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A Characterization of Extractable, Hydroxylated Fatty Acid Bearing Components in Legionella Pneumophila

Jonathan R. Lane
East Tennessee State University

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A characterization of extractable, hydroxylated fatty acid bearing components in *Legionella pneumophila*

Lane, Jonathan Ray, Ph.D.

East Tennessee State University, 1993

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300 N. Zeeb Rd.
Ann Arbor, MI 48106

A CHARACTERIZATION OF EXTRACTABLE, HYDROXYLATED FATTY ACID BEARING COMPONENTS IN *LEGIONELLA PNEUMOPHILA*

A Dissertation

Presented to

the Faculty of the Department of Microbiology

James H. Quillen College of Medicine

East Tennessee State University

In Partial Fulfillment

of the Requirements for the Degree

Doctorate of Philosophy in Biomedical Science

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by

Jonathan R. Lane

December 1993

APPROVAL

This is to certify that the Graduate Committee of

Jonathan R. Lane

met on the

21st day of October, 1993.

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council Associate Vice-President for Research and Dean, School of Graduate Studies, in partial fulfillment of the requirements for the degree of Doctorate of Philosophy in Biomedical Science, Microbiology.

William R. Mayberry, Ph.D
Chairman, Graduate Committee

Signed on behalf of the Graduate Council

Associate Vice-President for Research and Dean, School of Graduate Studies

ABSTRACT

A CHARACTERIZATION OF EXTRACTABLE, HYDROXYLATED FATTY ACID BEARING COMPONENTS IN LEGIONELLA PNEUMOPHILA

by

Jonathan R. Lane

Extraction of the lipids of Legionella pneumophila yields phases unlike those produced from other Gram-negative bacteria. A viscous interface forms between the aqueous (wash) and organic phases. More than half of the hydroxylated fatty acids were found distributed between the aqueous phase and the interfacial material, fractions in which such constituents have not been reported in other Gram-negative species.

It was further observed that after the material from the aqueous/interfacial phase was dissolved in methanol or chloroform/methanol (2:1 [V/V]), the addition of acetone would create a white, flocculent precipitate. Analyses showed that the supernatant contained fatty acids that were nonhydroxylated and the precipitate contained both nonhydroxylated and hydroxylated fatty acids.

The acetone precipitate could be further purified by column chromatography. Material was eluted from a silicic acid column with sequential additions of chloroform, acetone, and methanol. It was found that the methanol fraction contained the majority of the hydroxylated fatty acid containing material.

An improved method for extracting LPS-like material from Legionella pneumophila is presented. This study suggests that LPS-like material can be obtained from L. pneumophila in higher yield (6.4% of total cell weight), of higher purity (as indicated by SDS-PAGE), and by a simpler method than those previously reported.

SDS-PAGE profiles of purified (acetone precipitation and column chromatographic separation) LPS-like material extracted with chloroform/methanol (2:1 [V/V]) from L. pneumophila are identical to the previously reported profiles for L. pneumophila LPS.

The chemical analyses of the LPS-like material can only account for approximately one-half the isolated material weight. This is suggestive of a moiety that is as of yet undetectable by the means employed to characterize the LPS.

DEDICATION

My parents, Elijah and Edith Lane, provided to me both love and guidance. They have supported all endeavors I have ever undertaken.

My wife, Heather Renay Lewis Lane, has given purpose and meaning to my life. She will never fully understand the magnitude of her influence upon my life. I am most grateful for her unconditional love.

To Mom, Dad, and Heather, I dedicate this manuscript.

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CHAPTER 1

Introduction

Background Information

Legionella pneumophila—the Legionnaire's Disease Agent—was first described by Brenner et al. 1979. Although this organism showed the staining reactions and ultrastructural architecture typical of Gram-negative bacteria (Brenner et al. 1979; Keel et al. 1979), it was originally considered to be "an unusual Gram-negative microorganism" in that hydroxylated fatty acids were reported to be absent from the cellular fatty acid profiles (Finnerty et al. 1979; Moss et al. 1977; Moss and Dees 1979). More detailed examination showed that the organism contains, in addition to high levels of characteristic nonhydroxy fatty acids, significant levels of 3-monohydroxy fatty acids and novel 2,3-dihydroxy fatty acids. This latter class of compounds is rare in prokaryotes, found only in certain members of the genera *Legionella* (Mayberry 1981, 1984a, 1984b) and *Cytophaga* (Abbanat et al. 1988). The hydroxylated fatty acids are reported to be non-extractable by common lipid solvents and to remain bound to particulate cell matter, presumably cell surface macromolecules (Mayberry 1981, 1984a, 1984b).

An extensive literature has developed regarding the isolation and description of various cell-surface macromolecules and their relationshipto such biological

activities of the genus Legionella as virulence, endotoxicity, and antigenicity. Among the potential virulence factors of Legionella pneumophila are endotoxicity and the ability to live within normally phagocytic, bactericidal eukaryotic cells such as protozoa (Fields et al. 1986), monocytes, and macrophages (Daisy et al. 1981; Gabay and Horwitz 1985; Horwitz 1983a, 1983b; Horwitz and Silverstein 1980, 1981a, 1981b, 1981c; Weinbaum et al. 1984). Additionally, infection with living L. pneumophila or administration of killed cells induces the production of Tumor Necrosis Factor (cachectin) and Interferon in experimental animals (Blanchard et al. 1985, 1987). Furthermore, there is considerable interest in the identification and characterization of those antigenic determinants which elicit antibodies of varying specificity. Most of these studies have centered around moieties similar to lipopolysaccharide (LPS), as obtained by several methods. However, in none of these reports has there been presented a satisfactory accounting of the presence, concentration or relationship of the fatty acids to these macromolecules, suggesting that the fatty acids have not been sought, or when sought, have not been recognized. There are several Gram-negative cell components in which hydroxylated fatty acids may be associated (Tables 1 and 2).

All strains of Legionella examined to date have been shown to contain, in addition to the rather high levels of

Table 1. Cell components with which hydroxylated fatty acids may be associated: Components extractable with chloroform/methanol mixtures

Component	References
A. Sphingolipids, as found in Acholeplasma axanthum, Bacteroides spp., and Sphingobacterium spp.	A. Fritsche 1974, 1975; Kunsman 1972; Mayberry 1980; Mayberry and Smith 1983; Mayberry et al. 1973; Miyagawa et al. 1978; Plackett et al. 1970; Stoffel et al. 1975; White et al. 1969; Yabuuchi and Moss 1982.
B. Sulfonosphingolipids, as found in Capnocytophaga spp. and other gliding bacteria.	B. Abbanat et al. 1988; Godchaux and Leadbetter 1980, 1983, 1984.
C. Phosphonosphingolipids and related compounds, as found in Bdellovibrio bacteriovorus.	C. Steiner et al. 1973.
D. Aminoacyl lipids such as the ornithine-lipid of Paracoccus denitrificans.	D. Wilkinson et al. 1982.
E. Low molecular weight fragments of the Lipid A portion of lipopolysaccharide.	
F. Low molecular weight lipo-oligopeptides.	

Table 2. Cell components with which hydroxylated fatty acids may be associated: Non-extractable (bound) components

Component	References
A. Lipopolysaccharides (LPS), either S-form or R-form, obtainable by the extraction methods of Westphal, Galanos or Darveau and Hancock, as well as by other methods.	A. Darveau and Hancock 1983; Galanos et al. 1969; Luderitz et al. 1973; Rietschel 1976; Sonesson et al. 1989; Westphal and Jann 1965; Westphal et al. 1955; Wilkinson 1977; Wilkinson et al. 1973; Wollenweber et al. 1980.
B. Proteins contaminating the LPS fractions obtained by the above methods.	
C. Polysaccharide contaminants of the LPS fractions.	
D. Lipoprotein, such as that described in the Enterobacteriaceae, that, being covalently linked to the peptidoglycan, should remain with the particulate (insoluble) material after boiling with detergent or extraction by the above methods.	D. Braun and Bosch 1972; Braun and Hantke 1972; Braun and Rehn 1969; Braun and Siegelin 1970; Braun et al. 1970; Lugtenberg et al. 1977.
E. A novel lipoprotein that is not linked covalently to peptidoglycan and would be extractable by one of the above methods.	
F. Peptidoglycan itself.	
G. Some other, as yet undescribed cell component, in the cell envelope, the cytoplasmic membrane or the cytoplasm of the cell.	

the branched-chain nonhydroxy fatty acids which characterize this genus, low (relative to the nonhydroxy acids), but significant levels of monohydroxy fatty acids. Nine species contain not only nonhydroxy and monohydroxy fatty acids, but also 2,3-dihydroxy fatty acids, a class of compounds unusual in prokaryotes (Mayberry 1981, 1984a, 1984b; Mayberry, personal communication).

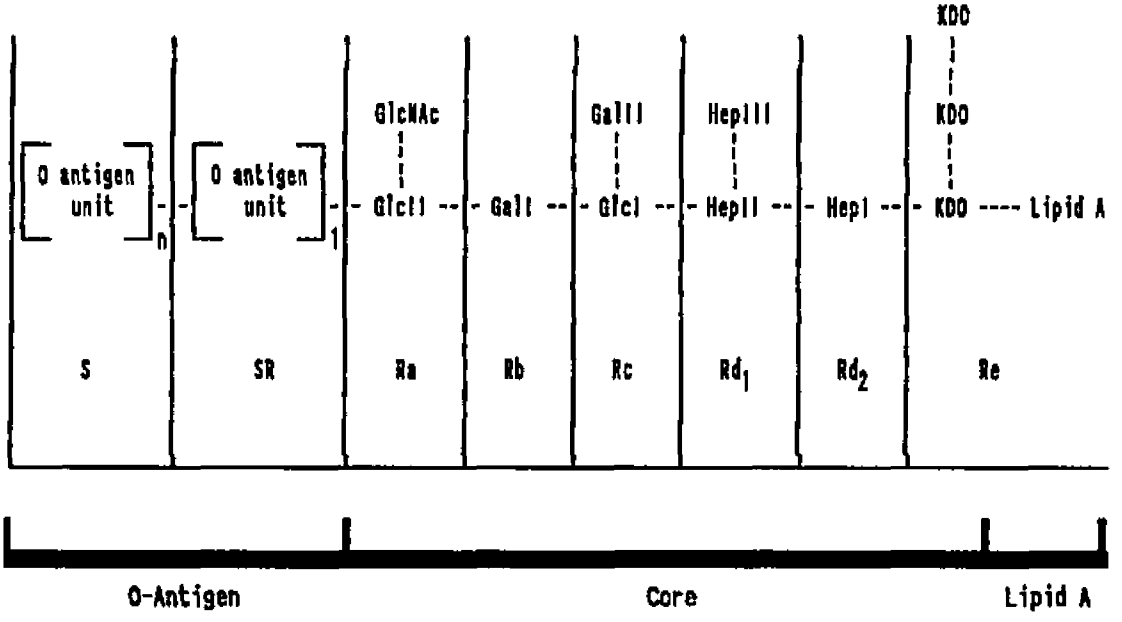
In Legionella spp. most of the nonhydroxy fatty acids, a small fraction of the monohydroxy fatty acids and virtually none of the dihydroxy fatty acids appear to be extractable by lipid solvents (Mayberry 1981, 1984a, 1984b). This suggests that the hydroxylated fatty acids are bound to non-lipid macromolecules, possibly including cell surface components.

There are several methods for the extraction of LPS from Gram-negative bacteria. Most of these have been developed using enteric bacteria and/or pseudomonads as model systems. One of the most popular is the hot phenol/water extraction procedure of Westphal et al. (1955, 1965). At temperatures above 70°C, the system is monophasic. After cooling, it separates into precipitable cell debris, a phenol phase containing mainly protein and an aqueous phase containing LPS, glycans, and potential contaminants such as nucleic acid.

Because investigators found phenol-soluble, hydrophobic LPS in phenol phases of Westphal extractions of some

bacteria, Galanos et al. (1969) developed an unheated, lipophilic extraction procedure using phenol/chloroform/petroleum ether. This method is intended to increase the yield of R-form lipopolysaccharides, which are ultimately obtained as water-soluble preparations devoid of protein, nucleic acid, glycans, and S-form lipopolysaccharides. Lipopolysaccharides are classified according to the length of their polysaccharide side chains, the so-called O-antigen. S-form lipopolysaccharides, also designated "Smooth LPS," contain long polysaccharide side chains that increase the hydrophilicity of the LPS. The increase in hydrophilicity tends to allow the S-form LPS to partition into the aqueous phase of extraction procedures. R-form lipopolysaccharides, also designated "Rough LPS," "lipo-oligosaccharides" (LOS) (Hitchcock et al. 1986) or "glycolipids" (Galanos et al. 1969), contain truncated polysaccharide side chains. The shortened polysaccharides tend to make R-form LPS a lipophilic molecule that is more likely to partition into organic solvents. The lipopolysaccharides of Salmonella typhimurium are considered to be typical of the LPS from many Gram-negative bacteria. A schematic diagram (adapted from Hitchcock et al. 1986) of S. typhimurium smooth LPS and rough LPS mutants is shown in Figure 1.

Another entity to which the hydroxylated fatty acids could be bound is a lipoprotein contained in the cell



Where, S is smooth LPS with varying number of O-antigen units;

SR is semirough LPS with only one covalently bound O-antigen unit;

Ra LPS is composed of only Lipid A and core polysaccharide;

Rb, Rc, Rd_1, and Rd_2 LPS are core mutants without any O antigen units; and

Re is composed of Lipid A and two to three molecules of KDO, Re is also called deep rough or heptoseless LPS.

FIGURE 1. Schematic diagram of the smooth and rough LPS of Salmonella typhimurium.

envelope. The lipoprotein of Escherichia coli, as described by Braun (1973), is covalently attached to the peptidoglycan by the ϵ-NH_2 of its carboxy-terminal lysyl residue. At the amino-terminus, a cysteinyl residue is substituted with a glycerol by a thioether linkage. This modified amino-terminus has three sites for acylation by fatty acids: the two free hydroxyl groups of the glycerol, capable of supporting ester linkages and the α-NH_2 of cysteine, capable of supporting an amide linkage. Acylation of these sites would make the amino-terminus of this protein strongly lipophilic. Because the lipophilic end of the protein is buried in the outer membrane and the other end is attached to the peptidoglycan, it is thought that the lipoprotein acts as an anchor for the outer membrane. Since this component is covalently linked to the peptidoglycan (the "murein sacculus" which provides shape and rigidity to the bacterial cell), it remains with the particulate material after bacterial cells are extracted with boiling detergent.

The hydroxylated fatty acids of Pseudomonas aeruginosa and Escherichia coli, species that are considered to be representative of many Gram-negative species, generally are not extractable by lipid solvents and have been shown to reside almost exclusively in LPS components of the cell envelope. The hydroxylated fatty acids are linked to these moieties via ester (alkali-labile) and amide (alkali-stable) bonds (Wilkinson 1977).

In early studies of L. pneumophila, Wong et al. (1979) isolated, in low yield, a material identifiable as LPS by the classic Westphal method. Its endotoxicity was reduced, as compared to LPS isolated from other Gram-negative bacteria and it was reported to be devoid of hydroxylated fatty acids (Wong et al. 1979). Subsequent studies have confirmed that little of the LPS can be found in the aqueous fraction of the hot phenol/water extraction and that other methods are preferable. Otten et al. (1986) found that some antigenic, endotoxic material could be found in the phenol phase of the Westphal extraction procedure. They also obtained an increased yield of more active material from the Galanos "hydrophobic" extraction procedure. These workers reported low levels of fatty acids, including "trace" amounts of a component which was identified as straight-chain 3-hydroxytetradecanoate (Otten et al. 1986).

In the relatively recent study of LPS from L. pneumophila, (Sonesson et al. 1989), the method of Darveau and Hancock (1983) and the method of Galanos et al. (1969) were sequentially applied to isolate the LPS of L. pneumophila. Lipid A was cleaved by 0.1 M acetate buffer, pH 4.4, at 100°C for 1 hour. Signficant quantities of the hydroxylated fatty acids were found in the Lipid A fraction. By weight, the Lipid A consisted of 20 percent carbohydrates, 28 percent nonhydroxylated fatty acids, 30

percent monohydroxylated fatty acids, and 5 percent dihydroxylated fatty acids.

Preliminary Research

Cells of Legionella pneumophila were extracted and partially fractionated by well-documented procedures that were developed for other Gram-negative species (Lane 1990). Fractions reported to contain moieties such as lipids, "smooth" and "rough" lipopolysaccharides (S-LPS and R-LPS), and lipoproteins were isolated. In addition to those fractions expected to contain compounds of interest, those fractions normally discarded in these methods were examined for fatty acids, neutral carbohydrates, phosphate, and protein.

Extraction, by the Folch method, of the lipids of L. pneumophila yielded phases unlike those produced from other Gram-negative bacteria. A viscous interface formed between the aqueous (wash) and organic phases. More than half of the hydroxylated fatty acids were found distributed between the aqueous phase and interface material, fractions in which such constituents have not been reported in other Gram-negative species.

Extraction of L. pneumophila by procedures designed to isolate LPS-like components resulted in the distribution of hydroxylated fatty acids between the cell residues (i.e., nonextractable) and the phases expected to contain R-LPS,

with little material in the phases expected to contain S-LPS.

There appeared to be no segregation of monohydroxy from dihydroxy fatty acids among the various fractions derived from the extractions. This suggests that these two classes of compounds are linked to similar cellular moieties. An interesting ancillary finding was that most fractions containing extracted hydroxylated fatty acids also contained unexpectedly high levels of protein, phosphate, and carbohydrate.

Furthermore, unique nonhydroxylated fatty acids with chain lengths of approximately 30 carbons seem to be present in the interfacial material as obtained by the extraction method of Folch. Recently, 27-oxo-octacosanoic acid, 27-hydroxy-octacosanoic acid, and heptacosane-1,27-dioic acid have been found in the phenol-chloroform-petroleum ether extracts of L. pneumophila (Moll et al. 1992) and were presumed to be constituents of the lipopolysaccharide. That these components are associated with LPS is suggested by the occurrence of Lipid A variants with 27-hydroxyoctacosanoic acid in the lipopolysaccharides from the family Rhizobiaceae (Hollingsworth and Carlson 1989; Bhat et al. 1991).

