MONTANA STATE UNIVERSITY

Teichoic acids of Listeria monocytogenes
by Nicholas Ward Hether

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry
Montana State University
© Copyright by Nicholas Ward Hether (1983)

Abstract:
A crude cell wall fraction of Listeria monocytogenes (LCWF) and a purified fraction (PF), prepared from LCWF by nuclease and protease digestion, have a broad range of bio-logical activities while a third fraction of base hydrolyzed PF (BHPF) retains only a limited number of biological activities. The cell wall fractions are chemically uncharacterized. The cell wall polysaccharides of Listeria are of uncertain nature. Chemical constituents of cell wall fractions were analyzed by thin layer chromatography, gas liquid chromatography, amino acid analysis and colorimetric analytical methods. Amphiphilic molecules were extracted from LCWF by phenol-water partition. Remaining cell walls were extracted with base and trichloroacetic acid for cell wall linked polysaccharides. Polysaccharides were isolated and purified by gel filtration and ion exchange chromatography, and analyzed by thin layer chromatography, gas liquid chromatography, nuclear magnetic resonance spectroscopy and colorimetric analytical methods. LCWF contained amino acids, sugars and lipids. PF also had sugars and lipids, but differed from LCWF because only peptidoglycan amino acids were found. BHPF was depleted in lipid, contained only peptidoglycan amino acids and had large quantities of sugars. A lipoteichoic acid (LTA) with glucose, galactose, fatty acids, glycerol and phosphate in respective molar ratios to phosphate of 0.06, 0.08, 0.17, 0.94 and 1.0 was isolated. LTA was structural type 1 and contained 19±2 1,3 phosphodiester linked glycerols per chain. Galactose disaccharide glycosyl substitution occurred about once per chain. The LTA lipid moiety was a glycolipid containing fatty acids, galactose, glucose and glycerol in respective molar ratios of 2:1:1:1, and may have been a phosphoglycolipid. A teichoic acid (TA) containing N-acetyl glucosamine, ribitol, rhamnose and phosphate with respective molar ratios to phosphate of 0.5, 0.5, 0.9 and 1.0 was isolated. TA contained a glycoside with rhamnose and ribitol in 2:1 molar ratio. A tentative structure for TA was proposed, but structural detail remains to be determined. Listeria cell wall fractions had biochemical differences reflecting differences in biological activity. Listeria contained teichoic acids typical of gram positive bacteria.

TEICHOIC ACIDS OF *Listeria monocytogenes*

by

Nicholas Ward Hether

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Biochemistry

MONTANA STATE UNIVERSITY
Bozeman, Montana

March 1983

ii

APPROVAL

of a thesis submitted by

Nicholas Ward Hether

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for admission to the College of Graduate Studies.

_3/17/83_
Date

_____
Chairperson, Graduate Committee

Approved for the Major Department

_3/17/83_
Date

_____
Head, Major Department

Approved for the College of Graduate Studies

_3-23-83_
Date

_____
Graduate Dean

STATEMENT OF PERMISSION TO COPY

In presenting this thesis in partial fulfillment of the requirements for a doctoral degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library. I further agree that copying of this thesis is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for extensive copying or reproduction of this thesis should be referred to University Microfilms International, 300 North Zeeb Road, Ann Arbor, Michigan 48106, to whom I have granted "the exclusive right to reproduce and distribute copies of the dissertation in and from microfilm and the right to reproduce and distribute by abstract in any format."

Signature _____
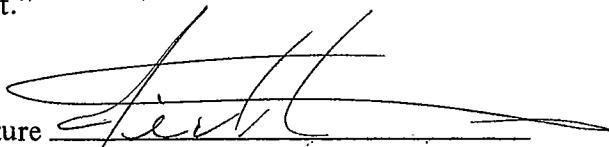
Date ___17 March, 1983___

. . . One of the facts hewn to by science is that every river needs more people, and all people need more inventions, and hence more science; the good life depends on the indefinite extension of this chain of logic. That the good life on any river may likewise depend on the perception of its music, and the preservation of some music to perceive, is a form of doubt not yet entertained by science.

Aldo Leopold

. . . for a man is rich in proportion to the number of things which he can afford to let alone.

Henry David Thoreau

. . . Awake, my sleeper, to the sun,
A worker in the morning town,
And leave the poppied pickthank where he lies;
The fences of the light are down,
All but the briskest riders thrown,
And worlds hang on the trees.

Dylan Thomas

VITA

Nicholas Ward Hether was born on July 3, 1948 in Cedar Rapids, Iowa, son of Gordon and Audrey Hether. He attended Gavitt High School in Hammond, Indiana, and graduated from Westwood High School, Mesa, Arizona, in 1966. He served four years in the United States Navy, received a commendation for hazardous duty, and was discharged honorably in 1970. He graduated with honors in 1973 from Mesa Community College, Mesa, Arizona, and earned a Bachelor of Arts with honors in chemistry from Arizona State University in 1975. From 1970 to 1974 he was the laboratory night shift supervisor at Mesa General Hospital and was a department supervisor at National Health Laboratories, Phoenix, in 1975 and 1976. He was assistant administrator and director of the laboratory and x-ray departments of Ashton Memorial Hospital, Ashton, Idaho, from 1976 to 1978. In 1977 he was elected to the board of directors of Ashton Health Services, Inc. He started graduate school at Montana State University in 1978. He is married to Carol Belohlavek.

