Fall 1967

THE CHEMISTRY OF VITREOSCILLA CELL ENVELOPES

GERARD O'LEARY JR.

68-6824

O'LEARY Jr., Gerard Paul, 1940-

THE CHEMISTRY OF VITREOSCILLA CELL ENVELOPES.

University of New Hampshire, Ph. D., 1967
Microbiology

University Microfilms, Inc., Ann Arbor, Michigan

THE CHEMISTRY OF *VITREOSCILLA* CELL ENVELOPES

by

GERARD PAUL O'LEARY JR.

B. S., Mount Saint Mary's College, 1962

M. S., New Mexico State University, 1964

A THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Doctor of Philosophy

Graduate School

Department of Microbiology

September, 1967

This thesis has been examined and approved.

George J. Hageage Jr.
Thesis Director, George Hageage Jr., National Institute of Health

Miyoshi Ikawa
Miyoshi Ikawa, Prof. of Biochemistry

Theodore E. Metcalf
Theodore Metcalf, Prof. of Microbiology

A. E. Teeri
Arthur Teeri, Prof. of Biochemistry

William Chesbro
William Chesbro, Assoc. Prof. of Microbiology

9/15/67
Date

This thesis is dedicated first and foremost to my parents. It is also dedicated to Mr. Rodrick Labrie in the hopes that its resurrection in three years will participate in his realization that the pursuit of knowledge is food for all men and that only through some degree of compatability can and should a worthwhile recognition, by an intellectual society, be attained.

ACKNOWLEDGMENTS

The author wishes to express his sincerest gratitude to Dr. George J. Hageage Jr. for his continued guidance and perserverance during this study and the preparation of this manuscript.

The author is also indebted to Dr. Miyoshi Ikawa whose constant guidance in chemical analysis and interpretation was greatly appreciated.

Recognition is given to Mr. Paul Forest for his stimulating discussion and his help in analytical procedures.

Thanks are also extended to Dr. William Chesbro, Dr. Jeffrey Burnham, and Emmory Clippert for their use of equipment and photographic assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The chemical analysis of bacterial cell walls has given investigators much insight towards an understanding of bacterial physiology and taxonomy. The procaryotic cells have been divided into two major groups on the basis of their gram reaction. This reaction resides in the chemical differences of the cell envelope. Thus, the chemical composition of gram-negative envelopes consists of lipoprotein, lipopolysaccharide, and mucopeptide as compared to the cell wall of gram-positive organisms which contain teichoic acids and mucopeptide. In addition the structural rigidity of the bacterial cell was found to reside in one specific layer and this layer was later characterized as a N-acetyl-glucosamine-N-acetyl-muramic acid repeating backbone usually containing D-alanine-D-isoglutamine-L-lysine or DAP-D-alanine-D-alanine side chain. These peptide side chains could also be connected to each other via another peptide molecule.

More recently, the outer wavy layer of the gram-negative envelope has been shown to be responsible for much of their toxic nature. The antigenic specificity found with different species of the same genus, i.e. Salmonella, has been chemically traced, at least in part, to a small side chain of sugars present in the endotoxic molecule. This outer layer of the cell wall is now known as the lipopolysaccharide layer. In many cases, it is the changing of a single monosaccharide

which imparts to that species or strain its particular antigenicity.

As early as 1945, Landsteiner realized the role chemical analysis of the cell wall would place in the scheme of bacterial taxonomy. It was through the chemical and immunological analysis of the Salmonella that 1,000 serotypes of the genus were described. The more chemical knowledge we attain about the gram-negative organism, the more types we discover. Even though some differences may be slight, this is still a step forward in a better understanding of their taxonomic relationship to one another and even to the eucaryotic cell.

The Vitreoscilla belong to that distinct group of procaryotic protests whose locomotion is accomplished by a type of gliding action. This type of motility is suggested to be caused by rhythmatic contractions taking place in the wall of the organism. Therefore, this investigation of the chemical composition of the cell envelopes of Vitreoscilla was undertaken to determine if (1) there was some major difference between gram-negative non-gliders and gliders, in hopes of relating this difference to some type of contractile mechanism, and (2) to determine if the chemical composition of the cell envelope could be of taxonomic value.

LITERATURE REVIEW

The Vitreoscilla are a group of procaryotic protists which are motile by means of a unique system known as gliding. These organisms, first isolated by Pringsheim and Robinow in 1947 from cow droppings, form trichomes which vary in length up to 100 u and are 1.2-1.5 u in width. Trichomes attaining a large enough length rarely remain straight but bend irregularly forming aggregates resembling curled hair (Pringsheim, 1949). On the basis of their trichomes locomotion and gliding motility, Pringsheim (1951) placed them in the order Beggiatoales, family Vitreoscillaceae where they remain to this date (Bergey's Manuel of Determinative Bacteriology, 1957).

The gliding locomotion exhibited by the Vitreoscilla is by no means restricted to these organisms or to the members of the family Beggiatoaceae, but is characteristic of the Myxobacteria and the Myxophyceae as well. Adanson (1767) was the first to describe this type of translocation and Cohn (1870-1) also described short filaments, composed of sections, which moved in an extremely slow manner over a solid surface, presumably a type of gliding movement.

One of the most widely accepted theories of the gliding mechanism involves the initiation of some type of contractile wave which is continuous along the whole length of the

organism. Weibull (1960) is of the opinion that this is probably associated with the cell envelope, but that it may be initiated from within the protoplasm itself. Costerton, Murray, and Robinow (1961) also hold to the contraction theory of movement and from their ultrastructure studies have brought forward the hypothesis,

> That the motility of these organisms is a function of orderly waves of contraction in an elastic outer layer of their cell walls. A rhythmic microscale undulation of this superficial layer could account for the gliding motility of Vitreoscilla in contact with a surface, for an apparent inability to swim in fluids, and for the rebound phenomenon when a solid surface is touched.

Kellenberger and Ryter (1958) were two of the first investigators to describe a multilayered envelope for gram-negative bacteria. Their thin-sections revealed the cell wall of Escherichia coli B to be composed of three 20-30 Å layers which they considered as being, "constituted of polysaccharide which is coated inside and outside either by protein, lipidic groups, or both." Weidel, Frank, and Martin (1960), working with E. coli, independently confirmed Kellenberger and Ryter's multilayered cell wall structure, however, Murray (1963) demonstrated that the cell wall of Spirllium serpens contained in addition to the three layers, a thin taut layer found between the plasma membrane and the three outer layers. More recent investigations have shown this multilayered wall structure to be typical for most gram-negative bacteria including E. coli B (Murray, Steed, and Elson, 1965), Veillonella (Bladen and Mergenhagen, 1964), and Thiobacillus thiooxidans (Mahoney and Edwards, 1966).

The ultrastructure of Vitreoscilla cell envelopes were investigated by Costerton et al. (1961) and by Hageage (1963), and were shown to be similar in appearance to those of other gram-negative, non-gliding bacteria. The cell envelope may be divided into three layers as seen in cross-section under the electron microscope. The outer portion was described as a wavy dense-light-dense layer measuring about 50 Å in thickness. Between this outer layer and the plasma membrane was a dense layer measuring approximately 30 Å in width while the total thickness of the wall varied between 140-400 Å due to the folding of the outer layer.

Bolle and Kellenberger (1958) were the first investigators to study the action of detergents on the cell envelope. They found that 1% lauryl sulfate removed the outer layer of the cell envelope. Similar results were obtained by Martin and Frank (1962), and they concluded that what remained after detergent action was the structural mucopeptide which is responsible for the rigidity of the whole cell. The existence of this rigid inner layer was confirmed by Weidel (1961) during his investigations of the chemical structure of the cell envelope of E. coli B.

The general structure and gross chemistry of gram-negative envelopes can then be represented by Weidel's three layered sandwich structure consisting of an outer layer of lipoprotein, a middle layer of lipopolysaccharide, and an inner layer of structural mucopeptide lying next to the plasma membrane. A chemical view of the cell wall of rough and

smooth strains of E. coli strain Lilly and V_2, respectively, was put forth by Wardlaw (1964). It is Wardlaw's contention, based upon the previous work of White (1928-1929),

> that the roughness or smoothness of a gram-negative organism is related to the proportion of the cell envelope surface occupied, respectively, by hydrophobic lipid or hydrophilic polysaccharide, the smooth strain having the latter in preponderance. The smooth strain V_2, which contains nine times more lipopolysaccharide than the rough strain Lilly, is shown as having the lipopolysaccharide extruding through the lipo-protein layer which it overflows and to some extent covers up. In Lilly, the lipopolysaccharide is scanty and occupies scarcely any of the cell.

In the past three years much interest has developed concerning the role of multivalent cations in the cell wall of gram-negative bacteria. The initiation of this line of research can be traced to the studies of Eagon and his co-workers (Carlson and Eagon, 1965; Asbell and Eagon, 1966; and Eagon, Simmons, and Carlson, 1965). They observed that osmoplasts of Pseudomonas aeruginosa could be formed and that the osmoplasts so formed could be restored to their rigid rod shaped form by the addition of divalent cations. They speculated that the removal of the cations, Ca^{++}, Mg^{++}, and Zn^{++}, caused the disruption of cross-linkages in the cell wall and was evidence for a non-peptidoglycan layer responsible for cell wall rigidity. Electron microscopy has also confirmed these results although the actual area of the cell wall affected by divalent cations has not been completely elucidated. Brown (1964) has implicated the structural organization of lipo-protein-type membranes while Asbell and Eagon (1966) stated that the divalent cations are involved in the

non-enzymatic assembly of substituents of lipopolysaccharide or lipoprotein or both. Divalent cations may therefore play an important role in the structural integrity for the genus Pseudomonas and marine bacterial forms. Whether this situation also applies to other gram-negative organisms remains to be seen.

The chemical analysis of gram-negative bacterial envelopes has been accomplished by means of isolating either fragmentary particles of their cell walls or by the isolation and purification of the entire cell envelope, including the plasma membrane. Recently, MacLeod (personal communication) has separated the gram-negative wall from the cytoplasmic membrane utilizing a marine Pseudomonas. MacLeod related that under certain ionic conditions the organism forms a true protoplast by sluffing off its cell wall which can be readily collected by centrifugation. This accomplishment will reopen the chemical analysis of true gram-negative wall and membrane structures for at least some gram-negative marine species.

Vincenzi (1887) was the first to isolate the cell wall of a bacterium. The substance isolated was an alkali resistant structure of the cell and not the entire wall. With the advent of the electron microscope in the early 1940's, it became evident that new methods were necessary for obtaining gram-negative envelopes. Thus, workers turned to mechanical disruption of bacterial cells (Salton, 1964).

The use of violent agitation by King and Alexander (1948) along with the incorporation of smooth glass beads

yielded bacterial hulls freed from cytoplasmic constituents. It was the introduction in 1948 of the Mickle tissue disintegrator and Dawson's (1949) use of glass beads with the apparatus which led to a quantitative procedure for the isolation of cell wall material. The technique was at first limited to the disruption of gram-positive organisms, however, Salton and Horne (1951) employing glass containers for the Mickle, found this method to be satisfactory for the isolation of gram-negative envelopes. Cummins and Harris (1958) also used this method of isolation to obtain cell walls from a variety of bacterial organisms.

Sonic and ultrasonic disintegration has been widely employed for the disruption of microbial cell systems. Salton (1953) employed sonic disruption to obtain walls from Pseudomonas fluorescens, Spirillum serpens, and Bacterium coli (sic). This procedure has also been used by Ikawa and Snell (1960) for the disruption of gram-positive cells. The use of sonicators, however, has presented the problem of possible "solubilization" of material during sonication. That some solubilization occurs has been shown by Roberson and Schwab (1960). This was not true solubilization however, since sedimentation of the material could be accomplished by the use of high centrifugal fields. The practicality of using sonic disruption for large scale wall isolation depends upon the strength of the wall structure and in general gram-negative cells are more susceptible to sonication than gram-positive cells.

Foster, Cowan, and Magg (1962) described an explosive decompression system used to disrupt cells of Serratia marcescens. Ribi, et al. (1959) developed a pressure cell disintegrator which disrupted cells by forcing them through a needle valve under high pressure while Salton (1956) and Brown (1961) used autolysis for obtaining cell walls. Osmotic lysis has also been employed extensively with marine bacterial forms. Christian and Ingram (1959) obtained undamaged envelopes from Micrococcus halodenitrificans by shaking the cells in distilled water. However, Brown and Shorey (1962) found complete dissolution of Halobacterium by this method. It was suggested by Brown (1962) that this was due to the dependency of the wall structure on divalent cations. Bacterial lysis by lysozyme, as a means of obtaining cell wall material, has been adequately reviewed by Alderton, et al. (1945) and Salton (1958) although these methods are rarely used for gram-negative bacteria. Utilization of various types of bacterial lysis for the procurement of cell wall material has been accomplished in the past. These methods which include lysis by agents of microbial origin, lysis due to induced metabolic disturbances, and bacterial lysis by means of virulent phage and the action of penicillin, have been adequately reviewed by Welsch (1958), McQuillan (1958), and Weidel and Primosigh (1958) respectively, and need not be dwelt on here.

Once the investigator has obtained a cell wall or cell envelope fraction, the preparation must be purified. The cell homogenate is centrifuged at approximately 3,000-4,000 x g for 10 minutes which sediments whole cells plus any large

aggregated particulate matter. The fragmented wall material is then separated from the cytoplasm by centrifugation at 10,000 x g to 20,000 x g for 15 minutes (Salton, 1964). Generally, gram-positive walls are cleaned of cytoplasmic debris and ribosomes by treatment with trypsin and other proteolytic enzymes (Salton, 1953), while gram-negative envelopes are cleaned by continued washing. Salton and Horne (1951) purified the cell walls of some gram-negative bacteria by washing several times with distilled water. A similar procedure was used to obtain clean envelope preparations of Vibrio fetus (Keeler, 1965) but Salton (1953) found that a more rapid and efficient removal of cytoplasmic debris could be obtained if he first washed the wall fragments in 1M NaCl prior to the distilled water washings. The walls were then washed with distilled water until no trace of the chloride ion could be detected by the silver nitrate test. Suspension of E. coli whole cells in a wash solution consisting of either 1M phosphate buffer, pH 7.0 or 1% NaCl prior to disruption, facilitated the removal of the cytoplasmic material from the envelope fractions (Salton and Horne, 1951).

Albertsson (1958) described the behavior of various particles including bacterial cell walls in phosphate-polyethlene glycols (PEG) and dextrin-PEG systems. Through the use of PEG and dextrin-PEG systems together with other liquid two phase systems, a variety of material could be isolated. This system can serve as an additional step, along with centrifugation techniques, for the isolation of a more

homogeneous cell wall fraction. The use of sucrose zone centrifugation was introduced by Roberson and Schwab (1960). The walls were centrifuged in a gradient of 1.025 to 1.30 density from top to bottom. This method, which gives a very reproducible and homogeneous wall preparation, was also used by Salton (1964) for the separation of cell walls from chromatophores of Rhodospirillum.

Many investigators have set certain standards for determining wall or envelope homogeniety and purity. Salton and Horne (1951) developed the use of ultra-violet absorption spectra of isolated cell walls. They subjected their wall sample to a continuous spectrum from 240 to 340 mu and then noted peaking at 260 and 280 mu as indications of protein and nucleic acid contamination. However, Barkulis and Jones (1957) showed that contamination is still present with this system. Salton (1956) also examined his cell wall preparations in the electron microscope and felt that this technique was "the most useful single guide to the homogeneity of the material". Electrophoretic analysis was suggested by Roberson and Schwab (1960) as a means of determining purity. They also favored the use of sucrose zone centrifugation over differential centrifugation. They felt that the whole cells absorbed some sucrose, thus increasing their sedimentation differential between the whole cells and the cell walls. Most investigators feel that the use of U.V. spectrum and electron microscopy are the best and quickest methods available for checking the purity of a cell wall preparation.

The unveiling of the chemical constituents of bacterial cell walls has shown that the two groups of bacteria distinguished by the gram reaction have corresponding differences in their cell wall composition. In contrast to the cell walls of gram-positive organisms the gram-negative envelopes were found to be composed of a wide variety of chemical constituents.

In a comparative study of the chemical make-up of the walls of gram-positive and gram-negative bacteria, Salton (1953) found the envelopes of gram-negatives to contain a much wider array of amino acids. Working with E. coli and Salmonella pullorum, he found high concentrations of alanine, aspartic acid, glutamic acid, serine, glycine, threonine, lysine, valine, leucine, and isoleucine. All the above mentioned amino acids were also common to gram-positive walls with the exception of threonine and serine. Gram-negative envelopes also contained proline, arginine, methionine, phenylalanine, and diaminopimelic acid which are not common to gram-positive walls although they may be found in isolated cases. Pseudomonas aeruginosa and Salmonella bethesda also contained this array of amino acids as reported by Collins (1963). Qualitatively, the envelopes were identical but quantitatively very different. The presence of diaminopimelic acid (DAP) was first discovered, isolated, and characterized by Work (1949, 1951) and is now known to be a typical component of the mucopeptide layer of bacterial walls (Primosigh, et al., 1961).

In general, gram-negative envelopes contain less amino sugar than gram-positive walls. Three percent amino sugar was

reported by Salton (1953) for the envelopes of E. coli while Gelby, et al. (1958) found 16-22% amino sugar in the walls of M. lysodeiktecus. Weibull and Bergstrom (1958) reported that 7-9% of the dry weight of Bacillus megatherium cell walls was amino sugar. The gliding organisms contain even lower percentages of amino sugar. The investigation of Collins (1964), using a marine bacterium, Cytophaga fermentans, indicated that only 0.4% of the dry weight of its envelope was amino sugar. Myxococcus xanthus has been shown to contain only 0.1% amino sugar in its cell envelope.

The values obtained for the carbohydrate content of cell envelopes have also been variable. Collins (1963) reported 8% total carbohydrate for Pseudomonas aeruginosa while Salton (1953) reported 16% carbohydrate for the envelopes of E. coli. Most gram-negative envelopes average between 10-20% carbohydrate while the rest of the cell envelope may be accounted for by protein which averages between 55 to 65% of the dry weight.

Salton (1964) has reviewed the literature concerning the monosaccharide units found in isolated envelopes of gram-negative bacteria. In all cases reported, glucose was a major constituent of the envelopes while galactose was also prevalent in most envelopes analyzed. Some Salmonella and Shigella were found to possess mannose, rhamnose, and pentoses while Collins (1963) reported obtaining glucosamine, muramic acid, glucose, mannose, and rhamnose from the envelopes of Pseudomonas aeruginosa NCTC 6750 and Salmonella bethesda. Three strains

of Salmonella typhosa, H-901, 0-901, and Vi-1, were chemically investigated by Ghatak, Verme, and Misra (1966) for the presence of monosaccharide constituents. They found the common hexoses, plus the pentoses arabinose and rhamnose.