Review of Standard Extraction Procedures

Folch Extraction Procedure

The extraction procedure of Folch et al. (1957), in which cells are extracted with chloroform/methanol (2:1 [V/V]), is used to isolate lipids from tissues or bacterial cells. The flow diagram for a typical Folch extraction procedure is shown in Figure 2. All fractions, with exception of the organic solvent fraction, are normally discarded, because the assumption is that they contain little or no extractable lipids.

Westphal Extraction Procedure

The extraction procedure of Westphal et al. (1955), in which cells are extracted with hot aqueous/phenol, is a method used to isolate S-form bacterial LPS. The flow diagram for a typical Westphal extraction procedure is shown in Figure 3. The aqueous phase from the extraction is considered to contain the LPS. The phenol phase contains bacterial protein and has been shown to contain LPS-like components from some bacteria. The pellet is normally discarded.

Galanos Extraction Procedure

The extraction procedure of Galanos et al. (1969) was developed to isolate R-form LPS devoid of other bacterial components such as S-form LPS, nucleic acids, other sugar-containing moieties, and protein. A flow diagram for a

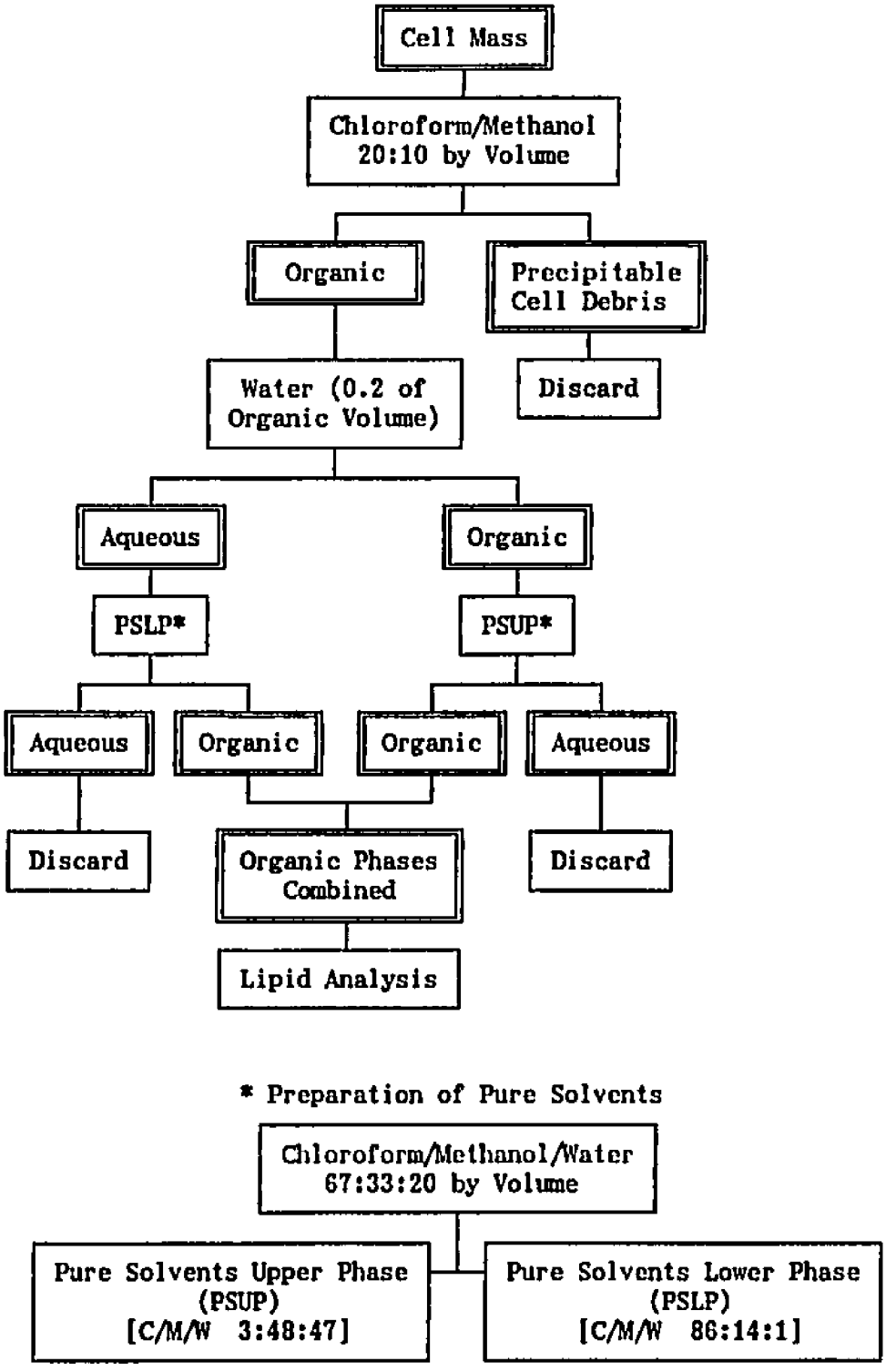


Figure 2. Flow diagram of Folch extraction procedure, as usually performed.

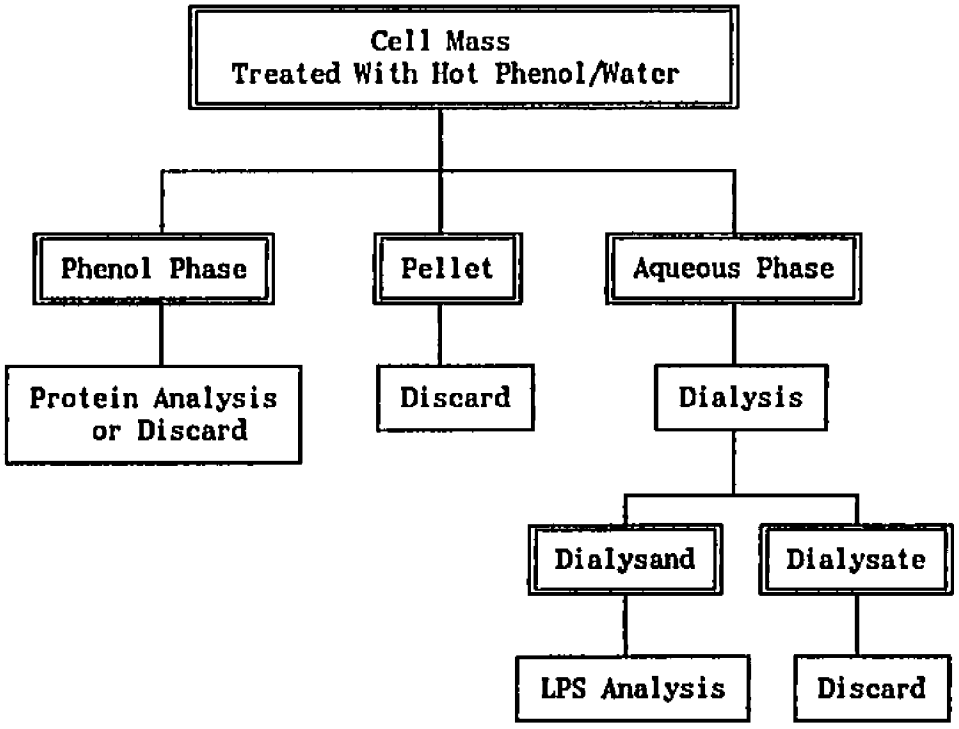


Figure 3. Flow diagram of Westphal extraction procedure, as usually performed.

typical Galanos extraction procedure is shown in Figure 4. "Glycolipids" are precipitated from the phenol solution that results from a phenol/chloroform/petroleum ether extraction. With exception of the precipitated "glycolipid," all fractions are normally discarded.

Braun Extraction Procedure

The extraction procedure of Braun (1973) consists of boiling bacterial cells in 4% sodium dodecylsulfate (SDS) for 4 hours. The flow diagram for a typical Braun extraction procedure is shown in Figure 5. The supernatant and water washes of the remaining pellet are discarded. The pellet is then used for lipoprotein analysis. The extraction procedure of Darveau and Hancock (1983) is similiar to that of Braun. Instead of analyzing the pellet, this procedure uses the supernatant solution as a source of LPS-like constituents, which are purified by an extensive series of differential ultracentrifugations.

Acetic Acid Hydrolysis

Mild acid hydrolysis of bacterial LPS with 0.2 M acetic acid has been shown to cleave the Lipid A component from the polysaccharide chain (Wilkinson et al. 1973). This method is normally applied to LPS after it has been isolated by one of the above mentioned procedures and is used to yield "pure" Lipid A. The flow diagram of a typical acetic acid extraction procedure is shown in Figure 6.

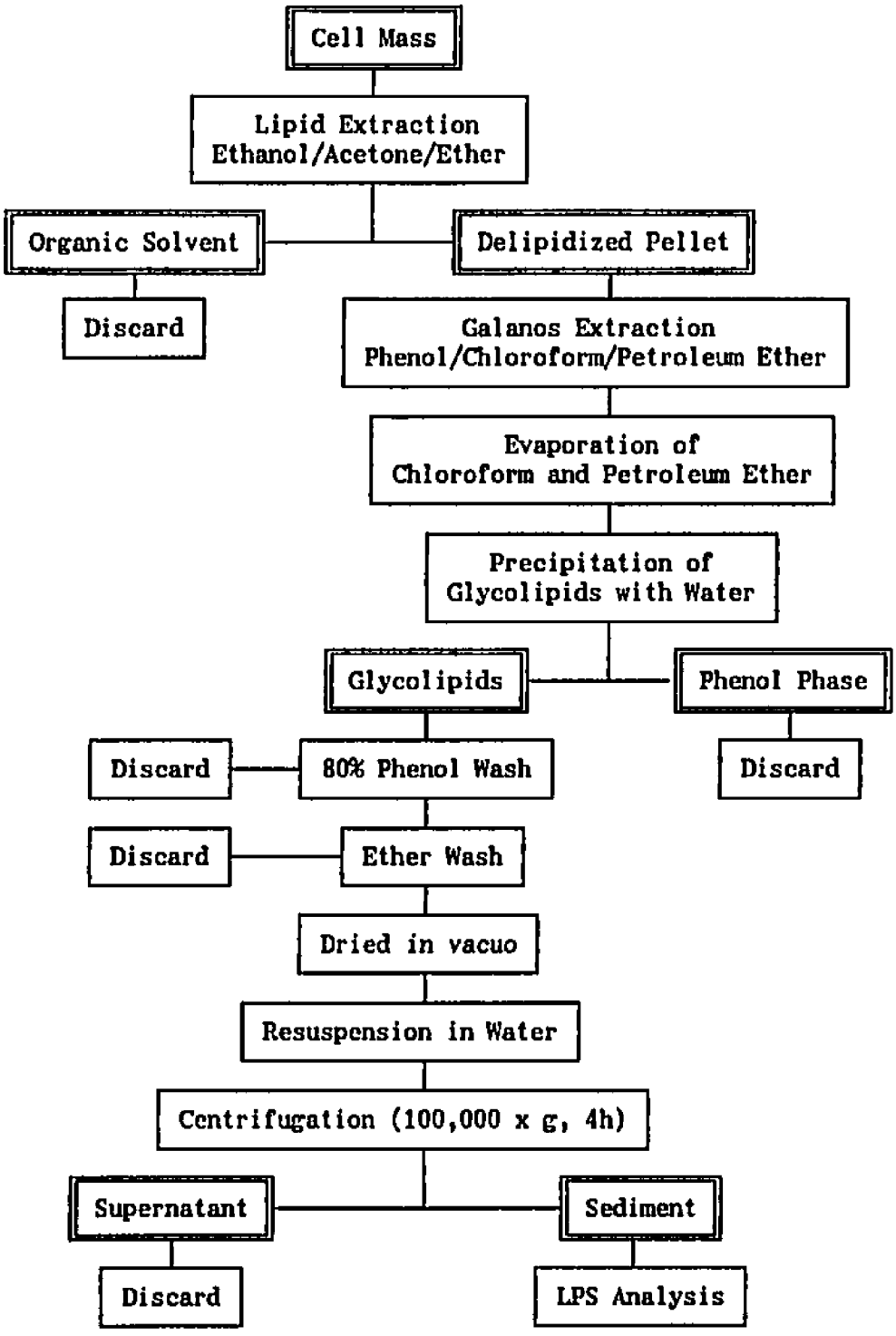


Figure 4. Flow diagram of Galanos extraction procedure, as usually performed.

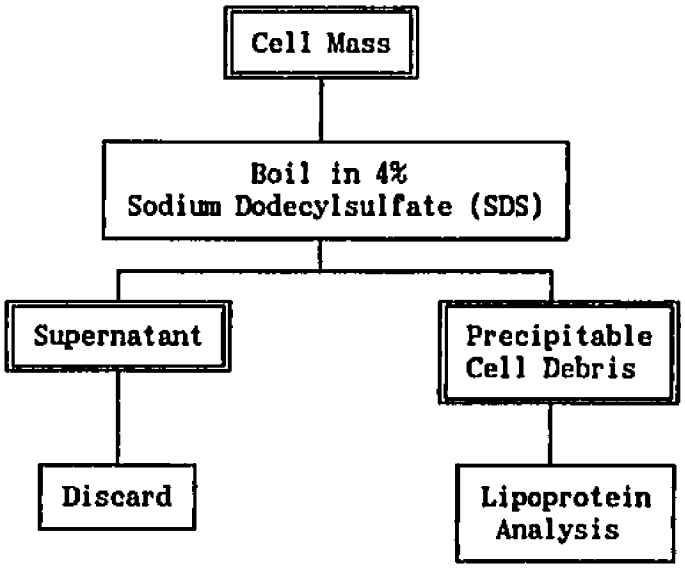


Figure 5. Flow diagram of Braun extraction procedure, as usually performed.

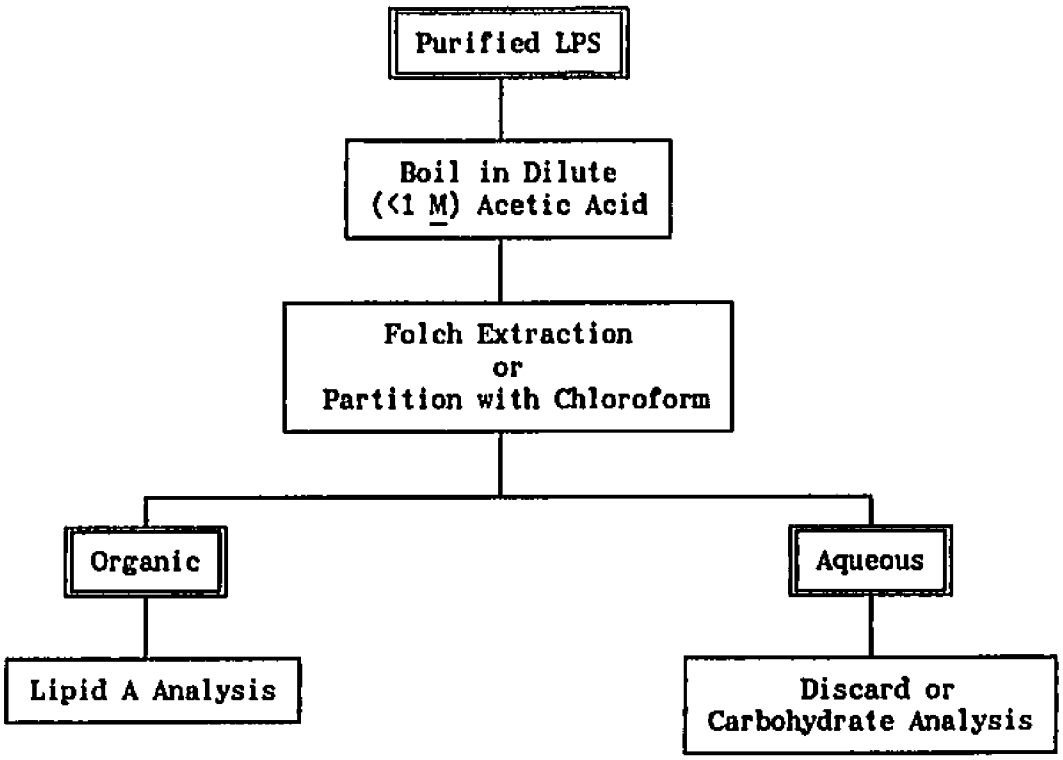


Figure 6. Flow diagram of acetic acid extraction procedure, as usually performed.

Objective

The objective of the proposed research was to examine the potentially novel cell components in Legionella pneumophila strain Philadelphia 1. The intent was to characterize the hydroxylated fatty acid bearing moeities as completely as possible. As a minimum, the molar ratios of the fatty acid classes, carbohydrates, phosphates, and proteins were to be determined. Composition analyses (profiles) of fatty acids, amino acids, and carbohydrates were conducted, as applicable.

Emphasis was placed upon the combined aqueous and interfacial material obtained by the extraction procedure of Folch. However, alternative extraction procedures for obtaining cellular components also were investigated. It was believed that a new extraction procedure could be developed to isolate those materials found in the interfacial material.

It was anticipated that from results obtained, some insight into the locations of the hydroxylated fatty acids could be gained. It was hoped that this research would lead to the determination of the cellular moieties to which, and the type of linkage (amide or ester) by which, the fatty acids are bound.

Of particular interest was the dihydroxylated fatty acid class. This class is apparently without precedent among prokaryotes, and may suggest a novel cell-surface

component. Such a component may have broad implications in the realm of bacterium/host interactions, whether immunologic or pathogenic.

CHAPTER 2

Materials and Methods

Reagents

Growth Medium Components

Yeast extract (YE), tryptic soy broth (TSB), and agar were obtained from Difco Laboratories, Detroit, MI. Charcoal, (N-[2-acetamido]-2-aminoethanesulfonic acid (ACES), and α-ketoglutarate (α-KG) were obtained from Sigma Chemical Company, St. Louis, MO.