ACKNOWLEDGMENT

TABLE OF CONTENTS

TABLE OF CONTENTS—Continued

TABLE OF CONTENTS—Continued

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

A crude cell wall fraction of *Listeria monocytogenes* (LCWF) and a purified fraction (PF), prepared from LCWF by nuclease and protease digestion, have a broad range of biological activities while a third fraction of base hydrolyzed PF (BHPF) retains only a limited number of biological activities. The cell wall fractions are chemically uncharacterized. The cell wall polysaccharides of *Listeria* are of uncertain nature. Chemical constituents of cell wall fractions were analyzed by thin layer chromatography, gas liquid chromatography, amino acid analysis and colorimetric analytical methods. Amphiphilic molecules were extracted from LCWF by phenol-water partition. Remaining cell walls were extracted with base and trichloroacetic acid for cell wall linked polysaccharides. Polysaccharides were isolated and purified by gel filtration and ion exchange chromatography, and analyzed by thin layer chromatography, gas liquid chromatography, nuclear magnetic resonance spectroscopy and colorimetric analytical methods. LCWF contained amino acids, sugars and lipids. PF also had sugars and lipids, but differed from LCWF because only peptidoglycan amino acids were found. BHPF was depleted in lipid, contained only peptidoglycan amino acids and had large quantities of sugars. A lipoteichoic acid (LTA) with glucose, galactose, fatty acids, glycerol and phosphate in respective molar ratios to phosphate of 0.06, 0.08, 0.17, 0.94 and 1.0 was isolated. LTA was structural type 1 and contained 19 ± 2 1,3 phosphodiester linked glycerols per chain. Galactose disaccharide glycosyl substitution occurred about once per chain. The LTA lipid moiety was a glycolipid containing fatty acids, galactose, glucose and glycerol in respective molar ratios of 2:1:1:1, and may have been a phosphoglycolipid. A teichoic acid (TA) containing N-acetyl glucosamine, ribitol, rhamnose and phosphate with respective molar ratios to phosphate of 0.5, 0.5, 0.9 and 1.0 was isolated. TA contained a glycoside with rhamnose and ribitol in 2:1 molar ratio. A tentative structure for TA was proposed, but structural detail remains to be determined. *Listeria* cell wall fractions had biochemical differences reflecting differences in biological activity. *Listeria* contained teichoic acids typical of gram positive bacteria.

INTRODUCTION

Listeria monocytogenes was first described in detail by Murray et al. [1] as the etiologic agent of an epidemic disease in an animal colony used for bio-medical research. The intervening half century has seen much effort devoted to understanding listeric infections and the nature of *Listeria* pathogenesis. In spite of the effort, many aspects of this interesting bacteria are incompletely understood or unknown.

Listeria monocytogenes is a gram positive, non-acid fast, non-spore forming, diptheroid-like bacillus of widespread geographical and environmental occurrence [2]. *Listeria* has been isolated from streams, mud, dust, sewage, silage, plants, slaughterhouse waste, ticks, flies, crustaceans, fish, birds and over forty species of mammals including humans [2,3,4]. Reflecting such an ubiquitous presence, *Listeria* is also pathogenic for a wide range of mammals. It can be an important pathogen in wild populations [3], is an economically significant pathogen in domestic livestock [2] and is an opportunistic pathogen for humans [2].

At an early stage clinicians recognized that the elderly, pregnant women and newborn infants were more susceptible to *Listeria* infection than other population groups [2,5]. In France and South Africa neonatal listeriosis is still the most common form of infection, and the incidence of infection is increasing [5]. In other parts of the world the distribution and incidence of *Listeria* infection has changed dramatically over the past twenty years. Although the elderly, pregnant women and neonates are still high risk groups, other population groups have surpassed them in the incidence of listeriosis within the United States and in Sweden [5].

Louria et al. [6] first associated listeriosis with malignant disease. The advent of aggressive chemotherapy and radiation therapy has prolonged survival of individuals with malignant disease [7]. The incidence of listeriosis in cancer patients has increased in parallel with prolonged survival [5,7]. In one study [8], the incidence of listeriosis increased while the incidence of infection by other opportunistic pathogens decreased. Organ transplantation, and especially kidney transplantation, has become a more common procedure since 1965 [7,9]. Listeriosis is one of the most common bacterial diseases among renal transplant patients, and *Listeria* is the most common cause of bacterial meningitis in this patient group [7,9]. Such forms of listeriosis as cerebritis and endocaritis were once thought to be extremely rare, but they now appear to be increasing in frequency [7]. *Listeria* meningitis, except in selected groups, has historically been considered quite rare [5]. In New York, *Listeria* meningitis is the second most common gram positive bacterial meningitis, is only slightly less common than *Neisseria* meningitis and has an overall mortality rate of 62 percent (%) [5]. *Listeria* has come to be recognized as a much more important bacterial pathogen than had previously been believed [5,7,8,9].

Listeria is a facultative intracellular pathogen capable of living and multiplying within phagocytic cells [10]. It is similar to such diverse bacteria as *Mycobacterium* species, *Salmonella* species and *Brucella abortus* in this regard [10]. Specific resistance to such intracellular pathogenic bacteria as *Listeria* is a cell-mediated process [11,12] in which humoral responses do not seem to play a major role [10]. Attempts to prevent listeric infection by development of killed cell vaccines have not met with high success [13,14, 15,16]. No ready means appear extant to protect high risk patients from listeric infection which is in sharp contrast to successful vaccines available against other pathogenic bacteria.

One of the hallmarks of listeric infection, which occurs in many but not all host species [2], is an increase in the number of monocytes in the peripheral circulation [1,2]; a monocytosis. Hence the species designation. Isolation of the monocytosis producing

agent (MPA) in a lipid-polysaccharide complex by Stanley [17] was the first indication that extractable entities in the *Listeria* cell might play a role in pathogenesis. MPA was also found in lipid extracts [18,19,20,21,22] which prompted studies of the lipid composition of *Listeria*. Fatty acids of *Listeria* are typical of gram positive bacteria [23,24], and no unusual phospholipids [25] or glycolipids [22,25,26] are present. MPA is extractable in aqueous solvents [27,28], and is present in a high molecular weight polysaccharide fraction which resembles an amphiphile and contains lipid and phosphate [28]. MPA remains incompletely characterized. Some extracts of MPA decrease host antibody formation against foreign antigens [20,21,29], but the immunosuppressive activity is distinct from MPA [28]. The immunosuppressive agent has not been identified.

Most bacteria contain high molecular weight amphiphiles at or near the cell surface, and the type of amphiphile present is characteristic of the major groups of bacteria [30,31, 32]. Lipopolysaccharides (LPS) are the major outer membrane amphiphiles of the gram negative bacteria [30,32]. The gram positive bacteria have lipoteichoic acids (LTA) which are structurally distinct from LPS [31,32]. Both LPS and LTA have biological activities which are unique to the particular amphiphile class [32]. LPS contains several compounds which serve as biochemical markers for presumptive identification of LPS. The markers of LPS include hydroxy fatty acids, heptose and 2-keto-3-deoxyoctanoic acid [30,32].

A cell surface component from *Listeria* has many of the biological activities of classical lipopolysaccharide endotoxins [33,34,35,36], but contains none of the biochemical markers associated with LPS [37]. Others [38,39,40,41,42] have extracted from *Listeria* an endotoxin-like component which has both the biochemical markers and many of the biological activities of LPS. Glycerol lipoteichoic acids act as heterophile antigens in the gram positive bacteria [43]. *Listeria* cross reacts with antibody prepared against glycerol teichoic acid [44]. Extracts from *Listeria* absorb anti-LTA antibody prepared against LTA of *Lactobacillus fermenti* [45]. Teichoic acids were not detected in a chemical examination

of *Listeria* cell walls [46]. The identity and nature of the major amphiphiles of *Listeria* seems uncertain while it is clear that they possess biologic activities which may play an important role in *Listeria* pathogenesis.