The unique features of the gram-negative envelope with regards to monosaccharide units are their location in the outer lipopolysaccharide layer. The chemistry of this layer will be discussed later and it suffices here to mention the presence in the envelope of Sal. typhimurium a heptose and a dideoxy sugar (Herzberg and Green, 1964). Even more recently, Raff and Wheat (1966) found a 3 amino sugar present in Citrobacter freundii 8090. Their preliminary evidence suggested a structure of 3-amino-3,6 dideoxyhexose. With respect to monosaccharides, the gliding bacteria have been neglected except for Mason and Powelson's (1958) report concerning Myxococcus xanthus. They found galactose, glucose, hexosamine, and rhamnose after hydrolysis of envelopes in 2N HCl at 100° C for 4 hours. Cytophaga fermentans has also been shown to contain 0.03% glucosamine and 0.1% muramic acid (Collins, 1964).

The lipids of gram-negative envelopes have only recently come under investigation. Lipid solvent soluble material accounts for approximately 10 to 20% of the cell envelope of most gram-negative bacteria (Salton, 1953 and Herzberg and Green, 1964), although Mason and Powelson (1958) reported up to 50% lipid for the envelope of Myxococcus xanthus. The lipids and fatty acids of envelopes have not been given much attention with the exception of the work

mentioned above. However, the investigations of Marr and Kaneshiro (1960) and Robrish and Marr (1962) into the cell wall lipids of Azotobacter vinlandii (agrilis) revealed that their envelopes contained 28% lipid. The lipids consisted of 10 to 20% neutral lipid and 70 to 75% phosphatides. The chief component of the neutral portion was the free fatty acid, palmitoleic acid. Palmitoleic acid accounted for more than 50% of the total while the remaining portion contained glycerides and other esters. The glyceride portion consisted almost entirely of phosphatidylethanolamine. The phospholipid associated fatty acids were myristic, palmitic, palmitoleic, and C 18 monoenoic acids. Kates (1964) in his review of bacterial lipids, states, "However, we may conclude from the high proportion of phosphatidylethanolamine that this phosphatide is probably present both in the cell wall and in the membrane." Once again, we see the complexity derived from having a system where the wall and membrane cannot be separated from each other. It can be stated, that most investigators concerned with cell wall chemistry have not determined the fatty acid content of gram-negative cell envelopes.

The study of bacterial endotoxins has been a topic of research since the early 1940's. Morgan and Partridge (1941, 1942) isolated and examined the O antigenic complex from Bacterium typhosum (sic). They found the lipopolysaccharides (endotoxin) to be chemically composed of a complex lipid, polysaccharide, and protein or peptide-like complex. This complex could be extracted from whole cells or purified cell walls. They also believed the protein moiety was necessary

for endotoxic potency. In 1952, Luderitz and Westphal indicated their investigation and isolation of a bacterial substance from cell walls having antigenic characteristics. In 1954, Westphal and Luteritz demonstrated that essentially protein-free lipopolysaccharide retained its ability to stimulate the immune response and thus showed the protein portion unnecessary for toxic responses. Similar results were obtained by Ribi, et al. (1961) with isolated lipopolysaccharide from Salmonella abortus equi and Shigella flexneri which had very low nitrogen and lipid properties but which showed a good immune response.

All Enterobacteriaceae and most gram-negative bacteria produce some sort of endotoxin. Probably the most studied endotoxins or lipopolysaccharides are those isolated from the Salmonella bacillus. The Salmonella have approximately 1,000 serotypes as defined by immunochemistry and these serotypes are based upon two specific antigenic components, the thermolabile H flagellar antigen and the thermostable O somatic antigen. Cummins (1956) showed that the O antigen was a constitutuve part of the bacterial cell wall. The chemical analysis of the O and H antigens, of which the O is lipopolysaccharide, has led to the 1,000 serotypes of Salmonella. Thus the lipopolysaccharide complex is not only important for its endotoxic properties but as a method of classification of bacterial types. Landsteiner (1945) said,

> The frequent overlapping reactions of Salmonella bacilli have not been the object of chemical investigation. One may expect that such studies will provide information on the apparent mosaic structure of antigens. Evidence for the existence of separate chemical

entities underlying the serological reactions may be gained by demonstrating several specific groupings in homogeneous, well purified polysaccharides.

It was through the pioneer work of Morgan and Goebel (1945) that intensive chemical investigation was undertaken. The monosaccharides of different Salmonella lipopolysaccharide complexes were investigated by Kaufmann, et al. (1960). They found sixteen chemotypes with regard to the differences in the chemical composition of the O antigen polysaccharide, the simplest of these being composed of glucosamine, galactose, glucose, and heptose. Thus from the investigations of Morgan, Partridge, Westphal, Kauffmann, and Ribi active investigation into the qualitative and quantitative chemical composition of bacterial lipopolysaccharides was initiated. The initial chemical investigation was undertaken to determine the specific chemical substances responsible for the antigenic specificity of endotoxins. As an outgrowth of these investigations came the structural and biosynthetic theories of lipopolysaccharides.

It has been shown by a number of workers that the medium used for growth of the organism influences the chemical composition and toxic properties of the lipopolysaccharide obtained. Fukushi, et al. (1964) showed that the type of medium and extraction procedure greatly influence the chemical and toxic properties of Serratia marcescens lipopolysaccharides. Using Ribi's (1961) M-9 medium for growth and two extraction procedures, aqueous ether and trichloroacetic acid, they showed that the TCA extracted material was lower in percent nitrogen but higher in percent phosphorus, carbohydrate and

hexosamine. The lethality for mice (L.D. 50) was lower for TCA extracted material than for aqueous ether extracts. Extraction comparisons were also made by Nowotny, et al. (1963) using Serratia marcescens Bizio, Salmonella typhosa 0901, and Escherichia coli K-12. Nowotny employed five extraction procedures: trichloroacetic acid (TCA), Westphal and Luderitz's hot phenol and water (PHW), Ribi's (1959) water-saturated ethyl ether (AQE), and cetyltrimethyl-ammonium bromide in 5% solution (CTB). From their data, it became evident that no one method can be generally applied to the isolation of bacterial lipopolysaccharides either from an antigenic, toxic, or chemical point of view. It seems the binding forces of the lipopolysaccharide complexes are strikingly different and thus the difference in extractability. Any attempt to correlate different results becomes an impossible task, due to the inconstancy of the methods used. Anacker, et al. (1964), using three extraction procedures of both whole cells and cell walls of Escherichia coli 0113, showed the diversity of chemical results that can be obtained. They reported that not only the method of extraction but the method of purification can effect the chemical composition and molar ratios obtained for the same organism. Whole cells extracted with TCA, dialyzed, and precipitated with ethanol, resulted in 1.6% of the LPS as heptose. By extracting with phenol and water and the water phase dialyzed, the heptose increased two fold to 3.2%. Purification of the dialyzed material by four centrifugations at 105,000 x g resulted in an additional

increase in heptose to 4.2% of the total weight extracted. The total hexosamine also fluctuated with the extraction methods employed: TCA, 11%; aqueous ether, 14%; hot phenol-water (HPW), 9.7%. Upon centrifugation, the HPW extracted hexosamine dropped to 6.1%. It is thus apparent that the type of extraction and purification procedure used dictates the chemical results obtained.

Alaupovic, et al. (1966) dramatically demonstrated that the method used determines the percent recovery of the individual sugars. Using chromogenic and non-chromogenic strains of Serratia marcescens N.R.C. No. 8926 and elaborate extraction and purification procedures, they found the percent anthrone positive carbohydrate to vary from 18.7% to 24.1%, glucosamine varied from 2.44% to 4.0%, heptose from 5.3% to 8.3%, and uronic acid from 6.9% to 10.3%. Variable percentages in the amount of lipopolysaccharide isolated from E. coli 602 have also been shown by Kasai (1966) to be due to differences in fractionation procedures used. The major consistent fact that remains is the qualitative data for the chemical compositions. The structural chemical components of the lipopolysaccharides studied to date have been qualitatively determined by most workers (Fukushi, et al., 1964; Anacker, et al., 1964; Luderitz, Staub, and Westphal, 1966; Ribi, et al., 1962; and Westphal and Luderitz, 1964). In a review article by Luderitz, Staub, and Westphal (1966) it was shown that the major monosaccharides common to most known lipopolysaccharides were glucosamine, heptose, galactose, glucose, and 2-keto-3-deoxyoctonate (KDO).

The least investigated portion of the lipopolysaccharide molecule is the lipid portion designated lipid A by Westphal and Luderitz (1954). They took a phenol-water purified polysaccharide from S. abortus equi and hydrolyzed for 3 min. at 100 C in mineral acid. The product obtained after neutralization was called lipid A, of which approximately 26% was chloroform soluble. Upon further hydrolysis with 5N HCl for 15 hr. at 100 C, they obtained hexosamine, an aminodicarboxylic acid, glycerol, and phosphoric acid. This, to my knowledge, was the first real attempt to elucidate the chemical composition of the lipid moiety. Kasai and Yamamo (1964) have stated that the lipids obtained from E. coli communis 602 have two characteristics upon thin layer chromatography. The chromatograms showed lipid components with high RF values and also some with low RF values. They state, "Accordingly, the lipid components with high RF values appeared to play an important role for the toxic activity of lipopolysaccharide". Using naturally occurring hapten from E. coli 0113 which had been extracted with 0.5M TCA from lyophilized material, Anacker (1964) found eight definite fatty acid peaks upon gas-liquid chromatography. The eight peaks corresponded to 2, 12, 14, 16, 18, 20, and 22 carbon acids and a peak between the 20 and 22 carbon acids was also noted. According to Anacker's (1964) data, phosphorus, heptose, 2-keto-3-deoxyoctonate, and long chain fatty acids may be an integral part of the endotoxin but are lacking in native hapten from E. coli 0113. The lipid A fractions of

E. coli communis 602, S. typhi 0901, S. paratyphi B 8006, and Sh. flexneri type Y were investigated by Kasai and Yamano (1964) and showed identical chromatograms on silica gel plates. It was shown that the fractions contained at least eight different lipid components, A1, through A8. Kasai stated that the relative ratios of these lipid components found in the lipid A may well vary with the cultural conditions employed and with the method of isolation of the lipid A fraction. Although they found a common number of lipid fractions, they also found that in H. pertussis the lipid fraction A2 was missing, so it is not quite as common as one might like.

Burton and Carter (1964) studied the lipid A components of E. coli 0111 B4. The lipopolysaccharide was prepared by cold aqueous phenol extraction. The crude LPS was suspended in 2% (w/v) distilled water and stirred for several hours in the cold. It was then precipitated by 1M NaCl and the precipitate suspended in distilled water and centrifuged. This material was then hydrolyzed with 0.1N HCl by refluxing for 35 min. and extracted with chloroform. The lipid A obtained in this manner contained glucosamine, long chained fatty acids, O-acetyl substituents, phosphate, calcium, and magnesium. Gas-liquid chromatography of the methyl esters of the fatty acids yielded four major peaks and two to four minor peaks. These results are in good agreement with Kasai's silica acid column chromatography analysis reported above. The four major fatty acids found were lauric, myristic, palmitic, and B-hydroxymyristic acid. A peak was also obtained behaving like B-hydroxydecanoic acid on vapor-phase

chromatography. Working with Serratia marcescens and E. coli 0111, Nowotny (1963) made the observation that the linkages between the O antigen and the rest of the molecule were of different hydrolytic strengths. Previously, Nowotny, et al. (1963) found a wide range of differences in the strength of linkages holding the endotoxic O antigens on the cell wall for different gram-negative families. In this particular report, Nowotny not only showed differences in binding forces holding O antigen complexes but that the ease of splitting of the polysaccharide portion from the lipid structure also differed from strain to strain. A chromogenic strain of S. marcescens liberated its lipid in 5 minutes under 0.1N formic acid while the non-chromogenic strain required 35 to 45 minutes. It was also found that Pseudomonas readily liberated its lipid A portion but, under identical conditions, E. coli K-12 lambela had to be hydrolyzed for at least 60 minutes. It, therefore, is evident that the liberation of lipid A from the endotoxin depends not only on the hydrolysis procedure but also the time factor and binding forces present in different bacterial endotoxic species. However, it can not be assumed that the lipid present in the wall or the isolated lipopolysaccharide is in the same form as observed after hydrolysis because of the number of sensitive ester linkages destroyed during acidic hydrolysis.

Six fatty acids were found in S. marcescens and seven in E. coli 0111 lipid A structures by Nowotny (1966) using paper chromatography. Gas chromatographic analysis also

revealed minor amounts of other fatty acids. The number of fatty acids present agreed well with the previously described findings of other investigators. Kasai (1966) has confirmed Nowotny's work and states that the lipid A fraction is a mixture composed of at least eight to ten fatty acids and that the relative amounts of these components vary within bacterial species, with cultural conditions and the method of isolation. Kasai has found that some fatty acids of E. coli and Bordella are liberated as early as two minutes during acid hydrolysis. It was found that the trimethylsilyl (TMS) chromatography of E. coli 602 showed a peak corresponding to B-hydroxymyristate. Gas-liquid chromatography yielded peaks for laurate, myristate, palmitate, and B hydroxymyristate. Thin layer chromatography on alkaline silica gel plates of LPS from E. coli, S. paratyphi B, and Sh. flexneri showed at least 14 lipids present, the additional fractions being sub-lipid components separated from the fractions previously designated A_3, A_4, A_5, and A_7 by Kasai and Yamano (1964). B-hydroxymyristate was found as a common component among all lipid A fractions studied but in addition an unidentified peak represents B-hydroxydecanoate. Bordella pertussis strains all seem to lack the lauric acid which is common to most other bacteria studied. Alaupovic, et al. (1966) found the fatty acid composition of both lipid A fractions from chromogenic and non-chromogenic strains of S. marcescens remarkably similar. The major acids found were B-hydroxymyristic, lauric, myristic, and palmitic acids. Four minor acids were also present but unidentified.

Thus, the number of fatty acids and the same four major types found seem fairly common to most bacterial species. One interesting fact is that 10% of the fatty acids from the chromogenic strain was acetic acid. It now appears that the lipid portions are composed chemically of glucosamine, fatty acids, and phosphorus; the major types of fatty acids being B hydroxy acids, lauric, myristic, and palmitic acids with usually four minor fatty acids present. These results are qualitative for most endotoxins, quantitation at present being a risky subject.

The normal monosaccharide units reported for most all lipopolysaccharides consists of heptose, glucose, galactose, glucosamine, and 2-keto-3-deoxyoctonate. The above sugars may be supplemented with other monosaccharides depending on the O antigen structure, which varies from organism to organism. 3,6-Dideoxy sugars have been reported as being found in the O antigenic side chain of lipopolysaccharides. For example, Salmonella typhimurium contains a 3,6 dideoxy-d-galactose now known as abequose (Osborn, 1963) and Heath, et al. (1966) reported another dideoxy sugar, colitose, located on the antigenic side chain of E. coli 0111. These sugars plus others such as mannose and rhamnose have been observed associated with this structure. The monosaccharides of the polysaccharide core and side chain have been reviewed by Luderitz, Staub, and Westphal (1966).

The 2-keto-3-deoxy-octonate (KDO) component was discovered by Heath and Ghalambor (1963) who demonstrated its presence in lipopolysaccharide extracts from E. coli 0111 B4

and J5. The molecule has since been found in all LPS's examined. Perry and Adams (1967) have identified KDO recently as a 3-deoxy-D-manno-octulosonic acid.

The heptose which is usually found in the lipopolysaccharide backbone as a heptose-phosphate polymer was first discovered by Jesaitis and Goebel (1952) from somatic antigen of Shigella sonnei. Osborn (1963) characterized the heptose from Sal. typhimurium as a L-glycero-D-mannoheptose.

A phospholipid has been implicated by Rothfield, et al. (1966) to be involved with the enzymatic synthesis of core lipopolysaccharide of Sal. typhimurium. This P-lipid was identified as phosphatidyl ethanolamine and has since been reported for other lipopolysaccharide complexes. Grollman and Osborn (1964) reported another phosphorylated compound, O-phosphorylethanolamine, isolated from Salmonella typhimurium, and claimed that it was not a degradation product of the phosphatidyl ethanolamine. They suggested that the O antigen contains an internal core polysaccharide common to all enteric bacteria in which O-phosphorylethanolamine could be a major component.

Sutherland and Wilkinson (1966) reported that the A3 strain of Klebsiella aerogenes does not contain glucose which is common to all other LPS molecules. They have stated that the absence of glucose indicates that the high content of galactose must be directly linked to a heptose-phosphate core. Uronic acids have also been found associated with different lipopolysaccharides. Recently, Adams and Martin (1967) isolated glucuronic and mannuronic acids from S. marcescens LPS with 90% phenol extraction.

The calculations of component molar ratios and studies with rough mutants has shed much light on the biosynthesis of the LPS molecule. This has led a few investigators to postulate a structural arrangement for the molecule. Working with a Sal. typhimurium deficient in phosphoglucose isomerase, Osborn (1963), found incomplete polysaccharide synthesis. The polysaccharide contained only heptose, phosphate, and KDO. This evidence, along with molar ratios, suggested to Osborn a molecule containing a heptose-phosphate backbone with periodic chains of a glucose-galactose polymer. The complete backbone structure was connected to a KDO molecule which was somehow connected to the lipid portion of the molecule. It was later found by Osborn, et al. (1964) that the glucose-galactose polymer contained another mole of glucose and a mole of N-acetylglucosamine. Therefore, the postulated core portion of the molecule became an N-acetylglucosamine-glucose-galactose-glucose polymer. The core structure has been extended by Rothfield, et al. (1966) and found to include another mole of galactose which seems to be attached to the first glucose off the KDO-heptose backbone. Elucidation of the glycosidic linkages of the core molecule was presented by Rothfield and Takeshita (1966) who showed that the newly discovered galactose linked 1---6 to the glucose while the galactose contained in the straight chain portion was linked 1---3 to the same glucose molecule.

Rothfield and Takeshita (1966) have also found a phospholipid associated with the backbone portion of the molecule. The P-lipid was identified as a phosphotidyl

ethanolamine. It was shown that the interaction of the lipid and lipopolysaccharide is necessary to permit the soluble transferase enzyme, the enzyme involved in the hooking of the core sugars to the backbone, to bind to its lipopolysaccharide substrate.

Nikaido and Nikaido (1965) described a lipid intermediate involved also in the synthesis of the O antigenic side chain of Sal. typhimurium lipopolysaccharide. This lipid carrier was suggested by Osborn and Weiner (1967) to be a complex polyglycerol phosphatide. The same lipid intermediate has been found for Sal. newington O antigen (Dankert, et al., 1966). The authors also showed the O antigen to be a mannose-rhamnose-galactose polymeric repeating unit. Salmonella typhimurium possesses a slightly altered O antigen containing one mole of abequose associated with the mannose-rhamnose-galactose chain as determined by Weiner, et al. (1966). The O antigen structure of Sal. typhimurium has also been confirmed by Osborn (1966). Robbins (1967) has isolated the lipid carrier involved in O antigen side chain synthesis of Sal. typhimurium and has shown it to be a polyisoprene pyrophosphate.

