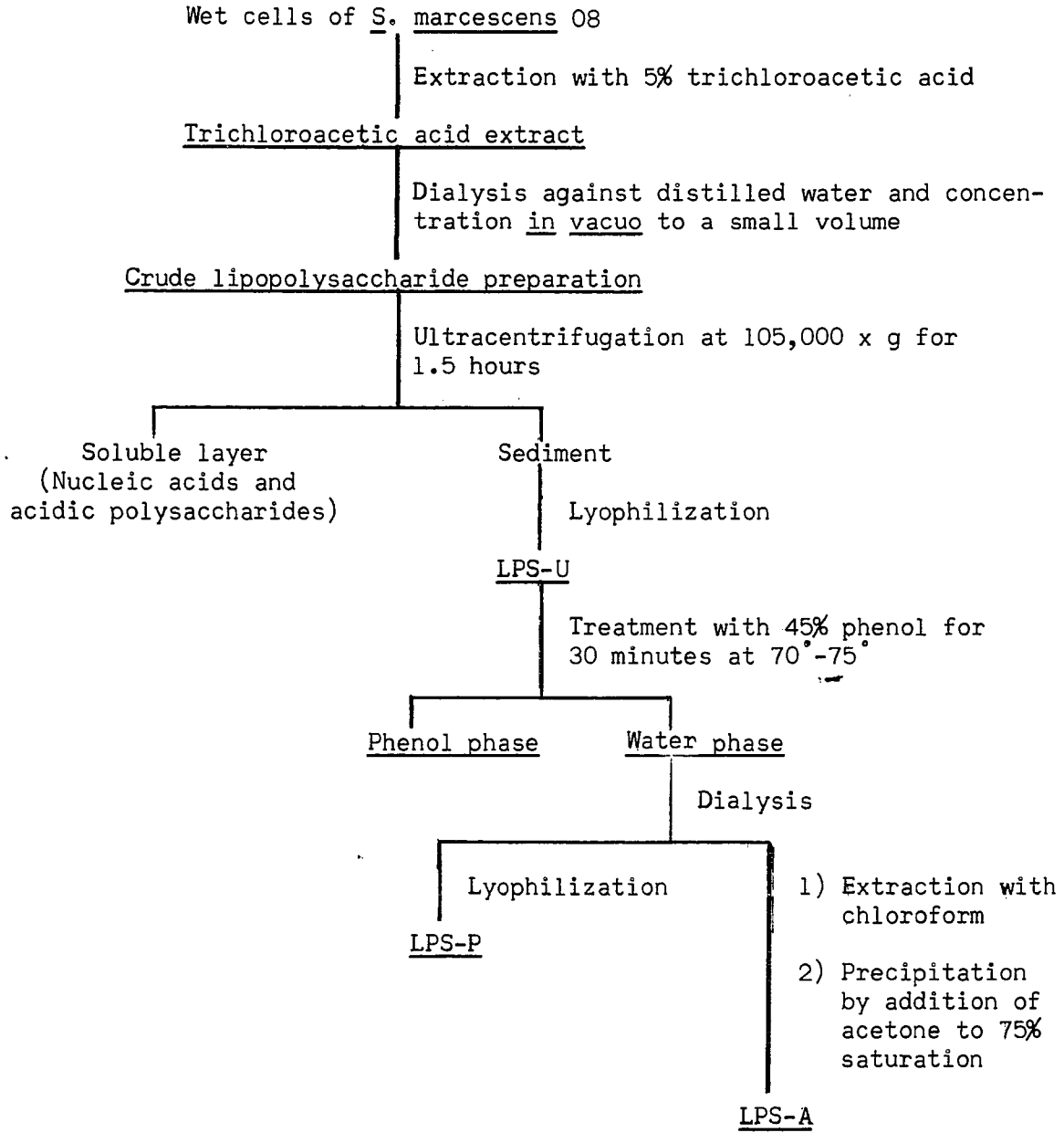


Figure 3. Procedure for the isolation and fractionation of lipopolysaccharides.

a 45% aqueous phenol solution for 30 minutes between 70-75° according to the method of Westphal et al. (31). The reaction mixture was cooled and the phenol and water phases were separated by centrifugation (2,000 rpm) at 4°. The phenol phase was washed with equal volumes of water three times. The combined water phases were exhaustively dialyzed and concentrated by vacuum distillation to 100 ml. A small portion was lyophilized yielding the partially deproteinized lipopolysaccharide (LPS-P). The remaining suspension 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 at 4°. In order to collect a white, dry precipitate (LPS-A) the overlayering solvent was removed by decantation and the last traces of acetone and water were eliminated by evaporation and lyophilization, respectively.

In a separate experiment, LPS-P (2 g) was extracted twice with 45% phenol in the same manner as described earlier. After chloroform extraction and acetone precipitation, fraction LPS-AM was obtained (Figure 4).

Alkaline polysaccharide (LPS-AP) was prepared according to the method of Staub (37). Three grams of LPS-A were dissolved in 150 ml of 0.25 N NaOH. The solution was stirred for 5 hours at 58-60°. Following the pH adjustment of the solution to 6.5, it was extracted three times with chloroform. The aqueous phase was dialyzed against 1.5 liters of distilled water for three days, changing water every 24 hours. The alkaline polysaccharide was obtained by lyophilization (Figure 5).

Isolation of Compounds from Phenol Phases

The material from phenol phase I was isolated according to the

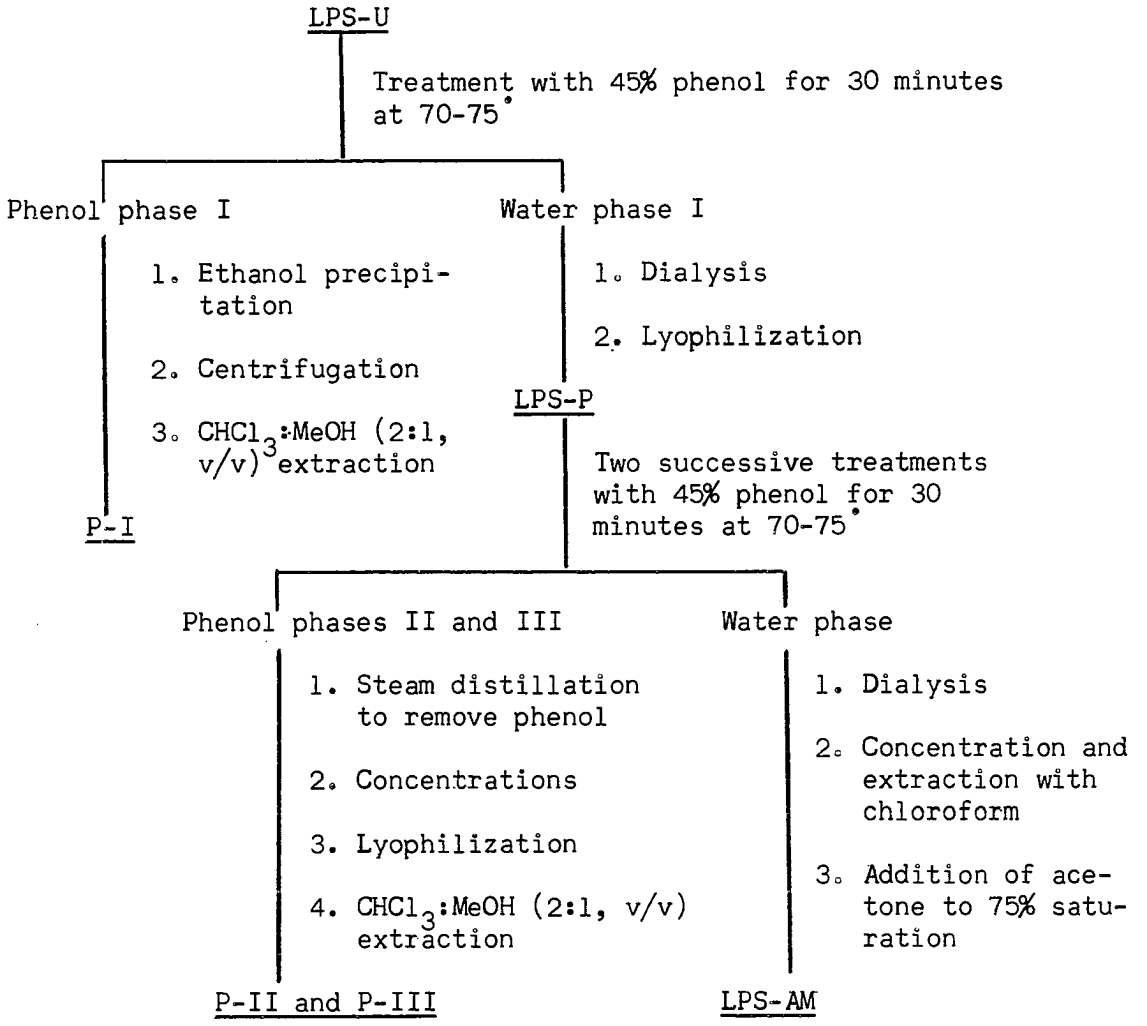


Figure 4. Procedure for the isolation of fractions P-I, P-II, P-III and LPS-AM.

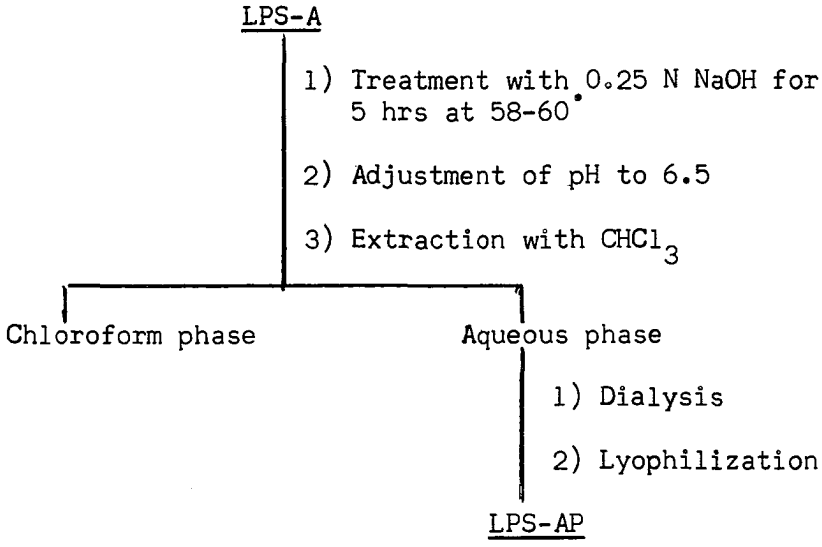


Figure 5. Procedure for the preparation and isolation of LPS-AP.

method of Wober and Alaupovic (133). The phenol solution was washed several times with distilled water to remove the remaining lipopolysaccharides and other water soluble compounds. Then, 9.5 volumes of ethanol were added and the mixture was allowed to stand overnight at 4°. The precipitate was collected by centrifugation and washed several times with water and ethanol. To remove free lipids, the precipitate was extracted exhaustively by chloroform-methanol (2:1, v/v), yielding fraction P-I (Figure 4).

Phenol phases II and III were submitted to steam distillation to remove phenol. The remaining aqueous suspensions were concentrated and lyophilized. The residues were freed of lipids by chloroform-methanol (2:1, v/v) extraction to yield P-II and P-III, respectively.

The chloroform-methanol phases were concentrated in vacuo to dryness and the residues were treated with warm acetone. The acetone insoluble residue was removed by filtration. Both acetone soluble and acetone insoluble fractions were characterized by thin layer and gas-liquid chromatography.

Isolation of the Lipid Moiety

LPS preparations (LPS-A and LPS-AP) were hydrolyzed with 0.1 N HCl. For each gram of LPS fraction 100 ml of acid were used. The acid was preheated to 85-90° and the sample added with stirring. The mixture was refluxed for 30 minutes, cooled in an ice bath and extracted with three portions of redistilled chloroform. The combined extracts were washed exhaustively with deionized water until last traces of acid were removed. The extracts were concentrated in vacuo, yielding fractions A-C and AP-C, respectively.

The lipid fractions were purified by repeated treatments with boiling acetone. The acetone insoluble material (fraction A-AI or fraction AP-AI) was removed by filtration and the acetone soluble portion (fraction A-AS or fraction AP-AS) was evaporated in vacuo to dryness (Figure 6).

Isolation of Amino Sugar Phosphate and Amino Sugars

Twenty milligrams of fraction A-AI were hydrolyzed with 8 ml of 2 N HCl for 6 hours under reflux. The hydrolysate was extracted three times with equal volumes of diethyl ether to remove fatty acids. After removal of HCl by neutralization and drying in NaOH dessicator, the sample was redissolved in 2 ml of water and submitted to ion exchange chromatography on Dowex 50-H^+ (X 4, 200-400 mesh), column size (1 cm x 20 cm). The neutral and acidic sugars were eluted with 300 ml of deionized water ("neutral fraction") and the amino sugars were eluted with 300 ml of 0.33 N HCl ("acidic fraction").

Isolation and Characterization of Fatty Acids

Fifty milligrams of A-AI were hydrolyzed with 25 ml of 5 N HCl for 20 hours under reflux. The hydrolysate was extracted three times with equal volumes of chloroform. The combined chloroform extracts were washed three times with water and dried with anhydrous sodium sulfate. The chloroform was evaporated in vacuo and the residual fatty acids were converted to methyl esters for GLC analysis. Prior to GLC analysis, small portions of methyl esters were submitted to acetylation and hydrogenation. Fraction AP-AI was analyzed in the same manner.

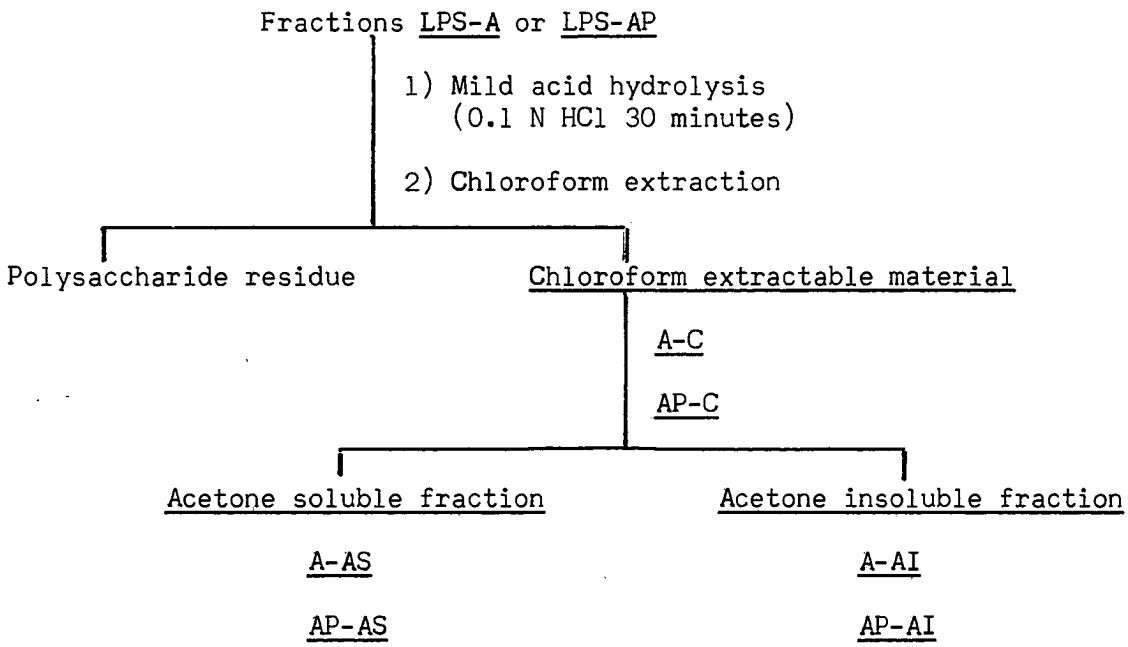


Figure 6. Procedure for the isolation of lipid moieties from LPS-A and LPS-AP.

Acetylation. The methyl esters of fatty acid (1-3 mg) were mixed with 1 ml of redistilled pyridine and 0.5 ml of anhydrous acetic anhydride and the mixture was allowed to stand in the dark overnight. The excessive acetic anhydride was eliminated by the addition of 2 ml water and the mixture was extracted three times with equal volumes of diethyl ether. The combined diethyl ether extracts were washed with water. The traces of water were removed by filtering the extract through anhydrous sodium sulfate. The solvent was evaporated *in vacuo* and the acetylated product was dissolved in hexane for GLC analysis.