The cell wall or peptidoglycan of *Listeria* contains biochemical components characteristic of bacterial cell walls [46,47,48,49], exhibits turnover typical of gram positive bacterial peptidoglycans in general [50], and has a structure that does not appear to be unusual [51]. The *Listeria* cell wall does exhibit a wide range of biologic activities in spite of the apparent mundane biochemical nature.

A crude *Listeria* cell wall fraction (LCWF) acts as a mitogen [52], as a polyclonal B cell mitogen [52] and as an immunologic adjuvant [53,54,55]. LCWF can activate complement by the alternative pathway [56], and can induce macrophages to become tumoricidal [57]. Also, LCWF can increase resistance to bacterial infection [15,57]. LCWF must be administered to test animals more than five days before challenge of the test animals with viable bacteria for the increased resistance to be manifested [15]. However, if LCWF is given five days or less before or accompanying challenge of test animals with viable bacteria there is a decrease in resistance to bacterial infection [56,57]. A purified *Listeria* cell wall fraction (PF), which is prepared from LCWF by treatment with proteases and nucleases [56], retains all of the biological activities associated with the crude cell wall fraction [56,57,58,59]. The ostensible loss of protein and nucleic acids in the preparation of PF would seem to preclude the role of such constituents in the biological activities. If PF is hydrolyzed under mild basic conditions, the resulting cell wall fraction (BHPF) looses all activities except the ability to induce tumoricidal activity and the ability to decrease resistance to bacterial infection [57]. Material lost in the preparation of BHPF seems to be responsible for the bulk of the biological activities while BHPF itself retains other properties of equal or greater importance and interest.

5

Summary of the Problem

Listeria monocytogenes is set apart from other common bacterial pathogens because it can survive and grow within phagocytic cells. *Listeria* is an important pathogen for humans and no methods are available to prevent listeric infections in high risk patients. A variety of extracts obtained from *Listeria* display biological activities yet the natures and identities of the active components remain unknown. The nature of the cell wall amphiphiles is unclear, but they have activities of interest and importance. Cell wall fractions of *Listeria* display biological activities which may play a role in the pathogenesis of *Listeria*. Further, the loss of activities in the preparation of one cell wall fraction from another suggests that many of the observed activities may be attributable to particular compounds or compound classes. Biochemical characterization of the active cell wall fractions, however, does not exist.

Statement of the Objective

The objective of the research described in this thesis is to provide answers to two questions.

1. Are there biochemical differences among the biologically active cell wall fractions of *Listeria monocytogenes* which reflect differences in biological activity?

2. Are teichoic acids present in the cell wall of *Listeria monocytogenes*?

EXPERIMENTAL PROCEDURE

Bacteria and Cell Wall Fractions

Cells of a virulent strain of *Listeria monocytogenes* serotype 1 were used uniformly throughout the course of the research described. The *Listeria* cell wall fractions were a gift from Priscilla A. Campbell and two of the fractions (LCWF and PF) were prepared in her laboratory. The crude cell wall fraction (LCWF) was composed of sonically disrupted, washed cells which contained no viable organisms [15]. A purified fraction (PF) was prepared from LCWF by digestion with nucleases and proteases [56]. A third cell wall fraction of base hydrolyzed PF (BHPF) was prepared by hydrolysis of PF in 0.1 M ethanolic NaOH for 30 min at ambient temperature [57]. Insoluble material was removed by centrifugation at 23,300 xg for 15 min at 4°C. The pellet (BHPF) was washed five times with distilled water, lyophilized and stored at −20°C until use. The supernatant liquid was examined for lipids as described in the lipid analysis section of the experimental procedures. Figure 1 outlines the preparation of the cell wall fractions.

Lipoteichoic Acid Extraction

Lipoteichoic (LTA) was extracted from the crude *Listeria* cell wall fraction (LCWF) and Figure 2 outlines the LTA extraction and isolation scheme. Multiple samples of LCWF were suspended in chloroform-methanol (2:1 v/v) at a mass to volume ratio of 1:20 [60]. The slurries were stirred at ambient temperature for 2h and filtered through Whatman GF/C glass microfibre filters (1.2 μmeter pore size) (Whatman, Ltd., England) in coarse scintered glass funnels. Insoluble material was suspended in a second volume of chloroform-methanol (2:1 v/v) and extracted a second time in the same manner. Pooled

LCWF   (*Listeria* Cell Wall Fraction)
          sonically disrupted, killed, washed cells

nuclease and protease digestion

PF   (Purified Fraction)

0.1 M ethanolic NaOH, 30 min, R.T.

BHPF   (Base Hydrolyzed Purified Fraction)
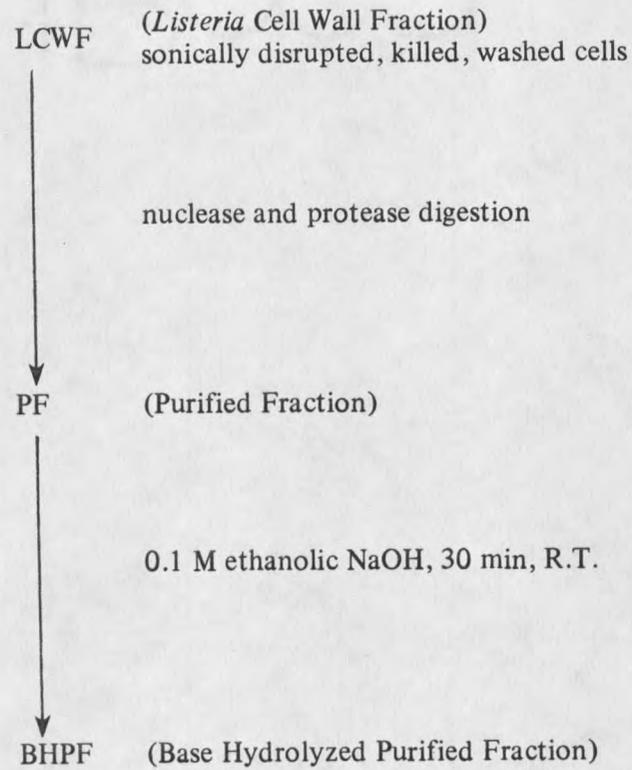
Figure 1. Preparation of *Listeria* Cell Wall Fraction.