Escherichia coli has been studied with regard to its lipopolysaccharide structure. The backbone structure remains constant and is the same as that of Salmonella, but there does seem to be slight differences in the core chain polysaccharide. The glucose-galactose-glucose-N acetylglucosamine polymer remains constant but as Heath, et al. (1966) reported,

there are two molecules of a 3,6 dideoxy sugar, colitose, located at the second glucose and also linked terminally to the N acetyl glucosamine. The only differences then between E. coli 0111 B4 and Salmonella is the addition of colitose at the antigenic side chain. However, E. coli K-12 differs from E. coli 0111 B4 in that the core polysaccharide of E. coli 0111 B4 contains the proximal glucose in association with the heptose-phosphate backbone but with a change in the side chain polysaccharide, while E. coli K-12 contains a $(\text{rhamnose-galactose})_n$ polymer.

For the past four years, a large number of publications have appeared concerning the chemistry of Serratia lipopolysaccharide, but as yet no structure has been proposed. Preliminary investigations (Forest, personal communication) indicates that Serratia does not contain an O antigenic side chain but probably does possess a core structure similar to those already described.

The lipid portion of the lipopolysaccharide molecule is the least investigated structure. A structure was reported for Salmonella by Nowotny (1961) indicating the possibility of a poly-D-glucosamine-phosphate chain. Based upon the isolation of 6-P-D-glucosamine, 4-P-D-glucosamine, and 1-peptido-4-P-D-glucosamine, he postulated a peptide connected to the poly-glucosamine phosphate with fatty acids connected to the hydroxyl groups of the amino sugar. Escherchia coli strain 0111 B4 has been structurally examined by Burton and Carter (1964) and found to contain a molar

ratio of 2:1 for nitrogen and phosphorus. Their results, using sodium borohydride reduction and alkaline degradation, indicated that two glucosamine molecules were linked glycosidically. On the basis of the above information, they proposed a lipid A structure having a glucosamine backbone with staggered glycosidic and phosphodiester linkages, with fatty acids located at the free hydroxyl groups of the glucosamine. Changes in the position of the glycosidic bonds or of the phosphodiester bonds, respectively, would allow for a number of variations of the two structures proposed.

With the identification of phosphatidyl ethanolamine associated with lipopolysaccharide synthesis, Rothfield, et al. (1966) became interested in the possibility that its function might be related to solubilization of the biosynthetic system and micelle formation. They investigated, by electron microscopy, the structure of isolated lipopolysaccharide. Employing the negative staining technique of Horne, they found the isolated LPS of S. typhimurium to be a relatively homogeneous population of hollow spherical structures. Phosphatidyl ethanolamine was then added to the isolated LPS and the solution heated, cooled and examined under the electron microscope. They found that the particles became bound to the leaflets of phosphatidyl ethanolamine yielding a string-like arrangement. The electron microscopy of Veillonella parvula lipopolysaccharide has also revealed a particle predominately circular in shape. These ranged in diameter from 250 to 1400 Å (Mergenhagen, et al., 1966). Hageage (1967) also reported round particles from phenol-water extracts of Flexibacter.

His electron micrographs indicated that three types of structures were present, round A particles similar to those previously reported, B particles which seem to be material released from the A particle, and an amorphous material designated C particle. This same type of material has been seen in Serratia marcescens after 40% TCA extraction and acetone precipitation (Ikawa, 1967, personal communication). Negative staining and examination under the electron microscope of extracellular lipopolysaccharide by Taylor, et al. (1966) yielded long threads forming bunches of parallel filaments. The filaments also showed intermittent blebs within the filaments. These same filaments and bead like structures were also noted by Korczynski, et al. (1967) in lipopolysaccharide from Ferrobacillus feroxidans and by Ikawa (personal communication) in preparations from Serratia marcescens.

MATERIALS AND METHODS

Organisms

Four strains of Vitreoscilla and one strain of Escherichia coli were employed during this investigation. Three strains of Vitreoscilla sp., designated UM3, LCI, and UNH-L, were obtained from Dr. George Hageage while the fourth strain, Vitreoscilla stercoraria ATCC 15218 was obtained from the American Type Culture Collection. All Vitreoscilla cultures were maintained on YAT agar medium consisting of 0.15% (w/v) yeast extract, 2.5% (w/v) sodium acetate, 2.0% (w/v) tryptone, 0.75% (w/v) K_2HPO_4, and 1.5% (w/v) Bacto Agar and the pH. was adjusted to 7.8 with NH_4OH. Incubation was at 25 C with transfers to fresh media made every 30 days. Escherichia coli B/r3 was maintained on T-Soy agar and incubated at 37 C. The source of all organisms used in this study may be found in Table 1.

Preparation of Cell Envelope Material

The Vitreoscilla strains were grown in YAT broth in four liter flasks containing 2.5 l of medium at 25 C with vigorous shaking on a Brunswick rotary shaker. All cultures were harvested during their mid-log phase of growth at 4 C in a Serval RC-2 continuous flow centrifuge at 17,000 x g. The whole cells obtained were washed twice in 1% NaCl (pH 7.0) and either used immediately or frozen at -70 C for later use.

Table 1

Cultural Designation and Source of Organisms Studied.

Culture Designation	Source
Vitreoscilla sp. strain UM3	Isolated from pasture cow dung at the University of Maryland, Oct. 15, 1961
Vitreoscilla sp. strain UNH-L	Isolated from cow dung at the University of New Hampshire in October, 1964
Vitreoscilla sp. strain LCI	Isolated from pasture cow dung in London, Canada, July 30, 1961
Vitreoscilla stercoraria ATCC 15218	Isolated from dung by Pringsheim and Robinow in May, 1946 (Pringsheim, 1951) and obtained from ATCC
Escherichia coli B/r3	Obtained from the University of New Hampshire stock culture collection.

Approximately 2.5 g wet weight of washed cells were resuspended in 30 ml of 1% NaCl and sonicated for 3-4 minutes on a MSE sonicator at 15-17 Kc. Observations of the sonicate under phase microscopy indicated 95-99% breakage for this time interval for both Vitreoscilla and E. coli B/r3. The sonicate was centrifuged at 480 x g for 20 minutes to remove whole cells. The whole cell pellet was then either resuspended and resonicated or discarded. The envelope fraction was centrifuged at 3,020 x g for 20 minutes to remove any trace of whole cells and non-envelope particulate matter. The presence of whole cells was checked for by phase microscopy and the above procedure continued until no trace of whole cells remained. The crude envelope fraction was packed at 27,000 x g for 20 minutes and the supernatant discarded. The envelopes were resuspended in 1% NaCl and washed three times. After the third washing, the envelopes were suspended in distilled water (10 mg of envelopes in 20 ml of water) containing 0.01 mg/ml of DNase and RNase. The mixture was allowed to incubate at 37 C in a water bath for 3 hours after which the suspension was centrifuged at 27,000 x g. The envelope pellet was washed twice more with 1% NaCl and then resuspended in distilled water and washed until no trace of the chloride ion could be detected with silver nitrate reagent. The purified envelopes were finally lyophilized and stored under dessication.

As a control on the chemical analysis and purification procedure, samples of E. coli B/r3 were also subjected to the envelope purification of Salton and Horne (1951). The

purity of the cell envelope fraction was determined according to the procedure of Salton and Horne (1951). A slightly turbid envelope suspension was checked for the presence of U.V. absorbing material at 260 and 280 mu by running a continuous spectrum curve from 230 to 360 mu on the Beckman DU spectrophotometer. Envelopes were also placed on formvar grids shadowed with tungsten oxide, and observed for impurities electron microscopically.

Determination of Percent Cell Envelope of Whole Cells

Cells were grown, harvested and sonicated as previously described. Undisrupted whole cells were removed from the suspension by centrifugation and the supernate considered as total cell material. Three 15 ml samples were placed in predried, preweighed aluminum weighing dishes (Fischer Aluminum weighing dishes) and dried at 100 C for 12 hours. The dried pan containing the total cell material was placed in a dessicator under vacuo for 48 hours and weighed. This yielded the weight of whole cells per ml of sonicate. The remaining volume of sonicate was subjected to the cell envelope purification procedure. The purified envelopes were taken up in 50-80 ml of distilled water and three 15 ml aliquots were weighed as previously described. The percent cell envelope was calculated from the data obtained.

Determination of Bound, Free, and Total Lipid

Total lipid was determined gravimetrically by a modification of the method of Buchfield and Stores (1962).

Approximately 30 mg of purified envelope material was placed in a teflon screw cap test tube, combined with 20 ml of redistilled acetone and sonicated in a Sonogen sonic bath for one half hour. The envelope residue was separated by centrifugation at 30,000 x g on a Serval RC-2 centrifuge using the S-43 head. The acetone soluble portion was placed in a preweighed, ethanol cleaned aluminum weighing dish and taken to dryness at 50 C. The pan was then dried for 48 hours in vacuo and weighed. The envelope residue was sonically extracted four times with 20 ml of redistilled chloroform-methanol (2:1 v/v) and the chloroform-methanol soluble portion pooled. After the fourth extraction, the material was filtered through fat free filter paper and the combined supernatants taken to dryness and weighed. The chloroform-methanol and acetone soluble material taken together constituted the extractable free lipid portion of the cell envelopes.

The filter paper containing the envelope residue was then placed in a flask and refluxed in 30 ml of 2N KOH for six hours. After saponification, the material was made acidic and extracted with chloroform-methanol (2:1 v/v). The entire material was filtered and allowed to separate into two phases. The chloroform-methanol soluble fraction was removed and the acid phase was reextracted with chloroform-methanol. The combine extracts were combined and washed three times according to the method of Folch (1957). A one phase system was obtained on the addition of a small quantity of methanol and the whole system was taken to dryness at 50 C and weighed as before.

This constituted the bound lipid fraction. The combined weight of the free and bound lipid was considered to be the total extractable lipid of the cell envelopes.

Isolation of Vitreoscilla Lipopolysaccharide

Lipopolysaccharide was isolated and purified by a modification of Luderitz and Westphal's (1952) procedure. Cells were grown and harvested according to the procedure previously described with the exception that distilled water was substituted for the 1% NaCl. The washed cells from 15 l of culture were resuspended in 200 ml of distilled water and mixed in a Serval Omni-mixer for 30 seconds at 100 volts in order to break up any clumps present. The suspension was placed in a 60 C water bath and allowed to come to temperature. Two hundred millaliters of hot phenol (60 C) was added to the suspension and it was returned to the omni-mixer for 15 minutes at 100 volts. The material was then placed in an ice bath and cooled rapidly to 20 C. The cooled material was transfered to 250 ml glass centrifuge bottles and centrifuged for 30 minutes at 1,500 x g in an International centrifuge using the swinging bucket #824 head. The centrifugation separated the material into four phases; a water soluble upper phase, a fluffy interfacial material, a phenol soluble lower liquid phase, and an insoluble pellet. The upper water soluble lipopolysaccharide layer was syphoned off, being careful not to contaminate it with any interfacial material. This supernatant was centrifuged in a Serval RC-2 centrifuge at 3,500 rpm employing the GSA head to insure complete removal of the interfacial

material. The supernatant was dialyzed in the cold room against distilled water in a four liter flask, changing the distilled water every eight hours for approximately 36 hours, or until all traces of phenol was removed. The supernatant contained the crude lipopolysaccharide.

Purification of this crude material was accomplished by lyophilization and ultra-centrifugation. The dialyzed supernatant was shell frozen at -20 C in a methanol bath and lyophilized, thus producing a white fluff material. This white material was dissolved in 100 ml of distilled water and centrifuged in a Beckman model L-2 ultracentrifuge for 2 hours at 40,000 rpm using the number 50 head. The lipopolysaccharide was deposited as a clear gel-like pellet. All pellets obtained in this manner were pooled, resuspended in 50 ml of distilled water and recentrifuged as before. The gel material obtained by the second centrifugation was pooled, taken up in a small amount of distilled water, and lyophilized. The material obtained from the second lyophilization was designated as purified lipopolysaccharide.

The lipid A fraction of the lipopolysaccharide was isolated by a modification of Alaupovic's et al. (1966) 0.1N acetic acid (1:1 w/v) and stirred with a magnetic stirrer for five minutes. The solution was transferred to test tubes in 10 ml amounts and hydrolyzed in a boiling water bath for 30 minutes. The hydrolysates were pooled and allowed to stand in a cold room for 1¼ hours at 4 C after which one half its volume of redistilled chloroform was added and mixed

thoroughly on a magnetic stirrer for about five minutes. Centrifugation of this solution in 250 ml glass centrifuge bottles at 1,200 rpm in an International centrifuge for 30 minutes resulted in a three layer separation. The bottom chloroform soluble layer was carefully pipetted off, washed with distilled water (v/v) and recentrifuged at 1,200 rpm for 30 minutes. The washed chloroform portion was then taken to dryness on a rotary flash evaporator after which the free fatty acids were dissolved out by the addition of 10 ml of redistilled acetone. The material was once again centrifuged and the acetone soluble material saved. The insoluble portion was reextracted with acetone, centrifuged and combined with the above portion, taken to dryness, and weighed for the amount of free fatty acids. The remaining insoluble portion was completely dissolved in redistilled chloroform, taken to dryness and weighed as total chloroform soluble lipid. The total obtained from both the acetone and chloroform soluble portions was designated as the total amount of lipid A extractable from the lipopolysaccharide.

Quantitative Chemical Determinations

Total protein was estimated colorimetrically by the procedure of Lowry, et al. (1951) using bovine serum albumin (Calbiochem.) as the standard. Color intensity was measured on the Klett Summersen photoelectric colorimeter as were all other colorimetric determinations unless otherwise specified. Protein was measured at 660 mu. The amino acids were determined on the Beckmann amino acid analyzer. Approximately 5

to 10 mg samples were prepared for application to the column by taking lyophilized envelopes, placing them in hydrolysis tubes with 10 ml of 6N constant boiling HCl. The hydrolysis tubes were placed in a dry ice and acetone bath until the contents were completely frozen. While in the frozen state, the tubes were flushed three times with nitrogen. The tubes were finally evacuated and sealed. The sealed tubes were placed upright in an oven at 110° C and hydrolyzed for 22 hours. The hydrolysate was cooled in an ice bath and the contents filtered through a fine sintered-glass filter into a pyrex test tube. The filtrate was then reduced to dryness by freeze drying and the dried sample taken up in buffer and applied to the Beckman amino acid analyzer (Miller, 1964).

The determination of carbohydrates also required hydrolysis. The hydrolysis of cell envelopes was carried out according to the procedure of Salton (1953). Carefully weighed samples (approximately 10 mg each) were hydrolyzed in 2N HCl at 100 C for four hours, passed through a sintered glass filter, and the pH adjusted to 6.5 with 1N NaOH. The samples were taken to dryness in vacuo over P_2O_5 and rehydrated to a volume of 10 ml with distilled water. Envelope hydrolysates prepared by the above procedure were used for all carbohydrate quantitations involving the cell envelopes. Total carbohydrate was determined colorimetrically by the anthrone procedure as published by Pelczar, Hansen, and Konetzka (1961) using glucose (Calbiochem.) as the standard. Measurements were taken on the Klett-Summerson Colorimeter at 620 mu. Hexosamines were determined by the Horowitz, Ikawa, and Fling (1950) modification of

the Elson-Morgan (1949) test system. Hexosamines were calculated against a glucosamine HCl standard from the Sigma Chemical Company. Kimax 16x150 mm screw cap tubes sealed with teflon lined caps were used during the boiling step. Colorimetric readings were made in the Klett at 530 mu. The reducing power of the hydrolysates as percent reducing sugar were estimated employing the Folin Malmros (1929) method and were expressed in relation to a glucose standard. During the boiling procedure of the assay, 150x18 mm capped test tubes were used. The samples were assayed at 530 mu.

Purified lipopolysaccharides were hydrolyzed with 2N HCl for four hours at 100° C, filtered through a sintered glass filter, and dried under reduced pressure over NaOH pellets. The redissolved material was then used for both the quantitative estimation of monosaccharide units and for paper chromatography. The quantitation of total carbohydrate, hexosamine, and phosphorus were determined as previously described. The determination of galactose and glucose was performed employing Galactostat and Special Glucostat from the Worthington Biochemical Corp., Freehold, N. J. The galactose standard was obtained from Calbiochem. The presence and amount of 2-keto-3-deoxyoctulosonic acid (KDO) was determined by the method of Weissbach and Hurwitz (1959) employing 2-deoxy-D-ribose (Sigma Chemical Co.) as the standard. The quantitation of the heptose, L glycero-D-manno-heptose, was determined by Osborn's (1963) modification of the Dische (1953) cysteine-H_2SO_4 reaction. The L glycero-D-mannoheptose used was graciously supplied by Dr. Richtmyer, National Institute of Health, Bethesda, Maryland.

Inorganic phosphate was determined colorimetrically according to the method of Bartlett (1959) using Fisher reagent grade KH_2PO_4 as the standard. Klett measurements were taken with the number 64 red filter at 660 mu. Microbial assay procedures were used for the choline and inositol assays. Choline was measured by the method of Horowitz and Beadle (1943) using Difco Assay Medium while inositol was assayed as described by Campling and Nixon (1954) employing Difco Assay Medium KB. The uronic acid content of the lipopolysaccharide was assayed by the method of Bitter and Ewins (1961) using glucuronic acid (K & K Laboratories) as a standard.

Quantitative Analysis of Metallic Ions of Cell Envelopes

The metallic ions of cell envelope preparations were determined by spectrochemical analysis by the University of New Hampshire Engineering Experiment Station. Determinations were made on five milligrams of lyophilized cell envelopes mixed with graphite.

Chromatographic Methods

A. Paper Chromatography

Cell envelopes were hydrolyzed in 2N H_2SO_4 at 100 C for 4 hours for chromatography. The hydrolysate was filtered through a fine sintered glass filter, brought to pH 6.5 with solid barium hydroxide, and taken to dryness in vacuo over P_2O_5. The dried material was resuspended in a small volume of distilled water and applied to chromatograms while a constant stream of warm air was passed over the paper.

Lipopolysaccharide was hydrolyzed for chromatography as previously described. Chromatography of all sugar components was carried out on Whatman No. 1 paper applying 30 ug or more of the standard solutions with a microsyringe. The initial sugar separation was attained using Smith's (1960) n-butanol-acetic acid-water (4:1:1 v/v) solvent in a descending system for 20 hours. Duplicate chromatograms were sprayed with aniline hydrogen phthalate and silver nitrate sprays as described in Block, Durrum, and Zweig (1956). The detection of glycerol was accomplished with the above system employing a four hour solvent flow period and detected with the periodate benzidine spray system.

Cell envelopes hydrolyzed as previously described for application to the amino acid analyzer were also chromatographed in a two dimensional system. The chromatograms were developed in one direction using phenol-water (110:15 v/v) in a 1M NH_4OH atmosphere and in the second direction with lutidine-water (65:35 v/v). All chromatograms were sprayed with ninhydrin for the detection of amino acid spots. Amino sugars were separated using the procedure developed by Mukerjee and SriRam (1964). Chromatograms were developed in ethyl acetate-pyridine-n-butanol-butyric acid-water (10:10:5:1:5 v/v) for 24 hours at room temperature in a descending manner. The presence of amino sugars were initially observed with ninhydrin and later confirmed with the Elson Morgan and silver nitrate reagents.

The separation of glucose from galactose was accomplished using a descending system of ethyl acetate-pyridine-water

(120:50:40 v/v) for 20 hours. As before, duplicate chromatograms were sprayed with aniline hydrogen phthalate and silver nitrate reagents.