Solvents

All solvents were obtained from Fisher Scientific Company, Fair Lawn, NJ. Chloroform and methanol were redistilled before use; petroleum ether, diethyl ether, hexane, heptane, phenol, and pyridine were used as supplied.

Derivatization Reagents

Trimethylchlorosilane (TMCS), hexamethyl disilazane (HMDS), bis(trimethylsilyl)trifloroacetamide (BSTFA), and trifloroacetic anhydride (TFAA) were obtained from Sigma and were used as supplied.

Internal Standards

D,L-12-Hydroxystearic acid (n-C18:0_h), heneicosanoic acid (n-C21:0), and myo-inositol were obtained from Sigma. 9,10-Dihydroxystearic acid (n-C18:0_{h2}) was obtained from Applied Science Laboratories, Inc., State College, PA.

Other Chemicals

Mineral acids, bases, and various salts were obtained from Fisher. Acrylamide/bisacrylamide (29:1 [V/V]), TRIS (2-amino-2-hydroxymethyl-1,3-propanediol), TEMED (N,N,N,N,-tetramethylethylenediamine), ammonium persulfate, glycerol, 2-mercaptoethanol, bromophenol blue, Ficoll 400, EDTA-disodium salt, xylene cyanol, ethidium bromide, and Coomassie Brilliant Blue G-250 were also obtained from Fisher. Silica Gel H was obtained from Sigma. Unisil (activated silicic acid, 100-200 mesh, acid washed, controlled particle size) was obtained from Clarkson Chemical Company, Inc., Williamsport, PA. Sodium dodecylsulfate (SDS) was obtained from Bio-Rad Laboratories, Richmond, CA.

Analytical Reagents

Phenol-reagent solution, 2 N (Folin-Ciocalteau), was obtained from Fisher. Ascorbic acid, bovine serum albumin (BSA), 2-thiobarbituric acid (4,6-dihydroxypyrimidine-2-thiol), periodic acid, and dimethyl sulfoxide (DMSO) were obtained from Sigma. Sodium arsenite, citric acid, and formaldehyde solution (37% [V/V], histological grade) were obtained from Fisher. Protein assay dye reagent concentrate was obtained from Bio-Rad. Sodium borohydride was obtained from Eastman Kodak Company, Rochester, NY. Silver nitrate was obtained from Sargent-Welch Scientific Company, Skokie, IL.

Water

All water was distilled with an all-glass still with deionized water as feed.

Organism

Bacterial Strain

The culture of Legionella pneumophila ssp. pneumophila strain Philadelphia 1 was provided by Dr. W. R. Mayberry, James H. Quillen College of Medicine, East Tennessee State University, Johnson City, Tennessee.

Bacterial Lipopolysaccharides (LPS)

Lipopolysaccharide from Legionella pneumophila ssp. pneumophila strain Philadelphia 1 was kindly provided by Dr. Anders Sonesson, Center for Environmental Biotechnology, Knoxville, Tennessee. The LPS was extracted using the phenol/chloroform/petroleum ether (2:5:8 [V/V/V], PCP) method (Galanos et al. 1969) from cells previously washed with 95% ethanol, acetone, and diethyl ether. LPS was precipitated from the PCP-extracts using diethyl ether and subsequently subjected to enzyme digestion steps using DNase, RNase, and Protease K. The LPS extract contained 3% ([W/W]) polyhydroxybutyrate and 5% ([W/W]) of protein as contaminants. The LPS was not soluble in water but soluble in chloroform/methanol 2:1 ([V/V]).

Lipopolysaccharides from Escherichia coli were purchased from Sigma. The three types were: S-LPS isolated

from E. coli serotype 055:B5 by the method of Westphal et al. (1955), Rc-LPS isolated from E. coli J5 by the method of Galanos et al. (1969), and Ra-LPS isolated from E. coli EH100 by the method of Galanos et al. (1969).

Growth and Harvesting of Bacteria

Legionella pneumophila cells were grown on buffered charcoal yeast extract media supplemented with α-ketoglutarate (BCYE-α) (Feeley et al. 1979) for 60 hours at 37°C. At harvest, cells were scraped from the plates, suspended in distilled water and subjected to centrifugation at 2500 x g for 45 minutes at 4°C. The pellets were washed free from residual medium by resuspension and centrifugation from distilled water. The pooled cell mass was freeze-dried.

Fractionation Procedures

Total Fatty Acid Analysis

Fatty acid methyl esters (FAME) were formed by the method of Mayberry and Lane (1993). This method involves three steps: alkaline saponification, acid hydrolysis, and esterification.

Alkaline Saponification. A weighed amount of each organism was placed in 16x125 mm tubes with Teflon[1]-lined screwcaps. 1.0 ml methanol and 0.1 ml of 12 M KOH were added; the samples were heated 1 hour at 55°C.

Acid Hydrolysis. To the samples, after alkaline methanolysis, internal standards, 5.0 ml distilled water, and 1.5 ml concentrated HCl were added to obtain a final solution of 2 M HCl. Each sample was then overlaid with 1 ml heptane. The upper layer of heptane provided a nonpolar area in which released cyclopropyl and unsaturated fatty acids were protected from acid degradation, which has been suggested to be a potential source of analytical error (Vulliet et al. 1974; Lambert and Moss 1983). The samples were heated at 100°C for 12 hours in a sand bath.

After hydrolysis, samples were transferred to 18x150 mm tubes. The samples were partitioned by the addition of 10 ml chloroform. The aqueous layers were removed and adjusted in volume to 25.0 ml. The organic layers were washed two additional times with distilled water and dried on a vortex evaporator. The aqueous and organic layers were analyzed as described below in the Analytical Methods section.

Conversion of Fatty Acids to Methyl Esters (FAME). 10 ml of chloroform and 0.25 ml acetic anhydride (to act as a water scavenger) were added to each dried organic layer derived from sequential alkaline methanolysis-acid hydrolysis. The samples were vortexed. To each sample, 5.0 ml methanol and 0.1 ml concentrated HCl (acid catalyst) were added. Each sample was heated at 55°C for 30 minutes, then washed 3 times by partition against distilled water. The

organic phases were dried and the above procedure was repeated to ensure complete esterification.

Extraction of Unbound Lipids

Folch Extraction Procedure. Weighed amounts, typically 100 mg, of lyophilized cells of each organism were extracted with chloroform/methanol by the method of Folch et al. (1957), resulting in at least three fractions: aqueous phase, organic phase, and pellet. Aliquots of the aqueous phases, organic phases, and pellets of each fraction were subjected to alkaline methanolysis and acid hydrolysis, and analyzed as described in Analytical Methods. A flow diagram of the Folch extraction procedure, as employed, is shown in Figure 7.

Methanol and Water Extractions. Weighed amounts, typically 100 mg, of lyophilized cells were extracted with 10 ml methanol. Other samples were similiarly extracted with water. Each sample was heated for 18 hrs at either 55°C or 100°C. The supernatant was removed by filtering the extraction system through a glass fiber filter. The filter cake was resuspended in the appropriate solvent and the extraction procedure was repeated for an additional 18 hrs. As before, the extraction system was filtered through a glass fiber filter. The resulting supernatants of each

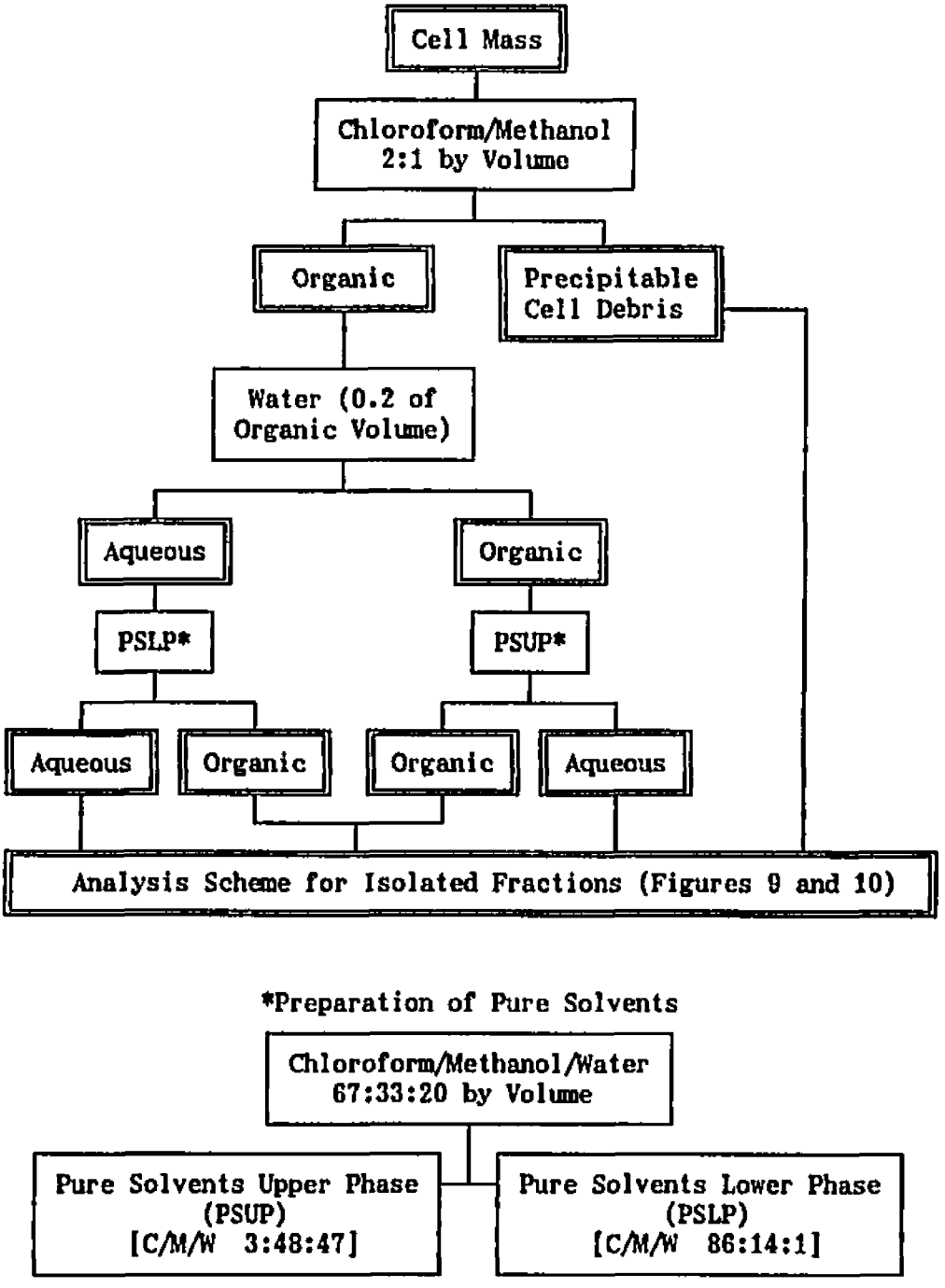


Figure 7. Flow diagram of Folch extraction procedure, as employed in this study.

extraction were pooled and dried in vacuo. The filter cakes were allowed to air dry. The supernatants were reconstituted in 1 ml of the appropriate solvent. Acetone, 10 ml, was added and the mixtures were centrifuged at 2500 rpm for 30 min. The supernatant solutions were aspirated from the precipitates. The precipitates and the supernatant solutions were dried in vacuo. Aliquots of the resulting precipitates, supernatant solutions, and pellets of each fraction were subjected to alkaline methanolysis and acid hydrolysis, and analyzed as described in Analytical Methods. A flow diagram of the methanol and water extraction procedures, as employed, is shown in Figure 8.

Chromatographic Separation

Column Chromatography. Extraction products (material that precipitated in acetone) was further purified by chromatography over acid-washed silicic acid. Approximately 5 g silicic acid (previously washed in 1.0 M HCl) in a methanol slurry was packed in a glass-wool plugged 50 ml buret. The column was then washed twice with 50 ml additions of chloroform. Samples were reconstituted in methanol, mixed with silicic acid, and the mixtures dried in vacuo. The sample preparations were then layered on top of a chromatographic column packed with acid-washed silicic acid. Fractions were eluted with 50 ml sequential additions

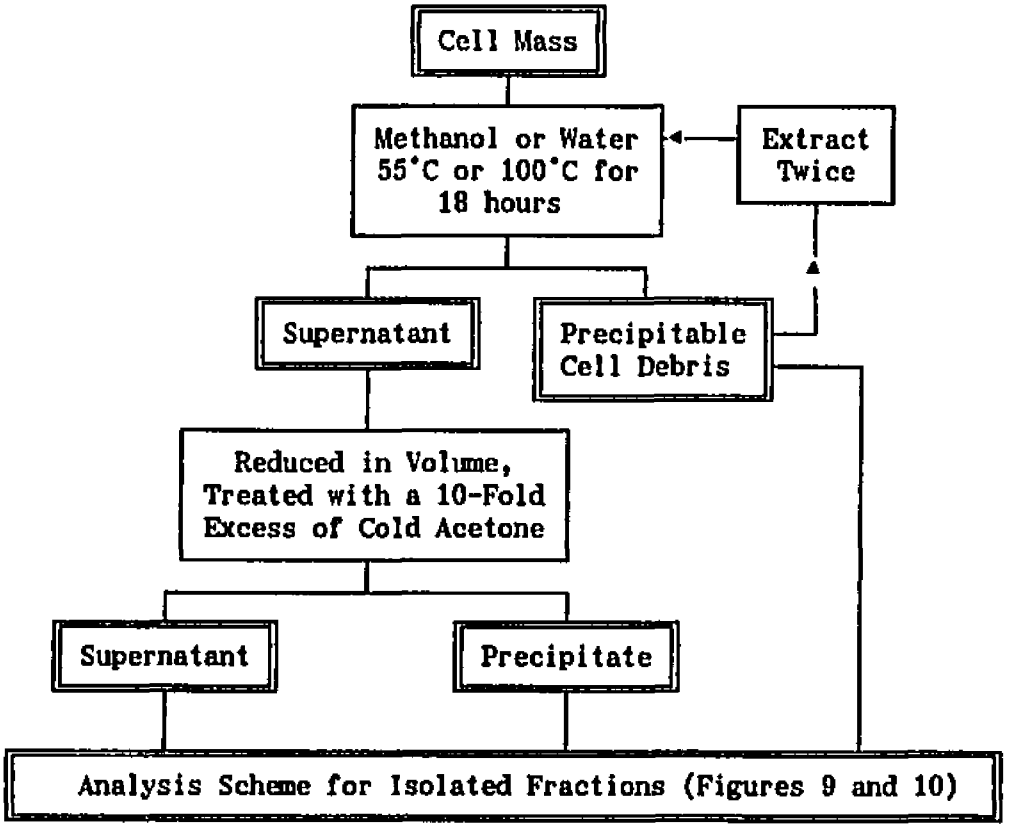


Figure 8. Flow diagram of methanol or water extraction procedure, as employed in this study.

of chloroform, acetone, and methanol, at flow rates of approximately 2 ml/min.

Flash-Column Chromatography. Extraction products were also purified by flash-column chromatography. Samples were dried in silicic acid and the coated silicic acid was placed in glass-wool plugged champagne columns (Supelco, Bellefonte, IL). Fractions were eluted from the columns under reduced pressure with 20 ml sequential additions of chloroform, acetone, and methanol.

Analytical Methods

A flowchart of Analytical Methods is presented in Figures 9 and 10.

Fatty Acid Analysis

Internal Standards. Approximately 500 nanomoles each of n-henicosanoic acid (n-C21:0), D,L-12-hydroxystearic acid (n-C18:0_h), and 9,10-dihydroxystearic acid (n-C18:0_{h2}) were added to each sample prior to acid hydrolysis. These compounds served as internal standards for nonhydroxylated fatty acids, monohydroxylated fatty acids, and dihydroxylated fatty acids, respectively. Identification of cellular fatty acids was based on retention times relative to those of the internal standards. Calculations of the concentrations of cellular fatty acids were based on peak areas relative to those of the internal standards (IS),

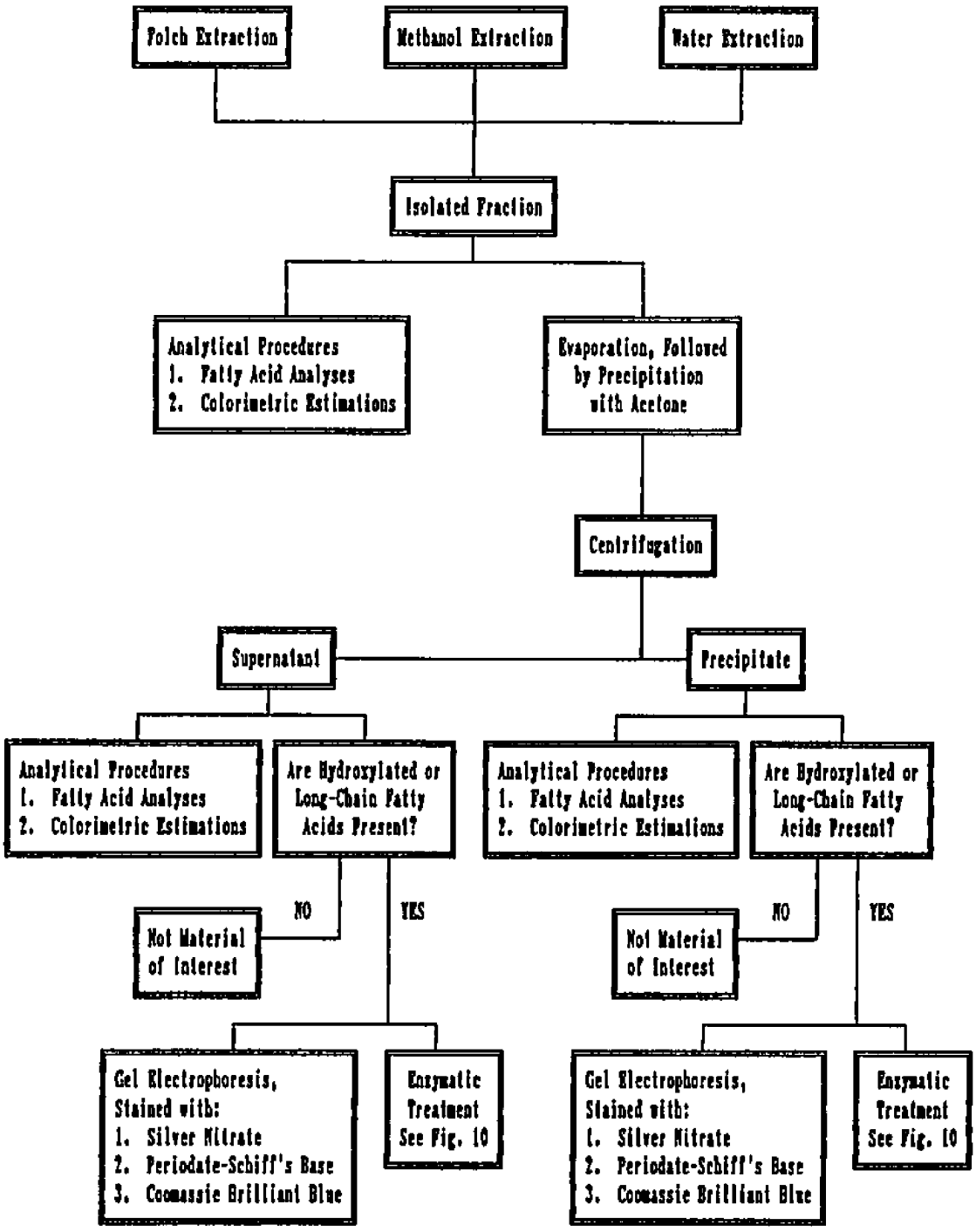


Figure 9. Analysis scheme for isolated fractions.