LCWF

chloroform-methanol (2:1 v/v) ————————————→ Lipids
extraction x2

Residual
Cell Walls

45% Aqueous Phenol, 65°C ————————————→ Aqueous Phase
*Crude Lipoteichoic Acid

Residual
Cell Walls

(saved for later
 extraction of
 Teichoic Acids)

Dialysis,
Gel filtration on
Sepharase 6B

*Lipoteichoic Acid

* Analyzed for
1. Phosphate
2. Glycerol
3. Sugars
4. Amino Acids
5. Nucleic Acid

Ion exchange
chromatography
on DEAE-Sephadex
A-25

*Lipoteichoic Acid

Figure 2. Lipoteichoic acid extraction and isolation.

organic extracts were reduced in volume on a rotary evaporator, transferred to vials, swept with nitrogen and stored at $-20°C$. The remaining insoluble cell wall material was transferred to preweighed 50 ml beakers, dried at ambient temperature for 24h and weighed.

LTA was extracted from the lipid depleted cell walls in hot aqueous phenol [41]. Cell wall samples were suspended in distilled water and warmed to $65°C$ in a water bath. An equal volume of hot ($65°C$) 90% aqueous phenol (J. T. Baker Chemical Co., Phillipsburg, NJ) was added to give a final mass to volume ratio of 1:50 and a final phenol concentration of 45%. The mixture was shaken for 8 min at $65°C$ and then centrifuged at 10,000 xg for 30 min at $4°C$ to separate the phases. The aqueous phases were removed and dialyzed in membrane tubing with a molecular weight cut off of approximately 3,500 (Spectrapor 3, Spectrum Medical Industries, Inc., Los Angeles, CA) for 24h against four 4 liter volumes of 3 mM NaN_3 in distilled water. Dialyzates were reduced in volume on a rotary evaporator, transferred to 13×100 mm screw cap tubes and lyophilized.

Lipoteichoic Acid Isolation

Lyophilized dialyzates of the crude phenol extracts were dissolved in distilled water (50 mg/ml), applied in 0.5 ml volumes to a 0.7 cm × 115 cm column of Sephanose 6B, and eluted with 0.1 M ammonium acetate (pH 6.8) which was also 3 mM in NaN_3 (Buffer A). Fractions of 1.0 ml were collected and analyzed for phosphate, total neutral sugars and absorbance at 260 nm. Tubes under the major peaks were pooled, reduced in volume and applied again to the same Sepharose 6B column. Fractions thus isolated by gel filtration were partitioned against three volumes of chloroform-methanol (2:1 v/v), dialyzed, lyophilized and weighted. The LTA fractions were dissolved in distilled water (10 mg/ml) and then analyzed for phosphate, glycerol, neutral sugars, amino sugars, amino acids, fatty acids, protein and nucleic acids.

LTA was also purified further by ion exchange chromatography [61]. LTA was eluted with distilled water from a 0.7 cm X 50 cm column of Sephadex G-10 to remove salts. Dried LTA was dissolved in 1.0 ml of 0.01 M Tris buffer (pH 8.0) (Sigma Chemical Co., St. Louis, MO) containing 0.2% Triton X-100 (Sigma) (Buffer B). The samples were applied to a 0.9 cm X 10 cm column of DEAE-Sephadex A25 (Pharmacia) previously equilibrated with buffer B. The column was eluted with a linear gradient of NaCl from 0 to 1.0 M in 200 ml of buffer B. Fractions of 1.0 ml were collected and every third tube was analyzed for phosphate. Tubes under the major peak were collected, reduced in volume and dialyzed as previously described. Dialyzates were reduced in volume to about 5 ml on a rotary evaporator using one drop of octanol as a surfactant and were then extracted ten times with 15 ml volumes of chloroform to remove detergent. The purified LTA was reduced in volume under nitrogen sweep at 40°C, lyophilized and analyzed for chemical constituents.

Lipoteichoic Acid Deacylation

Partial deactylation of LTA was done by the methods described by Wicken et al. [62]. Samples of LTA were dissolved in distilled water (1 volume), warmed to 37°C and mixed with 10 volumes of 0.2 M methanolic KOH also at 37°C. The solutions were held at 37°C for 15 min in a water bath, chilled on ice, diluted with water and passed over a column of Dowex 50 8-X (H⁺ form) (Sigma). The acidic eluents were extracted three times with chloroform, and the pooled organic extracts were analyzed for fatty acids. The aqueous phases were neutralized with 1.0 M NH_4OH and reduced in volume on a rotary evaporator. Concentrated deacylated lipoteichoic acid (dLTA) was analyzed by gel filtration chromatography as described in the lipoteichoic acid isolation section. The major phosphate peak was collected, treated as previously described, and analyzed for chemical constituents.

An alternative mild deacylation procedure was incubation of LTA at ambient temperatures for 16h in a mixture of LTA in water (1 volume) and concentrated NH_4OH (2 volumes). Ammonium hydroxide was removed on a rotary evaporator and fatty acids were extracted with chloroform following acidification as described above.

Gel Filtration in Detergent Buffers

LTA in 0.5 ml of buffer A was applied to a 0.7 cm × 25 cm column of Sepharose 6B previously equilibrated with buffer A. The sample was eluted with buffer A. Fractions of 1.0 ml were collected and analyzed for phosphate to determine the elution volume of the LTA. Tubes under the phosphate peak were pooled, reduced in volume and desalted on a Sephadex G-10 column. The desalted material was reduced in volume under nitrogen sweep at 40°C. The Sepharose 6B column was then equilibrated with five column volumes of 0.2% Triton X-100 in buffer A. The LTA sample was dissolved in 0.5 ml of the same detergent buffer, applied to the column and eluted with the detergent buffer. Fractions of 1.0 ml were collected and analyzed for phosphate. The experiment was then repeated using 0.5% Triton X-100 in buffer A as the eluting solvent.

Immunological Methods

Passive hemagglutination (HA) and hemagglutination inhibition (HAI) assays were conducted as described by Antonissen et al. [45]. The LTA from *Lactobacillus fermenti* used in these experiments was a gift from Dr. Anthony J. Wicken (University of New South Wales, Kensington, NSW, Australia) and is described elsewhere [62]. The rabbit anti-LTA serum employed in the HA and HAI assays was a gift from Dr. Robert W. Jackson (Southern Illinois University, Carbondale, IL). The antiserum was prepared against the polyglycerol phosphate moieties of LTA from *Streptococcus pyogenes* [63,64], and was heat inactivated and filter sterilized (R. W. Jackson, personal communication). Sheep red

blood cells (SRBC) (Colorado Serum Co., Denver, CO) were obtained from Dr. Norman D. Reed (Montana State University) and normal rabbit serum was a gift from Dr. James E. Cutler (Montana State University). Microtiter plates with U-shaped wells (Dynatech Laboratories, Inc., Alexandria, VA) were used throughout.