Hydrogenation. Hydrogenation of methyl esters of fatty acids was performed in a Brown's automatic-titrating hydrogenator (Delmar Scientific Lab., Maywood, Ill.) according to the method of Miwas *et al.* with a platinum catalyst and with sodium borohydride as the source of hydrogen (155). The catalyst was prepared by stirring a 2-propanol suspension of active charcoal (100 mg), diglyme (1 ml) and 0.2 ml of 0.5 M chloroplatinic acid in the hydrogenator. Hydrogen was generated from 0.2 ml of 1 M sodium borohydride. After hydrogenation was completed the content was then filtered through fat-free filter paper and the filtrate partitioned in a separatory funnel between hexane and distilled water. The hexane fraction was concentrated under nitrogen and analyzed on GLC.

Alkaline Hydrolysis of Fraction A-AI

Fraction A-AI (250 mg) was suspended in 50 ml of 1 N NaOH in 50% methanol. The suspension was refluxed for 6 hours. The methanolic solution was evaporated *in vacuo* to dryness, and the residue was redissolved in 100 ml of water. The aqueous solution was extracted with chloroform to yield the chloroform soluble material (AC-1) (Figure 7). Addition of

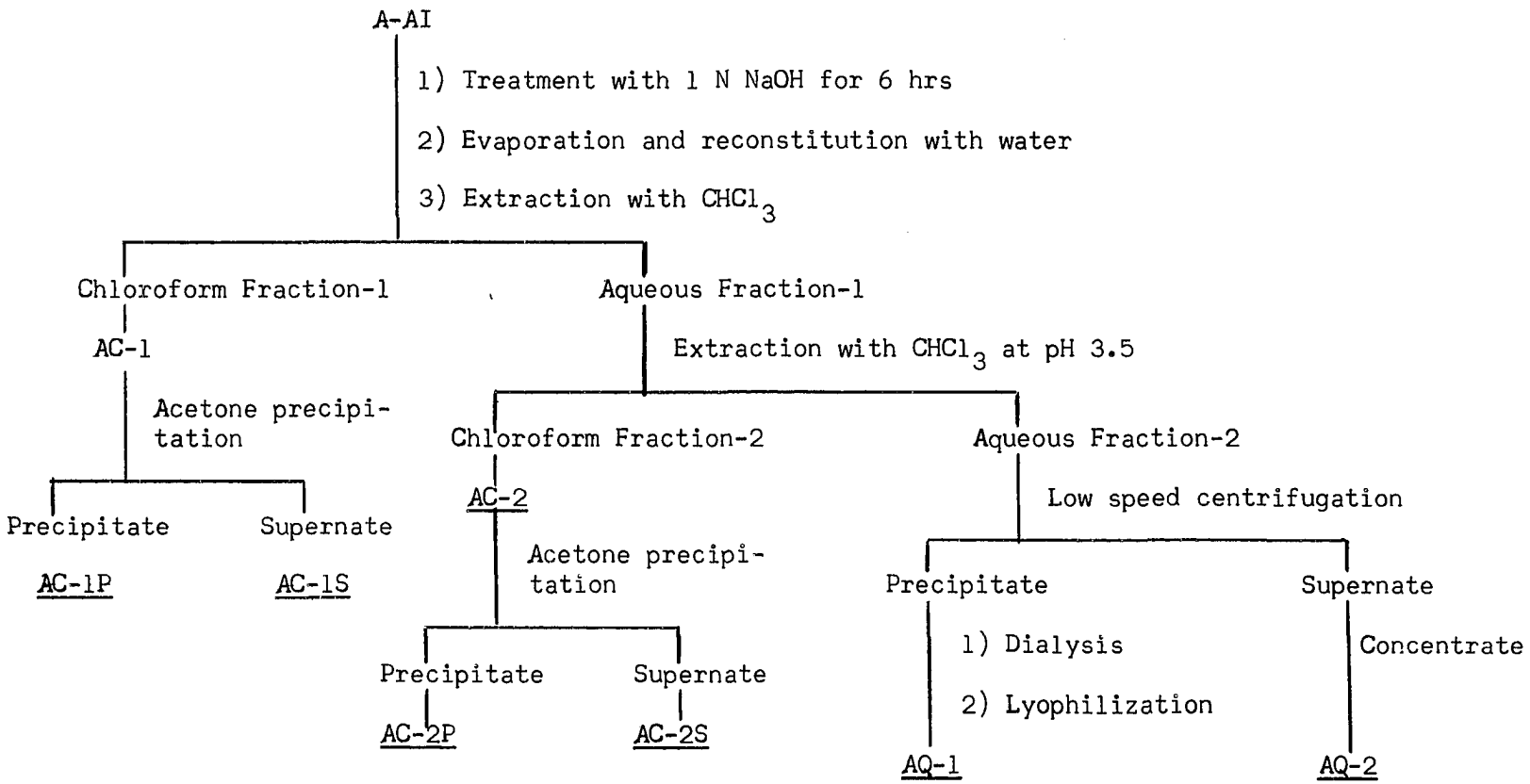


Figure 7. Procedure for the isolation of products of alkaline hydrolysis from fraction A-AI.

acetone to AC-1 resulted in a precipitate (AC-1P) which was separated from the supernate (AC-1S) by centrifugation. The aqueous layer (Aqueous Fraction-1) was neutralized and acidified to pH 3.5 with N HCl. The acidified solution was extracted three times with chloroform to yield the second chloroform soluble fraction (AC-2). Addition of acetone to AC-2 resulted in a precipitate (AC-2P) which was separated from the supernate (AC-2S) by centrifugation. Another precipitate (AQ-1) was obtained when the Aqueous Fraction-2 was submitted to low speed centrifugation. The supernate fraction was concentrated to 5 ml (AQ-2) and subjected to Sephadex G-25 column chromatography.

Sodium Borohydride Reduction of Fraction A-AI

Sodium borohydride (100 mg) dissolved in 5 ml of methanol was added dropwise to 200 mg of fraction A-AI suspended in 100 ml of methanol, and the reaction mixture was refluxed for 24 hours. Additional 100 mg of sodium borohydride were added and the refluxing was continued for another 24 hours. Methanol was removed in vacuo and acetone was added to destroy any excess sodium borohydride. Acetone was evaporated in vacuo, and the reduced products resuspended in 20 ml of water were submitted to low speed centrifugation (2,000 rpm). The sediment was dialyzed and lyophilized to yield fraction R-1 (Figure 8). The supernate was extracted three times with chloroform after the pH was adjusted to 3.5. The combined chloroform extracts were evaporated in vacuo to yield fraction R-C. The aqueous phase was centrifuged at 25,000 rpm for 30 minutes. The gel-like sediment was dissolved in water and lyophilized (fraction R-2). Addition of acetone to the supernate to 75% saturation resulted in the formation

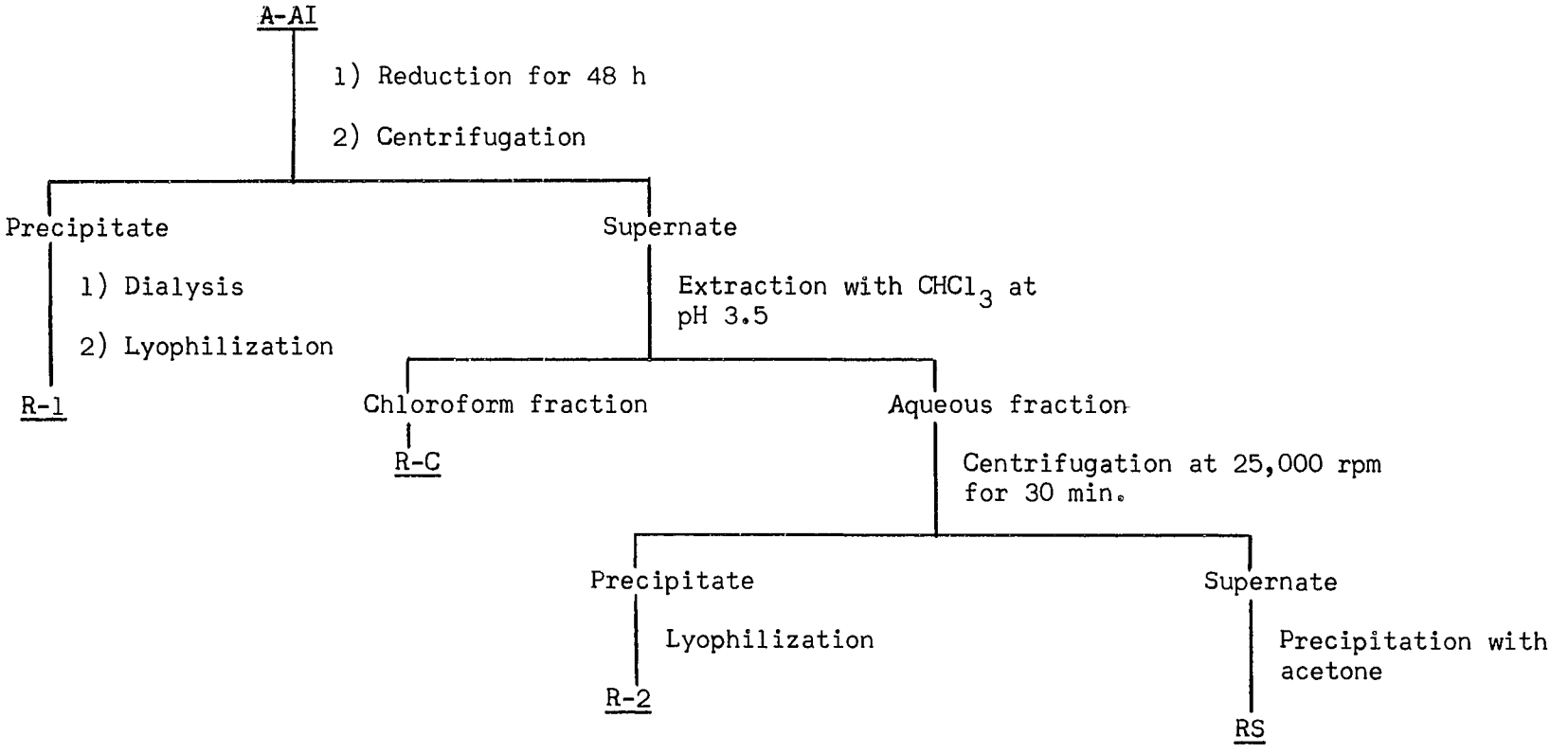


Figure 8. Procedure for the isolation of products obtained by sodium borohydride reduction of fraction A-AI.

of a precipitate (fraction RS) which was collected by centrifugation. The precipitate was redissolved in water and fractionated on a Sephadex G-25 column.

Silicic Acid Column Chromatography of Fraction A-AI

Further fractionation of the acetone insoluble lipid fraction (A-AI) was accomplished by silicic acid column chromatography. Silicic acid (Bio-Sil A, 100-200 mesh) was washed several times with redistilled chloroform and packed in a column with a 2 cm diameter, to a height depending on the amount of material to be fractionated. One gram of silicic acid was used for every 10 mg of lipid. The acetone insoluble lipid (200 mg of A-AI) was dissolved in a small amount of chloroform and applied onto the column. Elution was performed with successive portions of chloroform and mixtures of chloroform:methanol in the proportions 9:1, 8:2, 7:3, 6:4 and 5:5 (v/v), respectively. The entire elution procedure was carried out under nitrogen. The fraction obtained with chloroform:methanol (9:1) was rechromatographed with chloroform:methanol mixtures containing higher proportions of chloroform (99:1, 97:3, 95:5, and 93.7, v/v).

Simultaneous Determination of Glucosamine and Glucosaminol

Each lipid fraction (2-4 mg) obtained by silicic acid column chromatography was reduced with sodium borohydride (4-8 mg) in 5 ml of methanol for 48 hours. Methanol was evaporated *in vacuo* and the excess sodium borohydride was destroyed by the addition of a few drops of acetone. The residues were hydrolyzed with 2 N HCl (2-3 ml) for 16 hours

under reflux. The fatty acids were removed by extraction with diethyl ether, and the hydrolysate was freed of HCl in the usual manner. The residue was redissolved in 0.2 N citrate buffer, pH 3.26, and the content of glucosamine and glucosaminol was determined by the Beckman Model 120 C amino acid analyzer.

Hydroxylaminolysis

Hydroxylaminolysis was performed according to the method of Verheyden and Nys (156). Four volumes of 2.5% ethanolic solution of sodium hydroxide and three volumes of 2.5% ethanolic solution of hydroxylamine hydrochloride were mixed and the insoluble sodium chloride was removed by centrifugation. The lipid fraction (28 mg) suspended in 1 ml of ethanol and the alkaline hydroxylamine solution (14 ml) were mixed well in a glass tube and the suspension was stirred under nitrogen for 1.5 hr at room temperature. The insoluble material was recovered by centrifugation and washed by resuspending and centrifuging twice in 95% ethanol, once in 0.01 N acetic acid in 95% ethanol, and again in ethanol. The precipitate was dissolved in water and lyophilized.

CHAPTER IV

RESULTS

Preparation of LPS Complexes

Although the yield and chemical composition of LPS fractions depend on the methods of cultivation and extraction of the bacteria, the application of Boivin's isolation procedure followed by specific purification steps resulted in several complexes, the yields of which were fairly reproducible. The yields of LPS complexes from two batches of 900 gm of semi-wet cells are presented in Table 3.

The amjor difficulty encountered during the purification step was the removal of the endotoxically non-essential cytoplasmic contaminants. Nucleic acids and acidic polysaccharides were removed by ultracentrifugation of the crude TCA extracted preparation (Figure 3). When the resulting fraction LPS-U was extracted with hot aqueous phenol, the chromogenic material (prodigiosin) and traces of phospholipid were removed. The extraction of LPS-P with chloroform represented an essential step in removing not only the remaining part of the pigments but also the anti-foam material which was added to the bacterial culture medium. Without the chloroform extraction, the anti-foam material in fraction LPS-P would be carried along to fraction LPS-A and eventually to the chloroform soluble fraction following hydrolysis of LPS-A by mild acid.

TABLE 3

YIELDS OF FOUR MAJOR LIPOPOLYSACCHARIDE FRACTIONS FROM TWO BATCHES OF 900 GM SEMI-WET CELLS

Fractions	Yields in gm #1	#2
Crude LPS	41	39
LPS-U	23	21.5
LPS-P	14.5	13.5
LPS-A	10.0	8.5

The anti-foaming agent could be characterized by its infrared spectrum with absorption peaks at 820 cm^{-1}, 1015 cm^{-1}, 1093 cm^{-1}, and 1258 cm^{-1} characteristic of polydimethyl siloxanes (157). In contrast to the successful elimination of nucleic acids as evidenced by the absence of an absorption peak at 260 mμ and of acidic polysaccharides, a complete deproteinization of fraction LPS-U by the hot phenol treatment presented an extremely difficult task. Even after multiple phenol extractions, a very small amount of peptide-like material could still be found as a part of the LPS complex (LPS-AM). The presence of such impurities in fraction LPS-A complicated further the isolation and structural study of the bound lipid.

General Properties and Composition of LPS Fractions

The lyophilized LPS preparations were amorphous, and hygroscopic. The infrared spectra of LPS preparations (Figures 9 and 10) are characterized primarily by the absorption peaks at 1735-1725 cm^{-1}, 1660-1650 cm^{-1}, 1550-1540 cm^{-1} attributable to ester -C=O stretching, and $-\overset{O}{\overset{\|}{C}}-NH-$ and -N-H deformations, respectively. However, ester carbonyl absorption decreased substantially in LPS-AM and was completely absent in LPS-AP. The absorption band at 1000-1100 cm^{-1} can be regarded as characteristic of polysaccharides in general. The absorption at 1300-1250 cm^{-1} indicates the presence of sugar phosphates.

Glucose, galactose and glucosamine were identified by paper chromatography in all LPS fractions (Figure 11). Small amounts of mannose may be present in fractions LPS-U, LPS-P and LPS-A. On the other hand, all fractions contained also heptose and keto-deoxyoctulonic acid.