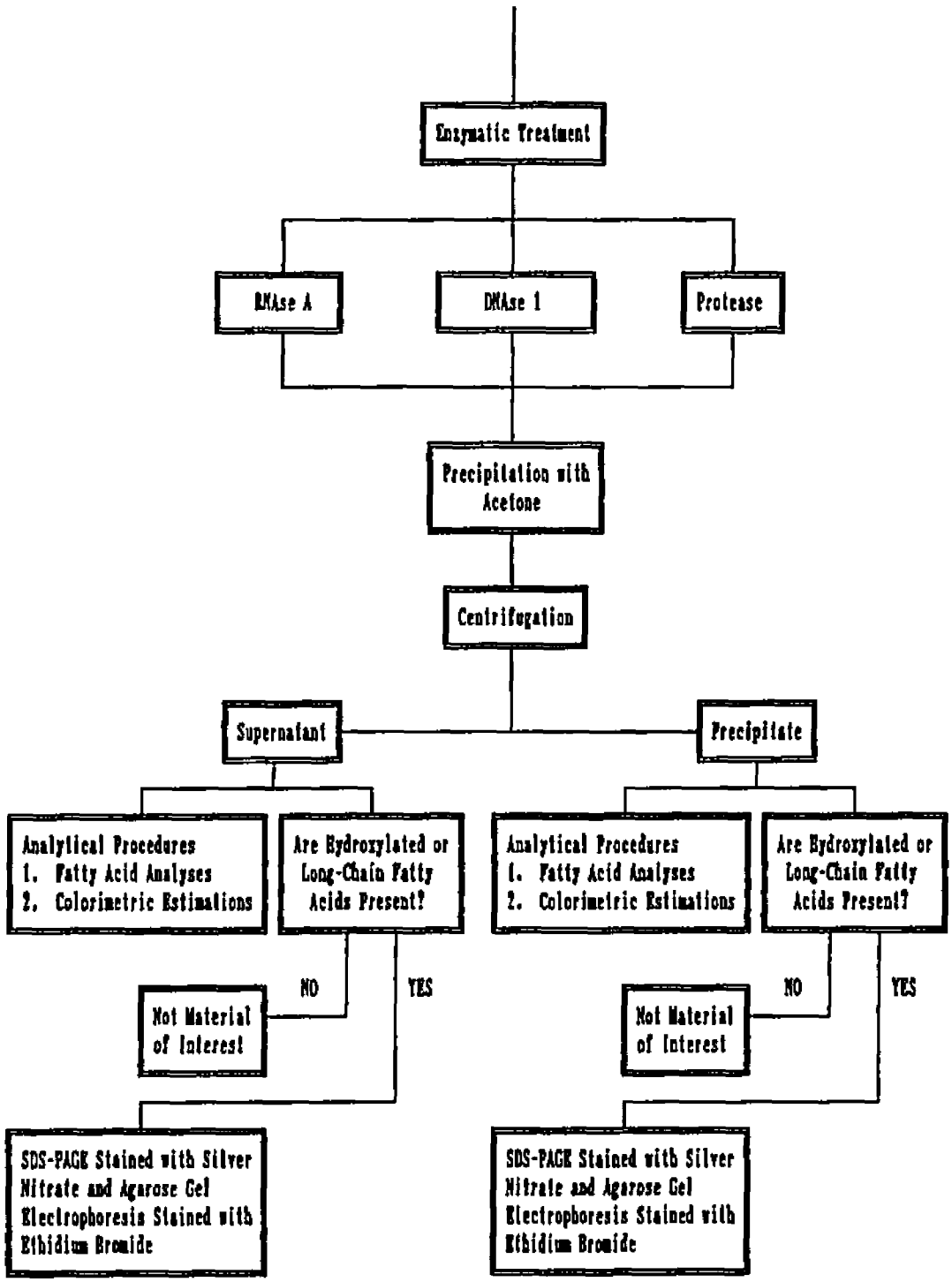


Figure 10. Enzymatic treatment and analyses of isolated material.

adjusted for the Relative Molar Response (RMR) of the individual components. As an example, the concentration of any component X is given by:

$$[X] = \frac{(Peak\ Area\ of\ X)/(RMR\ of\ X)}{(Peak\ Area\ of\ IS)/(RMR\ of\ IS)} * [IS]$$

Thin-Layer Chromatographic Separation of FAME. The dried FAME were suspended in a mixture of 1 ml chloroform and 1 ml petroleum ether. Each sample was streaked to TLC plates coated with 0.5 mm silica gel H. The plates were developed to 15 cm in chloroform solvent, air dried, and developed to 15 cm in a second solvent system of diethyl ether/hexane (60:40 [V/V]). Three bands were seen after the plates were sprayed with distilled water: nonhydroxylated fatty ester (nFAME) band at 12-15 cm, monohydroxylated fatty ester (hFAME) band at 7-10 cm, and dihydroxylated fatty ester (h_2FAME) band at 3-5 cm. Plates were allowed to air dry. Bands were scraped from the plates, eluted with chloroform/methanol (3:2 [V/V]), and the eluates dried on a vortex evaporator.

Lipid A Analysis

Weighed amounts of E. coli S-LPS, L. pneumophila LPS, and L. pneumophila material recovered from the aqueous phase

of the Folch extraction (by acetone precipitation and column chromatography) were subjected to 0.2 M acetic acid for 1 hour at 100°C (Wilkinson 1977) and also to 0.1 M acetate buffer, pH 4.4, for 1 hour at 100°C (Sonesson et al. 1989). After centrifugation, the supernatants were removed and the pellets were washed with water and then with acetone. Gas chromatographic analyses were conducted on both the supernatants and precipitates. Aliquots of each fraction were also subjected to SDS-PAGE analysis.

Electrophoresis

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). The Laemmli SDS-polyacrylamide gel electrophoresis system (1970) incorporating 0.1% SDS in a 12% acrylamide/bisacrylamide (29:1 [V/V]) separating gel (pH 8.8) with a 4% acrylamide/ bisacrylamide (29:1 [V/V]) stacking gel (pH 6.8) was used in a Mini-Slab vertical slab-gel apparatus (Idea Scientific Company, Minneapolis, MN). The size of the slab-gels were 10 x 15 x 0.077 cm. Samples in water (or 0.15 M phosphate buffer for enzymatically digested samples) were diluted in 4X SDS sample buffer (0.04 M Tris-HCl buffer, pH 6.8, containing 4% SDS [W/V], 40% glycerol [V/V], 20% 2-mercaptoethanol [V/V], and 0.02% bromophenol blue [W/V]) yielding a 1X preparation. The mixtures were heated at 100°C for 5 min then briefly centrifuged. Samples were applied to gel by approximately equal weights. Gels were subjected to discontinuous vertical electrophoresis for

approximately 800 volt-hours. Gels were removed from the system and the bands visualized by the silver stain of Tsai and Frasch (1981), the periodate-Schiff stain of Segrest and Jackson (1972), or the Coomassie Brilliant Blue stain (Ausubel et al. 1993). Gels were photographed on a clear glass plate using a Nikon F3 35 mm format camera mounted to a copy stand illuminated from the base and sides. Photographic film used was Tmax Professional 100 (ISO 100, process black and white, Eastman Kodak Company, Rochester, NY).

Agarose-Gel Electrophoresis. Samples were diluted in 10X loading buffer containing 20% Ficoll 400, 0.1 M Na_2EDTA, pH 8, 0.25% bromophenol blue, and 0.25% xylene cyanol yielding a 1X preparation. Approximately equal weights of each samples were then placed in wells formed in a horizontal mini-gel (1.4% agarose [W/V] in 0.5X TBE (8 mM Tris, 9 mM boric acid, 0.25 mM EDTA), 6 x 11 x 1 x cm) and the gel was electrophoresed for approximately 45 min at 100 volts in 0.5X TBE in a Mupid electrophoresis apparatus (E. Y. Laboratories, Inc., San Mateo, CA). The agarose gel was removed from the electrophoresis system and placed in 200 ml 0.5x TBE containing 25 µl of a 10 mg/ml ethidium bromide solution. The gel was stained for 15 min with gentle rocking. Bands were visualized on a UV transilluminator and photographed with Polaroid Type 55 film.

Enzymatic Treatment of Extracted Material

Material (that partitioned into the Folch aqueous fraction and subsequentially purified by column chromatography) was treated to enzymatic degradation in 0.15 M phosphate buffer (Bobo and Foster 1964). The extracts were treated with protease (from Streptomyces griseus, 0.2 Kunitz units) at pH 8.6, DNAse-1 (28 Kunitz units) at pH 5.0, and RNAse-A (4 Kunitz units) at pH 7.6 for 18 hrs at 37°C. Samples were then precipitated with 10 volumes of acetone. Supernatants and precipitates were analyzed for fatty acids as outlined in Analytical Methods.

Materials purified by column chromatography from the Folch aqueous fraction, as well as the 55°C methanol and water extractions of L. pneumophila cells were also treated to enzymatic digestion as outlined above, except that RNAse-A and DNAse-1 digestion were done concurrently at pH 6.75. After enzymatic digestion, aliquots of each sample (without being precipitated with acetone) were analyzed by SDS-PAGE. The RNAse/DNAse treated samples were also analyzed by agarose-gel electrophoresis.

Spectrophotometric Determinations

Protein Estimations. Aliquots of each derived fraction were suspended in 10.0 ml distilled water. Protein estimations were performed on each sample by the methods of Lowry et al. (1952) and Bradford (1976).

2-Keto-3-deoxyoctonate (KDO) Estimations. Aliquots of each sample to be tested for KDO content were analyzed by the method of Karkhanis et al. (1977).

Carbohydrate Estimation. Each aqueous fraction that resulted from acid hydrolysis was subjected to carbohydrate estimation by the phenol-sulfuric acid method (Ashwell 1966; Dubois et al. 1956).

Phosphate Estimation. Each aqueous fraction that resulted from acid hydrolysis was ashed to ensure complete mineralization of any organic phosphate. Estimations were then conducted by the method of Ames (1966).

Gas Chromatography/Mass Spectrometry (GC/MS)

Instrumentation. FAME, amino acid butyl esters, and carbohydrates were analyzed using a Hewlett-Packard 5840A Gas-Liquid Chromatograph (Palo Alto, CA) equipped with a flame ionization detector, electronic integration, and a 25 m nonpolar capillary column (UltraPhase-1, Hewlett-Packard, Avondale, PA). The instrument was operated in the splitless mode under temperature programmed conditions. GC/MS analyses were conducted using a Hewlett-Packard 5890 GC interfaced to a 5970 Mass Selective Detector. Injection was with a 20:1 split ratio and programmed temperature GC separations were done on a 10 m nonpolar capillary column (UltraPhase-1).

Derivatization. The hydroxylated FAME were analyzed as O-trimethylsilyl (TMS) and O-trifluoroacetyl (TFA) derivatives (Mayberry 1981, 1984a, 1984b). The nonhydroxylated FAME were not further derivatized. Samples were reconstituted in n-heptane for GC injections.

Aqueous phases derived from the acid hydrolysis of extraction-products (extracted by chloroform/methanol (2:1 ([V/V]) and purified by acetone precipitation and column chromatographic separation), LPS from L. pneumophila, and E. coli S-LPS were analyzed by gas-liquid chromatography (GLC) and gas chromatography/ mass spectrometry (GC/MS) for amino acid and carbohydrate composition. Amino acid butyl esters reconstituted in n-heptane were analyzed as O-trifluoroacetyl (TFA) derivatives (Gehrke et al. 1968). Carbohydrates, were converted to methyl glycosides and alditols. The methyl glycosides were analyzed as O-trimethylsilyl (TMS) derivatives. The alditols were analyzed as acetate (Gunner et al. 1961; Sawardeker et al. 1965), O-trimethylsilyl (TMS), and O-trifluoroacetyl (TFA) derivatives. Samples were reconstituted in n-heptane for GC injections.

Colorimetry. Measurements were made on a Bausch & Lomb Spectronic 20 at the wavelength appropriate to each method.

Infrared Spectroscopy. Infrared spectroscopy was performed with a Perkin-Elmer Infrared Spectrophotometer 710B, Perkin-Elmer Corporation, Norwalk, CT.

Computer Analysis. Data processing was performed with an AT&T PC 6300 (AT&T Information Systems, Inc., Iselin, NJ) operating under MSDOS 4.01 (Microsoft Corporation, Redmond, WA). Calculations concerning FAME were conducted with BASIC programs written to fulfill specific needs. Other programs used were WordPerfect 5.1 (WordPerfect Corporation, Orem, UT) for word processing, REFLEX 1.1 (Borland International, Inc., Scotts Valley, CA) for database management, QUATTRO (Borland) for spreadsheet applications, PSPlot (PolySoft, Salt Lake City, UT), and PlotIt (Scientific Programming Enterprises, Haslett, MI) for graphics.

CHAPTER 3

Results

Typical profiles of nonhydroxylated and hydroxylated fatty acid methyl esters (FAME) derived from whole cells of Legionella pneumophila are shown in Figure 11. Relative mobilities of the fatty acid methyl esters are expressed in Equivalent Chain Lengths (ECL), a system in which the relative retention times of saturated straight-chain nonhydroxylated fatty esters are set equal to the carbon atom content of the acid (e.g., n-C16:0 has an ECL of 16.0). For ease of comparison, concentrations are expressed in terms of Relative Abundance, with the highest component in each class set to 100.

Lipopolysaccharides typically demonstrate a heterodispersed banding pattern ("ladder-like") in SDS-PAGE systems. Figure 12 shows a polyacrylamide (12%) gel stained with silver nitrate. Legionella pneumophila extracts (crude and purified by acetone precipitation and column chromatography) are visualized along with E. coli S-LPS, E. coli Ra-LPS, E. coli Rc-LPS, and material identified as L. pneumophila LPS (Sonesson 1989).

The periodate-Schiff method stains vicinal hydroxyl groups and free aldehyde groups. The high carbohydrate content of lipopolysaccharides enable staining by the periodate-Schiff method. Figure 13 shows a polyacrylamide (12%) gel stained by the periodate-Schiff method.

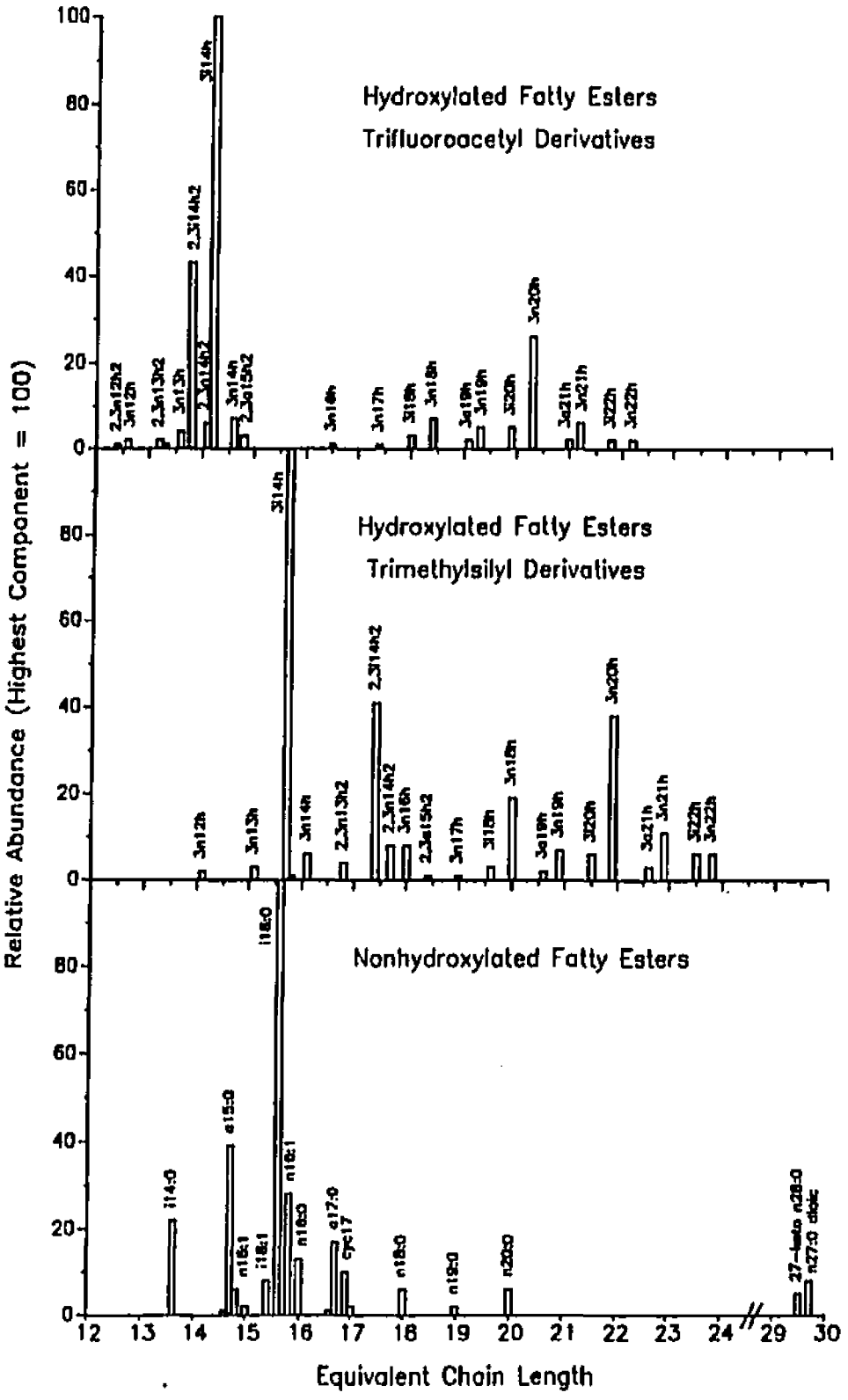


Figure 11. Typical gas chromatographic profiles of methyl esters of nonhydroxy and hydroxy fatty acids derived from whole cells of Legionella pneumophila.