B. Thin Layer Chromatography

Thin layer chromatography was applied to the isolated lipid A fraction of the lipopolysaccharide as well as to its hydrolysis products. Isolated lipid A was hydrolyzed by the method applied to the lipopolysaccharide and was used for both quantitative determinations and thin layer chromatography. For thin layer chromatography the hydrolysis products were dried and extracted with acetone, chloroform, and distilled water and chromatographed along with the complete lipid A. The absorbent was prepared by adding 30 grams of Silica-gel G (Brinksman Instrument Co.) to 69 ml of distilled water and spreading it at a thickness of 0.25 mm onto glass plates. The plates were heat activated and prerun in ethyl ether to remove impurities. Materials were spotted on the plates by means of a microsyringe employing warm air for rapid drying of material. Detection of spots was accomplished by charring at 140 to 150 C with 50% H_2SO_4 spray. Ninhydrin, aniline hydrogen phthalate, and molybdate sprays were also used to determine the presence of amino groups, carbohydrate, and phosphorus, respectively. A variety of solvent systems were used for the separations: chloroform ($CHCl_3$)-methanol (MeOH)-7N NH_4OH (60:35:6 v/v), $CHCl_3$-MeOH-7N NH_4OH (60:35:4 v/v), $CHCl_3$-MeOH-water (60:35:4 v/v), $CHCl_3$-MeOH-12N NH_4OH (55:30:10 v/v), $CHCl_3$-MeOH-12N NH_4OH (55:30:12 v/v), and $CHCl_3$-MeOH-11N NH_4OH. For the latter system, chloroform (55 ml) was

mixed with 30 ml of methanol and 11N NH_4OH added with constant stirring till a two phase system was reached. The solution was then brought back to a single phase by the addition of a few drops of methanol.

RESULTS

Cell Envelope Purity

A. The Use of U.V. Absorption Spectrum as an Indication of Envelope Purity

Isolated envelopes were subjected to several methods of purification. Five methods were selected for possible application to Vitreoscilla and the cell envelopes subjected to each procedure. These procedures involved washing the envelopes in buffers or sodium chloride. Envelopes washed in phosphate buffer and 1% NaCl at a pH of 7.0 showed the least amount of protein and nucleic acid contamination, although some peaking at 260 and 280 mu was noted (Figure 1). The addition of DNase and RNase to the procedure followed by several more washings, as described in Materials and Methods, yielded preparations with little contamination. The use of 1% NaCl and the enzymes, gave a preparation showing very little nucleic acid or protein as indicated by the reduction in absorption between 260 and 280 mu. When the wash solutions were spectrophotometrically analyzed (Figure 2), it was evident that the enzyme caused the release of a large amount of 260 mu absorbing material into the resulting supernatant which was greater than any of the 1% NaCl washes. The final washing with distilled water contained no detectable amount of Cl^- ion and showed very little removal of material from the envelopes. We, therefore, decided that the use of 1%

FIGURE 1

The Ultra-Violet Absorption Spectra of Cell Envelopes Isolated from *Vitreoscilla sp.* Strain UM3 and Purified by Four Different Methods.

- △ Envelopes washed in 1% Sodium Chloride and treated with DNase and RNase.
- ○ Envelopes washed in 1% NaCl only
- ● Envelopes washed in Tris buffer
- ▲ Envelopes washed in phosphate buffer

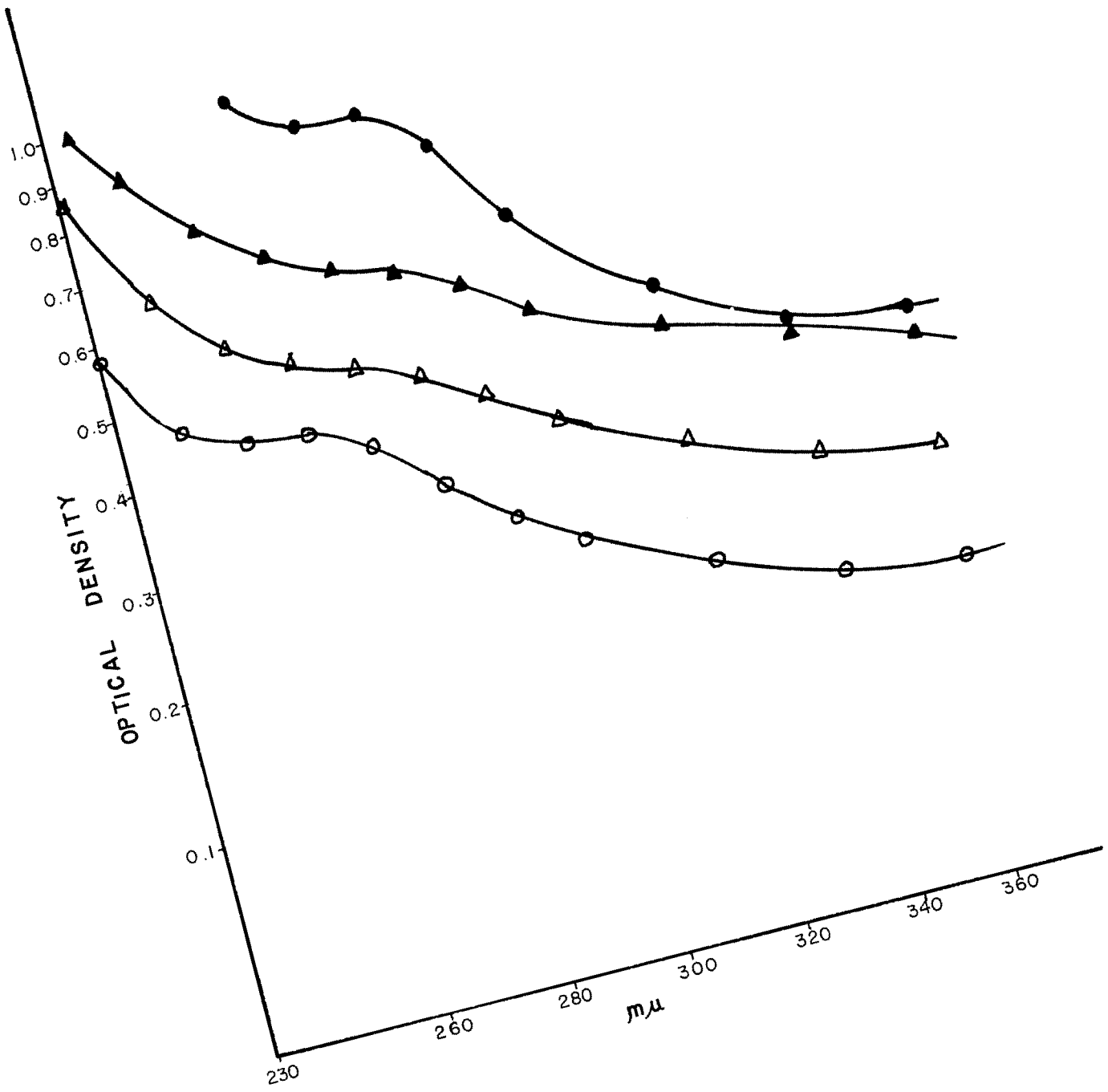

OPTICAL DENSITY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
230
260
280
300
320
340
360
mμ

FIGURE 2

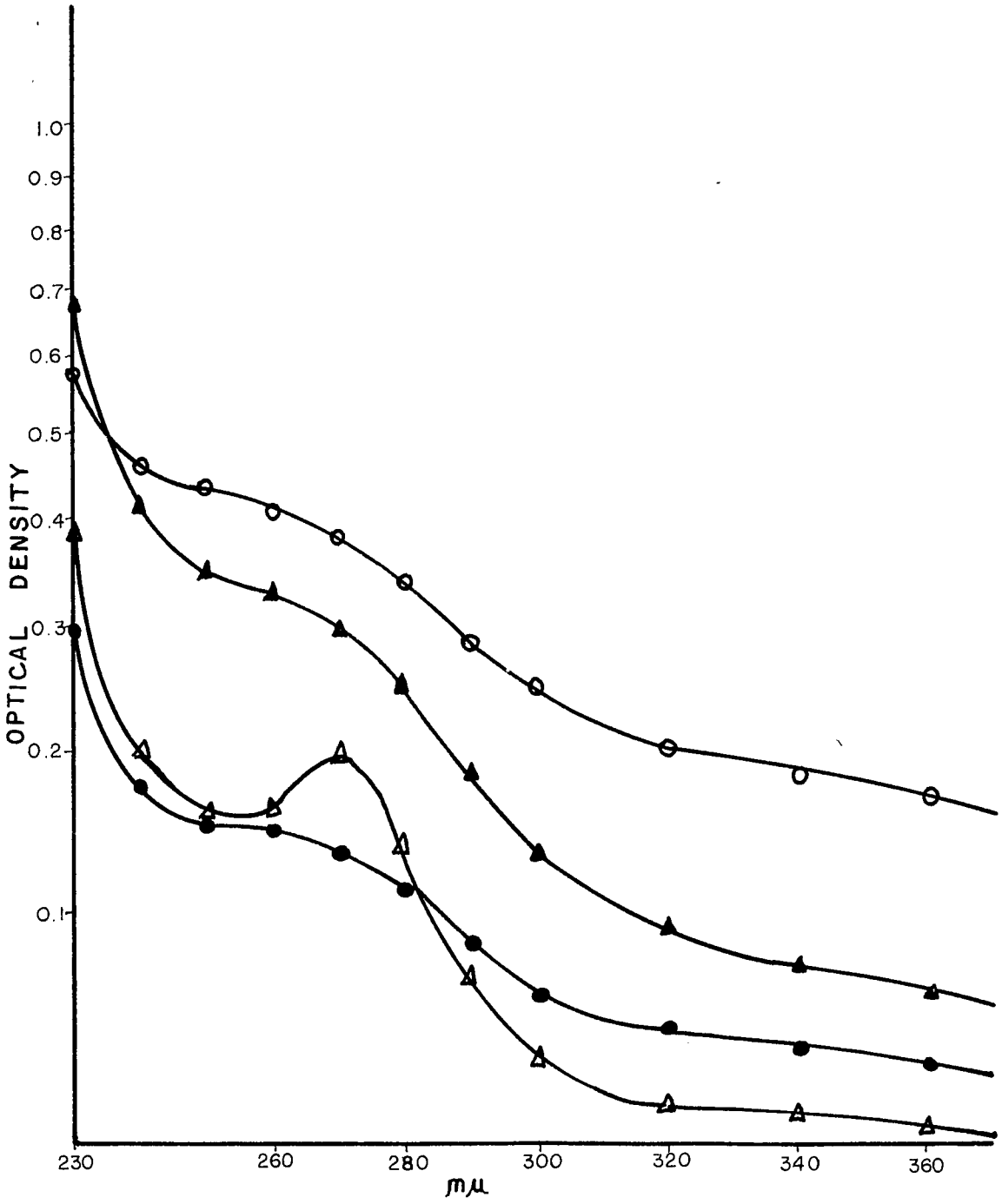
The Ultra-Violet Absorption Spectrum of the Cytoplasmic Fractions Obtained During Purification of Vitreoscilla sp. Strain UM3 Cell Envelopes.

▲ Supernatant from first envelope wash with 1% NaCl

● Supernatant from third envelope wash with 1% NaCl

△ Supernatant after treatment with DNase and RNase

○ Supernatant from final envelope distilled water wash


OPTICAL DENSITY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
230
260
280
300
320
340
360
mμ

NaCl in combination with nuclease treatment and distilled water washes gave the purist preparation of Vitreoscilla sp. strain UM3 envelopes. Vitreoscilla sp. strain LCI, UNH-L and Vitreoscilla stercoraia also showed similar results.

A comparison of our method with that of Salton and Horne's (1951) revealed that, at least for E. coli B/r3 and Vitreoscilla, the use of nuclease gave a purer cell envelope preparation than any single washing technique (Figure 3).

B. The Purity of Cell Envelopes as Seen by Electron Microscopy

Purified cell envelopes of Vitreoscilla sp. strain UM3 placed on a formvar grid and negatively stained with phosphotungtic acid (PTA), as seen in Figure 4, revealed little contaminating debris although the envelopes do possess many folds. Escherichia coli B/r3 envelopes (Figure 5) also showed a contamination-free preparation. One does note, however, the presence of flagella still attached to the cell envelope. These flagella are still attached after five minutes of sonication at 15 to 17 Kc and will have an effect upon the protein and amino acid results presented later in this text. Based upon the results obtained by U.V. absorption and electron microscopy, the cell envelopes were considered as clean preparations with the exception of the contaminating flagella found on E. coli B/r3.

FIGURE 3

The Ultra-Violet Absorption Spectra of Cell Envelopes from *Escherichia coli* B/r3, Prepared by Two Different Purification Methods.

- ▲ Cell envelopes prepared according to Salton and Horne (1951)
- ● Cell envelopes prepared according to the procedure outlined in Materials and Methods

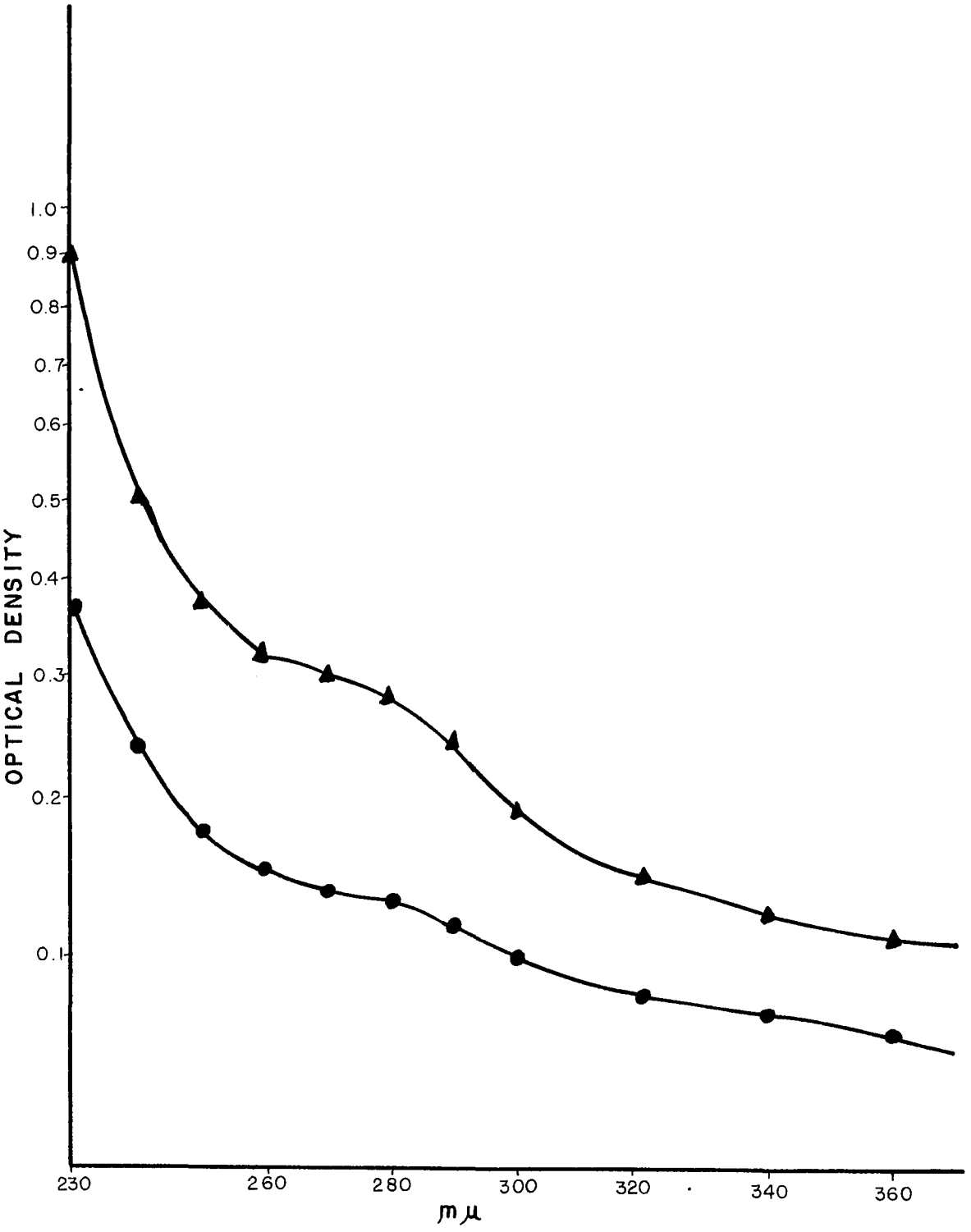

OPTICAL DENSITY
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
230
260
280
300
320
340
360
mμ

FIGURE 4

Negatively Stained Purified Cell Envelope Preparation of *Vitreoscilla sp.* Strain UM3. X 40,000.

FIGURE 5

Negatively Stained Purified Cell Envelope Preparation of Escherichia coli B/r3 Showing Flagella Still Attached After Five Minutes of Sonication. X 120,000.

Chemical Analysis of Cell Envelopes

A. Amino Acid Analysis of Cell Envelopes

Gram-negative cell envelopes are known to contain a wide array of amino acids as compared to their gram-positive counterparts. Gram-negative envelopes are normally composed of 12 to 18 amino acids. Vitreoscilla is no exception and contains at least eighteen amino acids. Table 2 presents the amino acid data obtained from the amino acid analyzer. Escherichia coli B/r3 and Vitreoscilla show the same array of amino acids with a few exceptions. The Vitreoscilla do not seem to contain methionine although a peak is present in the methionine area. The peak does not have the configuration normally found for methionine and may possibly be galactosamine or an analogue of methionine. This is considered unknown 2 in Table 2. The unknown peak 1 is between valine and isoleucine. Between these two amino acids three peaks were resolved in the following order: valine, glucosamine, unknown 1, unknown 2 (possibly an analogue of methionine), and isoleucine. It was thought that unknown 1 might be muramic acid but upon further investigation muramic acid was found to peak in the same area as aspartic acid. This analysis did not detect the presence of muramic acid in the envelopes tested. Diaminopimelic acid also was not detected. There remain two possibilities why the above two amino acids were not detected. First, they may be present in such small traces that they could not be analyzed, or second, the unknown peaks may be a derivative of these amino acids, causing them to appear in

TABLE 2.

Qualitative Amino Acid Analysis of Purified Cell Envelopes from *Escherichia coli* B/r3 and Four Strains of *Vitreoscilla*.

Amino Acids	*E. coli* B/r3	*Vitreoscilla* UM3	LCI	UNH-L	Stercoraia
Ala	+	+	+	+	+
Arg	+	+	+	+	+
Asp	+	+	+	+	+
Glu	+	+	+	+	+
Gly	+	+	+	+	+
His	+	+	+	+	+
Ileu	+	+	+	+	+
Leu	+	+	+	+	+
Lys	+	+	+	+	+
Met	+	±	±	±	±
Phe	+	+	+	+	+
Pro	+	+	+	+	+
Ser	+	+	+	+	+
Thr	+	+	+	+	+
Tyr	+	+	+	+	+
Val	+	+	+	+	+
Cys	+	+	+	+	+
Unknowns 1,2 and 3	-	N.D.	+	+	N.D.