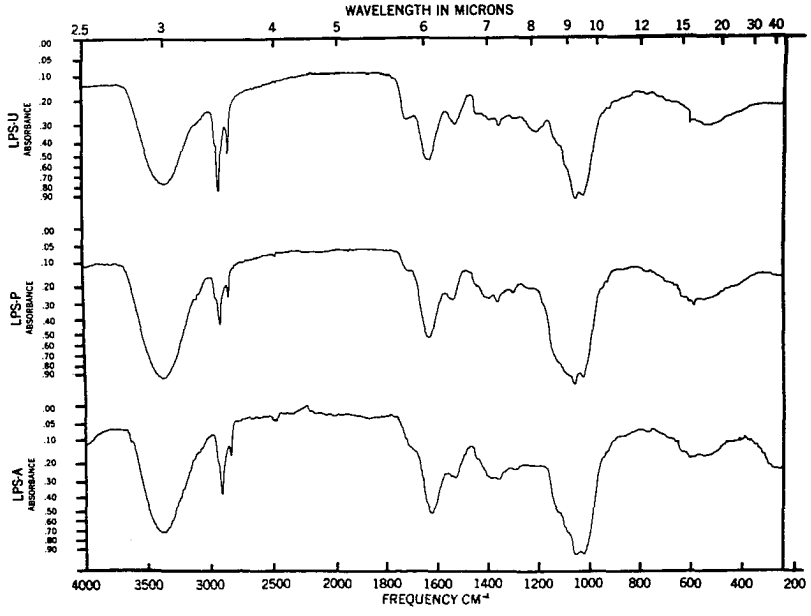


Figure 9. Infrared spectra of fractions LPS-U, LPS-P and LPS-A.

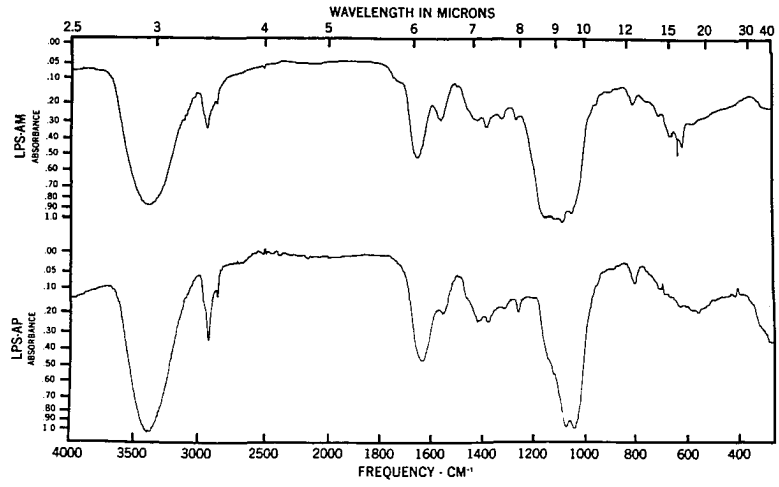


Figure 10. Infrared spectra of fractions LPS-AM and LPS-AP.

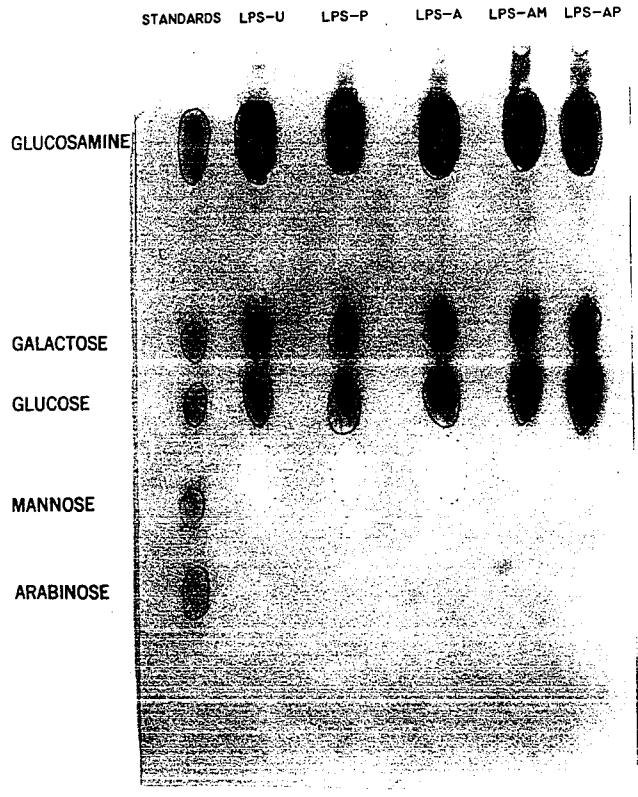


Figure 11. Paper chromatography of sugar components in lipopolysaccharide preparations.

Chromatogram was developed with solvent 1 and the spots were detected with alkaline silver nitrate.

The former was determined quantitatively by the cysteine-sulfuric acid method, while the latter, due to the lack of KDO standard, was estimated semi-quantitatively by the thiobarbituric test. The absence of muramic acid in all fractions clearly indicated that the mucopeptide or the murein layer of the cell wall was not extracted along with the lipopolysaccharide-peptide complexes.

Table 4 summarizes the quantitative composition of all preparations. Fractions LPS-U, LPS-P and LPS-A contained a high percentage of reducing sugars (29-40 per cent) and glucosamine (10-20 per cent), a substantial amount of fatty acids and heptose, and a relatively low content of uronic acids. The unexpected presence of uronic acid in these three fractions has yet to be explained. Whether they represent remaining contaminating acidic polysaccharides or whether they are an integral part of the lipopolysaccharide complex is still an open question. In comparison with LPS-U, fraction LPS-A shows a marked decrease in amino acid content and a significant increase in reducing sugars. This difference in the gross chemical composition may be explained by the removal of the protein moiety from LPS-U by a single phenol treatment.

The LPS fractions differed in quantitative rather than qualitative amino acid composition (Table 5). The acidic amino acids, aspartic and glutamic acids, were the major ones and the basic amino acids, lysine, arginine and histidine, were the minor amino acids in fractions LPS-U, LPS-A and LPS-AP. However, the situation was reversed in the case of LPS-P. The presence of ammonia in the hydrolysate suggests that either some of the glutamic acid and aspartic acid existed in the amide form or that some of the hexosamines were degraded. The straight chain acids

TABLE 4

CHEMICAL COMPOSITION OF LIPOPOLYSACCHARIDE FRACTIONS

Lipopolysaccharide Fractions	N %	P %	Reducing Sugars %	Anthrone positive carbohydrates %	Glucosamine %	Heptose %	Uronic Acid %	Fatty Acids %	Amino Acids %
LPS-U	5.75	1.83	29.0	13.6	13.5	5.8	2.1	9.1	7.35
LPS-P	4.00	0.93	31.2	17.4	10.3	6.1	Traces	7.0	1.35
LPS-A	5.43	1.10	40.4	11.6	21.2	8.6	Traces	8.0	0.53
LPS-AM	2.45	0.35	41.8	20.8	12.3	4.9	Traces	2.0	0.17
LPS-AP	3.30	0.74	30.0	25.0	17.0	8.9	0	3.8	1.07

TABLE 5

AMINO ACID COMPOSITION OF LIPOPOLYSACCHARIDE FRACTIONS

Amino Acids (μMoles/g)	LPS-U	LPS-P	LPS-A	LPS-AM	LPS-AP
Lysine	15	25	1.0	1.5	3
Histidine	3	21	1.0	2.5	5
Arginine	10	Trace	Trace	Trace	Trace
Ammonia	+	+	+	+	+
Aspartic acid	66	8	7.0	2.0	7
Threonine	23	1	Trace	Trace	1
Serine	30	2	1.0	1	6
Glutamic acid	43	3	2.0	Trace	7
Proline	13	Trace	Trace	-	-
Glycine	62	10	7.5	1.5	9
Alanine	44	5	4.0	1.0	8
Half-cystine	4	3	4.0	Trace	-
Valine	22	1	1.0	Trace	3
Methionine	1	1	Trace	Trace	-
Isoleucine	11	1	Trace	Trace	2
Leucine	25	1	1	Trace	4
Tyrosine	18	Trace	Trace	Trace	1
Phenylalanine	12	Trace	Trace	Trace	2

such as glycine and alanine constituted by far the major proportion of the neutral amino acids. Since acid hydrolysis causes a partial degradation of serine and threonine, the actual content of the hydroxy amino acids is probably greater than that shown in the table. Despite the fact that fraction LPS-AM was obtained by multiple phenol extraction of LPS-U, the whole spectrum of amino acids, though in much lower amount, was still present.

Physical and Immunochemical Properties of LPS Fractions

All three major fractions (LPS-U, LPS-P and LPS-A) were only slightly soluble in water. However, successive phenol treatment of parent endotoxic fraction LPS-U and alkaline treatment of LPS-A resulted in preparations (LPS-AM and LPS-AP) with increased water solubilities. The poor solubility of these fractions greatly hampered studies of their homogeneity and physical characteristics.

Sephadex G-200 thin layer chromatography of the LPS fractions indicated heterogeneity of these preparations (Figure 12). The parent fraction LPS-U was resolved into two spots; the major streaking component was very similar in its mobility to those of fractions LPS-P and LPS-A. However, these latter two preparations did not contain the faster moving component of LPS-U. In contrast, fraction LPS-AM displayed a single fast moving component differing in mobility from all other constituents of LPS fractions.

The schlieren patterns of the soluble components of LPS fractions showed single peaks with identical or very similar $s_{20,w}$ values (Figure 13).

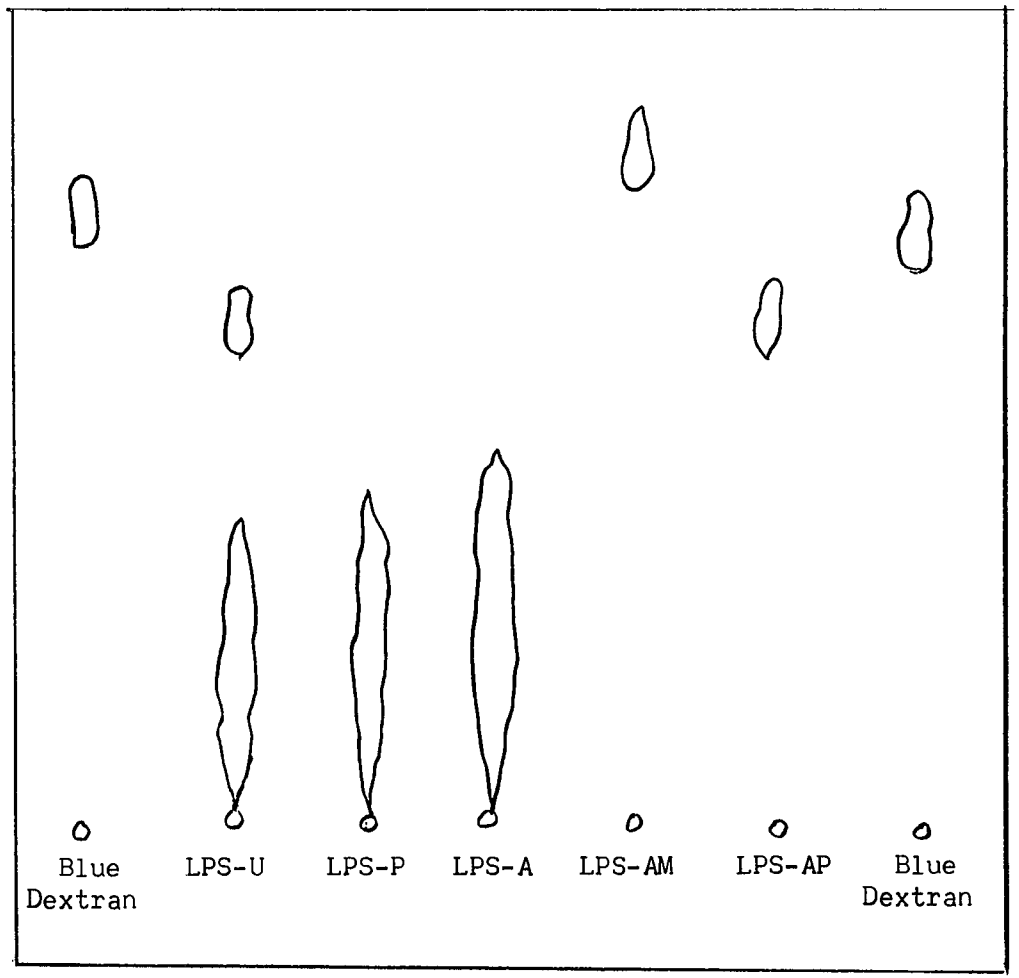


Figure 12. Sephadex G-200 thin-layer chromatography of lipopolysaccharide fractions.

Chromatogram was developed with an eluant solvent consisting of 0.15 M NaCl in 0.05 M phosphate buffer (pH 7) and stained with o-toluidine blue.

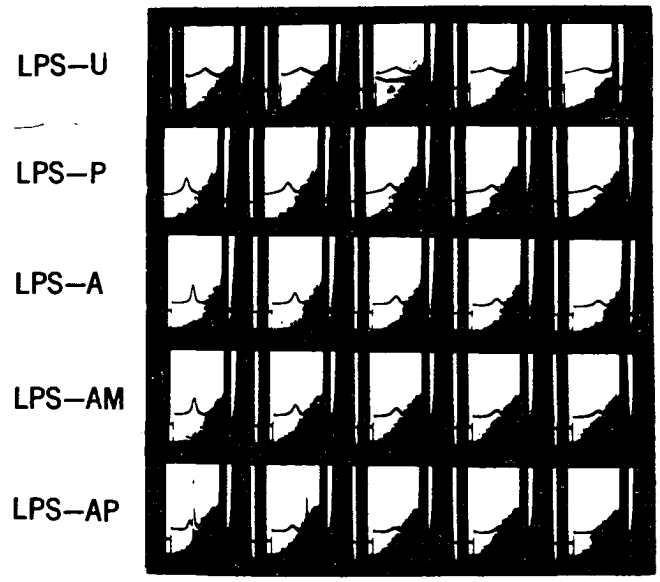


Figure 13. Ultracentrifugal schlieren patterns of lipopolysaccharide preparations.

All samples dissolved in 0.15 M NaCl were run in the synthetic boundary cell at 25°. The photographs were taken at 20 minute intervals after reaching a rotor speed of 52,640 rpm.

Except for fraction LPS-U, all preparations gave broad, single precipitin lines in immunodiffusion tests (Figure 14). The LPS-U, LPS-P and LPS-A fractions contained a common antigen. However, the fraction LPS-AM seemed to show a reaction of only partial identity with fraction LPS-A. The ultracentrifugal and immunochemical characteristics of LPS fractions are summarized in Table 6.

Effect of Phenol Extraction on the Chemical Composition and Biological Properties of LPS Fractions

In view of the capacity of aqueous phenol to dissociate and partition the protein-lipopolysaccharide complex, some undesirable side reactions may occur if a crude TCA extracted endotoxic preparation is subjected to further purification by hot phenol water extraction. The interest in such a study of the effects of phenol on the constituents of the lipopolysaccharide complex was prompted by the fact that this method was applied for the isolation of lipopolysaccharides without any critical appraisal of the possible consequences on the chemical composition, structure, or biological properties of the final products.

When fraction LPS-U was submitted to repeated phenol extractions, the resulting lipopolysaccharide fractions (LPS-A and LPS-AM) showed a marked decrease of amino acid and fatty acid content but an increase of sugar content (Table 4). The significant chemical changes produced by phenol treatment were reflected also in the LD_{50}-values of LPS fractions. In comparison with the parent endotoxic fraction (LPS-U), fractions LPS-A and LPS-AM exhibited diminishing toxic effects. Even at high doses (2000 µg/mouse) of LPS-AM preparation, there were no recorded deaths in successive experiments (Table 7).