SRBC's were washed three times with veronal buffered saline (VBS) (0.01 M sodium diethylbarbiturate in 0.14 M NaCl, pH 7.28) and mixed with *Listeria* LTA or *Lactobacillus* LTA to give final concentrations of 10% (v/v) cells in 1.0, 0.1, 0.01 and 0.001 mg LTA/ml 10% cells respectively. After incubation for 30 min at 37°C the respective cell preparations were washed three times with VBS and each preparation was adjusted to a concentration of 1% (v/v) in VBS. Control cells were treated in an identical manner with the omission of lipoteichoic acid.

For HA assays a 2 fold serial dilution of 0.05 ml anti-LTA serum or normal rabbit serum in VBS was prepared for each line of wells. The last well of each line received 0.05 ml of VBS only. To all wells of each line 0.05 ml of 1% LTA-coated or control treated SRBC's were added. The plates were shaken gently and the cells were allowed to settle for 4 to 6h at ambient temperature. The HA titer was read as the $-\log_2$ of the last serum dilution causing complete hemagglutination.

In the HAI assay, a 2 fold serial dilution of 0.04 ml of each test material was prepared in VBS for each line of wells. The last well of each line received VBS only. *Listeria* LTA, *Listeria* dLTA and phenol extract fraction 3 were tested at starting concentration of 100 μg, 10 μg, 1 μg and 0.1 μg/well respectively. After the addition of 0.04 ml of 1:120 diluted anti-LTA serum or normal rabbit serum to each well, the plates were gently shaken and incubated 1h at 37°C. To each well was added 0.08 ml of 1% (v/v) SRBC's coated with *Lactobacillus* LTA (1 mg/ml 10% SRBC) or control treated cells. The cells were allowed to settle 4 to 6h at ambient temperature, and the minimal concentration of test substance causing complete inhibition of hemagglutination was read.

Teichoic Acid Extraction

Cell wall material remaining after phenol extraction was washed five times in 95% ethanol followed by five washes with distilled water. The residual cell wall material was dried, weighed and teichoic acids (TA) were extracted by two separate methods [65].

Cell wall material was suspended in 0.5 M NaOH with a mass to volume ratio of 1:80. The suspension was stirred for 4h at ambient temperature. The supernatant was removed after centrifugation (10,000 xg, 30 min, 4°C) and neutralized with 0.5 M HCl. The neutralized crude base extract was then dialyzed in membrane tubing with a molecular weight cut off of approximately 3,500 (Spectropor 3, Spectrum Med. Ind.) for 24h against four 4 liter volumes of 3 mM NaN in distilled water. Dialyzates were reduced in volume on a rotary evaporator, transferred to 13×100 mm screw cap tubes and lyophilized.

For the second extraction method, cell wall material was suspended in 10% (w/v) aqueous trichloroacetic acid at a mass to volume ratio of 1:20 and was stirred at 4°C for 24h. After centrifugation (10,000 xg, 30 min, 4°C), the supernatant solution was mixed with five volumes of cold (–20°C) 95% ethanol and kept at –20°C for 16h. The cell wall residue was extracted with a second volume of trichloroacetic acid at 4°C for 48h and the supernatant solution was treated as before. The ethanol precipitates were recovered by centrifugation (20,000 xg, 30 min, –20°C), combined and dissolved in 5 ml of cold (4°C) 10% trichloroacetic acid. Insoluble material was removed by centrifugation (10,000 xg, 30 min, 4°C) and 25 ml of cold (–20°C) 95% ethanol was added to reprecipitate teichoic acid which was recovered by centrifugation and washed with alcohol and then ether. The alcohol-trichloroacetic acid solutions from the precipitation steps were combined, neutralized with 10 M NaOH, reduced in volume on a rotary evaporator and dialyzed as described above to give an additional teichoic acid preparation. The dialyzates were reduced in volume and dried under nitrogen sweep.

Residual Cell Walls
(After Phenol Extraction)

0.5 N NaOH, 4h, 22°C   10% TCA, 4°C
                                            extraction x2

                cell walls   cell walls

*Crude Teichoic Acid   *Crude Teichoic Acid

        neutralized,   cold ethanol
        dialyzed   precipitation

*Crude Teichoic Acid   *Crude Teichoic Acid

        Gel filtration on   Gel filtration on
        Sephacryl S-200   Sephacryl S-200

*Teichoic Acid   *Teichoic Acid

        Ion exchange   Ion exchange
        chromatography on   chromatography on
        DEAE-Sephadex A-25   DEAE-Sephadex A-25