N. D. - Values not determined.

different areas from the standards. Unknown 3 was detected as a very distinct peak between phenylalanine and ammonia and in closer association with ammonia than phenylalamine. The analysis did, however, detect the presence of glucosamine. The *E. coli* B/r3 envelope hydrolysate also showed allo-isoleucine and guanidine while neither could be found in *Vitreoscilla*. It was thought that the so called methionine peak might be galactosamine but this amino sugar could not be detected by chromatography and, therefore, is probably not present. As seen in Table 2, the four strains of *Vitreoscilla* were very similar in their amino acid content.

B. Paper Chromatography of Sugars

The absence of muramic acid, a known constituent of bacterial mucopeptide, in the amino acid analysis suggested that it may be present in small amounts if at all. A large quantity of *Vitreoscilla sp.* strain UM3 envelopes were hydrolyzed, concentrated, and spotted on paper chromatograms. Upon development and spraying of the chromatogram with ninhydrin (Figure 6), two ninhydrin spots appeared, corresponding to glucosamine and muramic acid. It should be noted that the muramic acid spot was faint even with the application of 200 ug of the hydrolysate, and is probably present at a very low concentration in the envelope. Since ninhydrin detects only the presence of amino groups, triplicate chromatograms were run and sprayed for the presence of amino groups, carbohydrate, and hexosamine by ninhydrin, aniline hydrogen phthalate, and the Elson Morgan reagent, respectively. The two unknown spots,

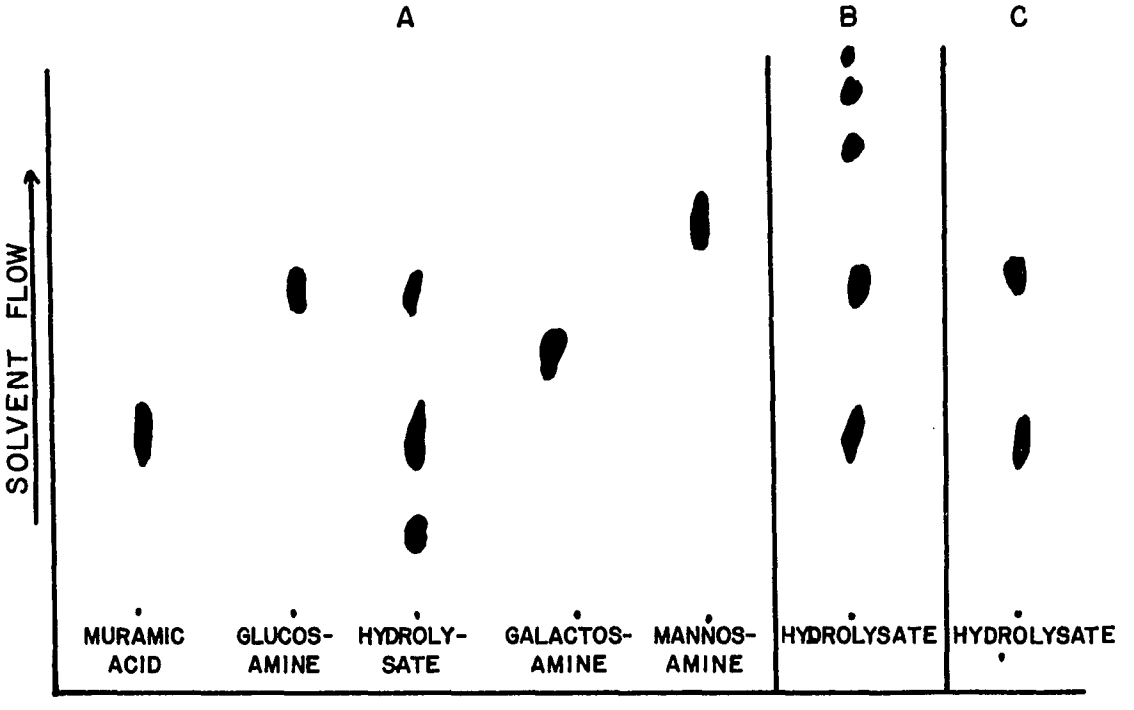
FIGURE 6

Paper Chromatography of *Vitreoscilla sp.* Strain UM3 Cell Envelope Hydrolysate Assayed for the Presence of Amino Sugars.

A. - Section sprayed with ninhydrin

B. - Section sprayed with aniline hydrogen phthalate

C. - Section sprayed with the Elson-Morgan reagent


A
B
C
SOLVENT FLOW
MURAMIC ACID
GLUCOS-AMINE
HYDROLY-SATE
GALACTOS-AMINE
MANNOS-AMINE
HYDROLYSATE
HYDROLYSATE

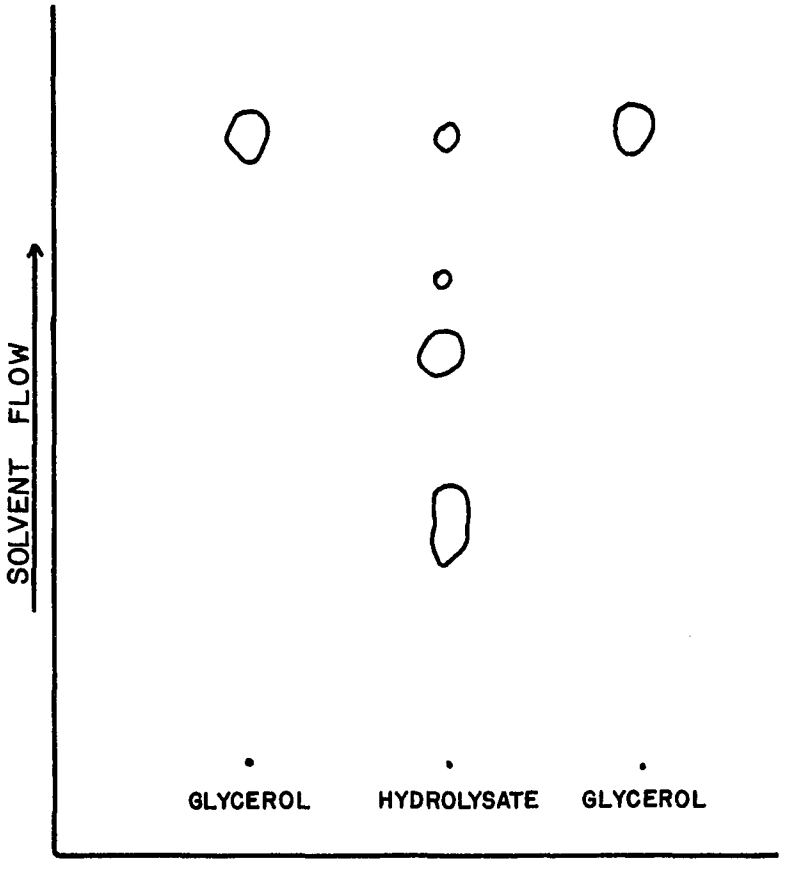
which corresponded to the known amino sugars, glucosamine and muramic acid, gave positive ninhydrin, carbohydrate, and Elson-Morgan hexosamine reactions. Thus positive identification of glucosamine and muramic acid in the cell envelope of Vitreoscilla was obtained. Evidently, muramic acid was not detected during the amino acid analysis because of the small amount of sample applied to the column. All Vitreoscilla strains, as indicated by paper chromatography, contained the two amino sugars. No galactosamine could be detected so that unknown peak 2 from the amino acid analysis was not galactosamine which was considered as one of the possibilities.

Upon initial separation in butanol-acetic acid-water (4:1:1 v/v) three aniline hydrogen phthalate positive spots appeared corresponding to glucosamine, glucose and/or galactose, and a fast moving component which, according to its migration pattern, was considered to be either mannose or glycerol. This component was subsequently identified as glycerol (Figure 7) by use of the periodate benzidine spray. Glucose and galactose were separated by employing ethyl acetate-pyridine-water (120:50:40 v/v) as the solvent system. The solvent was allowed to flow descendingly for 24 hours in order to insure good separation of glucose from galactose. When chromatograms were sprayed with aniline hydrogen phthalate and heated at 105 C three spots appeared. The separation indicated glucose, galactose, and glucosamine. When the chromatograms were reheated at 120-130 C for a few minutes a reddish spot developed between the glucose and galactose brown areas. A red spot developing with aniline hydrogen phthalate usually

FIGURE 7

Paper Chromatography of Vitreoscilla sp. Strain UM3 Cell Envelope Hydrolysate for the Presence of Glycerol.

Chromatogram sprayed with 1% periodate benzidine


SOLVENT FLOW
GLYCEROL
HYDROLYSATE
GLYCEROL

indicates the presence of a pentose although the pentoses included as standards turned from red to brown when heated at 120-130° C. The spot was later identified as D-glycero-L-manno-heptose, a component usually found in the lipopolysaccharide layer of gram-negative bacteria.

C. Quantitative Analysis of Cell Envelopes

A quantitative chemical analysis for gross constituents of the cell envelope was initiated for comparison with known gram-negative and gliding bacterial cell envelopes. Escherichia coli B/r3 cell envelopes prepared by Salton and Horne's (1951) method were also examined as a control on all analytical procedures. Colorimetric assays were performed for total amino sugar, reducing sugar, carbohydrate, protein, lipid, phosphate, inositol, choline, and percent envelope on a whole cell dry weight basis. Table 3 indicates the results of the aforementioned chemical analysis. The E. coli B/r3, which was used as a control, corresponded with previously published results, containing approximately 2.5% amino sugar, 11-12% carbohydrate, 63.4% protein, 1% phosphate, and 22.1% lipid which accounted for 98.2% of the cell envelope. The E. coli B/r3 cell envelopes prepared according to our purification procedure showed approximately the same values although the total percentage was 3.0% below the other method. This slight decrease could be accounted for from experimental error due to the inherent statistical deviations in each chemical procedure.

TABLE 3.

General Quantitative Analysis of *Vitreoscilla* and *Escherichia coli* B/r3 Cell Envelopes.

	Escherichia coli B/r3		*Vitreoscilla*			
	#1*	#2**	UM3	UNH-L	LCI	Stercoraria
% Amino Sugar	2.54	2.16	0.672	0.65	0.542	3.627
% Reducing Sugar	11.9	10.8	2.34	2.92	2.62	5.8
% Carbohydrate	11.2	12.5	3.49	3.26	3.32	2.55
% Protein	63.4	62.0	35.3	38.8	62.0	49.5
% Lipid	22.1	21.0	61.4	58.5	35.4	44.7
% Free Lipid	15.0	13.8	41.25	41.6	25.8	32.1
% Bound Lipid	7.1	7.22	20.15	16.75	9.15	12.6
% Dry Wt. of Envelopes	16.5	13.5	11.5	13.77	15.2	17.4
% Phosphate	0.805	1.105	0.225	0.271	0.341	0.407
% Inositol	0.002		0.003	0.002	0.003	0.004
% Choline Chloride	0.001		0.003	0.003	0.004	0.005
% Recovery	98.2	95.5	100.2	100.8	101.06	104.0

* Cell Envelopes prepared by the Method of Salton and Horne (1951).

** Cell Envelopes prepared by the Method used for the *Vitreoscilla*.

Vitreoscilla sp. strains UM3, UNH-L, and LCI all gave low values for Elson-Morgan amino sugar when compared to the E. coli B/r3 control. Vitreoscilla stercoraria was the only strain which possessed amino sugar in a concentration comparable to the control and other investigated gram-negatives. Total carbohydrate was also extremely low for a gram-negative organism. Most gram-negatives contain 10-20% anthrone carbohydrate while the Vitreoscilla values were 2-6% carbohydrate. The most extraordinary observation was the lipid content of these organisms. The Vitreoscilla total lipid fluctuated from 35.3% in Vitreoscilla sp. strain UM3 to 62.0% in Vitreoscilla sp. strain LCI, while the other two strains fell in between these values. Even though the lipid values fluctuated and were not consistent between strains, all values were consistently higher than those obtained for most gram-negative microorganisms. However, there was good correlation in the relationship of the bound and free lipid to total lipid within all strains of Vitreoscilla. The free lipid, as defined in the Materials and Methods, was 29% of the total lipid. By the use of this method, we have accounted for 99% of the total lipid. The protein content of the envelopes fluctuated in an inverse proportion to the lipid content. Vitreoscilla sp. strain UM3 contained 61.4% protein which is in good agreement with the results of E. coli B/r3 while the other strains were lower in protein content. The lowest protein content was exhibited by Vitreoscilla sp. strain LCI at 35.4% of the total content of the envelope fraction. The phosphorus content

was also low in relation to the E. coli B/r3 tested. Generally, the phosphorus content was one quarter that of E. coli B/r3 and most other gram-negatives while in the case of Vitreoscilla stercoraria, it was 0.407% or about one half that of E. coli B/r3 envelopes prepared by either method.

Due to the high quantity of lipid present, it was of interest to investigate the amount of inositol and choline present in these envelopes. Microbial assay indicated a relatively low content of both compounds and in some cases the values approached the lower limits of the assay system. This data indicates no relationship exists between these high lipid envelopes and the walls of Mycobacterium tuberculosis which are high in lipid and inositol.

The percent dry weight of the cell envelopes as compared to the dry weight of the whole cells were determined and corresponded to those found by other investigators for gram-negative bacteria. The Vitreoscilla envelopes range from 11.5 to 17.4% of the dry weight of whole cells.

Qualitative and semi-quantitative spectrochemical analysis were performed on purified E. coli B/r3 and Vitreoscilla cell envelopes. The results of this analysis appear in Table 4. Both the E. coli B/r3 and Vitreoscilla showed the same spectrochemical pattern. The large quantity of Na^+ was considered contamination obtained from the 1% NaCl wash, since during the final stages of purification with distilled water only the release of the Cl^- ion was tested for. It is very possible that not all the Na^+ ions were washed free of the envelopes. The one outstanding feature of

TABLE 4.

Qualitative Spectrochemical Analysis of the Cell Envelopes from *Vitreoscilla* and *Escherichia coli*.

Elements	*E. coli* B/r3	*Vitreoscilla* UM3	UNH-L	LCI	Stercoraria
Na	++++	++++	++++	++++	++++
Ca	++++	++++	++++	++++	++++
Fe	+++	+++	+++	+++	+++
Al	+++	+++	+++	+++	+++
Cu	++	++	++	++	++
Si	++	++	++	++	++
Mg	++	++	++	++	++
Cr	+	+	+	+	+
Ni	+	+	+	+	+
Mn	+	+	+	+	+

Symbols used:

++++ - large amount

+++ - medium amount

++ - small amount

\+ - trace amounts

this analysis is the finding of a medium amount of Al^{+++} in both genera. The high amounts of Ca^{++} and Mg^{++} are common to wall and envelope preparations but the amounts of Fe^{++}, Si^{++}, and Cu^{++} were not expected and remain unexplained.

Analysis of Isolated Lipopolysaccharide

A. Physical Properties of Isolated Lipopolysaccharide

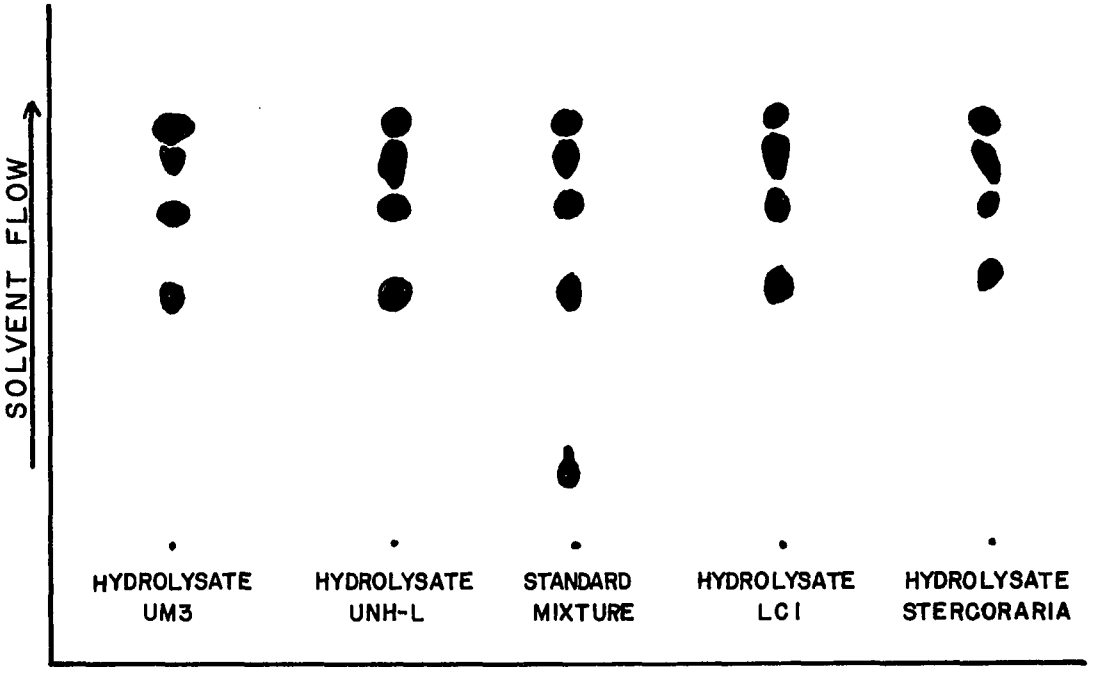
Physical differences in the lipopolysaccharides were noted during their purification. Vitreoscilla sp. strains UM3, UNH-L, and LCI all extracted as a fluff white textured material upon the initial lyophilization step in their purification. The three strains also produced a clear gel-like button after the final centrifugation step. The crude lyophilized LPS from Vitreoscilla stercoraria was much less when starting with equal quantities of whole cells. The first lyophilization step produced a small amount of a course textured powder which had a slight yellowish color. The final gel-button was also physically different. The button was not clear as seen with the other strains but grayish-white in appearance. After chemical analysis no definitive difference could be detected to account for the difference noted during its extraction.

B. Paper Chromatography of Sugars (Monosaccharide Units)

Paper chromatography of isolated lipopolysaccharides disclosed (Figure 8) the presence of four aniline hydrogen phthalate positive reacting spots. These four areas migrated the same as glucose, galactose, glucosamine, and

FIGURE 8

Paper Chromatography of a Standard Monosaccharide Mixture and Hydrolysate of *Vitreoscilla sp.* Strains UM3, UNH-L, LCI, and *stercoraria* Lipopolysaccharide.