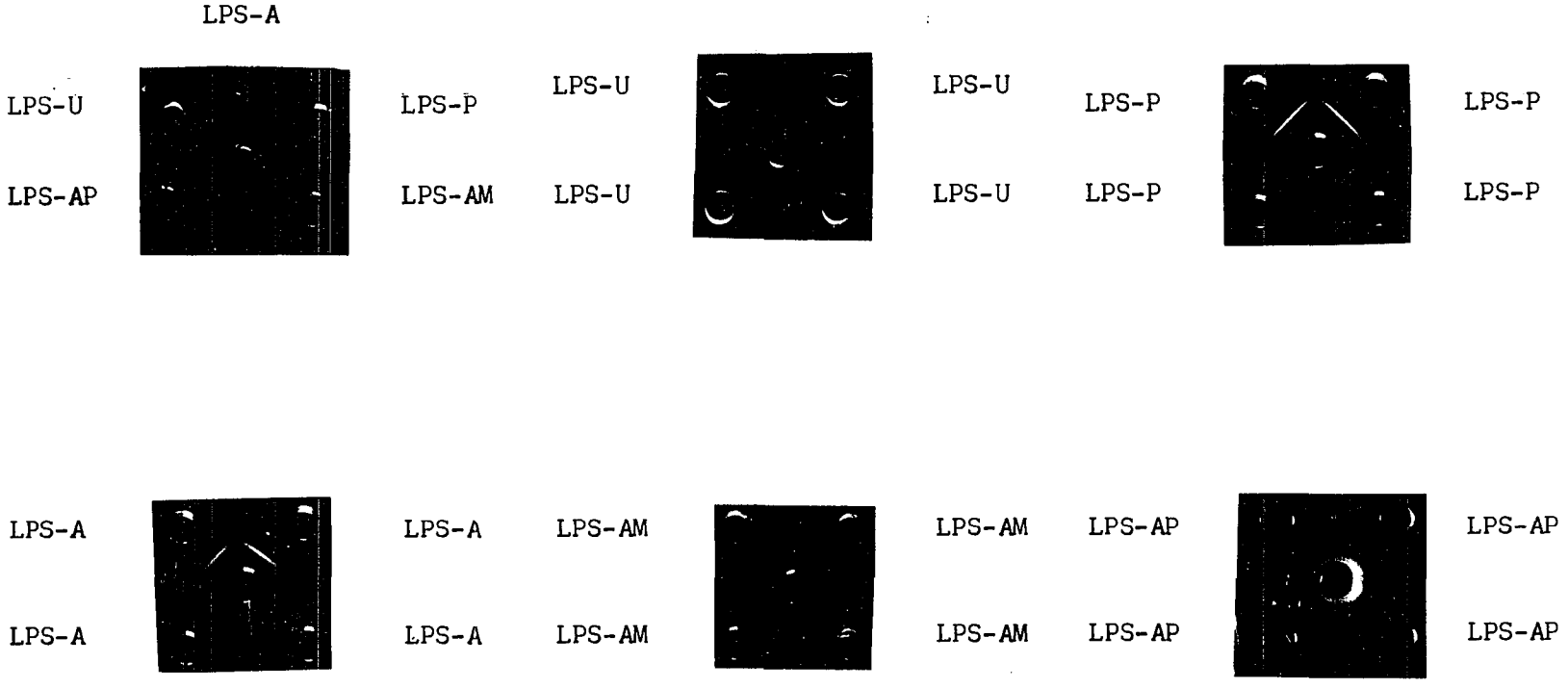


Figure 14. Immunodiffusion patterns of lipopolysaccharide fractions.

TABLE 6

ULTRACENTRIFUGAL AND IMMUNOCHEMICAL PROPERTIES OF LIPOPOLYSACCHARIDE FRACTIONS

Lipopolysaccharide Fractions	Solubility in water	$s_{20,w}$ (S)	Number of precipitin lines
			Anti-whole cells
LPS-U	Slightly soluble	1.5	Two lines
LPS-P	Slightly soluble	1.4	Single broad line
LPS-A	Slightly soluble	1.4	Single broad line
LPS-AM	Soluble	1.3	Single broad line
LPS-AP	Soluble	1.2; 10.5	No reaction

TABLE 7

TOXICITY OF LIPOPOLYSACCHARIDE FRACTIONS

Lipopolysaccharide Fractions	Lethality for mice (LD_{50}) μg/mouse
LPS-U	800-1000
LPS-P	1400
LPS-A	1400
LPS-AM	> 2000
LPS-AP	> 2000

Phenol treatment resulted not only in dissociation of loosely linked macromolecular entities (exemplified by the presence of glycolipoprotein in the phenol phase) but also in cleavage of covalently bound components of a singular macromolecular compound. This was clearly demonstrated by the decreased intensity of ester carbonyl absorption in the infrared spectrum of fraction LPS-A and an almost complete disappearance of that of fraction LPS-AM (Figures 9 and 10). Thus, the primary effect of multiple phenol treatment is characterized by the cleavage of ester bound fatty acids. However, the reduced contents of phosphorus, glucosamine and amino acids in fraction LPS-AM (Table 4) and the successful isolation of a glycolipoprotein from phenol phase I as reported by Wober and Alaupovic (154) prompted an investigation on the chemical composition of the constituents of remaining phenol phases.

Ethanol precipitation resulted in the isolation of a dark-purple amorphous material from the first phenol phase, but failed to yield any precipitable material from phenol phases II and III. However, slightly yellow substances were obtained by steam distillation and subsequent lyophilization of these two phenol phases. The residues were extracted with chloroform-methanol (2:1, v/v) and dried in vacuo. Fractions P-I, P-II, and P-III obtained in decreasing yields (7.1%, 2.5% and 1.7%, respectively) were insoluble in water, dilute acids and several organic solvents. They contained a peptide, fatty acids, glucosamine and phosphorus (Table 8). Fraction P-I had the highest content of amino acids (76%) and only traces of fatty acids, while fractions P-II and P-III had a substantial amount of both glucosamine and fatty acids. The amino acid composition is presented in Table 9. It shows a relatively high content

TABLE 8

PARTIAL CHEMICAL CHARACTERIZATION OF FRACTIONS P-I, P-II AND P-III

Fractions	Phosphorus %	Glucosamine %	Fatty acid %	Amino acid %
P-I	1.30	2.5	1.00	76.3
P-II	1.83	10.4	4.17	7.4
P-III	1.28	4.1	2.72	2.3

TABLE 9

AMINO ACID COMPOSITION OF FRACTIONS P-I, P-II AND P-III

Amino Acids (μMoles/g)	P-I	P-II	P-III
Lysine	244	26	11
Histidine	74	5	1
Arginine	181	20	5
Ammonia	+	+	+
Aspartic Acid	8260	89	24
Threonine	423	32	10
Serine	450	50	25
Glutamic Acid	5360	70	24
Proline	138	22	7
Glycine	660	83	37
Alanine	657	70	19
Half-cystine	Trace	Trace	Trace
Valine	380	32	9
Methionine	44	3	-
Isoleucine	256	12	5
Leucine	479	41	9
Tyrosine	240	20	3
Phenylalanine	229	17	3

of acidic amino acids and a very low content of cysteine in all three fractions. The major, identified fatty acids ("bound" fatty acids) are shown in Table 10. In order to separate the "free" fatty acids, chloroform-methanol extracts of phenol residues were treated with boiling acetone. The qualitative composition of "free" fatty acids detected in the acetone-soluble fraction is presented in Table 10. It differed very little, if any, from that of "bound" fatty acids. The thin-layer chromatographic analysis of the acetone-insoluble fraction indicated the presence of phosphorus-containing lipids and pigments.

The primary effect of multiple phenol treatment of the bacterial endotoxic preparations is the cleavage of ester bound fatty acids. However, the reduced contents of phosphorus, glucosamine and amino acids of fraction LPS-AM (Table 3) may indicate a more drastic degradation of lipopolysaccharide involving, for example, a partial removal of the polyglucosamine unit of Lipid A and specific peptides. That such degradation may have occurred, has been suggested by the results of Sepharose 4B gel-permeation column chromatography of LPS-AM (Figure 15). It resulted in the separation of two macromolecular components or fragments of similar size. The preliminary results have indicated some interesting differences in the chemical composition and structure of these compounds.

The Effect of Alkali on Chemical Composition and Biological Properties

Since the primary effect of multiple phenol treatment was the cleavage of ester bound fatty acids, it was of some interest to find out whether a mild alkaline hydrolysis of an endotoxic complex would result in similar or different degradation changes. The fraction LPS-A was

TABLE 10

COMPOSITION OF THE "FREE" AND "BOUND" FATTY ACIDS
OF PHENOL PHASES

Fractions	"Free" Fatty Acids	"Bound" Fatty Acids
1st phenol phase	N.D.	lauric, myristic, palmitic and β-hydroxy myristic
2nd phenol phase	capric, lauric, myristic, palmitic, and β-hydroxy myristic	capric, lauric, palmitic and β-hydroxy myristic
3rd phenol phase	capric, lauric, myristic, palmitic and β-hydroxy myristic	lauric, myristic, palmitic and β-hydroxy myristic

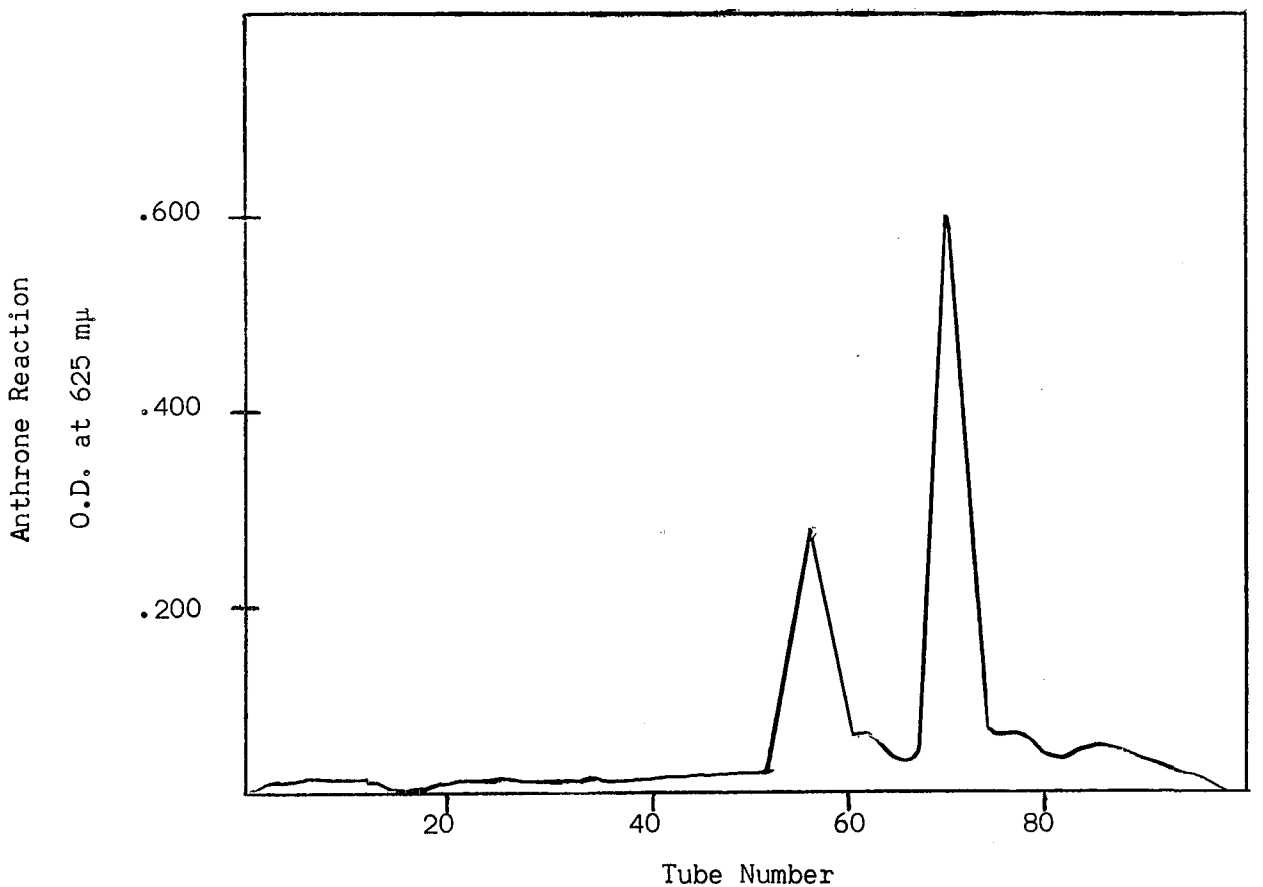


Figure 15. Gel-permeation chromatography of fraction LPS-AM on Sepharose 4B.

Experimental conditions: Column size (3x25 cm), eluted with 0.15 M NaCl in 0.05 M phosphate buffer (pH 7.0). Volume collected 2 ml per tube. Flow rate 16 ml per hr. Column was monitored by anthrone reaction.

treated with 0.25 N sodium hydroxide for 5 hours at 58-60°. The reaction mixture was adjusted to pH 6.5 and extracted exhaustively with chloroform. The aqueous phase was dialyzed against distilled water and the fraction LPS-AP was recovered by lyophilization (Figure 5). The chloroform fraction consisted of fatty acids. The solubility properties (Table 6) and the reduced fatty acid content (Table 4) of LPS-AP were very similar to those of LPS-AM. However, there were also some significant differences. The LPS-AP fraction showed two distinct peaks (1.2S and 10.5S) in the schlieren pattern (Figure 13) and had higher contents of glucosamine, amino acids and phosphorus and a lower content of reducing sugars. Both fractions LPS-AM and LPS-AP exhibited low toxicity (Table 7). However, the latter fraction also lost the ability to react with antiserum to whole cells (Figure 14). The absence of ester carbonyl absorption in the infrared spectrum of LPS-AP (Figure 10) clearly indicated that ester bound fatty acids were cleaved completely by the alkaline hydrolysis. The still measurable amount of fatty acids found in LPS-AP (Table 4) represents most probably the amide bound fatty acids of the lipid moiety, which seems to be less vulnerable to the action of mild alkaline hydrolysis than it is to the multiple treatments with phenol. However, the ultracentrifugally demonstrable heterogeneity of LPS-AP indicates that the effect of mild alkaline hydrolysis is not confined solely to the splitting of ester linkages. It is not known whether this action involves cleavage of an intramolecular covalent linkage or an intermolecular dissociation.

Preparation of the Lipid Moieties

In order to study the structure of the lipid moiety from the

lipopolysaccharides of S. marcescens 08 an endotoxic fraction (LPS-A) containing ester bound fatty acids and an inactive fraction (LPS-AP) devoid of ester bound fatty acids were used as starting materials. Fractions LPS-A and LPS-AP were refluxed with 0.1 N hydrochloric acid for 30 minutes and the reaction mixtures were cooled and extracted repeatedly with chloroform (Figure 6). The combined chloroform extracts were washed with water and concentrated in vacuo to yield fractions A-C (10-25%) and AP-C (3%). The chloroform extractable lipids were treated several times with boiling acetone. The acetone insoluble fractions A-AI (60%) and AP-AI (80%) were removed by filtration and the acetone soluble fractions, A-AS (40%) and AP-AS (20%), were evaporated in vacuo to dryness.

Characterization of Acetone Soluble and Acetone Insoluble Materials

The acetone soluble fractions (A-AS and AP-AS) consisted of a mixture of fatty acids. The characteristic β-hydroxy myristic acid was found in both acetone soluble fractions (Table 11), fraction A-AS had twice as much as fraction AP-AS. It seems, therefore, that part of the β-hydroxy myristic acid was removed from the lipopolysaccharide during the alkaline treatment of LPS-A.

The acetone insoluble fractions (A-AI and AP-AI) were tan-colored, waxy substances with M.P. 175-185° and 178°, respectively. Both fractions had limited solubilities in organic solvents. They were insoluble in benzene, diethyl ether, ethanol and methanol, but soluble in chloroform and pyridine. Spot tests indicated that they stained with molybdenum blue reagent and alkaline silver nitrate, but not with ninhydrin. The infrared spectra of these two fractions were quite similar (Figure 16).

TABLE 11

GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF FATTY ACIDS FROM THE ACETONE SOLUBLE FRACTIONS

Peak	Fatty Acid Esters	Relative Retention Time	Yields (%)	
			A-AS	AP-AS
1	Lauric acid	0.32	7.5	27.2
2	Myristic acid	0.55	24.8	26.0
3	U_1[a]	0.89	5.8	15.8
4	Palmitic acid	1.00	-	2.8
5	U_2[a]	1.11	-	-
6	U_3[a]	1.46	-	-
7	U_4[a]	2.73	3.6	-
8	β-hydroxy myristic acid	3.73	58.2	28.0

[a]U = unidentified peaks.

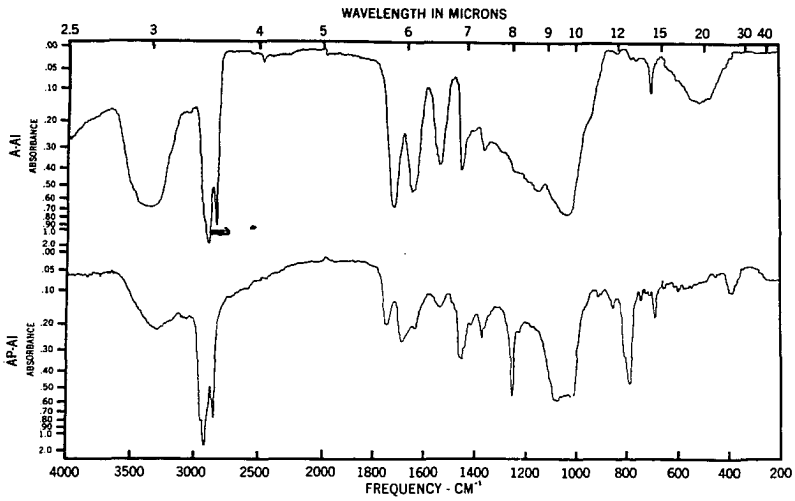


Figure 16. Infrared spectra of fractions A-AI and AP-AI.