Figure 12. SDS-PAGE (12.5% acrylamide) of silver-stained material from Legionella pneumophila.

Lanes:

A, E. coli S-LPS (6.1 µg);

B, E. coli Ra-LPS (5.0 µg);

C, E. coli Rc-LPS (5.0 µg);

D, L. pneumophila LPS (4.8 µg);

E, L. pneumophila LPS (9.6 µg);

F, L. pneumophila Folch aqueous (4.7 µg);

G, L. pneumophila Folch aqueous (9.3 µg);

H, L. pneumophila methanol (55°C) extract (6.5 µg);

I, L. pneumophila water (55°C) extract (7.1 µg);

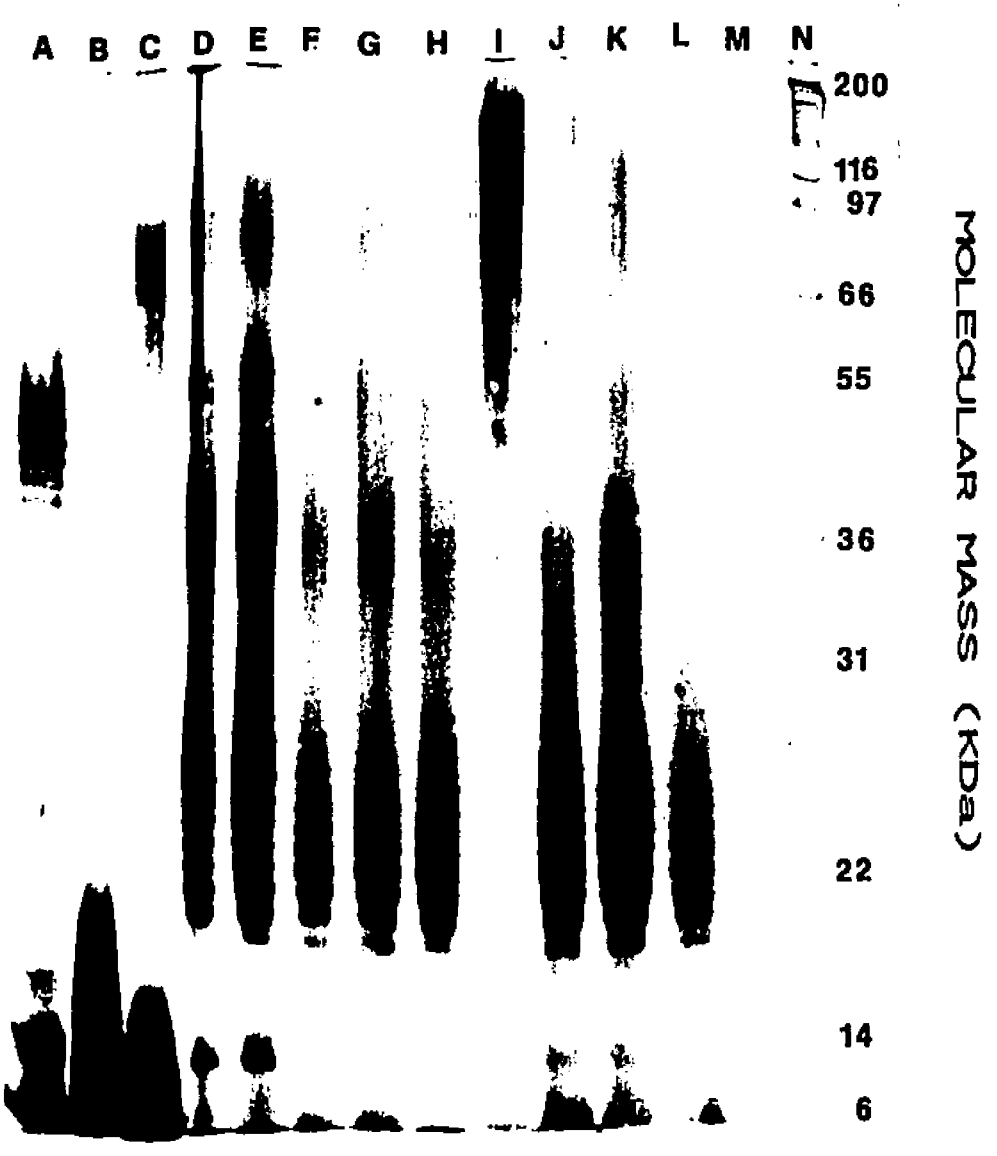
J, L. pneumophila Folch aqueous, column purified (5.9 µg);

K, L. pneumophila Folch aqueous, column purified (11.8 µg);

L, L. pneumophila methanol (55°C) extract, column purified (6.7 µg);

M, L. pneumophila water (55°C) extract, column purified (7.1 µg);

N, Protein molecular mass markers.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MOLECULAR MASS (KDa)

Figure 13. SDS-PAGE (12.5% acrylamide) of material from Legionella pneumophila stained by the periodate-Schiff method.

Lanes:

A, Blank;

B, Blank;

C, E. coli S-LPS (46 µg);

D, E. coli Ra-LPS (150 µg);

E, E. coli Rc-LPS (150 µg);

F, L. pneumophila LPS (144 µg);

G, L. pneumophila Folch aqueous (70 µg);

H, L. pneumophila methanol (55°C) extract (49 µg);

I, L. pneumophila water (55°C) extract (53 µg);

J, L. pneumophila Folch aqueous, column purified (88 µg);

K, L. pneumophila methanol (55°C) extract, column purified (50 µg);

L, L. pneumophila water (55°C) extract, column purified (35 µg);

M, Protein molecular mass markers;

N, Blank.

A B C D E F G H I J K L M N

Legionella pneumophila extracts (crude and purified by acetone precipitation and column chromatography) are visualized along with E. coli S-LPS, E. coli Ra-LPS, E. coli Rc-LPS, and material identified as L. pneumophila LPS (Sonesson 1989).

In order to visualize the protein composition of the various extracts, samples were separated by SDS-PAGE and stained by Coomassie Brilliant Blue. Figure 14 shows a polyacrylamide (12%) gel stained with Coomassie Brilliant Blue. Legionella pneumophila extracts (crude and purified by acetone precipitation and column chromatography) are visualized along with E. coli S-LPS, E. coli Ra-LPS, E. coli Rc-LPS, and material identified as L. pneumophila LPS (Sonesson 1989).

To determine the effects of protease treatment on the various extracts, samples were enzymatically treated overnight at 37°C. An aliquot of each sample was subjected to SDS-PAGE. Figure 15 shows the polyacrylamide (12%) gel stained with silver nitrate. Legionella pneumophila extracts (purified by acetone precipitation and column chromatography) are visualized with and without protease digestion.

The effects of nuclease treatment on the various extracts also were determined. Samples were enzymatically treated overnight at 37°C. An aliquot of each sample was

Figure 14. SDS-PAGE (12.5% acrylamide) of Coomassie Brilliant Blue-stained material from *Legionella pneumophila*.

Lanes:

A, *E. coli* S-LPS (19.7 µg);

B, *E. coli* Ra-LPS (65.0 µg);

C, *L. pneumophila* LPS (62.3 µg);

D, *L. pneumophila* Folch aqueous (30.2 µg);

E, *L. pneumophila* methanol (55°C) extract (21.1 µg);

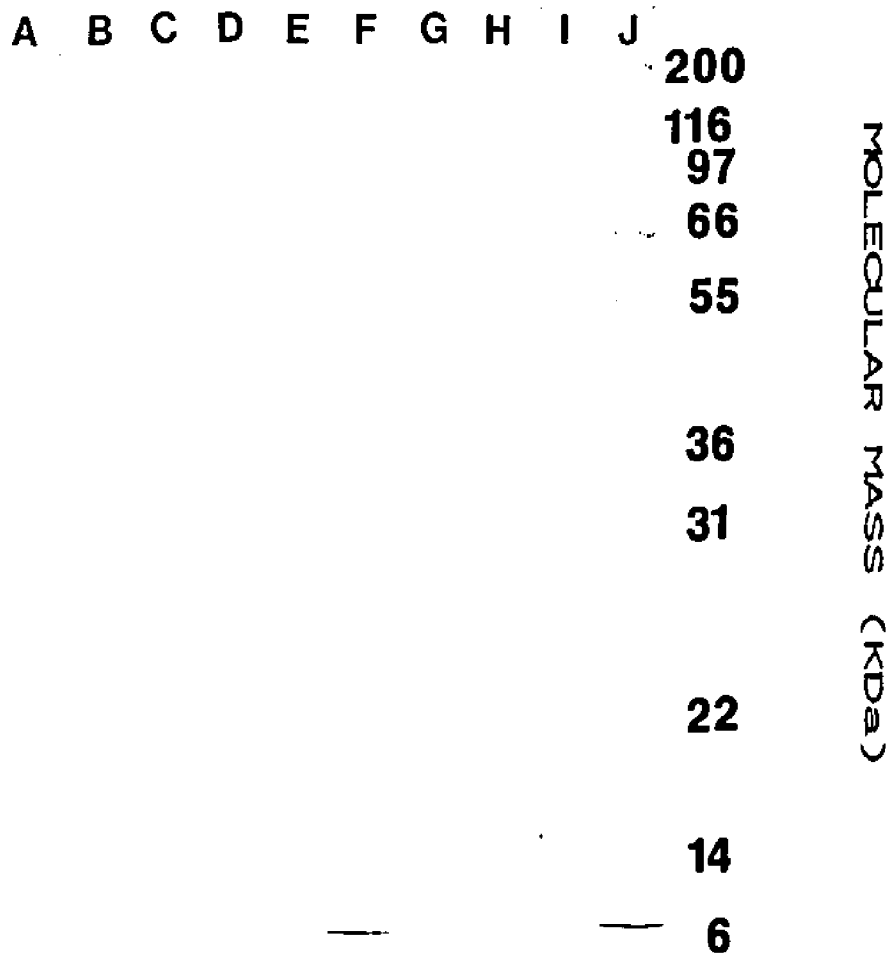
F, *L. pneumophila* water (55°C) extract (23.1 µg);

G, *L. pneumophila* Folch aqueous, column purified (38.4 µg);

H, *L. pneumophila* methanol (55°C) extract, column purified (21.7 µg);

I, *L. pneumophila* water (55°C) extract, column purified (15.3 µg);

J, Protein molecular mass markers.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MOLECULAR MASS (KDa)

Figure 15. SDS-PAGE (12.5% acrylamide) of protease-treated material from Legionella pneumophila. The gel was silver-stained.

Lanes:

A, Blank;

B, Bovine serum albumin (3.6 µg);

C, Protease (3.0 µg);

D, Bovine serum albumin (3.6 µg) and protease (1.4 µg);

E, Protein molecular mass markers;

F, L. pneumophila methanol (55°C) extract, column purified (2.5 µg);

G, L. pneumophila methanol (55°C) extract, column purified (2.5 µg) and protease (3.0 µg);

H, L. pneumophila water (55°C) extract, column purified (1.8 µg);

I, L. pneumophila water (55°C) extract, column purified (1.8 µg) and protease (3.0 µg);

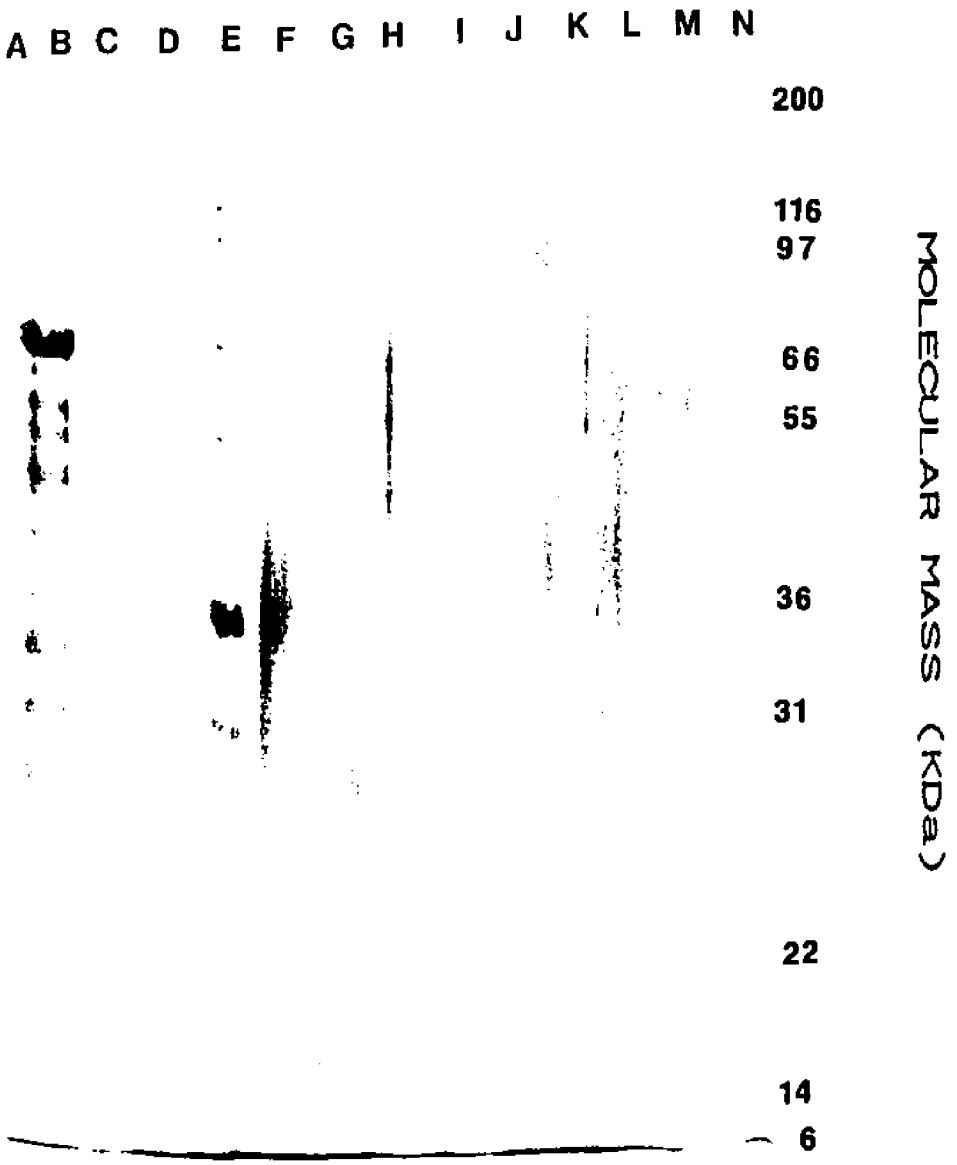
J, L. pneumophila Folch aqueous, column purified (4.4 µg);

K, L. pneumophila Folch aqueous, column purified (4.4 µg) and protease (3.0 µg);

L, L. pneumophila LPS (3.6 µg);

M, Blank;

N, Blank.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MOLECULAR MASS (KDa)

subjected to SDS-PAGE. Figure 16 shows a polyacrylamide (12%) gel stained with silver nitrate. Legionella pneumophila extracts (purified by acetone precipitation and column chromatography) are visualized with and without combined DNAse-1 and RNAse-A digestion.

An aliquot of each nuclease-treated sample also was applied to an agarose (1.4%) gel. After electrophoresis, the gel was stained with ethidium bromide. By such treatment, any nucleic acids contained in the sample should be visible. In Figure 17, Legionella pneumophila extracts (purified by acetone precipitation and column chromatography) are visualized with and without combined DNAse-1 and RNAse-A digestion.

Hydroxylated fatty acids are detectable in the Folch-extracted cell pellet of L. pneumophila. It is quite possible that the hydroxylated fatty acids are linked to nonextractable LPS macromolecules. Figure 18 shows a polyacrylamide (12%) gel stained with silver nitrate. Legionella pneumophila Folch aqueous extracts (purified by acetone precipitation and column chromatography) are visualized along with the debris left after the Folch extraction (and subsequentially extracted with methanol at 55°C). The supernatant and particulate matter that results from boiling L. pneumophila cells in 4% SDS for 4 hrs is also visualized. Material is shown with and without protease treatment.