SOLVENT FLOW
HYDROLYSATE UM3
HYDROLYSATE UNH-L
STANDARD MIXTURE
HYDROLYSATE LC I
HYDROLYSATE STERCORARIA

D-glycero-L-mannoheptose standards. In a few chromatograms, a faint indication of uronic acid was detected but could not be positively identified. Inspection of developed color intensity of individual spots indicated glucose, glucosamine, and D-glycero-L-mannoheptose in approximately the same concentration while the galactose content was less. Hydrolyzed lipopolysaccharides chromatographed for amino sugars showed the presence of only one amino sugar corresponding to the standard glucosamine. The presence of muramic acid could not be detected and therefore, we feel our lipopolysaccharide preparation free of mucopeptide. With ninhydrin the LPS hydrolysate from Vitreoscilla sp. strains UM3, UNH-L, and LCI showed two amino acids while Vitreoscilla stercoraria showed only one amino acid. It is not known whether these amino acids are connected to the lipopolysaccharide or were seen as contamination from another layer of the cell envelope, although if they were contaminants one would expect a larger number of amino acids to be present. Comparisons of the monosaccharides obtained from cell envelopes to those of the lipopolysaccharide are presented in Table 5. This table shows that the same monosaccharides are contained in both the envelope and the LPS fraction with the exception of muramic acid and glycerol which were not present in the lipopolysaccharide layer.

C. Quantitative Analysis of Isolated Lipopolysaccharides

Hydrolyzed and non-hydrolyzed lipopolysaccharide material from the four strains of Vitreoscilla were chemically

TABLE 5.

Qualitative Analysis of Vitreoscilla Lipopolysaccharide and Cell Envelopes.

	Lipopolysaccharide				Envelopes			
Sugars	UM3	UNH-L	LCI	stercoria	UM3	UNH-L	LCI	stercoraria
Glycerol	-	-	-	-	+	+	+	+
Glucose	+	+	+	+	+	+	+	+
Galactose	+	+	+	+	+	+	+	+
Glucosamine	+	+	+	+	+	+	+	+
Muramic Acid	-	-	-	-	+	+	+	+
D-Glyerol-L-Manno-heptose	+	+	+	+	+	+	+	+
Glucuronic Acid	±	±	±	±	N.D.	N.D.	N.D.	N.D.
Unknown Amino Acids								
# 1.	+	+	+	+				
# 2.	+	+	+	-				

N.D. - Not Detected

analyzed according to procedures described in Materials and Methods. Results of this experimentation appears in Table 6. Percent anthrone-positive carbohydrate was determined on hydrolyzed and non-hydrolyzed material. From the results, it is apparent that there was some destruction of sugar during hydrolysis. The average reduction was approximately 3% and therefore, the values obtained for nonhydrolyzed LPS were taken as a closer evaluation of the total carbohydrate. Some protein was detected in three of the four Vitreoscilla LPS preparations. It is not known whether this is an integral part of the molecule or contamination from the phenol extraction of whole cells. The percent protein as measured against the protein standard yielded color readings of 0 to 5 Klett units. The accuracy of the colorimeter is thought to be in the realm of $\pm$ 2 units and therefore in some cases the material may be protein free.

In order to get a truer relationship for a structural view of the lipopolysaccharide, the percent values for the sugars were converted to micromoles and appear in Table 7. The calculated micromoles indicate that none of the organisms tested could possess a heptose-phosphate-polysaccharide backbone because there is less phosphate than heptose. Uronic acid accounted for approximately 1.8 umoles per gram and was comparable to the amount of galactose found. From the above information molar ratio's were calculated in an attempt to obtain some insight on the lipopolysaccharide structure. Tables 8, 9, 10 and 11 report the molar ratios for Vitreoscilla sp. strains UM3, UNH-L, LCI and Vitreoscilla stercoraria

TABLE 6.

Quantitative Chemical Composition of Vitreoscilla Lipopolysaccharide.

Chemical Units	Strain UM3	UNH-L	LCI	stercoraria
% KDO	0.3615	0.254	0.402	0.320
% Non Hydrolyzed Carbohydrate	22.4	18.55	19.55	19.175
% Hydrolyzed Carbohydrate	14.53	14.70	18.75	18.23
% Phosphate	1.28	1.07	1.22	1.15
% Protein	0.0935	0.0	0.137	0.374
% Uronic Acid	4.44	3.44	3.30	3.44
% Glucosamine	7.23	7.35	7.05	7.06
% Heptose	9.95	12.68	12.95	12.60
% Galactose	2.85	2.59	2.39	1.57
% Glucose	4.42	4.06	5.02	4.61

TABLE 7.

The Chemical Composition of Vitreoscilla Lipopolysaccharide Expressed as uMoles per gram.

Chemical Units	UM3	UNH-L	LCI	stercoraria
KDO	0.269	0.1895	0.300	0.239
Heptose	4.74	6.04	6.16	6.00
Phosphate	4.13	3.45	3.94	3.72
Glucosamine	3.26	3.42	3.28	3.28
Carbohydrate	11.7	11.9	12.0	16.95
Glucose	2.54	2.36	2.91	2.59
Galactose	1.58	1.44	1.33	0.871
Uronic Acid	2.28	1.77	1.70	1.77

TABLE 8.

Component Molar Ratio's of Vitreoscilla sp. Strain UM3 Lipopolysaccharide.

	PER MOLE OF:						
	Pi	KDO	Uronic Acid	Heptose	Glucos-amine	Glu	Gal
Heptose	1.15	17.6	2.08	1.00	1.45	1.86	2.995
KDO	0.065	1.00	0.118	0.0825	0.106	0.106	0.1705
Glucosamine	1.27	12.2	1.43	0.688	1.00	1.28	2.06
Carbohydrate	2.84	43.5	5.15	2.47	3.59	6.74	7.40
Uronic Acid	0.55	8.48	1.00	0.48	0.70	0.898	1.44
Phosphate	1.00	15.4	1.81	0.873	1.26	1.63	2.62
Glucose	0.615	9.45	1.11	0.536	0.779	1.00	1.61
Galactose	0.383	5.87	0.694	0.334	0.485	0.622	1.00

TABLE 9.

Component Molar Ratio's of Vitreoscilla sp. Strain UNH-L Lipopolysaccharide.

PER MOLE OF:

	Pi	KDO	Uronic Acid	Heptose	Glucosamine	Glu	Gal
Heptose	1.75	31.8	2.68	1.00	1.76	2.56	4.19
KDO	0.055	1.00	0.107	0.032	0.055	0.0804	0.1315
Glucosamine	1.00	18.15	1.93	0.567	1.00	1.45	2.37
Carbohydrate	3.45	62.7	6.72	1.97	3.48	5.04	8.25
Uronic Acid	0.514	9.35	1.00	0.294	0.517	0.750	1.23
Phosphate	1.00	18.2	1.95	0.571	1.00	1.46	2.40
Glucose	0.684	12.45	1.33	0.392	0.690	1.00	1.64
Galactose	0.417	7.59	0.814	0.238	0.422	0.610	1.00

TABLE 10.

Component Molar Ratio's of Vitreoscilla sp. Strain LCI Lipopolysaccharide.

PER MOLE OF:

	Pi	KDO	Uronic Acid	Heptose	Glucos-amine	Glu	Gal
Heptose	1.57	20.3	3.62	1.00	1.88	2.11	4.64
KDO	0.076	1.00	0.117	0.049	0.091	0.103	0.226
Glucosamine	0.833	10.9	1.93	0.532	1.00	1.13	2.47
Carbohydrate	3.04	40.0	7.05	1.95	3.66	4.12	9.03
Uronic Acid	0.431	5.66	1.00	0.276	0.517	0.585	1.28
Phosphate	1.00	13.1	2.32	0.64	1.20	1.355	2.96
Glucose	0.74	9.70	1.72	0.472	0.888	1.00	2.19
Galactose	0.338	4.34	0.783	0.216	0.405	0.456	1.00

TABLE 11.

Component Molar Ratio's of Vitreoscilla stercoraria lipopolysaccharide.

PER MOLE OF:

	Pi	KDO	Uronic Acid	Heptose	Glucos-amine	Glu	Gal
Heptose	1.61	25.1	3.39	1.00	1.83	2.32	6.88
KDO	0.064	1.00	0.135	0.039	0.073	0.092	0.274
Glucosamine	0.881	13.7	1.85	0.547	1.00	1.265	3.77
Carbohydrate	4.55	71.0	9.58	2.83	5.16	6.55	19.4
Uronic Acid	0.476	7.40	1.00	0.297	0.539	0.684	2.04
Phosphate	1.00	15.5	2.10	0.620	1.13	1.44	4.27
Glucose	0.697	10.7	1.465	0.432	0.790	1.00	2.98
Galactose	0.234	4.01	0.492	0.145	0.268	0.336	1.00

lipopolysaccharide, respectively. The molar ratio data indicated that the backbone polysaccharide contains a polymer of approximately eighteen heptose units connected to one mole of KDO, while the heptose-phosphate ratio (1:1:5) precludes the heptose-phosphate (1:1 ratio) backbone commonly reported in the core portion of the molecule. Vitreoscilla sp. strain UM3 contained 1 uronic acid for every 2 heptoses and the uronic acid is present in approximately the same concentration as glucosamine, glucose, and galactose. Uronic acid thus seems to play an integral role in the lipopolysaccharide molecule of Vitreoscilla sp. strain UM3. All other strains tested also showed fair quantities of uronic acid. All Vitreoscilla showed a good degree of correlation as to their molar ratios, although Vitreoscilla stercoraria may have a slightly different structural arrangement.

Since all postulated structures for lipopolysaccharides include glucosamine and phosphate in the lipid A, it became necessary to isolate and chemically characterize this fraction in order to get a true value for the components present in the polysaccharide portion. The lipid A, as described in Materials and Methods, was determined gravimetrically as were its fractions. The lipid A was found to comprise 25% of the lipopolysaccharide for Vitreoscilla sp. strain UM3. The isolated lipid A from Vitreoscilla sp. strain UM3 was examined for its phosphate content. This fraction was found to contain 0.475% phosphate. Since the total phosphate for the complete LPS molecule was 1.28% (Table 6), this left 0.805% phosphate present in the polysaccharide portion. When these figures were

converted to moles, the lipid A contained 15.3 umoles and the polysaccharide portion, 26.0 umoles per gram. Glucosamine determination of the lipid A indicated it comprised 31.6% of this fraction which would make glucosamine 7.9% of the total lipopolysaccharide. The original determination of glucosamine in the whole molecule (Table 6) showed it to be 7.23%. It is therefore believed that all the glucosamine present in the lipopolysaccharide of Vitreoscilla sp. strain UM3 is contained in the lipid A fraction. The total moles of glucosamine for the lipid A or the complete LPS molecole can then be expressed as 3.41 umoles per gram. Upon the examination of the lipid A, no KDO, heptose, glucose, galactose, or uronic acid were found. Having this data in hand, one can accurately calculate the moles of the compounds contained in the polysaccharide portion of the lipopolysaccharide of Vitreoscilla sp. strain UM3 as shown in Table 12. The phosphate has been reduced to 2.60 umoles per gram while glucosamine has been completely eliminated from the polysaccharide portion. It was thus possible to determine the molar ratios for the constituents of Vitreoscilla sp. strain UM3 polysaccharide core. These results appear in Table 13 and indicate there are eighteen heptose units available for each mole of KDO. There is one phosphate per two heptoses, one glucose per two heptoses, one galactose per three heptoses and one uronic acid per every two heptoses. Glucose and uronic acid are in the same approximate concentration.

TABLE 12.

Chemical Composition of Vitreoscilla sp. Strain UM3 Polysaccharide Portion of its Lipopolysaccharide Molecule Expressed in Moles per 100 Grams.

Chemical Units	Moles per 100 Grams
KDO	2.69×10^{-3}
Heptose	4.74×10^{-2}
Phosphate	2.60×10^{-2}
Glucosamine	0.00
Glucose	2.54×10^{-2}
Galactose	1.58×10^{-2}
Uronic Acid	2.28×10^{-2}

TABLE 13.

The Molar Ratio's of Vitreoscilla sp. Strain UM3 Polysaccharide Portion of its Lipopolysaccharide Molecule.

PER MOLE OF:

	Pi	KDO	Heptose	Glucose	Galactose	Uronic Acid
Heptose	1.82	17.6	1.00	1.86	2.99	2.08
KDO	0.103	1.00	0.057	0.106	0.171	0.118
Phosphate	1.00	9.65	0.55	1.02	1.65	1.14
Glucose	0.98	9.45	0.536	1.00	1.61	1.11
Galactose	0.607	5.87	0.334	0.622	1.00	0.694
Uronic Acid	0.875	8.48	0.482	0.892	1.44	1.00

D. Thin Layer Chromatography of Isolated Vitreoscilla sp. strain UM3 Lipid A

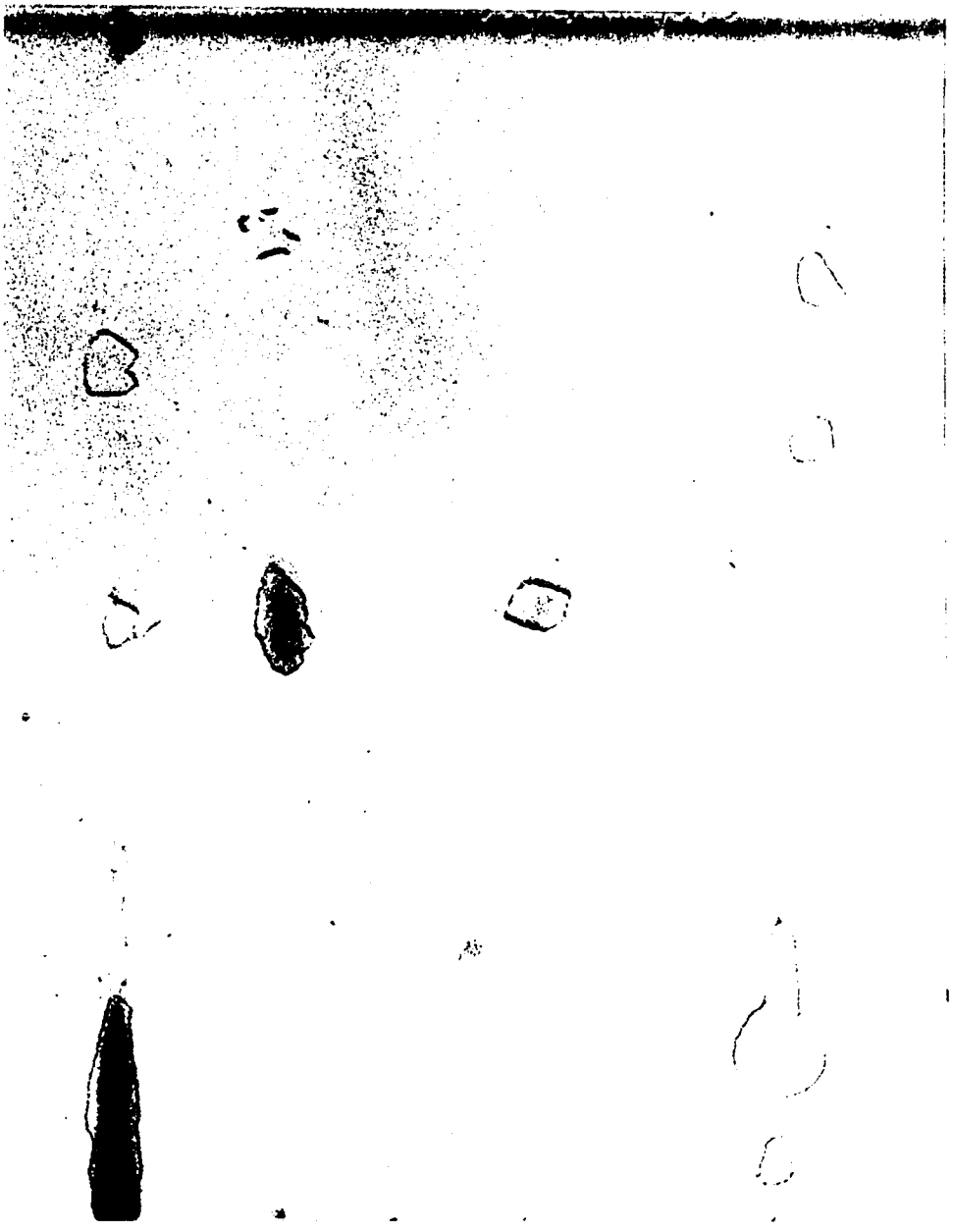
Thin layer chromatography was performed upon Vitreoscilla sp. UM3 lipid A utilizing a variety of solvent systems. Chromatographed with the lipid A in many cases were the acetone, chloroform, and water-soluble fractions obtained from the extraction of hydrolyzed lipid A. Figure 9 shows the migration pattern obtained when $CHCl_3$-MeOH-7N NH_4OH (60:35:4 v/v) was used as the solvent and the plates charred with 50% H_2SO_4. Lipid A was resolved into two slow moving components, two fast moving spots and one component which moved with the solvent front. The chloroform soluble portion of the hydrolyzed lipid A separated into two spots in the upper portion of the chromatogram while the acetone fraction yielded only one spot. This spot was present also in the lipid A and the chloroform fraction but absent from the water soluble fraction. When a duplicate chromatogram (Figure 10) was sprayed with ninhydrin and aniline hydrogen phthalate, lipid A showed a slight positive reaction to both reagents near the origin while neither the acetone or the chloroform soluble fractions showed positive reactions. The water soluble fraction of the hydrolysate showed a reaction to both sprays in the area corresponding to the second spot of the lipid A during charring. This indication of an amino group and a carbohydrate in the same area indicated the presence of an amino sugar, probably glucosamine, from the lipid A backbone.

In an attempt to increase resolution, the polarity of the solvent system was increased. The results seen in Figure

FIGURE 9

Thin Layer Chromatography of Vitreoscilla sp. Strain UM3 Isolated Lipid A and its Solvent Soluble Products.