They showed the presence of a hydroxy and/or amino group (3100-3600 cm^{-1}), an ester carbonyl group (1720-1740 cm^{-1}), an amide group (1655 cm^{-1}), a phosphate ester (1300-1250 cm^{-1}), the P-O-C group (1060-1090 cm^{-1}), a band characteristic of carbohydrates (1100-1000 cm^{-1}) and the methylene and methyl groups (2850-2920 cm^{-1} and 1465-1380 cm^{-1}). Adsorbosil-2 thin layer chromatography revealed a marked heterogeneity of both acetone insoluble fractions. The A-AI fraction showed a large, streaking spot at the origin and three well separated fast moving components.

The bound fatty acids were released from fractions A-AI and AP-AI by strong acid hydrolysis. Figure 17 shows a typical gas-liquid chromatogram of the methyl esters of fatty acids from A-AI. The fatty acid compositions of A-AI and AP-AI are shown in Table 12. In both lipid fractions, β-hydroxy myristic acid accounted for almost half of the total fatty acid content. The other identified fatty acids in the acetone insoluble fractions, lauric, myristic and palmitic acids were present in different amounts. The acetylation and hydrogenation experiments indicated that no other hydroxy fatty acid was detectable and that unsaturated acids were completely absent.

To study the water soluble component(s) fraction A-AI was hydrolyzed with 2 N HCl for 6 hours under reflux. After neutralization, the water soluble material was submitted to paper chromatographic analysis (Figure 18). With solvent system 1 at least five components were detected. A component which was ninhydrin positive and alkaline silver nitrate positive had the same R_f as D-glucosamine (Figure 18, A and B). Another component which remained at the origin gave positive reactions with ninhydrin, silver nitrate and Hanes-Isherwood phosphorus stain (Figure 18,

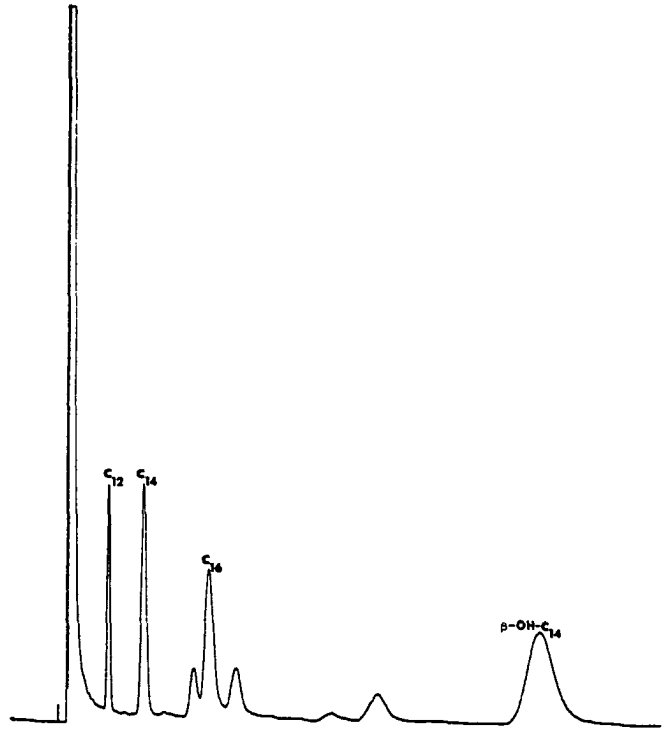


Figure 17. Gas-liquid chromatogram of fatty acids of fraction A-AI.

TABLE 12

GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF FATTY ACIDS
FROM A-AI AND AP-AI

Peak	Relative Retention Time[a]	Relative Retention Time[a] after Acetylation	Relative Retention Time[a] after Hydrogenation	Identification (Methyl Esters)	Yield % A-AI	Yield % AP-AI
1	0.32	No change	No change	C_{12} Lauric acid	6.5	4.4
2	0.55	No change	No change	C_{14} Myristic acid	14.0	9.7
3	0.89	No change	No change	Saturated, Unidentified	3.5	9.7
4	1.00	No change	No change	C_{16} Palmitic acid	20.5	Trace
5	1.11	No change	No change	Saturated, Unidentified	4.5	Trace
6	1.49	No change	No change	Saturated, Unidentified	Trace	9.7
7	2.73	No change	No change	Saturated, Unidentified	8.5	10.2
8	3.37	3.04	No change	Hydroxy acid β-hydroxy myristic acid	42.0	50.5

[a]Using methyl palmitate as reference.

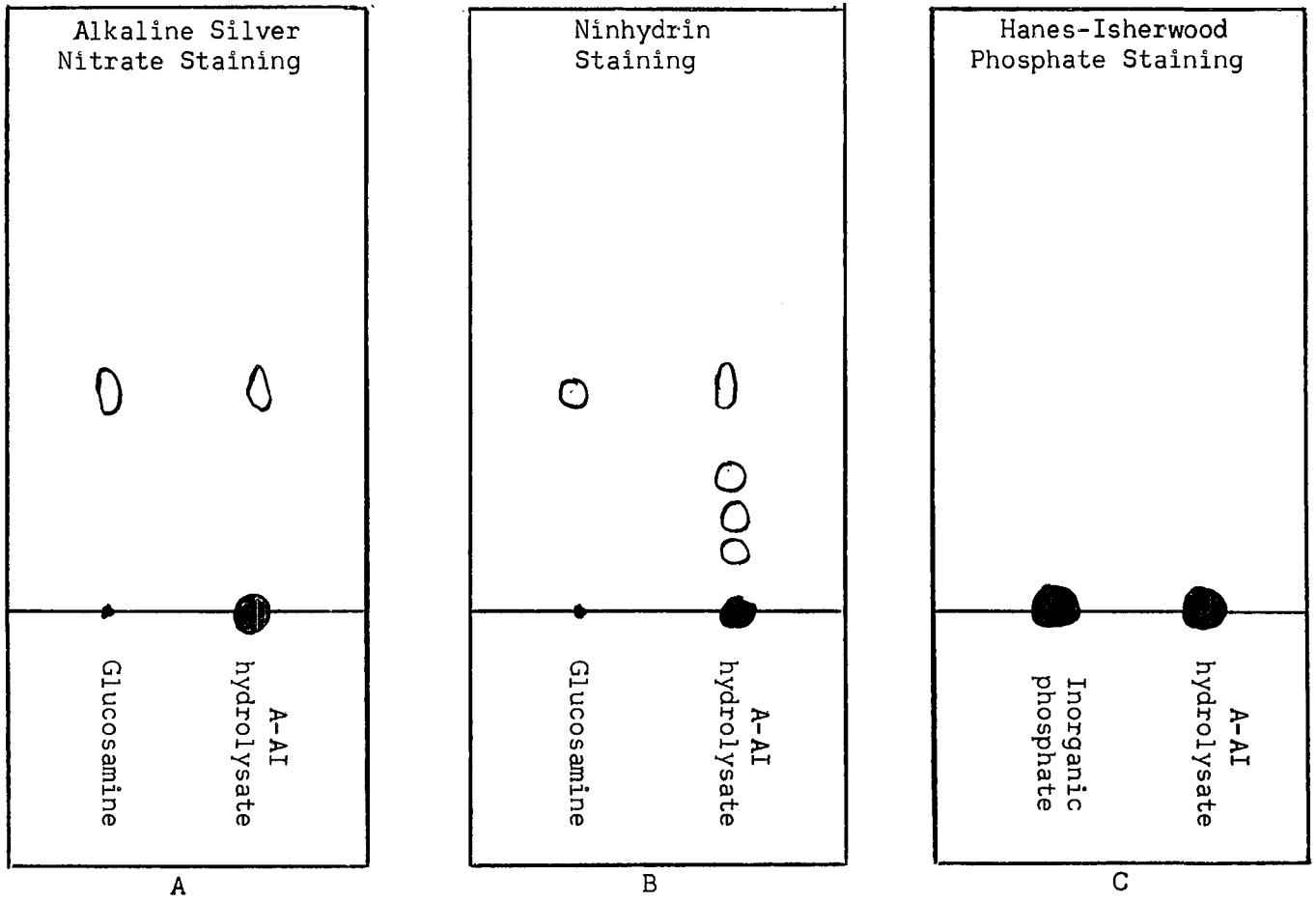


Figure 18. Paper chromatography of the acid hydrolysate from fraction A-AI. Chromatograms were developed with solvent system 1.

A, B and C). Three additional ninhydrin-positive spots (Figure 18, B) did not react with silver nitrate. Therefore, they were considered to be most probably amino acids. The absence of glycerol indicated that the phosphorus-containing lipid did not belong to the class of glycero-phosphatides.

In order to separate the hexosamine phosphates and glucosamine from other components such as amino acids, the neutralized hydrolysate of fraction A-AI was submitted to the ion-exchange (Dowex 50-H^+) column chromatography and a "neutral" and an "acidic" fraction were obtained by elution with water and 0.33 N HCl, respectively. Paper chromatography of "neutral" fraction with solvent system 3 indicated presence of three phosphorus-containing components (Figure 19, C). One of these spots was identified as inorganic phosphate; the remaining two spots gave positive reactions with ninhydrin and silver nitrate. Following additional hydrolysis of "neutral" fraction with 6 N HCl for ten hours, the neutralized hydrolysate contained only glucosamine and inorganic phosphate identified by paper chromatography with solvent systems 1 (Figure 19, A) and 3 (Figure 19, C). The "acidic" fraction contained glucosamine (Figure 19, B). It has been concluded, without further proof, that the phosphorus-containing compounds are most probably glucosamine phosphates. Thus, it has been demonstrated that the fraction A-AI contains fatty acids, glucosamine, glucosamine phosphates and amino acids.

Studies on the Alkaline Hydrolysis of Fraction A-AI

Because of the chromatographically demonstrated heterogeneity of fraction A-AI and its limited solubility in organic solvents, it was

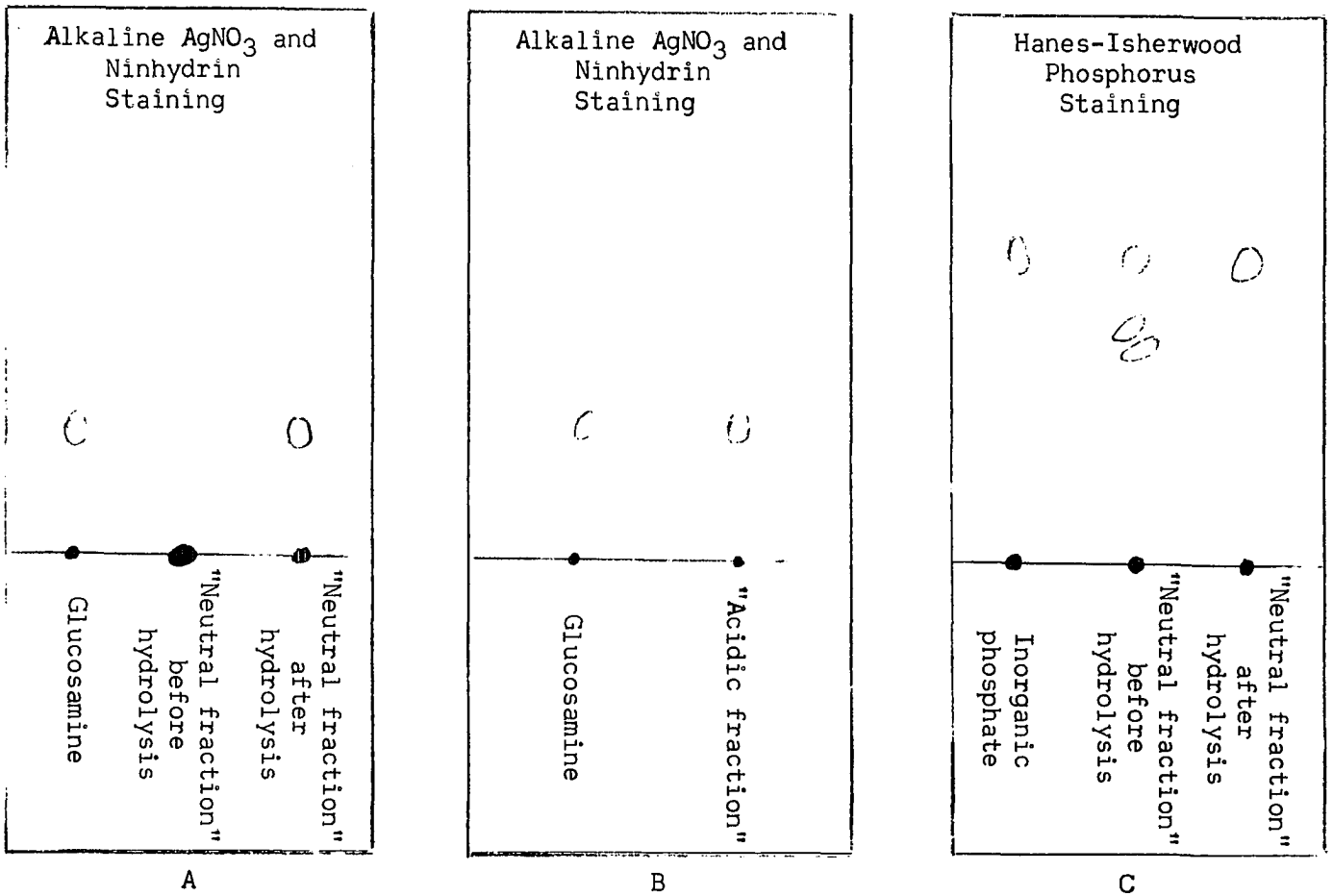


Figure 19. Paper chromatography of "neutral" and "acidic" fractions obtained by ion exchange column chromatography of fraction A-AI hydrolysate.

A and B chromatograms were developed with solvent system 1. C chromatogram was developed with solvent system 3.

thought that treatment with mild alkali might produce the deacylated, water-soluble carbohydrate skeleton of the lipid, which could be separated from fatty acids and other components by standard procedures. When fraction A-AI was hydrolyzed with 1 N NaOH for 6 hours, several chloroform- and water-soluble fractions were obtained (Figure 7). Out of 35% of the starting material recovered in the acetone soluble fractions (AC-1S 4%, and AC-2S 31%) of the chloroform extracts, only 70% was present in the form of fatty acids. The remaining material (30%) was considered to be unsaponifiable material. Only 2% of the original lipid was recovered as unchanged lipid (AC-1P). Fraction AC-2P was obtained as acetone insoluble material from the second chloroform extract in relatively good yield (15%). Fraction AQ-1 (11%) differed from fraction AC-2P in solubility as well as chemical composition (Table 13). While β-hydroxy myristic acid was the sole fatty acid found in fraction AC-2P, several fatty acids (lauric, myristic, palmitic and β-hydroxy myristic acids) were found in AQ-1.

The other water soluble fraction (AQ-2) was further fractionated on a G-25 Sephadex column (Figure 20). The results indicated that it represented a mixture of several completely deacylated water soluble fractions with different molecular sizes. Only one fraction (AQ-2-2) was obtained in salt-free form. It contained 11.6% glucosamine and 1.98% phosphorus.