Figure 16. SDS-PAGE of DNAse-1 and RNAse-A treated material from Legionella pneumophila. The gel (12.5% acrylamide) was stained with silver nitrate.

Lanes:

A, DNA (12.2 µg) and RNA (13.4 µg);

B, DNAse (3.5 µg) and RNAse (2.7 µg);

C, DNA (12.2 µg), RNA (13.4 µg), DNAse (3.2 µg) and RNAse (2.5 µg);

D, Protein molecular mass markers;

E, L. pneumophila methanol (55°C) extract, column purified (8.4 µg);

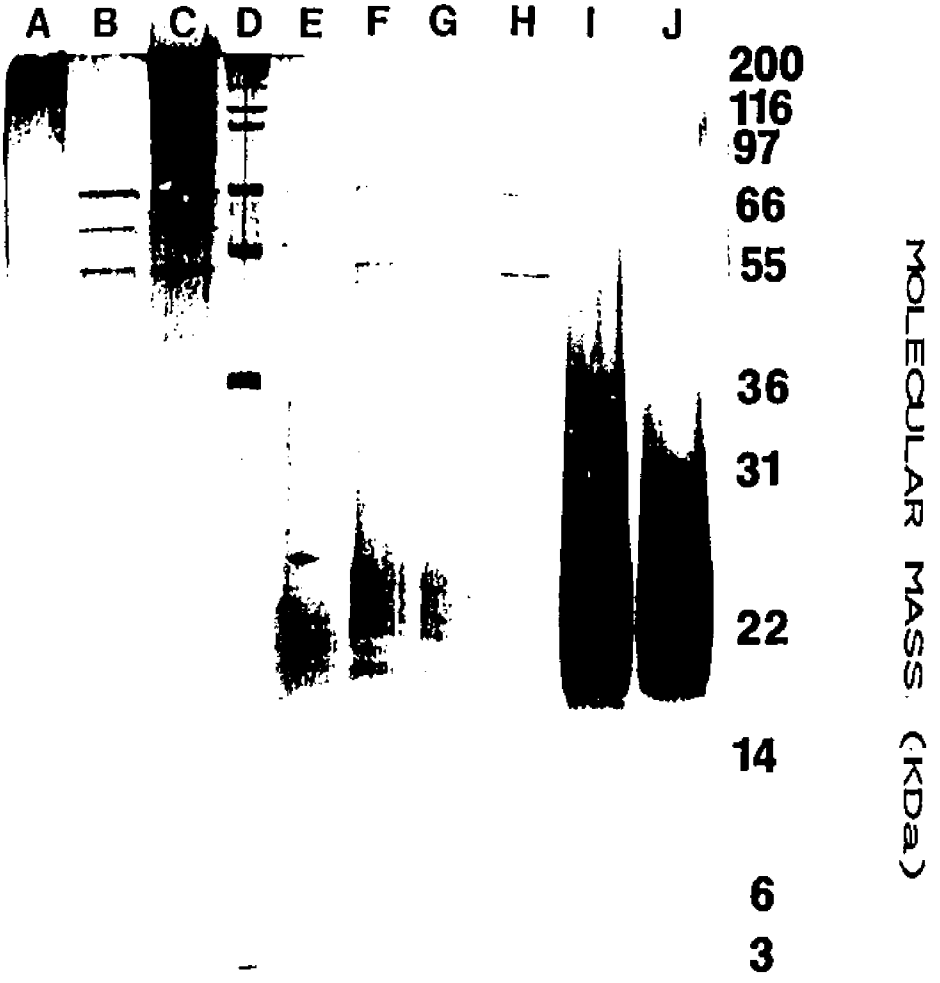
F, L. pneumophila methanol (55°C) extract, column purified (8.4 µg), DNAse (3.5 µg) and RNAse (2.7 µg);

G, L. pneumophila water (55°C) extract, column purified (5.9 µg);

H, L. pneumophila water (55°C) extract, column purified (5.9 µg), DNAse (3.5 µg) and RNAse (2.7 µg);

I, L. pneumophila Folch aqueous, column purified (14.8 µg);

J, L. pneumophila Folch aqueous, column purified (14.8 µg), DNAse (3.5 µg) and RNAse (2.7 µg).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MOLECULAR MASS (KDa)

Figure 17. Agarose-gel electrophoresis of DNAse-1 and RNAse-A treated material from Legionella pneumophila. The gel (1.4% agarose) was stained with ethidium bromide.

Lanes:

A, Blank;

B, Blank;

C, DNA (9.8 µg) and RNA (10.7 µg);

D, DNAse (2.8 µg) and RNAse (2.2 µg);

E, DNA (9.8 µg), RNA (10.7 µg), DNAse (2.6 µg) and RNAse (2.0 µg);

F, Lambda-BST (DNA Molecular Mass markers);

G, L. pneumophila methanol (55°C) extract, column purified (6.7 µg);

H, L. pneumophila methanol (55°C) extract, column purified (6.7 µg), DNAse (2.8 µg) and RNAse (2.2 µg);

I, L. pneumophila water (55°C) extract, column purified (4.7 µg);

J, L. pneumophila water (55°C) extract, column purified (4.7 µg), DNAse (2.8 µg) and RNAse (2.2 µg);

K, L. pneumophila Folch aqueous, column purified (11.8 µg);

L, L. pneumophila Folch aqueous, column purified (11.8 µg), DNAse (2.8 µg) and RNAse (2.2 µg);

M-Q, Blank.

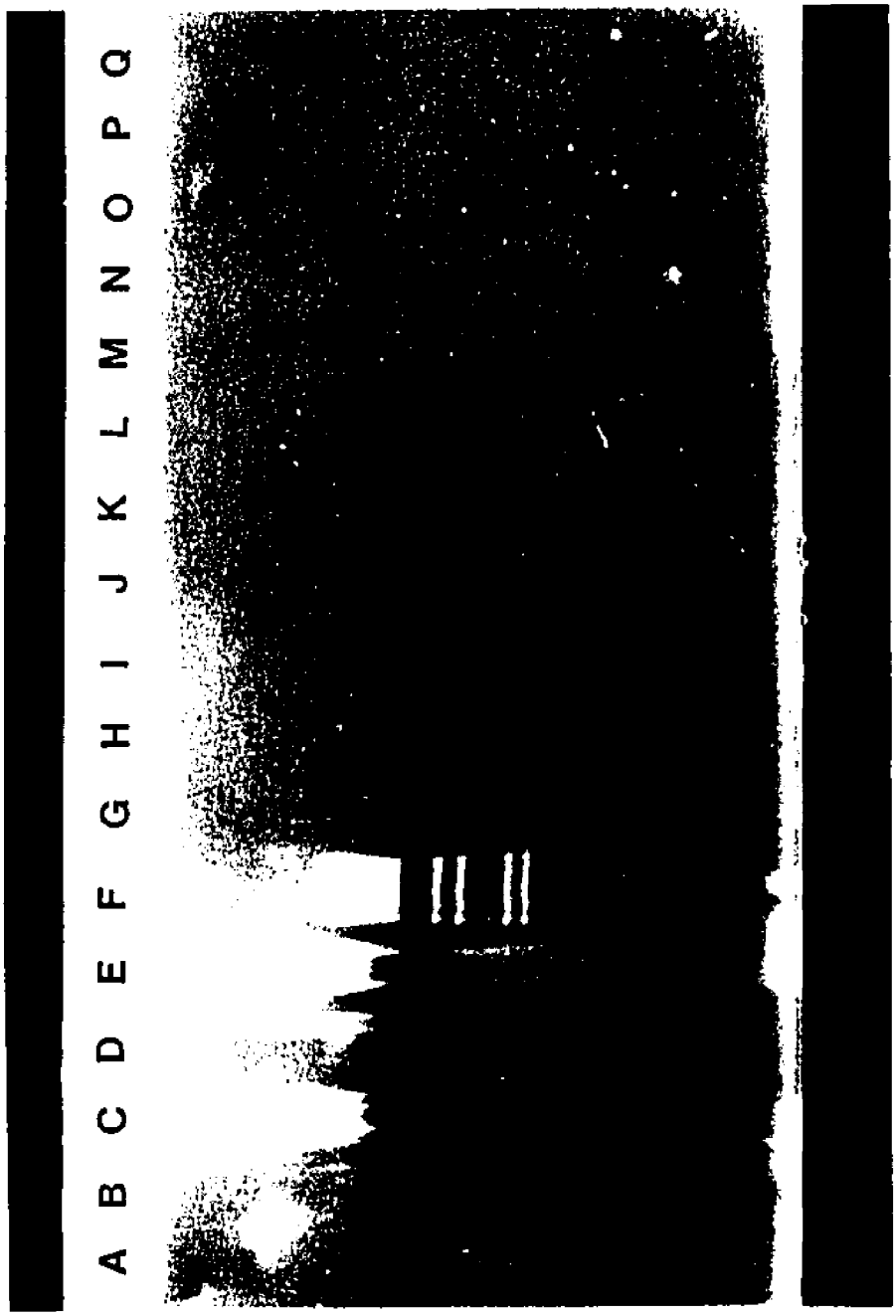
A B C D E F G H I J K L M N O P Q

Figure 18. SDS-PAGE (12.5% acrylamide) of silver-stained material from Legionella pneumophila.

Lanes:

A, Blank;

B, Protein molecular mass markers;

C, Folch aqueous, column purified (4.2 µg);

D, Folch aqueous, column purified (12.7 µg);

E, Folch and methanol extracted pellet (7.18 µg) and protease (0.1 µg);

F, Folch and methanol extracted pellet (21.5 µg) and protease (0.4 µg);

G, Folch and methanol extracted pellet (8.2 µg);

H, Folch and methanol extracted pellet (24.6 µg);

I, Pellet, after boiling in 4% SDS (3.1 µg) and protease (0.1 µg);

J, Pellet, after boiling in 4% SDS (9.3 µg) and protease (0.2 µg);

K, Pellet, after boiling in 4% SDS (3.1 µg);

L, Pellet, after boiling in 4% SDS (9.3 µg);

M, Supernatant, after boiling in 4% SDS (22.8 µg);

N, Supernatant, after boiling in 4% SDS (68.3 µg).

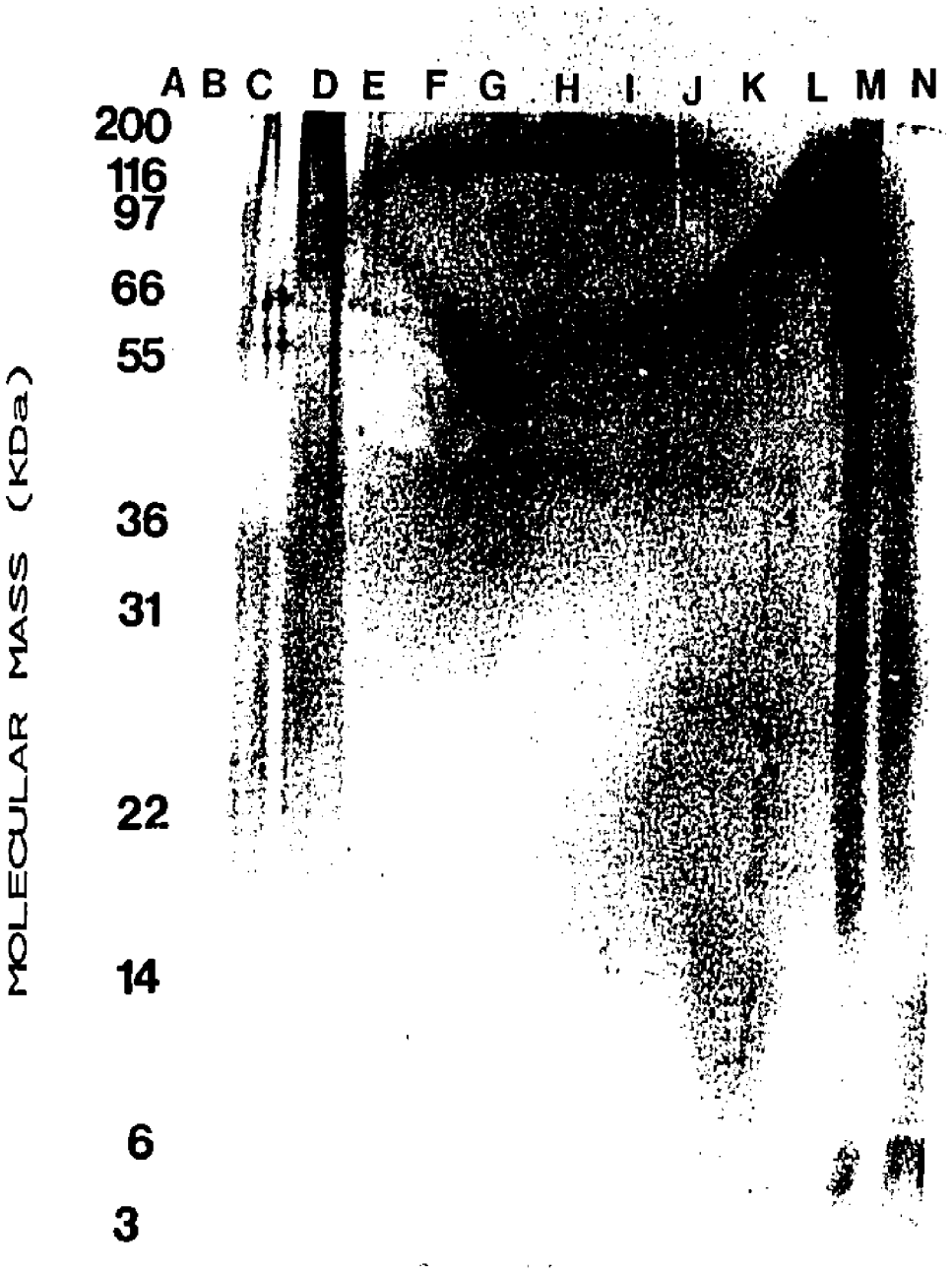
A B C D E F G H I J K L M N
200
116
97
66
55
36
31
22
14
6
3
MOLECULAR MASS (KDa)

Figure 19 shows a polyacrylamide (12%) gel stained with silver nitrate. Legionella pneumophila Folch aqueous material (purified by acetone precipitation and column chromatography) is visualized along with the debris left after the Folch extraction (and subsequentially extracted with methanol at 55°C). The two debris samples were as follows: 1, Incubated overnight at 37°C in 0.15 M phosphate buffer (pH 8.6) and subsequentially solubilized in 1% SDS; 2, Incubated overnight at 37°C in 0.15 M phosphate buffer (pH 8.6) in the presence of protease and subsequentially solubilized in 1% SDS. Hydrolysis products (precipitates and supernatant solutions) from treatment in acetic acid (0.2 M, 100°C, 1 hr) or acetate buffer (0.1 M, pH 4.4, 100°C, 1 hr) of Legionella pneumophila Folch aqueous materials (purified by acetone precipitation and column chromatography) are visualized along with E. coli S-LPS, and material identified as L. pneumophila LPS (Sonesson 1989) are also visualized.

Table 3 shows the distribution of protein, carbohydrate, phosphate, and the fatty acids in fractions derived from Folch extraction, methanol extraction (at both 55°C and 100°C), and water extraction (at both 55°C and 100°C) of Legionella pneumophila lyophilized cells. Each value is reported as amount per gram starting material (unextracted, dried whole cells).

Figure 19. SDS-PAGE (12.5% acrylamide) of silver-stained material from Legionella pneumophila.

Lanes:

A, Blank;

B, Blank;

C, Bovine serum albumin (3.6 µg);

D, Bovine serum albumin (3.6 µg) and protease (1.5 µg);

E, Folch aqueous, column purified (4.4 µg);

F, Folch aqueous, column purified (4.4 µg) and protease (1.5 µg);

G, Folch and methanol extracted pellet in 1% SDS (78.4 µg);

H, Folch and methanol extracted pellet in 1% SDS (89.6 µg) and protease (1.4 µg);

I, Folch aqueous, column purified, acetate buffer precipitate in 2% SDS;

J, Folch aqueous, column purified, acetate buffer supernatant;

K, Folch aqueous, column purified, acetic acid precipitate in 2% SDS;

L, Folch aqueous, column purified, acetic acid supernatant;

M, L. pneumophila LPS, acetic acid precipitate in 2% SDS;

N, L. pneumophila LPS, acetic acid supernatant;

O, E. coli S-LPS, acetic acid precipitate in 2% SDS;

P, E. coli S-LPS, acetic acid supernatant;

Q, Protein molecular mass markers.