*Teichoic Acid   *Teichoic Acid

        * Analyzed for
        1. Phosphate
        2. Sugars
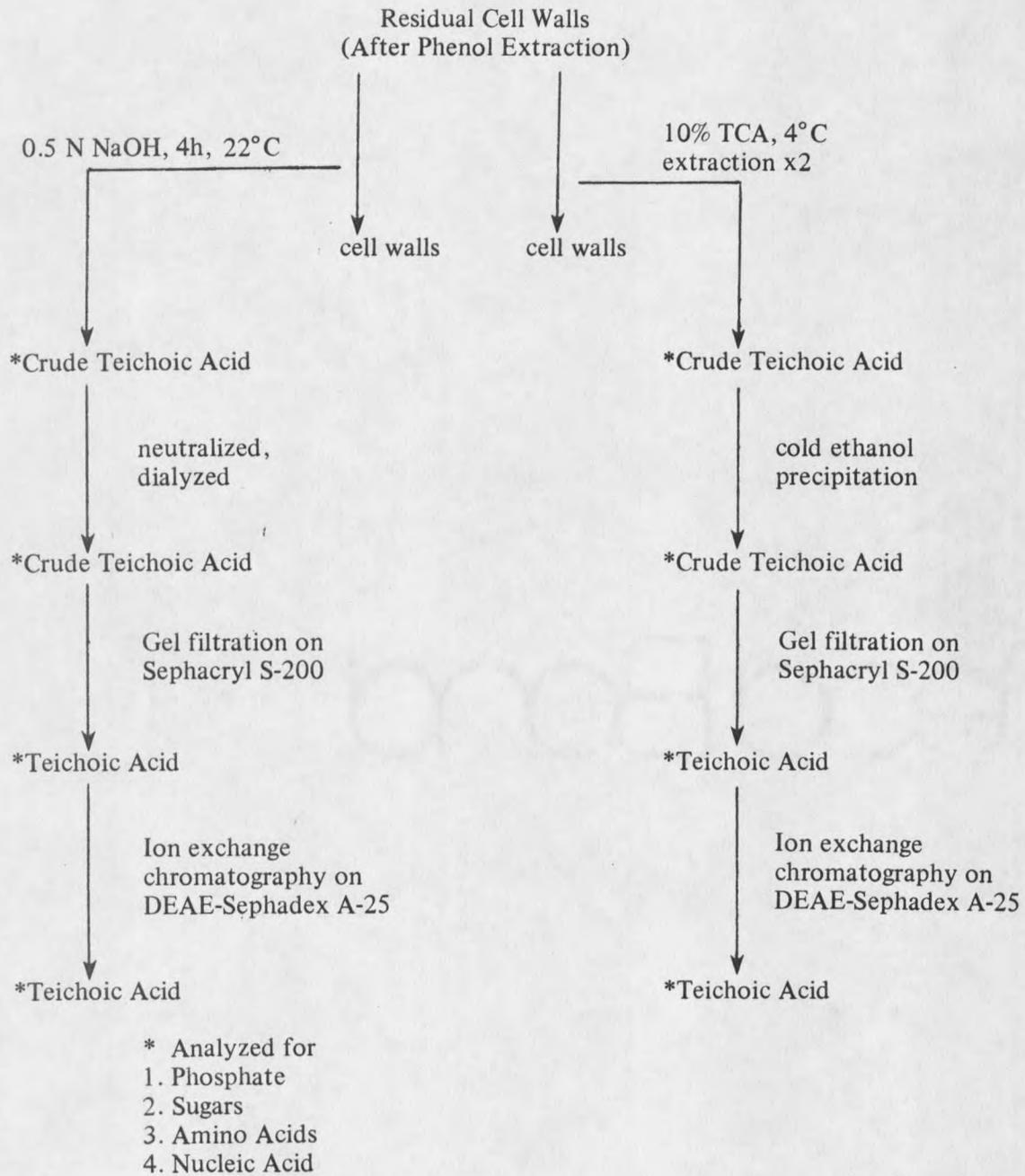   3. Amino Acids
        4. Nucleic Acid

Figure 3. Teichoic acid extraction and isolation.

Teichoic Acid Isolation

After the initial extraction procedures all TA samples were treated in an identical manner. Dried TA extracts were dissolved in distilled water (50 mg/ml), applied in 0.5 ml volumes to a 0.7 cm X 120 cm column of Sephacryl S-200 and eluted with buffer A. Fractions of 1.0 ml were collected and analyzed for phosphate and absorbance at 260 nm. Tubes under the major peak were pooled, reduced in volume and dialyzed as described in the TA extraction section. Pooled, lyophilized dialyzates were analyzed for chemical constituents. Samples of TA were also applied to 0.7 X 115 cm columns of Sepharose 6B and Sephadex G-50 and treated as described above.

TA extracts were further purified by ion exchange chromatography [65] Samples of TA containing approximately 100 μmoles of phosphate in 1.0 ml of distilled water were applied to a 0.9 cm X 10 cm column of DEAE-Sephadex A-25. The column was eluted with a linear gradient of LiCl from 0 to 0.7 M in 100 ml of distilled water. Fractions of 1.0 ml were collected and every third tube was analyzed for phosphate and sugars. Tubes under the major peak were pooled, dialyzed as described above, reduced in volume and lyophilized. Lyophilized material was weighed, dissolved in distilled water (50 mg/ml) and analyzed for chemical constituents.

Structure Evaluation Methods

Partial Hydrolysis

Several structural types have been described for lipoteichoic and teichoic acids. The fragmentation patterns produced under both acidic and basic hydrolysis conditions provide information about structure [65]. Samples of LTA and TA containing 0.5 mg to 2 mg of material (2.5 to 5 μmoles phosphate) were dried under nitrogen sweep at 50°C in the bottom centimeter of a 5.0 mm O.D. glass tube. The tubes were sealed after addition of 100 μl

of either 1.0 M HCl or 1.0 M NaOH to the respective samples. Hydrolysis for 3h was done either at 100°C in a sand bath or at 95°C in a boiling water bath.

Acid hydrolyzates were treated in two ways. For both methods the hydrolyzates were transferred to vials with several distilled water washes. In the first method acid was removed in an evacuated desiccator over NaOH pellets and anhydrous CaSO$_4$. For the second method the hydrolyzates were neutralized with 1.0 M NaOH and dried under nitrogen sweep.

Base hydrolyzates were also treated in two manners. In the first, hydrolyzates were passed over a 1.0 ml column of Dowex-50, 8-X (H$^+$ form), eluted with distilled water and dried under nitrogen. In the second, hydrolyzates were transferred to vials, neutralized with 1.0 M HCl and dried under nitrogen.

Thin Layer Chromatography of Partial Hydrolysis Products

Products of partial hydrolyses of LTA and TA were dissolved in 50 μl to 100 μl of distilled water and 1 μl to 5 μl volumes were spotted on 10 cm X 20 cm sheets of Baker-Flex Silica Gel 1 B (J. T. Baker Chem. Co.) 1.5 cm from the lower edge. Spotting was done under a stream of warm air from a hair dryer and spot diameters were held to 2 mm or less. Multiple sheets were spotted for each sample and both acid and base hydrolyzates were run concurrently on each sheet.

Standards were prepared to approximate the salt contents of hydrolyzates. Standards of glycerol-1-phosphate, glycerol-2-phosphate, glycerol, galactose, glucose, lactose (Sigma) and glycerol-1,3-diphosphate were run with LTA samples. With TA samples, ribitol, glucosamine, rhamnose, glucosamine-1-phosphate (Sigma), ribitol-1-phosphate and 1,4-anhydro-ribitol were used as standards.

TLC sheets were developed twice (16 to 18 cm) in 95% ethanol-ammonium hydroxide-water (6:3:1 v/v). Between developments the sheets were dried under a stream of warm

air. TLC sheets were also developed in n-propanol-ammonium hydroxide-water (6:3:1 v/v) [65]. Reducing substances were visualized with an alkaline silver nitrate spray [66]. Vicinal diols were detected with periodate benzidine [67], periodate Schiff [68] and alkaline permanganate [69]. Amines were detected with 0.25% ninhydrin in n-butanol. Phosphate esters were visualized with a modified ammonium molybdate spray [70]. Color development of the phosphate spray was enhanced by over spraying with a solution of 2% ascorbic acid in water-acetone (1:4 v/v) followed by heading at 100°C for 5 min and a second light overspray of water.