All three major fractions, namely, fractions AC-2P, AQ-1 and AQ-2-2 contained amino acids. Table 14 shows the results of the quantitative amino acid analyses. As in the case of the lipopolysaccharides, the products of alkaline hydrolysis contained the whole spectrum of the

TABLE 13

CHEMICAL COMPOSITION OF THE PRODUCTS OF ALKALINE HYDROLYSIS OF FRACTION A-AI

Fractions	Phosphorus	Glucosamine	Fatty acids	Amino acids
	%	%	%	%
AC-2P	1.74	11.0	14.5	2.0
AQ-1	1.21	5.3	22.8	1.4
AQ-2-2	1.98	11.6	0	2.1

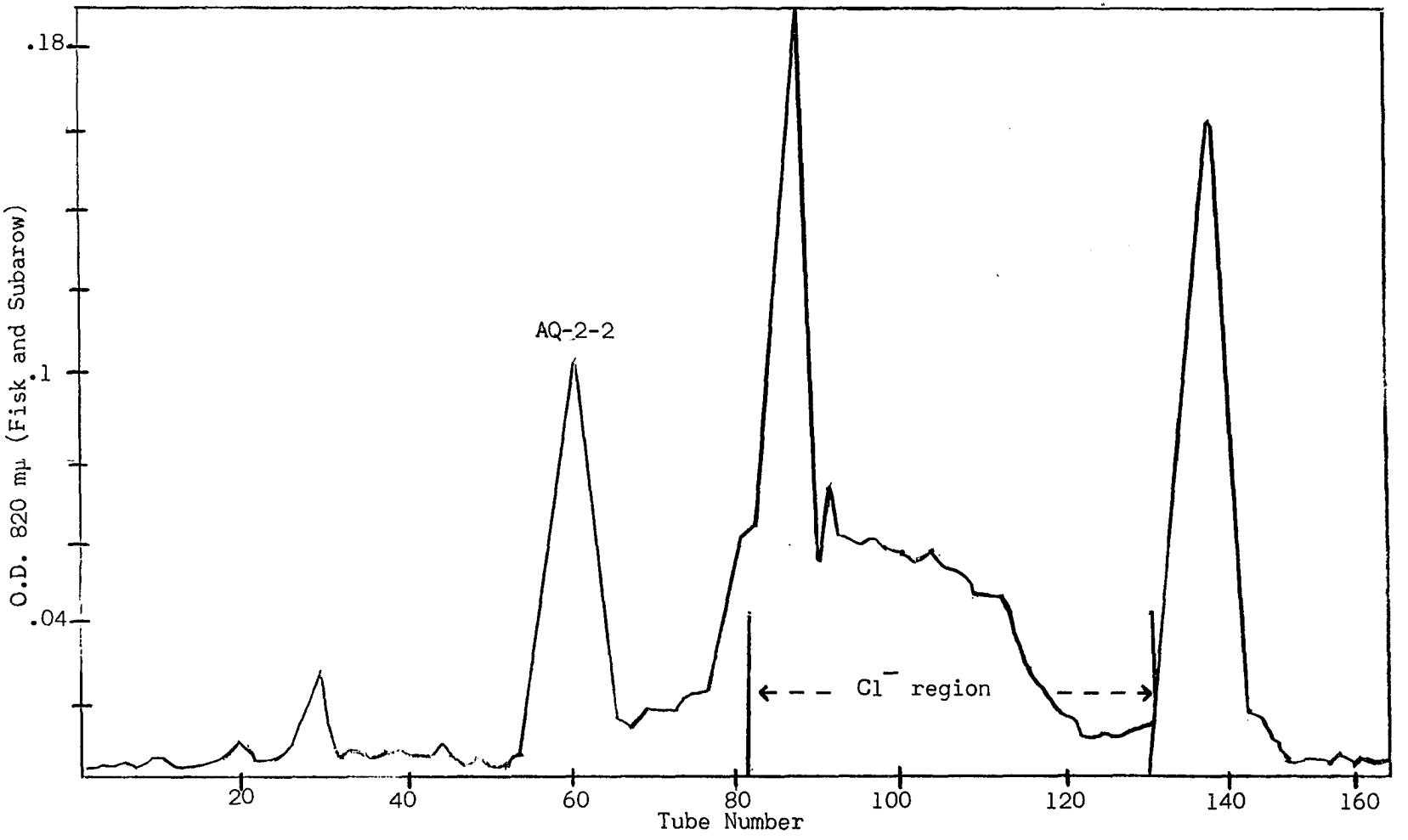


Figure 20. Gel-filtration chromatography of fraction AQ-2 on Sephadex G-25.

Experimental conditions: Column size (2.5x80 cm), eluted with deionized water. Volume collected 2 ml per tube. Flow rate 16 ml per hr. Column was monitored with phosphorus analysis.

TABLE 14

AMINO ACID COMPOSITION OF FRACTIONS AC-2P, AQ-1 AND AQ-2-2

Amino Acids (μMoles/g)	AC-2P	AQ-1	AQ-2-2
Lysine	8	15	10
Histidine	2	2.5	5
Arginine	Trace	Trace	Trace
Aspartic Acid	13	3.5	25
Threonine	1	2.5	4
Serine	9	15	8
Glutamic Acid	12	15	17
Proline	-	Trace	Trace
Glycine	44	18	20
Alanine	17	11	20
Half-cystine	2	Trace	-
Valine	4	3	7
Methionine	1	Trace	-
Isoleucine	4	2	4
Leucine	5	4	10
Tyrosine	1	Trace	3
Phenylalanine	Trace	Trace	4

common amino acids. The predominant ones were lysine, aspartic acid, serine, glutamic acid, glycine and alanine. Glycine and alanine were the major amino acids in fraction AC-2P and fraction AQ-2-2, in addition the aspartic and glutamic acids the major amino acids in fraction AQ-2-2. The variation of the amino acid patterns of these fractions indicate that there may be several different peptides present in crude lipid A preparations such as fraction A-AI.

Surprisingly, none of these fractions were susceptible to alkaline monophosphatase treatment nor was glucosaminol produced upon sodium borohydride reduction and subsequent acid hydrolysis. Considering the degradative action of alkali on sugars in general and amino sugars in particular, several reactions such as epimerization and other isomerization at the reducing end of the sugar, fragmentation, dehydration, rearrangements, and recombination of fragments may occur. In addition, deamination of the terminal reducing glucosamine unit of the polyglucosamine chain may result in the formation of glycitol rather than glucosaminol.

Reduction of Fraction A-AI by Sodium Borohydride

In an attempt to obtain its polyglucosamine skeleton devoid of ester bound fatty acids, fraction A-AI was submitted to a prolonged sodium borohydride reduction. It was hoped also that, if free, the aldehydic group of the terminal glucosamine unit would be reduced to a primary hydroxyl group. The procedure for the isolation of the reduction products is outlined in Figure 8. When 200 mg of A-AI was reduced with sodium borohydride in methanol for 48 hours an orange solution was obtained.

Methanol was evaporated *in vacuo* and the residue was suspended in water. Suspension was centrifuged at 2000 rpm and a sedimenting fraction, R-1, was obtained in a 22% yield. The supernate was adjusted to pH 3.5 and then extracted with chloroform to yield a chloroform soluble fraction (R-C, 30% yield). The aqueous phase was centrifuged at 25,000 rpm and a second sedimenting fraction, R-2, was obtained in a 16% yield. The addition of acetone to the supernate resulted in the precipitation and isolation of a water-soluble fraction R-S (20% yield).

The results of qualitative and quantitative analyses of fatty acids present in the chloroform-soluble fraction R-C are presented in Table 15.

Fractions R-1, R-2 and R-S were yellow amorphous solids differing in solubility properties and quantitative chemical composition (Table 16). Fraction R-S showed the typical fatty acid pattern. In contrast, fractions R-1 and R-2 contained only β-hydroxy myristic acid. The differences in fatty acid content seemed to have little effect on the solubility and physical characteristics of the fractions. In contrast to fractions R-2 and R-S, fraction R-1 was poorly soluble in water despite its relatively low fatty acid content (6%). However, all three fractions were soluble in alkaline solution with pH above 8.5. Analytical centrifugation of these three fractions dissolved in borate buffer solution (pH 8.7) indicated the presence of a single symmetric peak in each case (Figure 21). The high values for sedimentation coefficients indicated most probably the formation of macromolecular glycolipid-borate complex. This suggestion was further strengthened by detection of unusually high ash content of fractions R-1 and R-2 (14.17% and 16.96%, respectively).

TABLE 15

GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF FATTY ACIDS IN FRACTION R-C

Peak	Methyl Esters	%
1	Lauric acid	12.1
2	Myristic acid	26.5
3	U_1	9.8
4	Palmitic acid	13.9
5	U_2	11.0
6	U_3	4.4
7	U_4	Traces
8	β-hydroxy myristic acid	22.2

TABLE 16

CHEMICAL COMPOSITION OF FRACTIONS R-1, R-2 AND R-S

Fraction	N %	P_i %	Organic P %	Fatty Acids %	Glucosamine %	Glucosaminol %
R-1	4.18	0.60	2.30	6.0	12.40	1.40
R-2	3.37	0.80	3.16	20.0	17.40	5.80
R-S	2.85	0.95	1.33	18.2	10.40	0.75

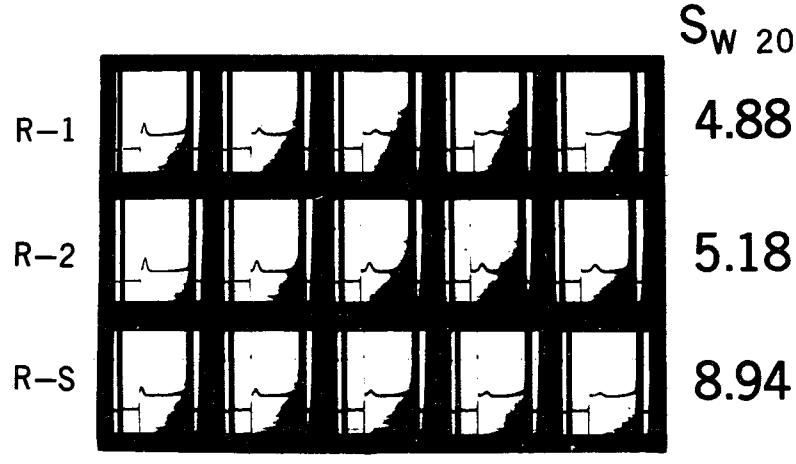


Figure 21. Ultracentrifugal schlieren patterns of fractions R-1, R-2 and R-S.

Fractions dissolved in borate buffer, pH 8.7, were run at 25°. The photographs were taken at 0, 4, 8, 16 and 24 minutes after reaching a rotor speed of 52,640 rpm.

The demonstration of glucosaminol after acid hydrolysis of these fractions indicated strongly that each fraction contained a terminal reducing glucosamine. Moreover, the calculation of the molar ratio of glucosamine to glucosaminol provided new information on the size of the polyglucosamine chains. These ratios as well as those relating total glucosamine to phosphorus and fatty acids are shown in Table 17. These suggest that sodium borohydride reduction of the lipid fraction A-AI results in a mixture rather than a single polyglucosamine chain; it seems that each chain, irrespective of size, contains an almost equimolar quantity of amide bound fatty acids and phosphate. Each fraction contained also a small but significant amount of peptides which differed characteristically in their amino acid composition (Table 18).

In attempting to demonstrate the nature of the phosphate linkage, all three fractions were treated separately with alkaline phosphatase and snake venom phosphodiesterase. While fraction R-1 was not susceptible to alkaline phosphatase, the treatment of fractions R-2 and R-S with the same enzyme resulted in the release of 67% and 81% of their organic phosphate, respectively (Table 19). The phosphodiesterase treatment of these two fractions followed by alkaline phosphatase failed to increase further the amount of released phosphate. Results of these experiments indicated that most of the phosphorus in A-AI was present as monophosphate. It seems, therefore, that the previously suggested occurrence of phosphodiester linkages between the glucosamine units in the polyglucosamine core is not very likely.

TABLE 17

MOLAR RATIOS OF THE MAJOR COMPONENTS OF FRACTIONS R-1, R-2 AND R-S

Fractions	Phosphorus:Glucosamine:Fatty acid					Glucosamine:Glucosaminol		
R-1	1	:	1	:	0.88	9	:	1
R-2	1	:	1	:	0.78	4	:	1
R-S	1	:	1.24	:	1.49	14	:	1

TABLE 18

AMINO ACID COMPOSITION OF FRACTIONS R-1, R-2 AND R-S

Amino Acids (μMoles/g)	R-1	R-2	R-S
Lysine	15	20	.003
Histidine	Trace	10	.001
Arginine	Trace	Trace	.001
Aspartic Acid	28	6	7
Threonine	17	Trace	2
Serine	21	11	4
Glutamic Acid	31	6	6
Glycine	31	9	6
Alanine	31	6	6
Valine	17	2	3
Methionine	-	-	-
Isoleucine	Trace	1.4	2
Leucine	21	3	4
Tyrosine	Trace	?	?
Phenylalanine	Trace	1	?

TABLE 19

ENZYMATIC REMOVAL OF PHOSPHATE FROM REDUCED PRODUCTS FROM FRACTION A-AI

Fraction	Inorganic phosphate (μg/mg) after		
	No treatment	Alkaline phosphatase	Phosphodiesterase followed by alkaline phosphatase
R-1	6.0	6.0	6.0
R-2	8	28.7	8
R-S	9.5	20.5	9.5

Fractionation of Fraction A-AI and Chemical Characterization of Its Major Fractions

Studies on the chemical characterization of the products obtained by mild alkaline hydrolysis and by sodium borohydride reduction of fraction A-AI have suggested that the heterogeneity of the lipid preparations, observed previously by Adsorbosil-2 thin-layer chromatography, may be due not only to a random distribution of ester bound fatty acids but also to the presence of polyglucosamine chains of various sizes. In order to establish whether these various polyglucosamines existed in the untreated lipid preparation (fraction A-AI), an attempt was made to separate these individual, acylated polyglucosamines by silicic acid column chromatography. The results of the chromatographic separation of fraction A-AI are shown in Table 20. Since Adsorbosil-2 thin-layer chromatography revealed a persisting heterogeneity of each fraction (Figure 22) the major fraction 2 was rechromatographed on silicic acid column by only slight increases in the polarity of eluting solvents (Table 21). The infrared spectra of all major fractions were similar to that of fraction A-AI except for a decrease in the intensity of ester carbonyl absorption and an increase in that of amide absorption with increasing polarity of fractions. Even fraction 2-2 showed still three components on Adsorbosil-2 thin-layer chromatography (Figure 23). The chemical composition (Table 22) shows that each major fraction contained glucosamine, fatty acids, amino acids and phosphorus. Although there were small amounts of anthrone-positive carbohydrates, no reducing sugars other than glucosamine were detected. Whereas glucosamine and phosphorus contents remained fairly constant, the fatty acid content decreased with increasing polarity of fractions. The β-hydroxy myristic acid accounted for more than two-

TABLE 20

SILICIC ACID COLUMN CHROMATOGRAPHY OF FRACTION A-AI

Fraction	Eluent Chloroform-Methanol	Yield %
1	100% chloroform	2
2	9:1	52
3	8:2	14
4	7:3	4
5	6:4	2
6	5:5	1

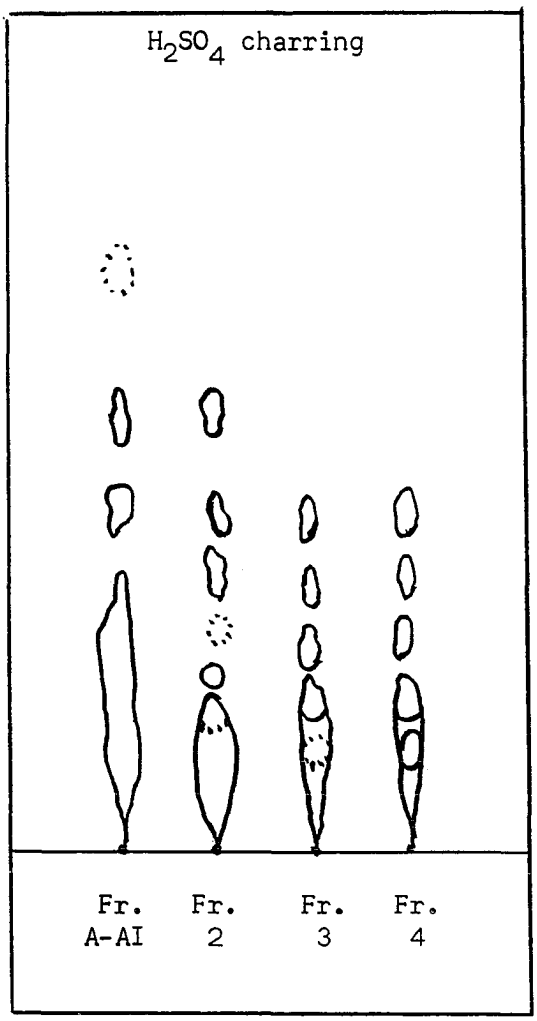


Figure 22. Adsorbosil-2 thin-layer chromatography of fractions A-AI, 2, 3 and 4.

Chromatogram was developed with a solvent system $CHCl_3$:MeOH:1 N NH_4OH 75:25:4.