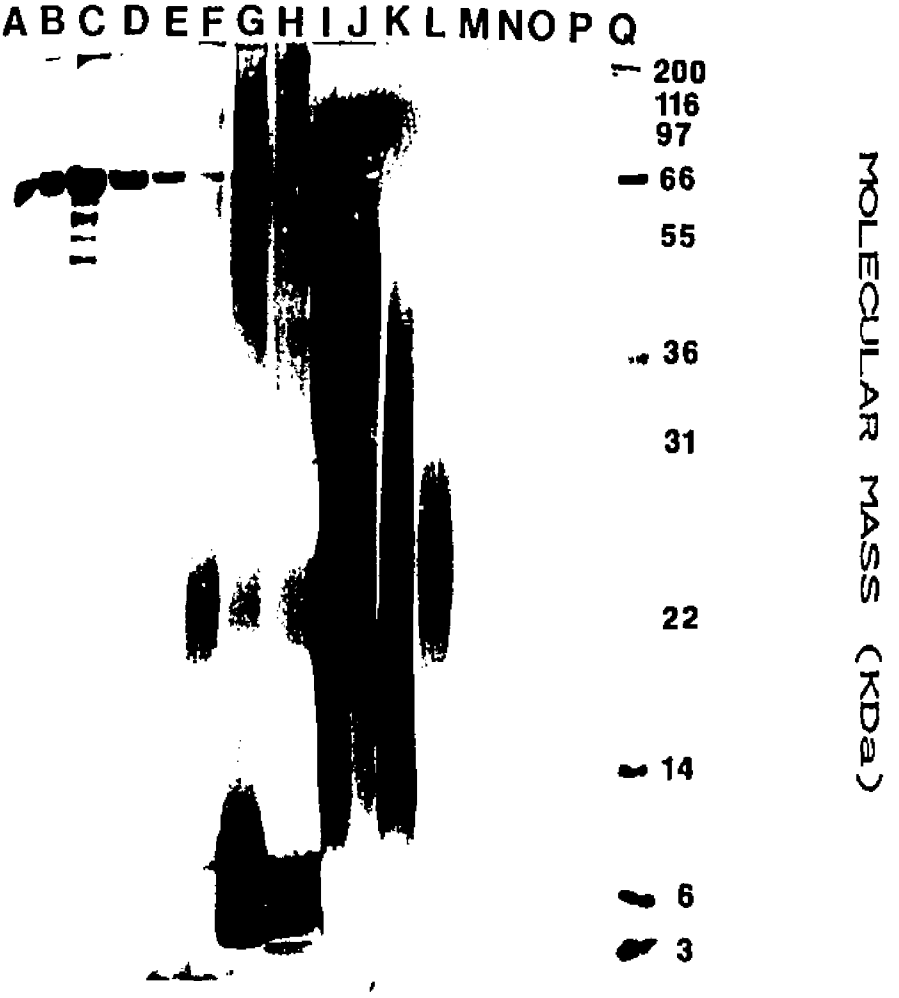
A B C D E F G H I J K L M N O P Q
200
116
97
66
55
36
31
22
14
6
3
MOLECULAR MASS (KDa)

Table 3. Distributions of major biochemical classes in fractions that result from extractions of *Legionella pneumophila*

	Expressed as Amount per gram Starting Material							
	Bradford Protein mg/g	Lowry Protein mg/g	Phenol-SO4 Carbohydrate µmole/g	Ames Phosphate µmole/g	Nonhydroxy FAME µmole/g	Monohydroxy FAME µmole/g	Dihydroxy FAME µmole/g	Total FAME µmole/g
STARTING MATERIAL								
Dried Cells	216	450	104	656	172	16	10	198
FOLCH EXTRACTION								
24°C								
Aqueous Fraction	3	4	14	19	25	5	2	32
Organic Fraction	2	0	2	30	103	3	0	106
Extracted Pellet	387	670	84	298	40	5	2	47
METHANOL EXTRACTIONS								
55°C								
Acetone Precipitate	8	12	12	59	20	4	2	26
Acetone Supernatant	1	54	0	36	134	2	1	137
Extracted Pellet	130	793	103	249	18	1	1	20
100°C								
Acetone Precipitate	0	37	18	57	7	1	1	9
Acetone Supernatant	2	81	2	42	137	2	0	139
Extracted Pellet	90	1038	83	269	19	8	3	30
WATER EXTRACTIONS								
55°C								
Acetone Precipitate	20	328	524	976	5	0	0	5
Acetone Supernatant	1	12	505	95	13	0	0	13
Extracted Pellet	386	596	1184	1224	168	9	1	178
100°C								
Acetone Precipitate	10	34	1097	1405	3	0	0	3
Acetone Supernatant	2	17	295	81	3	0	0	3
Extracted Pellet	317	573	706	436	183	10	2	195

After lipid analyses, it was found that the methanol fraction from column chromatographic separations contained the majority of the hydroxylated fatty acid containing material. The chloroform and acetone fractions did not contain any hydroxylated fatty acids. Trace amounts of hydroxylated fatty acids could be recovered when the silicic acid column was hydrolyzed.

Table 4 lists biochemical compositions of purified (after acetone precipitation and column chromatographic separation), hydroxylated fatty acid containing extracts from Legionella pneumophila. Samples were collected in the methanol eluates from the column chromatographic separations of crude extracts (acetone precipitates). The biochemical composition of the particulate cell debris (pellet) that remains after the Folch extraction of whole cells (and subsequentially extracted with methanol at 55°C) is also listed. Each value is reported as amount per gram of fraction weight.

Table 5 lists a comparison of Escherichia coli smooth lipopolysaccharide (S-LPS), material reported to be LPS from Legionella pneumophila (Sonesson et al. 1989), and L. pneumophila LPS-like material (purified material from the Folch aqueous phase). It was previously determined that E. coli S-LPS was not extractable with chloroform/ methanol (2:1 [V/V]) (Lane 1990). Solubility values for the E. coli S-LPS were determined from commercially obtained,

Table 4. Distributions of major biochemical classes in LPS-like material resulting from extractions of *Legionella pneumophila*.

	Bradford Protein mg/g	Lowry Protein mg/g	Phenol-SO4 Carbohydrate µmole/g	Ames Phosphate µmole/g	KDO mg/g	Nonhydroxy FAME µmole/g	Monohydroxy FAME µmole/g	Dihydroxy FAME µmole/g	Total FAME µmole/g
Folch Aqueous[a]	286	94	254	321	26	220	49	20	289
Water Extract[a]	11	552	196	2063	3	80	15	0	95
Methanol Extract[a]	327	100	211	887	29	359	83	28	470
Pellet after Folch then Methanol Extraction	582	903	49	308	3	14	4	1	19

[a]Purified by acetone precipitation and column chromatography

Table 5. A comparison of *E. coli* LPS[a], *L. pneumophila* LPS[b], and *Legionella pneumophila* LPS-like material[c]

	E. coli S-LPS	*L. pneumophila* LPS	*L. pneumophila* LPS-like material
Chloroform/Methanol 2:1 [V/V] Extractable	No[d]	—	Yes
Demonstrates "Ladder Banding" in SDS-PAGE	Yes	Yes, but the repeating units are approximately ½ the size of the units in *E. coli* S-LPS	Yes, but the repeating units are approximately ½ the size of the units in *E. coli* LPS
Soluble in Water	Yes	No	No
Soluble in Methanol	No	Yes	Yes
Soluble in Chloroform	No	No	No
Soluble in Acetone	No	No	No
Predominant Hydroxylated Fatty Acids (Percentage of Total LPS Fatty Acids)	3n14h (60)[e]	3i14h (11.1) 2,3i14h$_2$ (7.7)	3i14h (6.9) 2,3i14h$_2$ (6.9)
Predominant Nonhydroxylated Fatty Acids (Percentage of Total LPS Fatty Acids)	n12:0 (16)[e] n14:0 (20) n16:0 (4)	i16:0 (28.8) a15:0 (7.4) i14:0 (4.6)	i16:0 (25.5) n16:0 (9.8) n27:Odioic (7.2)
Percent Fatty Acid by Weight	25.6[f]	Nonhydroxy 9.7 Monohydroxy 8.6 Dihydroxy 1.6 Total 19.9	Nonhydroxy 6.6 Monohydroxy 1.5 Dihydroxy 0.6 Total 8.7
Hydroxylated Fatty Acid Linkage	Amide[f]	Amide suggested	Amide
Predominant Sugars (Percentage of Total LPS Weight)	Fucose (12.8)[f] Glucose (13.0) Galactose (8.7) Heptose (7.2)	Glucosamine (6.9) Glycerol (5.3)	See footnote[h]
Percent Carbohydrate by Weight	Neutral (52.6)[f] Amino (18.9)	Neutral (9.3) Amino (8.8)	4.6[i]
Percent KDO by Weight	5.9[f]	2.8	2.6
Percent Phosphate by Weight	2.5[f]	Not Reported	3.1
Percent of Whole Cell Weight	3.4[g]	1-4	6.4

[a]Extracted by the method of Westphal et al., solubilities determined by author from commercially obtained S-LPS,
[b]Sonesson et al. 1989,
[c]Aqueous fraction after extraction by method of Folch et al., purified by acetone precipitation and column chromatography,
[d]Lane 1990,
[e]Bryn and Rietschel 1978,
[f]Koga et al. 1985,
[g]Riedhardt et al. 1987,
[h]Sugars were not detectable under chromatographic conditions employed during this study,
[i]Reported percentage estimated by the phenol-sulfuric method.

phenol-extracted (Westphal et al. 1955) LPS. All other values for E. coli S-LPS were compiled from published reports (Bryn and Rietschel 1978; Koga et al. 1985; Niedhardt et al. 1987). Values for the L. pneumophila LPS were taken from those reported by Sonesson et al. (1989). SDS-PAGE banding patterns and solubilities for the L. pneumophila LPS were determined during the present study. Values for the L. pneumophila LPS-like material were generated from the presented research. L. pneumophila LPS-like material was purified, by acetone precipitation and column chromatographic separation, from the aqueous fraction of Folch extracted whole cells.

CHAPTER 4

Discussion

Preliminary research showed that extraction, by the Folch method, of the lipids of L. pneumophila yielded phases unlike those produced from other Gram-negative bacteria (Lane 1990). A viscous interface formed between the aqueous (wash) and organic phases. More than half of the hydroxylated fatty acids and unique, long-chain fatty acids were found distributed between the aqueous phase and interface material, fractions in which such constituents have not been reported in other Gram-negative species.

It was expected that hydrophilic cellular components bearing hydroxylated fatty acids could be isolated from L. pneumophila. The cellular lipids are normally expected to extract into nonpolar solvents. In L. pneumophila, a portion of the fatty acids can be extracted into relatively polar solvents. This finding tends to suggest that the fatty acids are linked to hydrophilic moieties. Furthermore, spectrophotometric estimations performed on the aqueous fraction from the Folch extraction indicate the presence of carbohydrate, phosphate, and protein.

The extraction of whole cells of L. pneumophila, E. coli, and P. aeruginosa by the method of Westphal et al. resulted in similar distributions of measured components among the fractions for each organism (Lane 1990).

Hydroxylated fatty acids were found in the aqueous phase where LPS is reported to be present (Westphal et al. 1955). This did not necessarily prove that the hydroxylated fatty acids of L. pneumophila were LPS-linked, but it did suggest that, at least in part, they track along with LPS as isolated by the method of Westphal et al. High concentrations of hydroxylated fatty acids were also detected in the phenol phase after the Westphal extraction of L. pneumophila cells. It has been suggested (Conlan and Ashworth 1985; Nolte et al. 1986; Petitjean et al. 1990) that the LPS from L. pneumophila is more lipophilic than the LPS from other Gram-negative bacteria. In preliminary research (Lane 1990), the concentrations of Bradford-detected protein and carbohydrate were reduced in the phenol phases from the extraction of the delipidized L. pneumophila pellets as compared to the whole cell extracts. A possible explanation is that the material found in the phenol phase from Galanos-extracted whole cells is related to the material found in the aqueous phase of Folch-extracted whole cells. In L. pneumophila, almost all of the carbohydrate and phosphate could be found in the phenol phase after the delipidized pellet was subjected to the Galanos extraction, instead of in the pellet, as occurred in E. coli and P. aeruginosa.

During this study, cells of Legionella pneumophila were extracted with chloroform/methanol by the method of

Folch et al. (1957). Cells were also extracted with water and with methanol. It was not unreasonable to expect polar cell components and associated fatty acids to extract into water and into methanol since fatty acids, carbohydrates, phosphates, and proteins have been found in the aqueous wash of the Folch system (Lane 1990).

The aqueous and interfacial phases from the chloroform/methanol (2:1 [V/V]) extraction of dried cells of L. pneumophila were pooled and dried under reduced pressure. In attempts to reconstitute the material in organic solvents, it was observed that the material would dissolve in methanol or chloroform/methanol (2:1 [V/V]). When acetone was added to the dried aqueous extract, the material turned white and would not dissolve. It was further observed that after the material was dissolved in methanol or chloroform/methanol (2:1 [V/V]), the addition of acetone would create a white, flocculent precipitate. Analyses showed that the supernatant contained fatty acids that were nonhydroxylated and the precipitate contained both nonhydroxylated and hydroxylated fatty acids. It was decided that acetone precipitation would be a powerful tool for both the concentration and the purification of moieties bearing hydroxylated fatty acids.

Since hydroxylated fatty acids can be found in the polar fraction of the Folch extraction method, it was believed that a polar extraction procedure could be

developed to allow more convenient isolation of material bearing hydroxylated fatty acids.

Dried cells of L. pneumophila were extracted with water. The reasoning was that since hydroxylated fatty acids could be found in the aqueous fraction from the Folch extraction system, water might be a solvent into which material bearing hydroxylated fatty acids would extract. Extractions were performed at both 55°C and 100°C for 18 hrs. Cell debris was removed by filtration The filtrate was reduced in volume by evaporation with heat under reduced pressure. Acetone (10-fold) was added to the concentrated extract. A white precipitate was collected after centrifugation. It was observed that the extraction at 55°C produced a low yield of precipitate while the extraction at 100°C produced an almost negligible amount of precipitate. Although the 55°C extraction provided a greater yield of material, most of the hydroxylated fatty acids could be recovered in the extracted pellet (for both the 55°C and 100°C extractions).

When dried cells of L. pneumophila were extracted with methanol at 55°C and 100°C, the 55°C extraction yielded more acetone precipitable material than the 100°C extraction (Table 3). Fatty acid analyses indicated that the 55°C extract contained more hydroxylated fatty acids than the extracted pellet. In contrast, the extracted pellet contained most of the hydroxylated fatty acids when

extractions were performed at 100°C. The methanol extracts contained high concentrations of both phosphate and nonhydroxylated fatty acids. These compounds indicate the the presence of extractable phospholipids. During the methanol extractions, cellular lipids are collected with the LPS-like material. In a Folch extraction, the LPS-like material is separated from the extractable lipids by partitioning the organic solvents with water.

It is curious that extractions at 55°C yield better results than those at 100°C. This result suggests that the moieties that bear hydroxylated fatty acids are in some manner sensitive to heat. It is quite possible that the higher temperature may cause the material to "clump" or coagulate.

Estimations of fatty acid, carbohydrate, phosphate, and protein content were performed on each extract. The extracts were then reduced in volume by evaporation and material precipitated by the addition of acetone. Again, estimations of fatty acid, carbohydrate, phosphate, and protein content were performed on each supernatant and precipitate.

All fractions that contained more than trace quantities of hydroxylated or long-chain fatty acids were examined by gel electrophoresis. Individual gels were stained by silver nitrate (shows lipopolysaccharides and proteins), periodate-Schiff (specific for vicinal hydroxyl groups and aldehyde

groups), and Coomassie Brilliant Blue (specific for protein).

SDS-PAGE is not directly applicable for determinations of the molecular weights of polysaccharides or glycoproteins. These macromolecules have a decreased binding of SDS as compared with standard proteins (Segrest and Johnson 1972). The lower SDS binding results in a decreased charge-to-mass ratio for glycosylated compounds versus standard proteins, a decreased mobility during SDS-PAGE, and thus a higher apparent molecular weight. However, with increased polyacrylamide gel cross-linking, molecular sieving dominates the charge-to-mass ratio in separating the compounds (Segrest and Johnson 1972) and values close to the real apparent molecular weights are approached.

Material isolated from L. pneumophila (Figure 12, Lanes D-M) is distinctly different from E. coli S-LPS (Figure 12, Lane A), Ra-LPS (Figure 12, Lane B), and Rc-LPS (Figure 12, Lane C). The banding patterns of the L. pneumophila extracts are tighter, with approximately two bands per each band in E. coli S-LPS, and visible in a lower molecular mass range (approximately 14-36 kDa, intensely stained bands 14-31 kDa) than the E. coli S-LPS (approximately 6-66 kDa, intensely stained bands 36-66 kDa). Materials isolated from L. pneumophila by both the Folch extraction and methanol extraction appear identical to the L. pneumophila LPS. A banding pattern was not evident in the water extracts.

Purification, by acetone precipitation and column chromatography, of the Folch aqueous fraction and methanol extract appears to remove a portion of the material that creates a "tailing" effect in the higher molecular mass range.

The periodate-Schiff staining method is specific for vicinal hydroxyl groups and aldehydes. After treatment by the periodate-Schiff method, it was observed that material isolated from L. pneumophila (Figure 13, Lanes F-L) stains differently than E. coli S-LPS (Figure 13, Lane C), Ra-LPS (Figure 13, Lane D), and Rc-LPS (Figure 13, Lane E). E. coli LPS stains intensely in concentrated areas. In contrast, L. pneumophila extracts stain weakly over a greater area. If the composition of the L. pneumophila extracts includes high quantities of amino sugars, the PAS stain would not intensely stain the material.

The LPS provided by Dr. Sonesson contained material that stained with Coomassie Brilliant Blue (Figure 14, Lane C). The stained area overlapped a portion of the area (LPS) that also stains with silver nitrate. However, the entire area which stains with silver nitrate was not stained by Coomassie Brilliant Blue. This could indicate contamination, by proteins, of the LPS. It is interesting to note that Gabay and Horwitz (1985) found only one protein contaminating L. pneumophila LPS (extracted by method of Darveau and Hancock (1983), pronase treated). The protein

concentration, as measured by the Lowry assay, was approximately 2% by weight. This protein had an apparent molecular mass of 28 kDa and was designated as the major outer membrane protein (MOMP).

It is also interesting that LPS could not be found in hot phenol/water extracts of Cytophaga johnsonae. Researchers have instead found six glycoproteins in C. johnsonae that possess LPS-like immunological properties (De Jong et al. 1991). Dihydroxylated fatty acids have been found only in certain members of the genera Legionella (Mayberry 1981, 1984a, 1984b) and Cytophaga (Abbanat et al. 1988). Nonhydroxylated fatty acids were found in the glycoprotein preparations, but no hydroxylated fatty acids were found. The method of fatty acid analysis was not reported (De Jong 1991). However, hydroxylated fatty acids could be present in the glycoprotein preparation. As shown by Mayberry and Lane (1993), acid hydrolysis is required to cleave the amide linkages of fatty acids. Treatment of the glycoproteins by alkaline saponification would not release amide linked fatty acids (the hydroxylated fatty acids in L. pneumophila are amide linked, data not shown). Alkaline saponification is widely, yet erroneously, accepted as the method of choice for the analysis for total cellular fatty acids. Unless the fatty acids of the glycoproteins were released by acid hydrolysis, the possibility still exists

that the glycoproteins do indeed contain hydroxylated fatty acids.