Hydrolysis of Lipoteichoic Acid in Hydrofluoric Acid

Hydrofluoric acid (HF) hydrolysis was done to release glycosides and glycolipids from LTA [71]. Samples of LTA (5 to 25 μmoles phosphate) were dried in 1.5 ml snap cap, polyethylene centrifuge tubes under nitrogen at 40°C. The tubes were then cooled to -20°C and 0.2 to 0.5 ml of cold (-20°C) concentrated (48% w/w) HF was added. Hydrolysis was done at 2°C after which the tubes were frozen in a dry ice acetone bath. HF was removed in a precooled (-20°C) evacuated desiccator over NaOH pellets and anhydrous $CaSO_4$ at -20°C. Dried hydrolyzates were dissolved in 0.3 ml of 0.05 M NH_4OH and lipids were separated by partion of the aqueous phase against three 1.0 ml volumes of chloroform-methanol (2:1 v/v) [60,71]. Three samples of LTA were hydrolyzed for 24, 48 and 96 hr respectively and inorganic phosphate was measured prior to lipid extraction to determine the best time for hydrolysis.

Ammonia was removed from water phases under nitrogen at 40°C. Water soluble material was applied in 0.5 ml to a 0.7 cm X 120 cm calibrated column of Bio-Gel P-2 and the column was eluted with 1.0 mM NaN_3. One milliliter fractions were collected and analyzed for sugars. Tubes under the sugar peak were pooled, reduced in volume, passed over a 1.0 ml column of Amberlite MB-1 (Sigma) and analyzed for chemical constituents.

The organic phases were reduced in volume under nitrogen at 40°C and analyzed by TLC in the following solvents: (A) hexane-diethyl ether-acetic acid (85:15:1 v/v) to detect free fatty acids, (B) hexane-diethyl ether-acetic acid (60:40:4 v/v) for mono and diacyl glycerol, and (C) chloroform-acetone-methanol-acetic acid-water (80:20:10:10:4 v/v) to resolve glycolipids. Glycolipids were isolated by preparative TLC on analytical plates developed in solvent C. Methanolic KOH (0.2 M, 45°C, 2h) was used to deacylate glycolipids after the addition of an internal lipid standard. The solutions were diluted with water, adjusted to pH 2 and the fatty acids were extracted. All other conditions were as specified in the Lipid Analysis section. The aqueous phase was neutralized and the glycoside was isolated as described above.

Hydrolysis of Teichoic Acid in Hydrofluoric Acid

Hydrolysis of TA in HF was done in the same manner as that described for LTA. Replicate samples of TA were hydrolyzed for varying time periods to determine the best hydrolysis times. After removal of HF the samples were examined as described for LTA on a Bio-Gel P-2 column. The fractions were analyzed for phosphate and sugars. Major peaks were pooled and analyzed for chemical constituents.

Base Hydrolysis of Teichoic Acid and Glycoside Isolation

Samples of TA containing 10 to 20 μmoles of sugar were dried in 13 mm X 100 mm screw cap tubes under nitrogen at 40°C. Samples were hydrolyzed in 0.5 ml of 1.0 M NaOH at 95°C in a boiling water bath for 3h [65]. Base was neutralized with 1.0 M HCl. Phosphate contents (organic and inorganic) were measured and aliquots were removed to determine the presence of free sugars by GLC. The neutralized hydrolyzates were reduced in volume to approximately 0.5 ml and applied to the calibrated Bio-Gel P-2 column. Elution conditions and fraction collection were as described previously. Fractions were assayed for phosphate and total sugars. Fractions under the sugar peaks which contained

no phosphate were pooled, concentrated, passed over a mixed bed ion exchange resin and analyzed for chemical constituents. The balance of the material eluting from the column near the void volume was pooled, concentrated and treated with a phosphomonoesterase. Inorganic and organic phosphate essays were done and the concentrated material was passed over the Bio-Gel P-2 column as described previously. Glycosides containing no phosphate were treated as described above and analyzed for chemical constituents. Material eluting near the void volume was pooled and lyophilized.

Estimation of Chain Length

LTA was treated with a phosphomonoesterase, and inorganic phosphate release was followed with time to determine whether terminal phosphomonoesters were present. LTA chain length was estimated on duplicate samples by the periodate consumption method of Critchley et al. [72]. LTA was dialyzed in membrane tubing with a molecular weight cut-off of 1,000 (Spectrapor 6, Spectrum Med. Ind.) for 12h against two 4 liter volumes of distilled water. Dialyzed LTA containing 22 μmoles of phosphate was dissolved in 15 ml of distilled water in a vial wrapped with aluminum foil and 15 ml of 0.2 mM $NaIO_4$ (J. T. Baker Chem. Co.) were added. The mixture was kept in the dark at room temperature. At intervals over a period of 40h periodate and formaldehyde contents were determined. Periodate consumed and formaldehyde produced were plotted against time and the curves were extrapolated to zero time. The values obtained from the graph, when compared to phosphate content, provided an estimate of chain length.

Excess periodate was destroyed with 1.0 ml of 10% $NaHSO_3$ and the residual LTA from the pooled samples was dialyzed as described above. The dried dialyzate was dissolved in 0.2 ml of distilled water in a conical bottom reaction vial. To remove terminal glycoaldehyde groups produced by periodate oxidation, 0.3 ml of a 1% solution of 1,1-dimethylhydrazine (adjusted to pH 6.0 with acetic acid) was added to the vial. The mix-

ture was sealed with a Teflon lined cap and held at 37°C for 24h. Hydrazine was removed by extraction into five 2.0 ml volumes of chloroform. The water phase was passed over a 1.0 ml column of Dowex-50 8-X (H$^+$ form) and the column effluent was dialyzed as described above. The dialyzate was dried, dissolved in buffer and treated with a phosphomonoesterase. Comparison of the amount of inorganic phosphate released by the enzyme to the total phosphate provided an estimate of chain length.

TA chain length was estimated by treatment with a phosphomonoesterase. As with LTA, the inorganic phosphate released from terminal phosphates of TA by the enzyme was compared to total phosphate content. The experiment was repeated four times.

TA chain length was also estimated by titration of the primary and secondary acidic groups [65,73]. TA containing 166 μmoles of phosphate was dissolved in 4.0 ml of 2.0 N NH$_4$OH and the solution was dried under nitrogen sweep at 40°C. Dried TA was dissolved in 5.0 ml of distilled water and passed over a 3.0 ml column of Dowex-50 8-X (H$^+$ form). The column was washed with 10 ml of distilled water. The eluate and washings were combined and adjusted to 20 ml with distilled water. The TA solution was titrated using a microburet with 0.097 M carbonate free NaOH which had been standardized by titrating a potassium acid phthalate primary standard to a phenophthalein end point. A plot of ΔpH/ΔV against V, where V was the volume of base used, gave a measure of the points of neutralization of the primary and secondary acidic groups. Comparison of the number of primary and secondary acidic groups gave an estimate of chain length.