TABLE 21

SILICIC ACID COLUMN CHROMATOGRAPHY OF FRACTION 2

Fraction	Eluent Chloroform-Methanol	Yield %
2-1	99:1	0.9
2-2	97:3	22.7
2-3	95:5	9.6
2-4	93:7	34.8
2-5	90:10	27.8

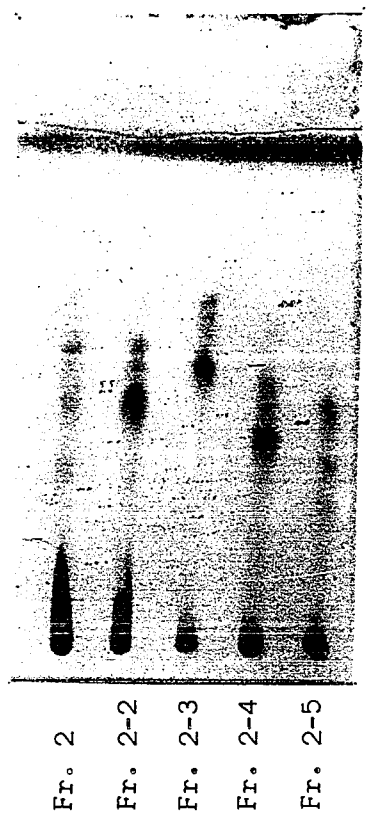


Figure 23. Adsorbosil-2 thin-layer chromatography of subfractions from fraction 2.

Chromatogram was developed with solvent system $CHCl_3$:MeOH:1 N NH_4OH 75:25:4 and charred with dilute sulfuric acid (1:1).

TABLE 22

CHEMICAL COMPOSITION OF FRACTIONS OBTAINED BY SILICIC ACID COLUMN CHROMATOGRAPHY OF FRACTION A-AI

Fractions	Amino Acids %	Fatty Acids %	Glucosamine %	Anthrone-positive carbohydrate %	N %	P %
2-2	16.0	40.0	16.7	1.6	3.1	1.6
2-3	0.5	N.D.	20.0	2.0	2.4	1.8
2-4	Trace	51.0	23.4	5.0	2.8	1.8
2-5	2.0	29.0	17.7	7.0	2.9	1.7
3	1.4	34.0	15.6	3.5	2.1	1.8
4	0.9	27.0	19.0	6.1	3.0	1.7

thirds of the total fatty acid content of less polar fractions (2-2 through 2-5); in the most polar fraction 5 it was present in only trace amount (Table 23).

It is somewhat surprising that the least polar fraction 2-2 contained a relatively large percentage and the most polar fraction 4 a relatively low percentage of amino acids. Except for fractions 2-3 and 2-4, there was very little difference in the qualitative amino acid composition of major fractions (Table 24). It is still not known whether the corresponding peptide(s) occur(s) as a separate entity(ies) in the mixture with A-AI or whether it is linked in some manner to the polyglucosamine chain. The extremely high molar ratio of glucosamine to individual amino acids in each subfraction and the virtual absence of amino acids in fractions 2-3 and 2-4 would seem to support the former alternative. However, a separation of peptides from polyglucosamine units has yet to be achieved.

Results obtained by calculating the molar ratios of glucosamine to glucosaminol and phosphorus (Table 25) have demonstrated the feasibility of separating the mixture of polyglucosamine units by silicic acid column chromatography into individual entities without prior cleavage of ester or amide bound fatty acids. Moreover, they have shown clearly that the acetone insoluble preparation (fraction A-AI) consists of a mixture of acylated polyglucosamine phosphate chains of various sizes. Fractions 2-2 through 2-4 represent, on the basis of number of glucosamine units, homogeneous lipid preparations; whether fractions 2-5 and 3 represent also homogeneous entities or overlapping mixtures of polyglucosamines of various sizes remains to be established. The amino acid-free fraction

TABLE 23

GAS CHROMATOGRAPHY OF THE METHYL ESTERS OF FATTY ACIDS FROM THE LIPID FRACTIONS OBTAINED BY THE SILICIC ACID COLUMN CHROMATOGRAPHY OF FRACTION A-AI

Peak	Methyl Esters	Fractions							
		2-2 %	2-3 %	2-4 %	2-5 %	2 %	3 %	4 %	5 %
1	Lauric acid	2.2	2.8	3.2	2.5	1.3	12.2	15.3	10.6
2	Myristic acid	16.7	18.4	12.4	Trace	-	2.6	3.8	Trace
3	U_1	6.3	24.2	6.9	3.2	3.0	26.1	19.1	53.1
4	Palmitic acid	11.8	8.7	6.9	6.6	0.9	4.0	7.2	36.7
5	U_2	-	-	-	Trace	2.6	8.2	15.6	Trace
6	U_4	-	-	-	-	4.1	Trace	Trace	Trace
7	β-hydroxy myristic acid	63.0	44.0	68.2	76.0	87.6	44.7	37.2	Trace

TABLE 24

AMINO ACID COMPOSITION OF LIPID FRACTIONS OBTAINED BY SILICIC ACID COLUMN CHROMATOGRAPHY OF FRACTION A-AI

Amino Acids (μMoles/g)	Fractions						
	2-2	2-3	2-4	2-5	3	4	5
Lysine	66	2	1	10	20	7	10
Histidine	23	1	1	2	2	2	2
Arginine	30	-	-	3	1	2	2
Aspartic Acid	89	2	Trace	10	12	8	9
Threonine	60	Trace	Trace	2	3	1	2
Serine	61	4	Trace	5	17	8	13
Glutamic Acid	133	1	Trace	11	4	7	9
Proline	47	-	-	5	Trace	Trace	Trace
Glycine	59	3	Trace	9	16	8	11
Alanine	92	1	-	10	4	7	13
Half-cystine	-	-	-	-	-	-	-
Valine	59	-	-	5	5	3	4
Methionine	5	-	-	Trace	1	1	2
Isoleucine	17	Trace	-	3	2	1	2
Leucine	84	Trace	-	6	6	4	4
Tyrosine	28	Trace	-	2	2	Trace	Trace
Phenylalanine	38	Trace	-	2	Trace	1	2

TABLE 25

MOLAR RATIOS OF GLUCOSAMINE, GLUCOSAMINOL AND PHOSPHORUS OF MAJOR FRACTIONS OBTAINED BY SILICIC ACID COLUMN CHROMATOGRAPHY OF FRACTION A-AI

Fractions	Elution Mixture $CHCl_3$:MeOH	Ratios	
		Glucosamine / Glucosaminol	Total Glucosamine / Phosphorus
2-2	97:3	1:1	1.7:1
2-3	95:5	1:1	1.8:1
2-4	93:7	1:1	2.2:1
2-5	90:10	2:1	1.2:1
3	80:20	5:1	2.2:1
4	70:30	6:1	1.5:1
5	60:40	N.D.	2 :1

2-4 contained equimolars of glucosamine and glucosaminol and one mole of phosphorus per two moles of glucosamine. Therefore, it has been assumed tentatively that fraction 2-4 consists of a polyacylated diglucosamine with a single phosphate group. In order to establish the nature of the linkage between glucosamine molecules, fraction 2-4 was submitted to various hydrolytic procedures as indicated in Table 26. Following each hydrolytic procedure the reaction mixture was reduced with sodium borohydride, hydrolyzed with strong acid and the content of glucosamine and glucosaminol was determined by amino acid analyzer. The mild acid hydrolysis would cleave the phosphodiester, but not the glycosidic linkage; hence, the hydrolysis of the phosphodiester linked disaccharide should have resulted in formation of two moles of glucosamine or, following the reduction of reaction mixture and hydrolysis, two moles of glucosaminol. However, the demonstration of equimolar amounts of glucosamine and glucosaminol suggested strongly the presence of glycosidic bond. The phosphodiester linkages can be cleaved also by mild alkaline hydrolysis with either 14% NH_4OH or 1 N NaOH at 100°. The alkaline hydrolysis of one mole of a phosphodiester-linked diglucosamine should result, in absence of any side reactions, in the formation of two moles of glucosamine or, following reduction with borohydride, in two moles of glucosaminol. However, the treatment of fractions 2-2, 2-4 and 2-5 with 14% NH_4OH (Table 26) followed by reduction with sodium borohydride and acid hydrolysis with 2 N HCl yielded 35-45% of the theoretical amount of glucosamine; not even traces of glucosaminol could be detected in the reaction mixture. A similar treatment of the same fractions with 1 N NaOH (Table 26) yielded only 10-20% of the theoretical amount of glucosamine

TABLE 26

AMOUNT OF GLUCOSAMINE AND GLUCOSAMINOL IN REDUCED LIPID FRACTIONS BEFORE AND AFTER ACID OR BASE HYDROLYSIS

	Fraction 2-2 µMoles/mg		Fraction 2-4 µMoles/mg		Fraction 2-5 µMoles/mg	
	Glucosamine	Glucosaminol	Glucosamine	Glucosaminol	Glucosamine	Glucosaminol
No hydrolysis	0.175	0.186	0.199	0.183	0.210	0.135
0.1 N HCl, 1 hr.	0.180	0.182	0.192	0.185	0.220	0.125
14% NH_4OH, 10 hrs.	0.120	-	0.133	-	0.191	-
1 N NaOH, 3 hrs.	0.090	-	0.038	-	0.083	-

and no glucosaminol. Although these results favor strongly the presence of a glycosidic rather than phosphodiester linkage, the relatively low yields of glucosamine pose some interesting questions regarding the stability of the glycosidically linked hexosamines to alkali. Generally, glycosidic bonds are stable to alkaline treatment. However, a stepwise degradation of oligosaccharides (158) may take place starting from the reducing end of the molecule at a rate depending on the position at which the reducing terminal monosaccharide is substituted. The glycosidic linkage (1 → 3) is very susceptible to alkali and can be easily cleaved. The rate of cleavage decreases with linkages (1 → 4) and (1 → 6); the linkages (1 → 2) are stable. This positional specificity can be explained by assuming that not hydrolysis, but a β-elimination reaction is responsible for the cleavage of glycosidic bonds. In cases where the reducing sugar is a hexosamine, the action of hot alkali results also in the elimination of ammonia. On the basis of these generalizations, it seems possible to reconstruct the reaction sequence for a hypothetical case involving alkali treatment of a diglucosamine linked through glycosidic linkages (1 → 3) or (1 → 4). In these cases, the liberated glucosamine would be deaminated simultaneously by the action of alkali and no reducible glucosamine would be available in the reaction mixture. The still unreacted portion, if any, of glycosidically bound diglucosamine would be then cleaved by acid hydrolysis and only glucosamine would be detectable by amino acid analyzer. The recovery of glucosamine, under these experimental conditions would depend solely on the reaction rates of β-elimination. Thus, the finding of only diminishing amounts of glucosamine, but no glucosaminol following alkali treatment of fractions

2-2, 2-4 and 2-5 suggest very strongly the occurrence of a glycosidic linkage between glucosamine units of various polyglucosamine chains. Whether these linkages are (1 → 3) or (1 → 4) has not been determined, as yet.

In order to establish the presence of a monophosphate ester linkage by enzymatic cleavage with alkaline monophosphatase, it was necessary to obtain first a water soluble diglucosamine substrate. To this end, fraction 2-4 was submitted to hydroxylaminolysis and a water soluble product containing 5% fatty acids (83% β-hydroxy myristic acid and 17% an unidentified fatty acid) and displaying, similarly to starting material, a glucosamine/phosphorus molar ratio of 2.2:1 was obtained in high yield (85%). The cleaved fatty acids were isolated as hydroxamate derivatives; β-hydroxy myristic acid was the predominant fatty acid. Treatment of this partially deacylated, water-soluble diglucosamine with alkaline phosphatase resulted in an almost quantitative release of the organic phosphorus (Table 27). The dephosphorylated product purified by ion-exchange column chromatography on Dowex 1-CO_3^{-2} showed no absorption bands characteristic of -P-O-C- (1060 cm^{-1}) or -P=O (1250-1300 cm^{-1}) group in its IR-spectrum.

An attempt to achieve a complete deacylation of a partially deacylated and completely dephosphorylated diglucosamine unit by prolonged hydroxylaminolysis has not been successful as evidenced by the presence of an amide absorption band at 1655 cm^{-1}).

TABLE 27

ALKALINE MONOPHOSPHATASE DEGRADATION OF THE PRECIPITATE FROM HYDROXYLAMINOLYSIS OF FRACTION 2-4

	Pi (μg/mg)	Total Phosphorus (μg/mg)	Organic Phosphorus (μg/mg)	Pi released (μg/mg)
Before addition of enzyme	2.1	15.9	13.8	-
After addition of enzyme	13.1	15.9	2.8	11

CHAPTER V

DISCUSSION

The results of this investigation show that the crude lipopolysaccharide preparation (LPS-U) extracted from a chromogenic strain of S. marcescens by trichloroacetic acid consists, like those isolated from several strains of E. coli (159) by aqueous phenol treatment, of a mixture of macromolecular compounds which can be separated by fractional ultracentrifugation into two distinct groups: nucleic acids plus acidic polysaccharides, and peptido-lipopolysaccharides. It has been shown (15) that acidic polysaccharides possess relatively low toxicity, thus representing most probably inert biological materials. This leaves then the group of peptido-lipopolysaccharides as the carrier of typical endotoxic characteristics.

Even after a single hot aqueous phenol treatment and subsequent solvent extraction of LPS-U, fraction (LPS-P) endowed with all typical endotoxic properties could still be obtained. Despite the successful removal of a glycolipopeptide (fraction P-I) and a considerable disruption of weaker linkages by phenol extraction of the peptido-lipopolysaccharide complex, there was no marked decrease in the toxicity of the resulting LPS fraction (LPS-P). Further extraction of this fraction with organic solvents had no effect on the endotoxic properties of the resulting prep-

aration (LPS-A).

Effect of Phenol Treatment on the Chemical and Biological Properties of LPS

Since the introduction of the aqueous phenol as a solvent for the extraction of LPS by Westphal et al. (32) in 1952, it has been tacitly assumed that this treatment causes little, if any, cleavage of covalently bound components of a macromolecular complex. This procedure has been applied routinely for the dissociation of protein from other naturally occurring macromolecules without any critical appraisal of the possible consequences on the chemical composition and structure of the final products. In order to evaluate critically the action of hot phenol on the peptido-lipopolysaccharide complex, the endotoxic fraction LPS-U was submitted to multiple extraction with hot aqueous phenol. It has been suggested previously (15, 133) that phenol treatment of fraction LPS-U may cause considerable disruption and dissociation of certain chemical linkages in addition to its well established function as a deproteinizing agent. Westphal et al. (32) have shown that fractions obtained by isoelectric precipitation of phenol phases from various Gram-negative bacteria consist of a protein practically free of phosphorus and carbohydrate. Wheat et al. (160) have found that the phenol-soluble fraction from Chromobacterium violaceum contains, in addition to protein, small amounts of glucosamine and fucosamine. More recently, Adams et al. (161) reported that it was possible to remove by a single phenol extraction all lipids from a lipopolysaccharide complex from S. marcescens, Temple strain, and to obtain a polysaccharide with firmly bound amino acid residues. This observation led to the suggestion that either the reported

lipopolysaccharide of S. marcescens was readily hydrolyzed or that the lipid and polysaccharide existed as a labile complex rather than as a covalently bound lipopolysaccharide. The results of experiments on the multiple phenol treatment favor strongly the former suggestion. The repeated phenol extractions resulted in the isolation of at least three glycolipopeptides (P-I, P-II and P-III) from the corresponding phenol phases and in the recovery of a water soluble fraction (LPS-AM) characterized by a markedly decreased fatty acid and glucosamine content. In addition, free fatty acids and other phosphorus containing lipids were also detected in the phenol phases. The three glycolipopeptides contained all the basic constituents of "lipid A" such as glucosamine, phosphoric acid and the characteristic fatty acids. If β-hydroxy myristic acid is accepted as a marker for 'lipid A', then it is not unreasonable to suggest that phenol extraction cleaved the bound lipid as well as the protein moiety from the peptido-lipopolysaccharide. It must be pointed out, though, that a small amount of amino acids was detected in the lipopolysaccharide fraction (LPS-AM) even after multiple phenol extraction. It is still not known whether these amino acids represent integral components of the endotoxic lipopolysaccharide or stem from the individual peptides linking peptido-lipopolysaccharide complex to murein as suggested recently by De Petris (162).

The removal of the lipidic groups from the LPS fractions resulted in an increased water solubility of macromolecular compounds and enabled their fractionation and separation by chromatographic methods. Gel-permeation column chromatography on Sepharose 4B indicated that fraction LPS-AM consisted of at least two macromolecular entities. Determination

of the physical-chemical as well as biological properties of these purified water soluble macromolecules would add further knowledge toward a better understanding of the relationship between structure and function of peptido-lipopolysaccharide.