Solvent system: $CHCl_3$-MeOH-7N NH_4OH (60:35:4 v/v)



<u>Vitreoscilla</u> Lipid A	$CHCl_3$ Soluble	Acetone Soluble	Water Soluble

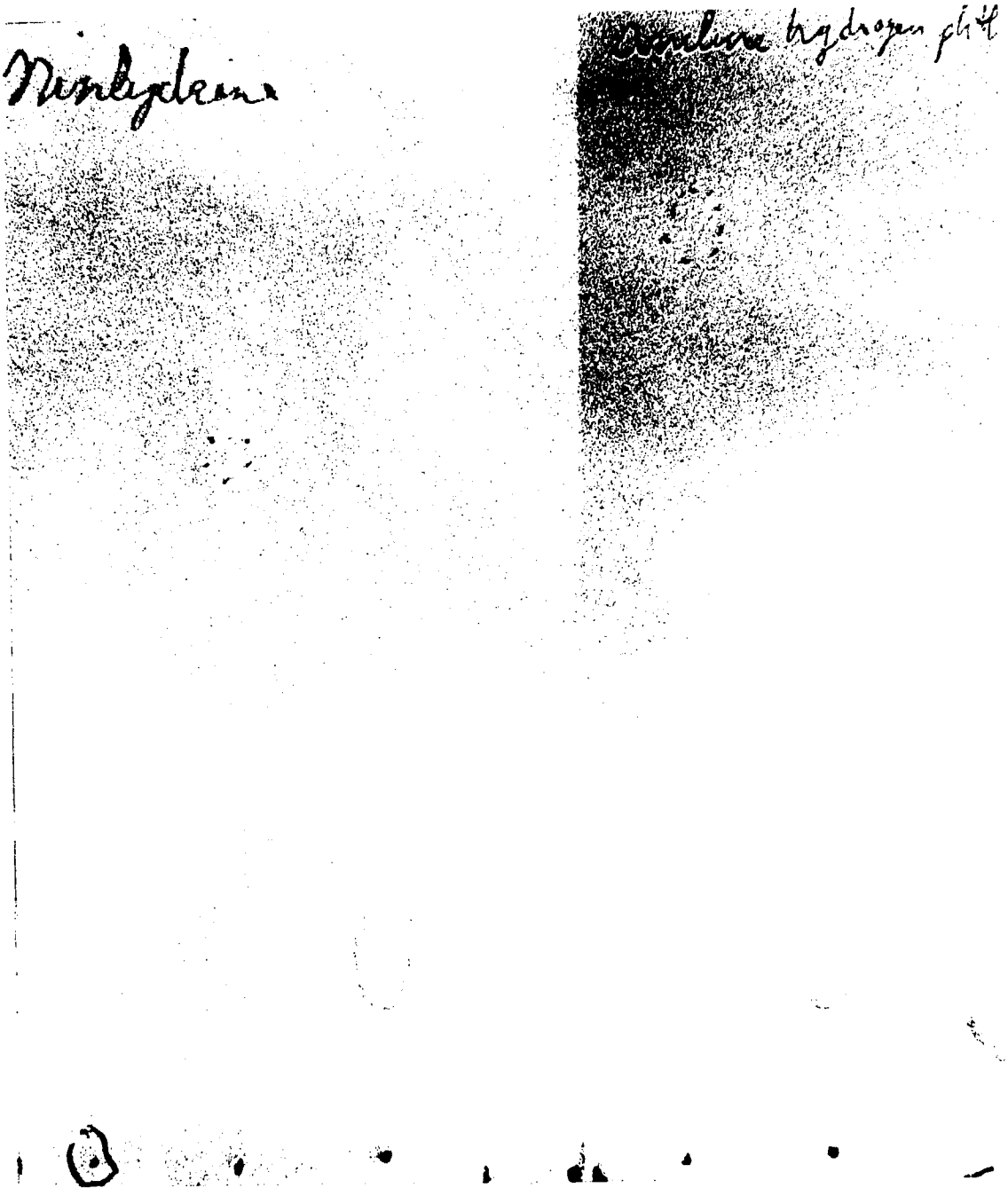
FIGURE 10

Thin Layer Chromatography of Lipid A Isolated from *Vitreoscilla sp.* Strain UM3 and its Solvent Soluble Fractions For the Presence of Carbohydrates.

Solvent system: $CHCl_3$-MeOH-7N NH_4OH (60:35:4 v/v)

A. - Detection with ninhydrin

B. - Detection with aniline hydrogen phthalate



11 showed the lipid A resolved into seven spots. The upper five spots probably are fatty acids. Increasing the polarity further by increasing the normality of the NH_4OH and its amount, caused a slightly better separation of the upper spots (Figure 12) although the number of spots remained at seven. A solvent system close to saturation with 11N NH_4OH showed the best separation that could be attained. The results obtained by this system and developed by H_2SO_4 charring appear in Figure 13. The lipid A was separated into eight distinct spots which is the same number obtained by most investigators (Kasai and Yamano, 1964; Burton and Carter, 1964; Nowotny, et al., 1963; and Alaupovic, et al., 1966). The spot previously reported associated with the solvent front was now detected in all fractions chromatographed except the water soluble fraction.

Chromatography of lipid A from Serratia marcescens showed nine spots. Four spots were found in common between the Vitreoscilla and Serratia lipid A fractions, including the spot found in the solvent front. These spots have not been identified except for the reaction of the lower spots to several spray systems. Chromatograms of lipid A and its water soluble hydrolysis products in the above solvent system were developed and sprayed with ninhydrin, aniline hydrogen phthalate, and molybdate sprays. The spot closest to the origin in Figure 13 reacted positively to all three sprays. This data indicates that this spot contains amino groups, a carbohydrate, phosphorus, and fatty acids. This spot possibly may be the glucosamine-phosphate found in the lipid A backbone.

FIGURE 11

Thin Layer Chromatography of Isolated Lipid A from *Vitreoscilla sp.* Strain UM3.

Solvent system: $CHCl_3$-MeOH-7N NH_4OH (60:35:6 v/v)

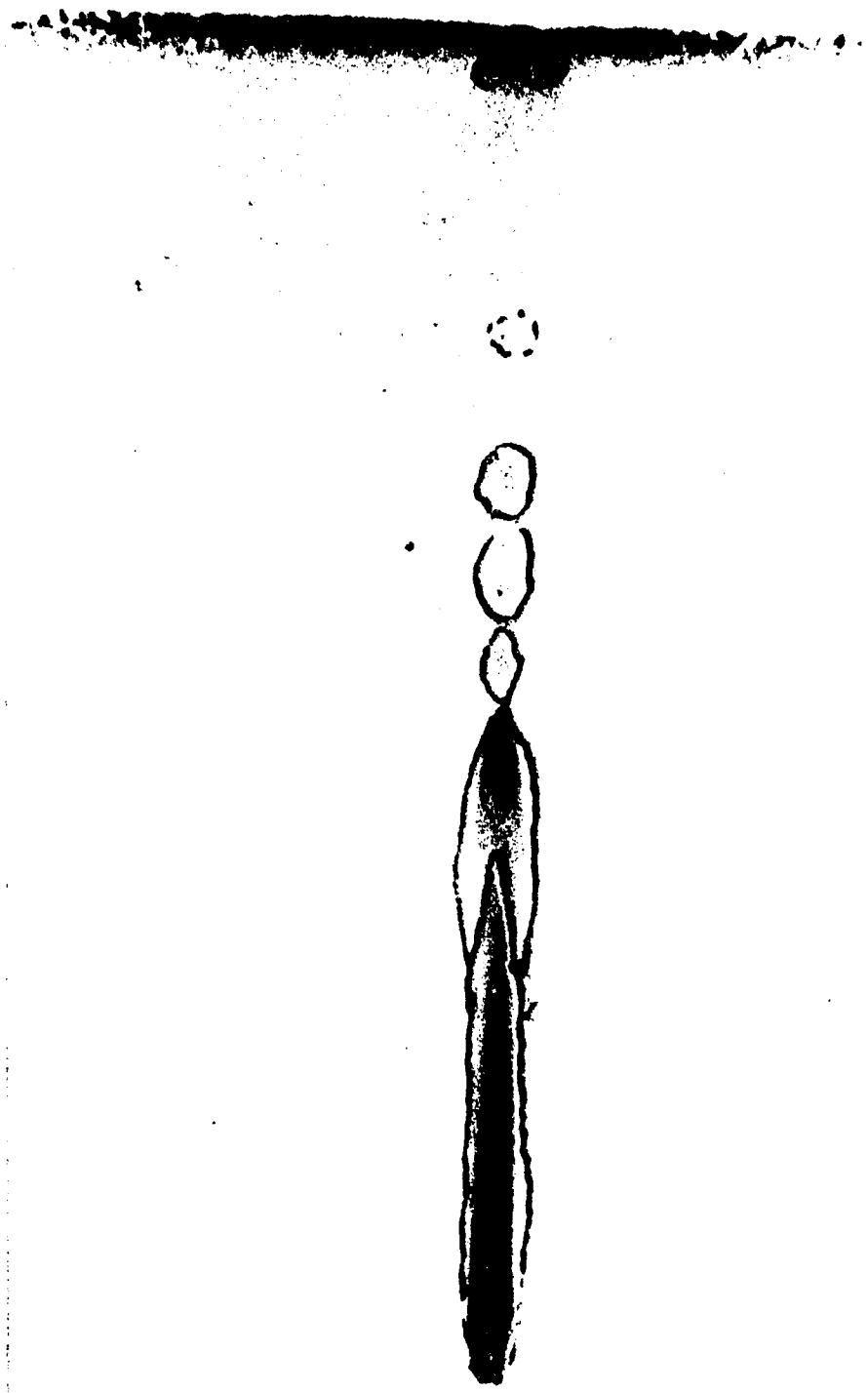
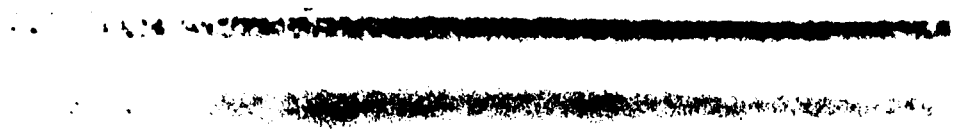


FIGURE 12

Thin Layer Chromatography of Vitreoscilla sp. Strain UM3 Lipid A employing $CHCl_3$-MeOH- 11N NH_4OH (55:30:12 v/v) as the Solvent System.



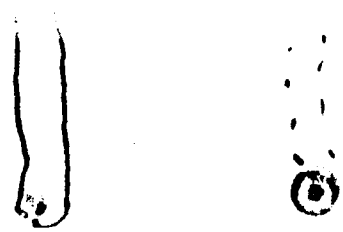
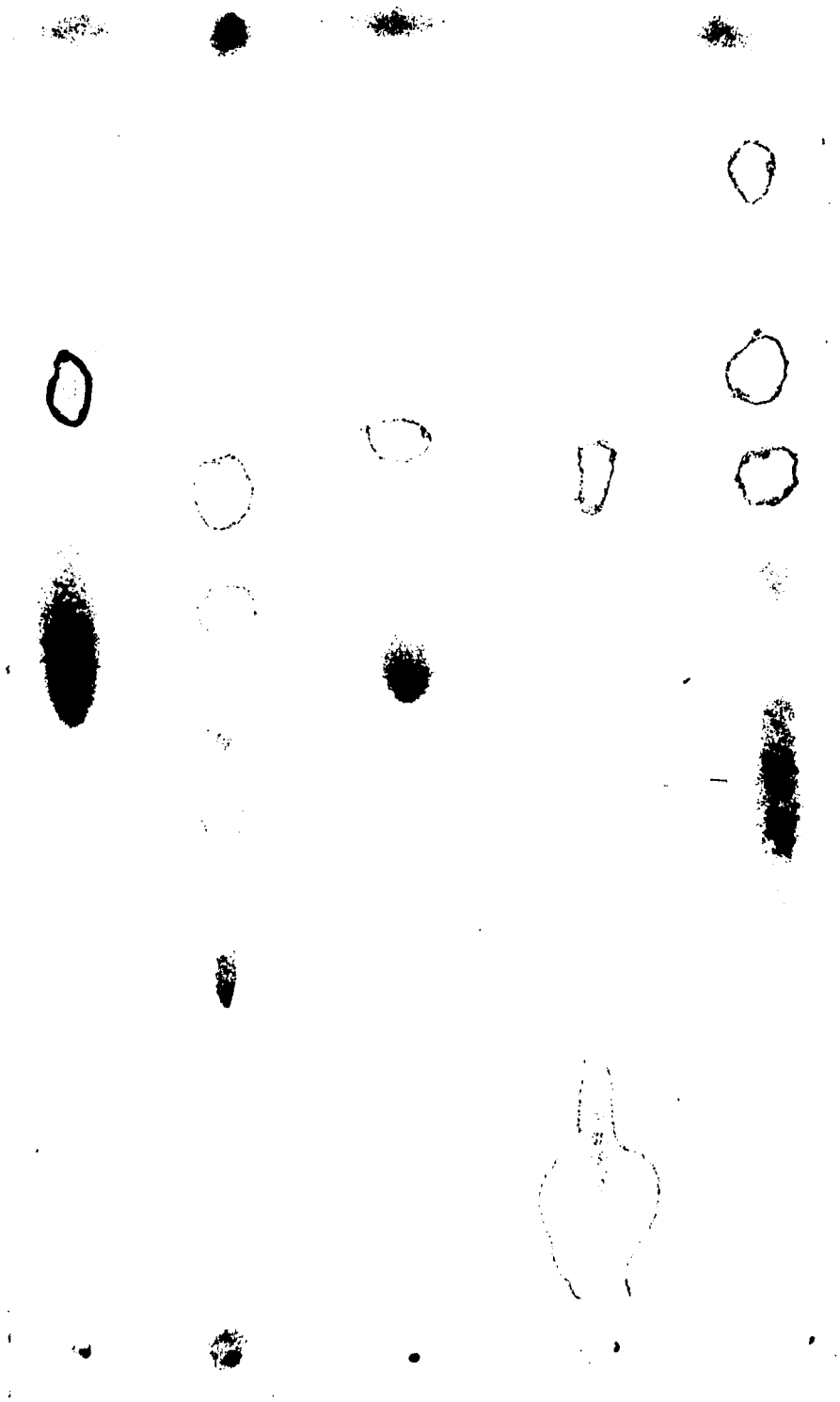


FIGURE 13

Thin Layer Chromatography of the Lipid A of *Serratia marcescens* and the Lipid A and its Soluble Fractions of *Vitreoscilla sp.* Strain UM3.

A chloroform-methanol solvent system (55:30 v/v) was used close to saturation with 11N NH_4OH.



$CHCl_3$ Soluble | Vitreoscilla Lipid A | Acetone Soluble | Water Soluble | Serratia Lipid A

E. Electron Microscopy of Isolated Lipopolysaccharide

Isolated lipopolysaccharide from the four strains of Vitreoscilla were negatively stained with phosphotungstic acid and examined by electron microscopy. The results presented are representative of all strains examined. Every preparation showed the typical isolated round bodies characteristic of lipopolysaccharide (Figure 14). These round particles have been designated A particles by Bladen, Gewurz, and Mergenhagen (1967) in a study of Veillonella lipopolysaccharide. In some cases an amorphous material was seen and usually in association with A particles (Figure 15). This amorphus looking material has been designated B particle by Bladen, et al. (1967) and was said to be material probably released from A particles. Neither A nor B particles have been chemically identified as yet.

The typical structure most prevalent for the lipopolysaccharide of Vitreoscilla is shown in Figure 16. This figure demonstrates a large number of strand-like structures which may periodically contain blebs or swellings within its strands and in some cases gives the preparation a "string of beads" appearance. Strands have been found in all Vitreoscilla preparations and appear as seen in Figures 17 and 18. The latter figure shows the presence of both A particles and strands. Figure 18 is the most representative of our results for the Vitreoscilla showing strands predominating over all other forms.

Studies at the National Institute of Health have shown that when some isolated lipopolysaccharides are reacted

FIGURE 14

Negatively Stained Lipopolysaccharide A Particles from Vitreoscilla sp. Strain LCI. X 120,000.

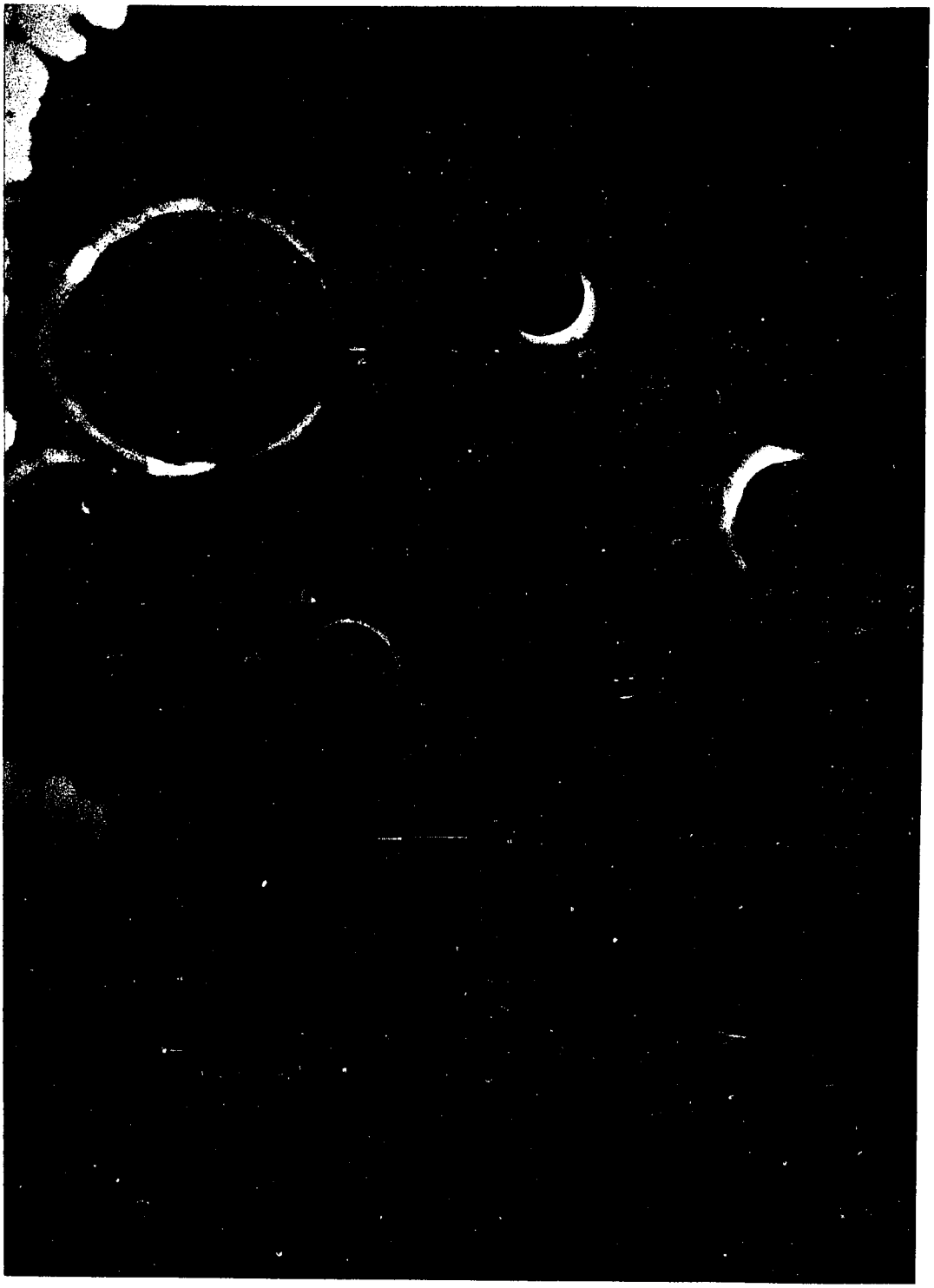


FIGURE 15

Negatively Stained Preparation of *Vitreoscilla sp.* Strain UNH-L Showing B Particles. X 120,000.