Nuclear Magnetic Resonance Spectroscopy

Natural abundance carbon-13 NMR spectra were obtained for both the lipoteichoic acid and teichoic acid in an effort to elucidate structure. Samples were dissolved in deuterated water at concentrations ranging from 25 to 120 mg/ml at least 24h before experimental runs to insure that complete hydrogen exchange had occurred. Samples were placed in

10 mm thin wall NMR tubes, and a sealed coaxial tube was inserted which contained tetra-methylsilane (TMS) in benzene. Chemical shifts of the carbons in unknown samples were determined relative to the external TMS standard. The broad band proton decoupled carbon-13 spectra at 62.83 MHz were obtained in a Fourier transform mode on a Bruker WM-250 MHz NMR spectrometer. The number of scans accumulated for each spectrum depended on the sample concentration, and was in the range of 2,000 to 15,000 scans. Parameters for carbon-13 NMR are in the Appendix.

Phosphorus-31 NMR spectra were also obtained for both teichoic acids using the method of Costello et al. [74]. Samples containing 50 to 200 μmoles of phosphate were dissolved in 2.0 ml of 10% deuterated water which was 0.1 M in sodium (ethylene-dinitrilo) tetraacetate (EDTA) and pH 7.0. Solvated samples were kept at ambient temperature for at least 24h before the experiments were done. Chemical shifts of phosphorus in the unknown samples were determined relative to 85% orthophosphoric acid in a coaxial system. Broad band proton decoupled phosphorus-31 spectra at 101.27 MHz were obtained in a Fourier transform mode. From 100 to 500 scans were accumulated for each spectrum. The Appendix contains the phosphorus-31 NMR parameters. For the lipoteichoic acid, all NMR experiments were done with the high molecular weight micellar form.

General Analytical and Miscellaneous Methods

Amino Acid and Amino Sugar Analyses

Amino acids were released from LCWF, PF, BHPF, LTA and TA samples by hydroly-sis in 6.0 M HCl for 16 to 24h at 100°C in evacuated sealed glass ampules in a sand bath. Acid was removed in an evacuated desiccator over NaOH pellets and anhydrous $CaSO_4$. Residual HCl was removed by adding distilled water to each sample followed by a second desiccation. Hydrolyzates were then dissolved in sample buffer (0.2 N sodium citrate, pH 2.2). LTA and TA were also examined for ester linked amino acids by hydrolysis of

LTA and TA samples in 0.1 ml of 1.0 M NaOH at 95°C for 3h in sealed 5 mm O.D. glass tubes in a boiling water bath. After addition of an equal volume of 1.0 M HCl to neutralize the base, the hydrolyzates were diluted to 1.5 ml with sample buffer.

Amino sugars were released by hydrolysis of respective cell wall or teichoic acid samples in 0.1 ml of 4 M HCl for 4h at 100°C in 5 mm O.D. sealed glass tubes in a sand bath. Hydrolyzates to be analyzed for amino sugars were neutralized with an equal volume of 4 M NaOH and diluted to 1.5 ml with sample buffer to avoid destruction of muramic acid which may occur during the drying step employed for analysis of amino acids [75].

Amino acid and amino sugar analyses were performed on a Beckman Model 120C amino acid analyzer using a modification of the method of Spackman et al. [76]. Long column runs were programmed with a temperature change from 30°C to 55°C after 15 min to resolve muramic acid and glutamic acid [77]. An internal standard of 2-amino-n-butyric acid (Sigma) was added to each sample. Commercial amino acid standards (Pierce Chemical Co., Rockford, IL) as well as standards of diaminopimelic acid, muramic acid and glucos-amine (Sigma) were prepared with the internal standard to determine relative molar responses. Total amino sugars were also estimated colorimetrically and identified by gas liquid chromatography (GLC).

Neutral Sugar Analysis

Neutral sugars were analyzed by GLC as the alditol acetates which were prepared by the method of Albersheim et al. [78] as modified by Griggs et al. [79]. Samples contain-ing 50 to 200 μg of sugars were dried in an ignition tube, dissolved in 1.0 ml of 2.0 M trifluoroacetic acid (TFA) and the sealed tubes were held at 120°C for 1h in a sand bath. TFA was removed on a rotary evaporator, the samples were dissolved in water and passed over a 1.0 ml column of Dowex-50 8-X (H$^+$ form). For some samples the ion exchange step was omitted to allow detection of amino sugars in the same chromatographic run

with neutral sugars. The column effluents were dried in vials and 1.0 ml of 1.0 mg NaBH$_4$/ml 1.0 M NH$_4$OH was added. After 1 to 16h, excess NaBH$_4$ was destroyed by dropwise addition of glacial acetic acid until effervescence ceased. The samples were then dried under nitrogen at 60°C. Benzene-methanol (1:5 v/v) (1.0 ml) containing one drop of acetic acid was added and after 10 min the solvents were evaporated under nitrogen at 60°C. The wash step was repeated four times with the omission of benzene. After the last evaporation, 1.0 ml of acetic anhydride was added and the samples were heated at 100°C for 2h. Acetic anhydride was removed by rotary evaporation at 40°C and the acetylated sugars were transferred to vials with three methylene chloride washes. Methylene chloride was removed under nitrogen and the samples were dissolved in chloroform for GLC analysis.

After removal of TFA from LTA and TA hydrolyzates, assays of total and inorganic phosphate were done. Samples which contained organic phosphate were treated with a phosphomonoesterase until greater than 95% of the phosphate was present as inorganic phosphate. The samples were then passed over a mixed bed ion exchange resin (1.0 ml of Amberlite MB-1, Sigma), reduced and acetylated as described above.

For conventional analysis of sugars, the hydrolysis, reduction and acetylation steps were done in sequence. Structural information was gained by altering the sequence or omitting steps. Reversing the sequence of the hydrolysis and reduction steps provided information about the sugars, if any, that were present at the reducing end of an oligo- or polysaccharide. Omitting the reduction step allowed detection of naturally occurring alditols.

Inositol (Sigma) was the internal standard in all cases. Standards containing glycerol, 2-deoxyribose, ribose, ribitol, rhamnose, glucose, α-methylglucose, β-methylglucose, galactose, mannose, glucosamine, galactosamine, D-glucohepdose, α-mannoheptitol, 2-keto-3-deoxyoctonate (Sigma) and 1,4-anhydroribitol were prepared with the internal standard