The repeated phenol-water extractions diminished significantly the toxicity of fraction LPS-AM. Most of the available information seems to indicate that toxicity depends mainly on the presence of sensitive linkages of fatty acid esters. Several attempts have been made to correlate the chemical structure and biological properties of endotoxins (163, 164, 1965). It appears from these studies that the toxic properties of intact endotoxin preparations depend upon certain minimal chemical and physical-chemical requirements. Ribi et al. (63) have concluded that the most active preparations consist of a polysaccharide complex and small amount of fatty acids, phosphorus and nitrogeneous material. They have suggested the possible presence of a small, as yet unidentified, acid sensitive component distributed throughout the endotoxin as the potential toxophore group. There is some indication that the active components are linked through ester bonds, since the treatment of lipopolysaccharide preparations by various esterolytic procedures causes a loss of toxicity (165). On the other hand, evidence has been presented indicating a certain critical size of aggregates of haptenic subunits as the necessary physical-chemical requirement for endotoxic activities (62, 64). These latter observations, however, can be explained by the fact that a loss of lipid groups may cause concomitant dissociation of the complexes, the subunits of which may be associated through hydrophobic bonds. Indeed, alkali treatment of LPS resulted in a water soluble heterogeneous prepa-

ration (LPS-AP) of very low toxicity and small amount of fatty acids. In contrast to the action of phenol, mild alkaline hydrolysis resulted mainly in the cleavage of ester bound fatty acids without an appreciable, concomitant removal of the core structure (polyglucosamine chains) of the lipid moiety. The loss of toxicity in fraction LPS-AP is consistent with the findings of the detoxification experiments of Nowotny who suggested that the cleavage of some ester groups in the endotoxin may be the cause for the loss of toxic effects and some other biological properties. Recently, in order to detect the active center(s) responsible for biological properties, a kinetic study of alkaline hydrolysis of an endotoxic preparation from S. marcescens has been performed (166). It was found that during the first hour of treatment the particles of an average molecular weight of 9 million had been degraded to particles of an average molecular weight of 3 million. This decreased extent of aggregation had no effect on the toxic properties during the short term treatment. However, prolonged treatment resulted in an increase of dissymmetry and loss of toxicity. Such conformational change in the endotoxic particles may possibly explain the inability of fraction LPS-AP to react with the anti-whole cell serum while phenol treated preparations, LPS-A and LPS-AM, did not lose such ability.

Therefore, it is suggested that a specific conformation of lipopolysaccharides maintained by the hydrophobic groups of ester linked long chain fatty acids may be the most important requirement for the elicitation of their typical endotoxic properties. In this connection, it is of interest to recall that the toxic "cord factor" from mycobacteria (167) is a fatty acid ester of a disaccharide (6,6'-dimycolotrehalose).

Whether the active center of lipopolysaccharide resides in the ester-bond, or in the size and conformation of the complex, remains to be explained.

Composition and Structure of the Lipid Moiety

It has been repeatedly demonstrated that the lipid moiety (lipid A) of the lipopolysaccharide contains D-glucosamine, phosphoric acid, fatty acids and, in some cases, amino acids (107, 110). However, the elucidation of the structure of lipid A represents presently one of the most challenging, unsolved problems of lipid chemistry. There are two opposite concepts regarding its basic structure. Nowotny has proposed that lipid A consists of an acylated poly-D-glucosamine linked through phosphodiester linkages (110). On the other hand, Burton and Carter have suggested that lipid A is an acylated, glycosidically linked diglucosamine phosphate (Figure 2, I) (107). These two views differ not only in respect to the type of linkage between glucosamine residues, but also in respect to the size of lipid molecule. Although the sensitivity of the linkage between the lipid and polysaccharide moieties to both inorganic and organic acids is generally known, no information is available concerning the stability of the linkages between the glucosamine residues to acid hydrolysis. The difference in size of glucosamine chains could possibly be explained by the difference in the procedures employed for the isolation of the lipid moiety.

Usually, the lipid moiety can be liberated under very mild acid conditions, by employing, for example, 0.1 N HCl for 30 minutes at 100°, or 0.1 N acetic acid for 2 hours at 100°, or 0.1 N formic acid for 30 minutes at 100°. The "releasing" (cleavage of lipid A from LPS) and "de-

grading" (cleavage of ester and/or amide bound fatty acids from lipid A) abilities of these three acids have been studied in detail (15, 168). Acids of the same concentration, but with decreasing acid strength were used, i.e., 0.1 N HCl, 0.1 N formic acid and 0.1 N acetic acid. The results indicated that the yield of the crude lipid (fraction C) obtained by these three hydrolytic methods differed considerably; as expected, the highest yield of crude lipid (18.5%) was obtained by HCl hydrolysis. However, the yield of formic acid preparation was lower (10%) than that of acetic acid preparation (15%). There appeared to be no relationship between acid strength and the lipid "degrading" power of these acids. The acetone insoluble material obtained by formic acid hydrolysis contained more fatty acids than those obtained either by the HCl or by the acetic acid treatments. However, the effect of these acids on the sizes of polyglucosamine chains has not been evaluated.

In the present study, only 0.1 N HCl was used for the hydrolysis of lipopolysaccharides and isolation of lipid A. The hydrolysis of the endotoxic fraction LPS-A and the non-toxic fraction LPS-AP resulted in the isolation of two different lipid fractions. Like analogous fractions from a strain of E. coli (107), they consisted principally of fatty acids, glucosamine and phosphate. However, amino acids were also found. The β-hydroxy myristic acid accounted for almost 50% of the total bound fatty acids in fraction A-AI and for 70% in fraction AP-AI. This slight discrepancy was due to the fact that in fraction LPS-AP, most of the ester bound fatty acids were already cleaved during the alkaline treatment of fraction LPS-A. A similarly degraded lipid fragment has been isolated also from a strain of E. coli (169). It contained a slightly higher

amount of β-hydroxy myristic acid (90%).

When the isolated crude lipid fraction (fraction A-AI) was hydrolyzed under stronger acid condition (2 N HCl, 4 hours) free inorganic phosphate and glucosamine-phosphates were released. This indicated a relative stability of linkage between glucosamine residues to mild acid hydrolysis.

Mild alkaline hydrolysis and sodium borohydride reduction of the crude lipid fraction (fraction A-AI) resulted in a mixture of totally and partially deacylated fragments of polyglucosamine core. Three groups of products were isolated: a chloroform soluble fraction containing the released fatty acids, a glucosamine derivative containing only β-hydroxy myristic acid (fraction AC-2P), and a mixture of completely deacylated products. One of the most important results of these deacylation studies was the realization that lipid A consists of polyglucosamine chains of various sizes. It was possible to estimate the approximate sizes of the reduced products by determining the glucosamine/glucosaminol ratio. However, it was not possible to conclude whether this heterogeneity had already existed in the lipid A preparations or whether it was caused by alkali or alkaline condition of sodium borohydride reduction. The relatively mild conditions of sodium borohydride reaction seemed to favor the former alternative, i.e., the existence of heterogeneity in lipid A.

It is important to emphasize that each lipid A fraction contained varying amounts of amino acids. This finding, contrary to the conclusion reached by Burton and Carter (107), supports similar results obtained by Nowotny (110) who suggested that a peptide may be covalently bound to the polyglucosamine chain.

Because of the heterogeneity of the crude lipid fraction demonstrable by TLC and the deacylation experiments, it was attempted to fractionate the acetone insoluble fraction (fraction A-AI) by silicic acid column chromatography. Several fractions were obtained by successive elution with chloroform-methanol mixtures of different proportions. However, Adsorbosil-2 thin layer chromatography revealed a persistent heterogeneity of each fraction. It was reported by Kasai (109, 113) that similar fractions from E. coli contained at least eight components demonstrated by silica gel G thin layer chromatography. Kasai explained the heterogeneity by suggesting that mild acid hydrolysis of lipid A resulted in its fragmentation into several components differing in the amount of ester and amide bound fatty acids. However, the possibility that some of the lipid components might be derived from several distinct lipopolysaccharides was not excluded. No data were presented to support this suggestion.

The results of the simultaneous determination of glucosamine and glucosaminol in the reduced lipid fractions indicated that there was a considerable difference in the size of polyglucosamine chains. The number of glucosamine units varied from two to nine. Thus, it was established for the first time that lipid A consists of a mixture of polyglucosamine chains of different sizes. Repeated silicic acid chromatography of the major fraction (Fraction 2) resulted in further fractionation. Two classes of compounds were obtained, characterized by the glucosamine-glucosaminol ratio of 1:1 and 2:1, respectively. However, even in fractions consisting of identical number of glucosamine units (fraction 2-2, 2-3, 2-4), several components could still be detected on Adsorbosil-2

thin layer chromatography (Figure 23). In this case, as suggested by Kasai, the heterogeneity of these fractions was probably due to the difference in the amount of fatty acids rather than to the difference in the number of residues in the polyglucosamine chains.

Differential hydrolyses with acids and bases followed by reduction of the diglucosamine units (fractions 2-2, 2-3, 2-4) revealed some important information concerning the linkages between glucosamine units. Mild acid would have hydrolyzed any phosphodiester-linked disaccharide resulting, upon reduction, in the formation of two moles of glucosaminol. However, the demonstration of equimolar amounts of glucosamine and glucosaminol favors strongly the presence of glycosidic bonds. The demonstration of glucosamine as a sole component in the reaction mixture following alkaline hydrolysis and reduction further substantiates the stability of the linkage between the diglucosamine units. As already previously discussed, the results of alkaline hydrolysis seem to suggest that the glycosidic linkage between glucosamine units is either (1 → 3) or (1 → 4).

In order to study the nature of the linkages between glucosamine units also by enzymatic means, it was necessary to prepare first deacylated, water soluble polyglucosamine chains by utilizing reagents which would split the ester or amide linkages, but would preserve the bonds between monosaccharides. It is generally known that reaction with hydroxylamine in alkaline alcoholic solution would remove ester bound and, occasionally, also the amide bound fatty acids. Since fraction 2-4 did not contain amino acids, it was ideally suited for the hydroxylaminolysis. The resulting water soluble precipitate containing a small amount of β-hydroxy myristate was found to be highly susceptible to the subsequent

action of alkaline monophosphatase. As in the case of the reduced products R-2 and R-S, about 80% of the organic phosphorus was released from partially deacylated fraction 2-4 by the enzyme. This experiment demonstrated that most of the phosphorus in lipid fraction 2-4 had been present as monophosphate. It is suggested, therefore, that the glucosamine units in the diglucosamine as well as in the higher polymers are linked glycosidically.

Possible Structure of the Native Lipid in the Lipopolysaccharide Molecule

The surprising demonstration that lipid A consists of a variety of polyglucosamine chains with an average 2:1 molar ratio of glucosamine to phosphorus raises some intriguing questions about the linkage of the lipid moiety in the lipopolysaccharide. Are the polyglucosamine chains components of a structural entity which is then linked to the polysaccharide moiety? Or, is each polyglucosamine chain bound separately to the polysaccharide moiety? At the present time, there is no experimental evidence to support either of these two structural alternatives. However, if one accepts the former alternative, namely, a lipid moiety consisting of branching polyglucosamine chains of various sizes, then the phosphodiester and glycosidic bonds represent the most probable links between these units. For further discussion, it is important to remember that the lipid moiety is obtained by the mild acid hydrolysis of lipopolysaccharide. Since it is well established that the glucosamine glycosides are very stable to mild acid hydrolysis (170, 171), the existence of the glycosidic linkages between various polyglucosamine chains is highly improbable. On the other hand, single phosphodiesters such as dimethyl

phosphate are stable under alkaline conditions and in the acid region down to pH 4, but are hydrolyzed at lower pH (172). The presence of hydroxy groups adjacent to the phosphate group markedly increases the rate of hydrolysis of phosphodiesters both under alkaline and acid conditions. Whereas N-acetyl glucosamine-1-phosphate is very labile (173), the glucosamine-6-phosphate is very stable to acid hydrolysis (174). Thus, the mild acid hydrolysis - used for the liberation of lipid A - may split the phosphodiester bonds between polyglucosamine chains, but preserve the monophosphate group linked to the C_6-position. On the basis of these deductions, it is suggested that the individual polyglucosamine chains may be bound into a lipid A entity through phosphodiester linkages (Figure 24). However, it is equally possible that individual polyglucosamine units - as separated by silicic acid column chromatography - are linked directly to the polysaccharide moiety. In either case, the lipid moiety is linked most probably through an acid-labile glycosidic bond to the KDO and polysaccharide moiety (87).

Results of the alkaline treatment of lipopolysaccharides demonstrated clearly the stability of the glycosidic linkage between lipid moiety and KDO to alkali.

Structural studies have revealed that the so-called lipid A or the lipid moiety of lipopolysaccharides from at least a chromogenic strain of S. marcescens consists of a mixture of polyglucosamine chains. The basic monosaccharide unit is principally the N-β-hydroxy myristoyl glucosamine phosphate. These units are bound, within each polyglucosamine chain, through (1 → 3) or (1 → 4) glycosidic linkages. The remaining hydroxyl groups are esterified with acetic acid and a variety of

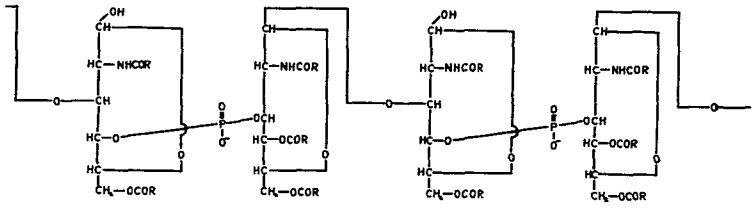


Figure 24. Possible linkage between polyglucosamine units in the lipopolysaccharide.

saturated long-chain fatty acids including also β-hydroxy myristic acid. The number of glucosamine units varies between two to nine; however, the principal polyglucosamine of lipid A of S. marcescens 08 consists of two glucosamine residues. The position and function of the monophosphate group has not been established. It is possible that it may represent either the link between various polyglucosamine chains or function as a bond between lipopolysaccharide and peptide moiety. However, the possible existence of separate, "native" polyglucosamine chains with its clearly implied heterogenicity would require not only a revision of the chemical and structural concept of lipid A but also of that of a lipopolysaccharide.

CHAPTER VI

SUMMARY

A nucleic acid-free lipopolysaccharide preparation extracted from whole cells of S. marcescens 08 by trichloroacetic acid was successively submitted to hot aqueous phenol treatment, chloroform extraction and finally to acetone-water precipitation. The fractionation procedure was monitored by determination of chemical composition, sedimentation coefficients, immunochemical patterns and toxicity of each isolated fraction.

Both multiple phenol and alkaline treatments of purified lipopolysaccharides resulted in the isolation of water soluble heterogeneous preparations of very low toxicity and small fatty acid content. Phenol treatment cleaved most of the ester and amide bound fatty acids and caused a further structural degradation of the endotoxic complex by at least partial removal of lipid A. On the other hand, the mild alkaline hydrolysis resulted mainly in the cleavage of ester bound fatty acids without concomitant removal of lipid A. It is concluded that the retention of toxic properties of lipopolysaccharides depends largely on the presence of ester bound fatty acids.

The phenol phases resulting from multiple hot aqueous phenol treatment of a purified preparation of lipopolysaccharide consist of a

mixture of free fatty acids and at least three different glycolipopeptides.

The main components of the lipid moiety (lipid A) from an endotoxic lipopolysaccharide preparation were glucosamine phosphate, a mixture of fatty acids and a small but significant amount of peptides. The fatty acids were identified as lauric, myristic, palmitic and β-hydroxy myristic acids. While most of the fatty acids were ester bound, β-hydroxy myristic acid was present in lipid moiety both as an ester and/or amide.

The silicic acid column chromatography of lipid A resulted in the isolation of several fractions each of which consisted of glucosamine, fatty acids, phosphorus and small but significant amounts of amino acids. The reduction of these lipid fractions with sodium borohydride followed by acid hydrolysis resulted in mixtures of glucosamine and glucosaminol which were separated and quantitated by amino acid analyzer. The results showed that each fraction consisted of a different number of glucosamine units. Differential hydrolysis of three subfractions indicated that the glucosamine units were linked through glycosidic bonds. The major fraction was found to contain two glucosamine units, one monophosphate group, as well as ester and amide bound fatty acids.

It is suggested that the lipid A from S. marcescens O8 represents a mixture of acylated and phosphorylated polyglucosamines varying in number of glycosidically bound glucosamine units.

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