Treatments with enzymes specific for protein and nucleic acids were performed on those fractions that contained hydroxylated or long-chain fatty acids. After enzymatic treatment, material was precipitated again with acetone. Fatty acid analyses were conducted on both the supernatant and precipitate. Since all of the hydroxylated fatty acids were in the precipitates after acetone treatment, only the precipitates were analyzed by electrophoresis.

The resolution of the gel in Figure 15 is of poor quality. However, protease digestion did not seem to affect the L. pneumophila extracts. The "ladder-like" banding that is characteristic of LPS is still visible after enzymatic treatment (Figure 15, Lanes F, G, J, and K).

Furthermore, enzymatic digestion with DNAse-1 and RNAse-A did not affect the L. pneumophila extracts (Figure 16). Visualization of nucleic acids in SDS-PAGE systems by silver nitrate does not yield definitive results. Nucleic acids are better visualized on agarose gels stained with ethidium bromide. There were no nucleic acids visible in the L. pneumophila extracts (Figure 17).

The only material observed in the Folch pellets (subsequently extracted with methanol) is a band at approximately 14-15 kDa (Figure 18, Lanes E, F, G, and H).

This band (along with material of smaller mass, approximately 6 kDa) is seen also in the LPS-like material from the Folch extraction (Figure 18, Lanes C and D). Both bands are resistant to protease treatment (Figure 15, Lanes G and K). These bands also are visible in previously reported SDS-PAGE analyses (Nolte et al. 1986). It is not clear what relationships, if any, exist between these bands and L. pneumophila LPS.

LPS-like material is visible in the Folch extracted pellets, when solubilized in 1% SDS, (Figure 19, lanes G and H) and probably accounts for the hydroxylated fatty acids that are detectable in the pellets. Precipitates (Lipid A) and supernatants (degraded polysaccharide) from the weak acid hydrolysis of E. coli S-LPS, L. pneumophila LPS, and L. pneumophila LPS-like material are also visualized in Figure 19. It is not surprising that no material was detected. It has been reported that Lipid A does not stain by silver nitrate (Hitchcock and Brown 1983) and the polysaccharide portion of the LPS can degrade under the hydrolysis conditions.

The chemical composition (Table 4 and Table 5) of the LPS-like material extracted from L. pneumophila by the Folch extraction by percentage weight is: 6.6% nonhydroxylated fatty acids, 1.5% monohydroxylated fatty acids, 0.6% dihydroxylated fatty acids, 4.6% phenol-sulfuric detectable carbohydrate, 2.6% KDO, 3.1% phosphate,

28.6% Bradford-detectable protein, and 9.4% Lowry-detectable protein. Depending on the value used for protein, only 28.4-47.6% of the total LPS-like weight can be accounted.

The previously reported (Sonesson et al. 1989) composition of the LPS by percentage of total weight is: 9.7% nonhydroxylated fatty acids, 8.6 monohydroxylated fatty acids, 1.6% dihydroxylated fatty acids, and 18.1% carbohydrate. Including the contaminants (5% protein and 3% polyhydroxybutyrate), only 46% of the total LPS weight can be accounted.

The chemical analyses of the L. pneumophila LPS-like material can only account for approximately one-half the isolated material weight. This is suggestive of a moiety that is not detectable by the means employed to characterize the LPS. The LPS-like material purified during this study accounts for only 16% of the whole cell fatty acids (Table 3). By fatty acid classes, 15% of the whole cell nonhydroxylated fatty acids, 31% of the whole cell monohydroxylated fatty acids, 20% of the whole cell dihydroxylated fatty acids are present in the LPS-like material.

Analyses of the long-chain keto and dicarboxylated fatty acids by GC/MS were identical to the recently reported findings of Moll et al. (1992). An additional observation is that these compounds are always (and possibly

exclusively) found in material that is identifiable as LPS by SDS-PAGE.

Aqueous phases derived from the acid hydrolysis of LPS-like material (extracted by chloroform/methanol (2:1 ([V/V]) and purified by acetone precipitation and column chromatographic separation), LPS from L. pneumophila (provided by Dr. Sonesson), and E. coli S-LPS were analyzed by gas-liquid chromatography (GLC) and gas chromatography/ mass spectrometry (GC/MS) for amino acid and carbohydrate composition. Carbohydrates were converted to methyl glycosides and alditols. The methyl glycosides were analyzed as O-trimethylsilyl (TMS) derivatives. The alditols were analyzed as acetate (Gunner et al. 1961; Sawardeker et al. 1965), O-trimethylsilyl (TMS), and O-trifluoroacetyl (TFA) derivatives. Samples were reconstituted in n-heptane for GC injections.

Carbohydrate standards (both amino and neutral sugars) could only be identified as alditol-TMS derivatives by gas chromatographic analyses. Poor results were obtained when standard sugars were analyzed as methylglycoside-TMS, alditol-acetate, and alditol-TFA derivatives. When the aqueous phases derived from the acid hydrolysis of LPS-like material (extracted by chloroform/methanol (2:1 ([V/V]) and purified by acetone precipitation and column chromatographic separation), LPS from L. pneumophila, and E. coli S-LPS were analyzed as alditol-TMS derivatives, no identifiable sugars

were seen. It is postulated that a more polar capillary column is required for carbohydrate analyses.

Amino acid butyl esters were analyzed as O-trifluoroacetyl (TFA) derivatives (Gehrke et al. 1968). No amino acid residues were detectable in the aqueous phases derived from the acid hydrolysis of LPS-like material (extracted by chloroform/methanol (2:1 ([V/V]) and purified by acetone precipitation and column chromatographic separation).

It has been extensively reported (Wong et al. 1979; Conlan and Ashworth 1985; Gabay and Horwitz 1985; Nolte et al. 1986) that the LPS of L. pneumophila whole cells does not partition in the aqueous phase of the hot phenol/water extraction procedure of Westphal et al.

Extractions with solvent mixtures high in phenol serve to isolate proteins and other cellular components. The extraction procedures of Westphal et al. and Galanos et al. use this principle to separate proteins from LPS-like material. In both extractions, L. pneumophila samples (as compared to E. coli and P. aeruginosa) consistently showed higher amounts of hydroxylated fatty acids in the phenol-containing phases (Lane 1990).

Alternate procedures have been routinely employed to extract the LPS from L. pneumophila. Such methods include proteinase-K digestion of whole-cell lysates, phenol/chloroform/petroleum ether extraction followed by LPS

precipitation and enzymatic digestion, "cold ethanol extraction" followed by ultracentrifugation, and SDS-EDTA extraction. The above methods are labor-intensive and low yields of LPS are recovered (as typified by the yields of 0.1% and 3.2% of cell dry weight reported by Petitjean et al. (1990), 1-4% of cell dry weight reported by Sonesson et al. (1989), and 0.4% of cell dry weight reported by Ciesielski et al. (1986)).

SDS-PAGE profiles of purified (acetone precipitation and column chromatographic separation) LPS-like material extracted with chloroform/methanol (2:1 [V/V]) from L. pneumophila are identical to the previously reported profiles for L. pneumophila LPS. These reports include LPS recovered from proteinase-K-digested whole cell lysates by the method of Hitchcock and Brown (1983) (Ciesielski et al. 1986, Nolte et al. 1986), "cold ethanol extraction" followed by incubation in SDS-EDTA as described by Darveau and Hancock (1983) (Gabay and Horwitz 1985, Ciesielski et al. 1986; Petitjean et al. 1990), hot phenol/water extraction by the method of Westphal and Jann (1965) (Conlan and Ashworth 1985; Petitjean et al. 1990), and phenol/chloroform/ petroleum ether (2:5:8 [V/V/V], PCP) extracts by the method of Galanos et al. (1969) (LPS provided by Dr. Sonesson).

This study suggests that LPS-like material can be obtained from L. pneumophila in higher yield (6.4% of total cell weight), of higher purity (as indicated by SDS-PAGE,

Figures 12, 13, and 14), and by a simpler method than those previously reported.

The Folch extraction of whole cells of L. pneumophila results in phases unlike those of other Gram-negative organisms (Figure 20, Lane 1990). A viscous interface forms between the aqueous and organic phases. An unexpected result was finding hydroxylated fatty acids in fractions in which Gram-negative cell constituents have not been reported (i.e. aqueous phase and interfacial material). Approximately one-fifth of the total cellular fatty acids and one-half of the total cellular hydroxylated fatty acids were recovered in these fractions (Lane 1990).

Because insignificant quantities of fatty acids are assumed to be present in the aqueous phase after the Folch extraction of E. coli and P. aeruginosa, this fraction is routinely discarded in typical applications of the Folch extraction (Folch et al. 1957). The present study has shown that significant quantities of the fatty acids are recoverable in the aqueous phase when L. pneumophila is extracted by the Folch method (Tables 3 and 4). The aqueous phase and interfacial material resulting from this extraction procedure are important fractions that contain LPS-like material.

An improved method for extracting LPS-like material from Legionella pneumophila is presented in Figure 21.

Figure 20. Folch extraction of Legionella pneumophila (approximately 1 g dried cell weight). The particulate cell debris has been removed by filtration and the organic phase (100 ml chloroform and 50 ml methanol) has been partitioned against water (30 ml).

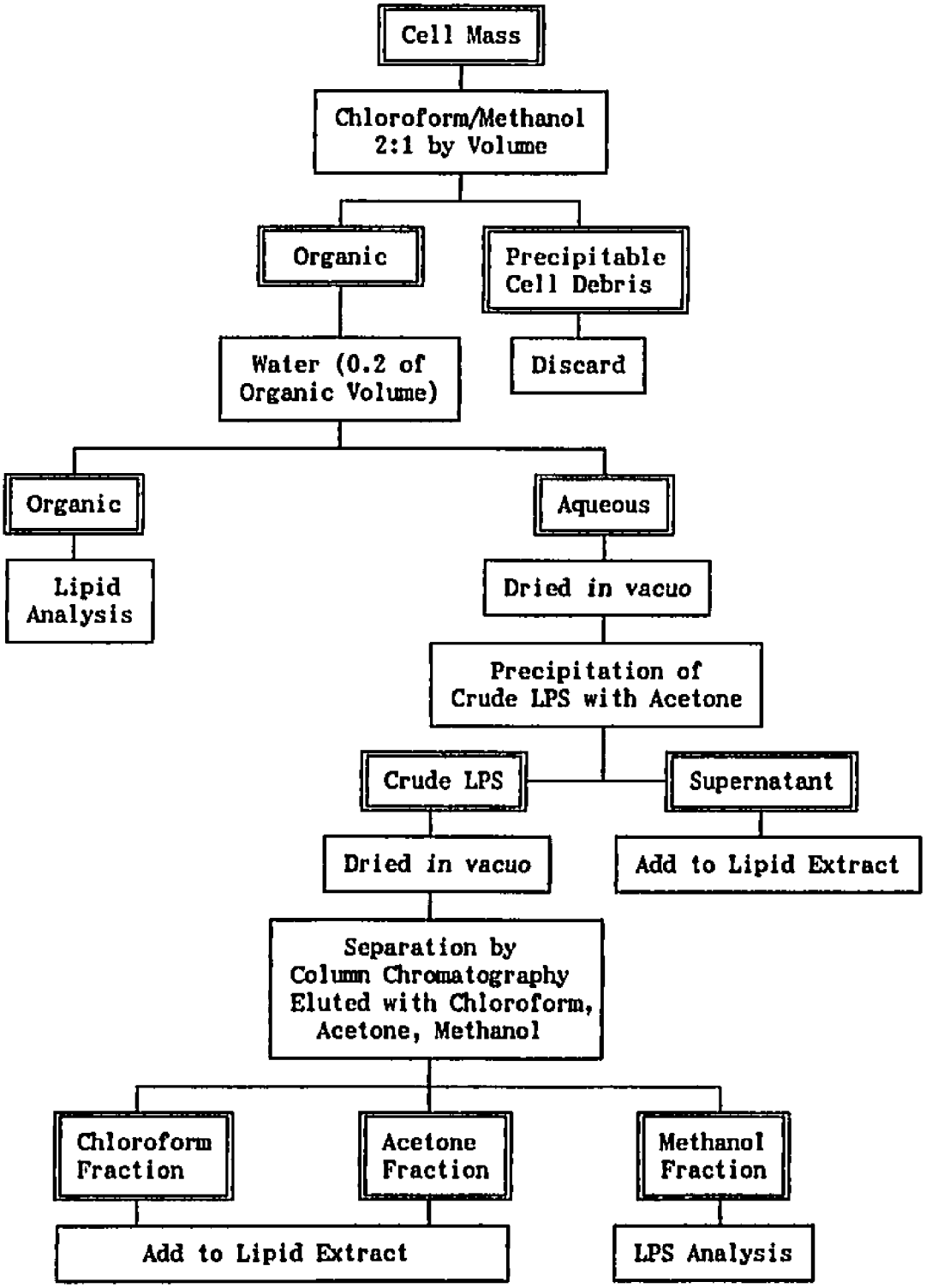

Figure 21. An improved method for extracting LPS-like material from Legionella pneumophila.

Whole cells are extracted with chloroform/methanol (2:1 [V/V]). Cell debris is removed by filtration or centrifugation. The organic extract is transferred to a separatory funnel and water (0.2 of the organic volume) is added. After vigorous shaking, the mixture is allowed to partition. An aqueous upper layer and an organic lower layer, separated by a small amount of interfacial matter, should appear after 1-2 hours. The lower layer is composed of extractable lipids and contains only trace amounts of hydroxylated fatty acids. The upper layer contains the crude LPS-like extract.

The upper layer is normally washed with Pure Solvents Lower Phase (PSLP) during a Folch extraction (Figure 2). If a such a wash is performed with the L. pneumophila extract, a white emulsion will form. This emulsion can be stable for several days. The aqueous fraction and any interfacial matter is collected as one sample. The aqueous volume is reduced by evaporation under reduced atmospheric pressure or by freeze-drying. Dried material is reconstituted in a minimal amount of water (methanol or chloroform/methanol (2:1 [V/V]) can also be used) and a ten-fold excess (by volume) of acetone is added. A white precipitate should immediately form. If no precipitation is observed, the material should be dried and reconstituted in a smaller volume of water. Material is precipitated with acetone and centrifuged for 30 min at 2500 rpm.

The supernatant contains lipid contaminants, but hydroxylated fatty acids are absent. The acetone precipitation procedure can be repeated to increase the purity of the crude LPS-like extract. After drying, the precipitate is soluble in methanol.

Acid-washed silicic acid is added to the crude LPS-like material and the slurry is thoroughly dried. The silicic acid coated with LPS-like material is layered over a chromatographic column packed with acid-washed silicic acid. Material is eluted by the sequential addition of chloroform, acetone, and methanol. Purified LPS-like material is eluted in the methanol fraction. The methanol is easily evaporated to yield purified LPS-like material.

Several limitations of the protein, phosphate and carbohydrate estimation procedures were encountered during the course of this study. The method of Lowry et al. estimates protein by reactions with both the peptide bonds and amino acid residues with phenol-like side chains (e.g., tyrosine). The amounts of protein in analyzed samples are estimated by their similarity to a standard protein (bovine serum albumin was used in this study). Analyzed proteins in samples that contain phenol and/or have high tyrosine content, as compared to the standard, can easily be overestimated.

Protein estimation by the method of Bradford is based upon a wavelength shift of a dye when complexed with

protein. This method is sensitive to alkali, so that protein in even weakly basic solutions can be overestimated.

All carbohydrate estimations were conducted on acid hydrolysates of samples. Some sugar degradation can be expected after acid hydrolysis, but it was reasoned that since percent carbohydrate concentration of each sample was based upon an acid hydrolyzed aliquot of starting material, the losses would cancel. The total concentration of carbohydrates is estimated by similar analysis of a glucose standard. The method is sensitive only to carbohydrates with a hydroxyl group in the 2-position. The amber color of an acid hydrolysate, rich in carbohydrates, is in the wavelength region at which determinations are conducted. Therefore, it must be recognized that a sample, high in sugar residues that have low phenol-sulfuric reacting component, could be overestimated in readable sugar content by an interference resulting from "the burnt sugar" color.

In conclusion, it has been shown that increased yields of material identifiable as LPS can be isolated from Legionella pneumophila. The LPS can be collected using a simple extraction procedure and appears to be of high purity (as observed by SDS-PAGE). The improved extraction procedure utilizes the amphophilic nature of the L. pneumophila LPS. Lipids (including the LPS) are extracted with organic solvents and then the LPS is partitioned into water. The LPS is further purified by

acetone precipitation and column chromatography. Analytical methods employed in this study can account only for approximately half of the weight of the LPS extract.

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VITA

JONATHAN RAY LANE

Personal Data:
Date of Birth: 02 September 1964
Place of Birth: Kingsport, Tennessee
Marital Status: Married (18 November 1990)

Education:
Public Schools, Blountville, TN

Brigham Young University, Provo, Utah; Microbiology, B.S., 1987

East Tennessee State University, James H. Quillen College of Medicine, Johnson City, Tennessee; Biomedical Sciences, M.S., 1990

East Tennessee State University, James H. Quillen College of Medicine, Johnson City, Tennessee; Biomedical Sciences, Ph.D., 1993

Professional Experience:
Graduate Research Assistant, East Tennessee State University, James H. Quillen College of Medicine, Johnson City, Tennessee; 1987-present

First Lieutenant (P), U.S. Army, Medical Service Corps

Publications:
Mayberry, W. R.; Lane, J. R. 1993. Sequential alkaline saponification/acid hydrolysis/esterification: a one-tube method with enhanced recovery of both cyclopropane and hydroxylated fatty acids. J. Micro. Methods 18:21-32.

Honors and Awards:
U.S. Army, 4-Year ROTC Scholarship, 1983
Academic Scholarship, 4-Year, Brigham Young University, Provo, Utah; 1983
Presidential Scholarship, King College, Bristol, Tennessee; 1983
Honor Graduate, Commandant's List, U.S. Army Medical Department Officer Basic Course, Fort Sam Houston, Texas; 1989
The Gamma Beta Phi Honor Society, 1992