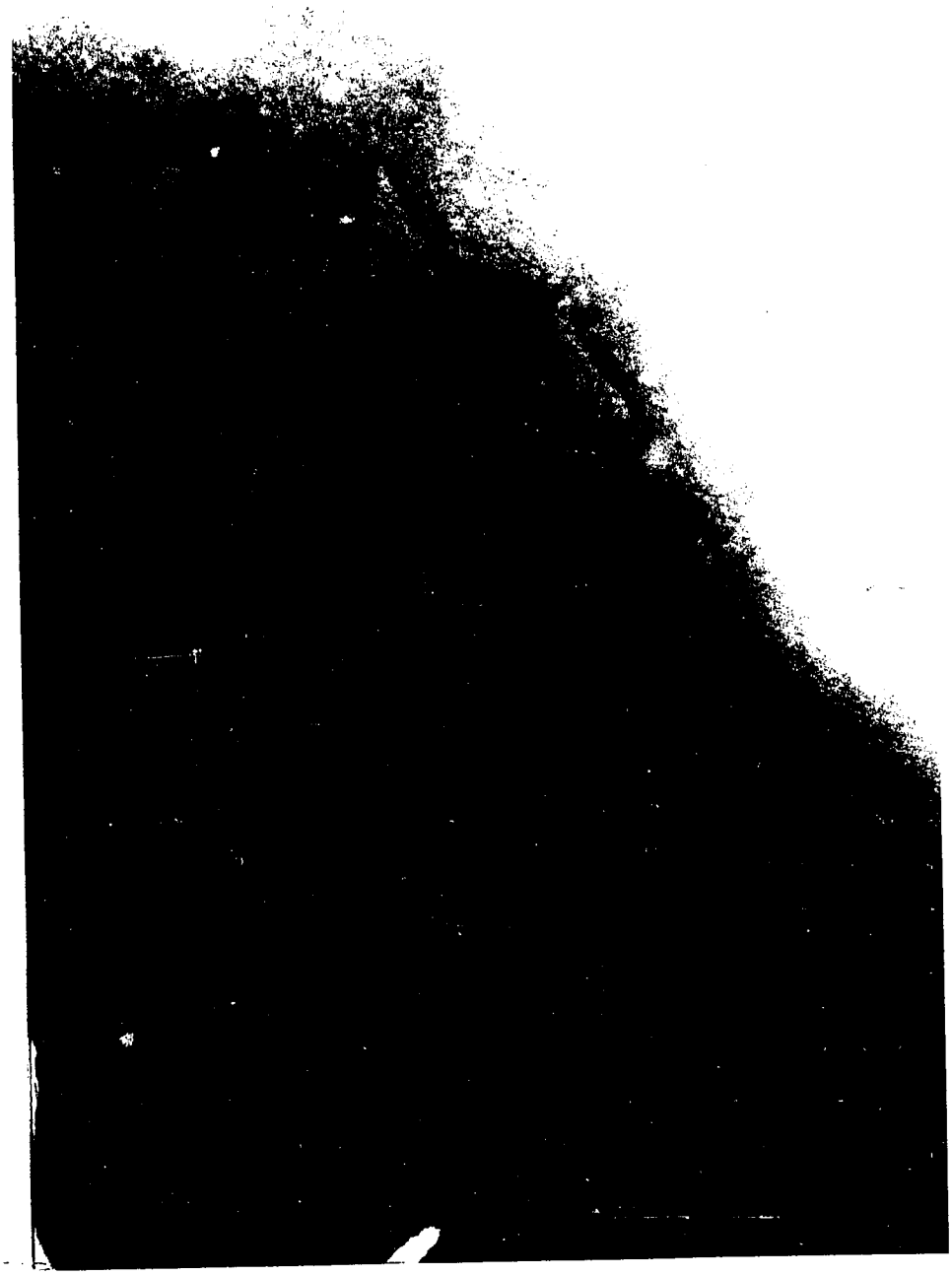


FIGURE 16

Negatively Stained Lipopolysaccharide from Vitreoscilla sp. Strain LCI Showing the LPS Material as Strands or Fibers. X 120,000.

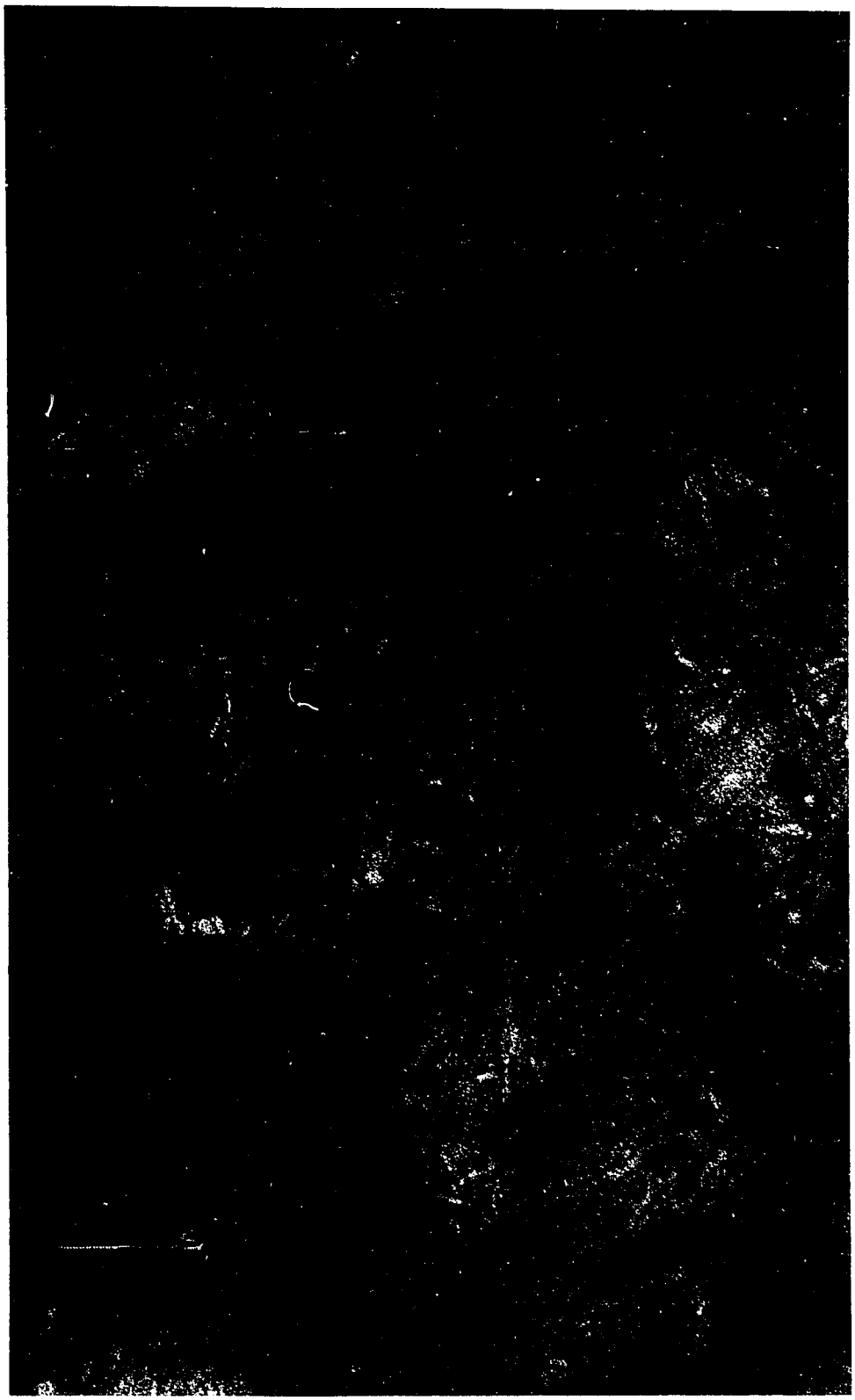


FIGURE 17

Negatively Stained Lipopolysaccharide from Vitreoscilla stercoraria X 120,000.

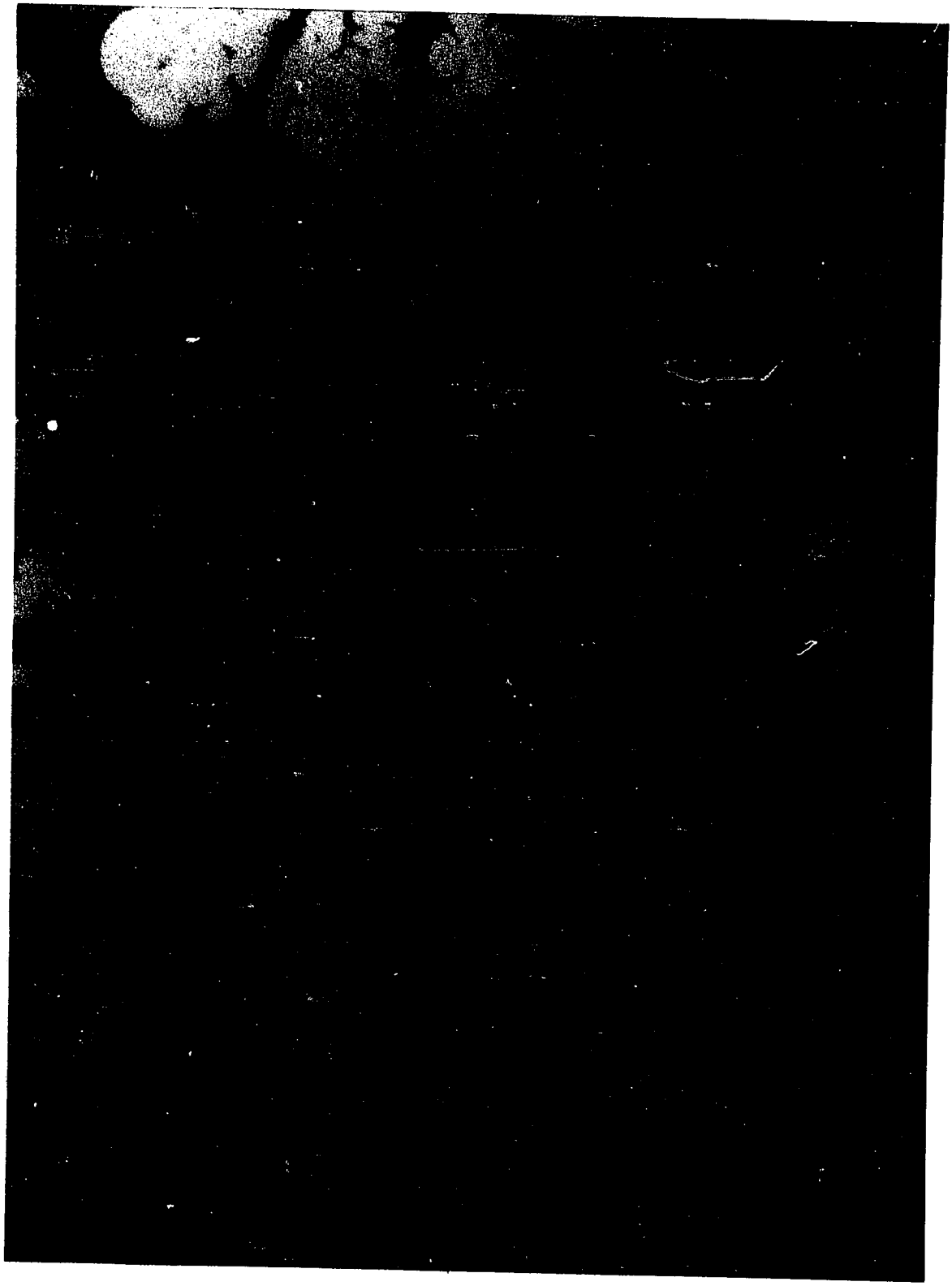


FIGURE 18

Negatively Stained LPS from Vitreoscilla sp. Strain UM3 Showing Both A Particles and Strands. X 120,000.

with guinea pig complement, lesions are produced in the lipopolysaccharide (Bladen, et al., 1967; Hageage, 1967). Upon subjecting Vitreoscilla sp. strain UM3 lipopolysaccharide to guinea pig complement (Figure 19), lesions could be seen by electron microscopy. These lesions, approximately 90 Å wide, appeared as small dark spots, due to PTA deposition, scattered throughout the preparation. Since PTA will deposit only in areas containing no material these lesions are thought to be holes produced by the action of complement upon the lipopolysaccharide (Bladen, et al., 1967). What complement is destroying, solubilizing, or releasing has not been determined.

FIGURE 19

Negatively Stained LPS from Vitreoscilla sp. Strain UM3 Treated with Guinea Pig Serum Showing Numerous Lesions. X 120,000.

DISCUSSION

Although the mechanism behind the gliding movement of some eucaryotic cells, notably desmids and diatomes (Drum and Hopkins, 1966) has been elucidated, the mechanism by which motile procaryons glide over a solid substrate still remains a mystery. Numerous theories have been proposed to explain this phenomenon although only four appear to have any merit. The osmotic theory (Weibull, 1960) states that movement is caused by rhythmic osmotic changes within the wall structure causing rhythmic swelling along its surface and thus propelling the organism in the direction of the wave. Burkholder (1935), put forward a theory based on surface tension differential. According to the theory, the organism would move only in the direction away from the area of the lowest surface tension. A third theory, the secretion theory, postulates that slime secreted from one end of the cell propels the organism in the opposite direction (Weibull, 1960). The fourth theory, contractility theory, at present has the largest following among investigators of gliding locomotion. This theory was put forward by Costerton, Murray, and Robinow (1961) in which they state, "that the motility of these organisms is a function of orderly waves of contraction in an elastic outer layer of their cell walls."

If contraction does take place, one would reasonably expect some variety of protein structure responsible for such

movement. This contension was held by Johnson and Baker as early as 1947 and by Bisset (1950) both of whom believe that some sort of protein fiber exist in the wall structure of these gliding organisms which contracts circumferentially and longitudinally causing the organism to glide. Graf (1965) has, recently, reported finding fibrillar bundles located in the cell wall of several aquatic strains of Myxobacteria. Graf believes these bundles consist of fibrillar units 100-200 mu long and 15-20 mu wide and are the contractile units responsible for gliding. Other investigators have also found rod-shaped bodies present in gliders which may be contractile units (Reichenbach, 1965, and Bradley and Dewar, 1966). Therefore, this investigation concerning the chemistry of Vitreoscilla cell envelopes was undertaken to determine if there was a major difference between the envelopes of gliding and non-gliding bacteria which could be related to their type of motility.

Upon the determination of total protein in Vitreoscilla envelopes and the comparison of these results with E. coli and other gram-negatives, no particular insight was gained for a contractile mechanism. It was expected that the Vitreoscilla might contain an elevated protein content such as found with Cytophaga fermentans (Collins, 1964). Comparative results from Table 14 indicate that Vitreoscilla cell envelopes contain from 35 to 62% protein, neither of which are greater than other gram-negatives. Due to the large fluctuation, it did not seem that the gliding mechanism could be related, at least, to total protein content of the cell envelopes.

TABLE 14.

Chemical Composition of Some Gram-Negative and Gliding Bacterial Cell Envelopes.

	PERCENT					
Organism	Amino Sugar	Reducing Sugar	Carbo-hydrate	Pro-tein	Lipid	Envelope Dry Wt.
A. aerogenes	2.0				14.6	
Sal. gallinarum	3.5				22.0	
Sal. bethesda				63.0	11.0	
Ps. aeruginosa		8.0		54.0	15.0	
E. dispar	5.0				12.6	
E. coli	3.0	16.0		60.0	22.0	15.0
E. coli B/r3*	2.54	11.9	11.2	63.4	22.1	16.5
E. coli B/r3**	2.16	10.8	12.5	52.0	20.5	13.5
Myxococcus xanthus	0.1			50.0		7-8
Cytophaga fermentans	0.4	6.5	7.0	75-85	10.8	
Vitreoscilla sp. UM3 **	0.672	2.34	3.49	35.3	61.4	11.5
Vitreoscilla sp. UNH-L **	0.65	2.92	3.26	38.8	58.5	13.7
Vitreoscilla sp. LCI **	0.542	2.62	3.32	62.0	35.4	15.2
Vitreoscilla stercoraria **	3.627	5.8	2.55	49.5	44.7	17.4

* Envelope prepared by the method of Salton and Horne (1951).

** Envelopes prepared by the method appearing in Materials and Methods.

The total lipid was correspondingly high for all Vitreoscilla as was also found for Myxococcus xanthus (Mason and Powelson, 1958), while Cytophaga fermentans contained only 10.8% lipid (Collins, 1964). The exceptionally high lipid content in the above mentioned gliding bacteria, as far as we know, can not be linked directly to gliding locomotion since contractibility has not been reported as a property of lipid material.

The possibility that the bacterial envelope does undergo some type of contraction would indicate that the envelope as a whole should be more flexible than other gram-negative microorganisms. The layer of the cell wall or envelope responsible for rigidity or flexibility resides in the mucopeptide structure. The murine sacculi or mucopeptide, as it is known, is chemically composed of N-acetyl-glucosamine linked to N-acetyl-muramic acid and encompasses the whole cell just above the plasma membrane. This study did not delve into the chemical structure of this layer but an indication of a lesser amount of mucopeptide in these organisms was obtained from the amino sugar content of the cell envelope which was found to be approximately one quarter that found in the envelopes of nongliding gram-negative bacteria, i.e., E. coli, (Salton, 1953) Pseudomonas aeruginosa, (Collins, 1963) and others (Salton, 1964). The amino sugar content of all Vitreoscilla tested were between 0.5 to 0.6% of the total cell envelope which concurs with White and Dworkins (1967) 0.11% found for Myxococcus xanthus and the results reported by

Collins (1964) for Cytophaga fermentans. This figure becomes even smaller when one subtracts from it the amount of glucosamine found in the lipopolysaccharide layer. The lipopolysaccharide layer contains about 0.1% of the total amino sugar of the cell envelope thus lowering the figure presented in Table 14 to 0.572% for Vitreoscilla sp. strain UM3. This makes the amino sugar content of the mucopeptide layer about one fifth that reported for other gram-negatives. These results are, however, in good agreement with those found for Myxococcus xanthus and Cytophaga fermentans.

A rebound phenomenon reported by Costerton et al., (1961) and later substantiated by Hageage (1963) indicated a flexion of some area of the organism. If the wall of gliders is flexible, it may contain less mucopeptide which could account for the low amino sugar found in their envelope. If the contraction takes place in the area of the membrane or directly below, the mucopeptide would not only have to keep the cell rigid but be flexible enough to allow the contractile wave to reach the surface upon which the organism was moving. This is one possible or functional reason why gliding bacteria would possess very little amino sugar as compared to other gram-negative microbes. This type of thinking is purely hypothetical but gives us one reason why these organisms may possess an abnormally low content of glucosamine and muramic acid. There still remains the possibility that contraction originates beyond the mucopeptide layer, either in the lipopolysaccharide, lipoprotein, or a layer which has not yet been detected.

Little data is available for comparison of Vitreoscilla envelope chemistry to other gliders. The available information shows good correlation between the low concentration of amino sugar, reducing substance, total carbohydrate, and phosphorus. The amino acids found associated with Vitreoscilla and Myxococcus show the same pattern with the exception that Vitreoscilla contains histidine. The amino acid data obtained for Cytophaga fermentans is not complete but the same general pattern is indicated. The gliding bacteria amino acid analysis is comparable to E. coli and in most cases to other gram-negative envelopes with the exception of possessing histidine, proline, and threonine. There also does not seem to be any consistency in the amounts or kinds of monosaccharide units found in gliders although this information is scanty.

The chemical information presented does not allow speculation as to the use of cell envelope chemistry as a taxonomic tool for distinguishing between gliding species. The low amino sugar may serve to distinguish gliders from non-gliders but their gliding motility is already their key distinguishing feature. More information on the cell envelope chemistry of many other gliders will be necessary in order to put forth a possible taxonomic relationship.

Our attention moved from the total picture to an investigation of isolated cell envelope layers of the four strains of Vitreoscilla. Since the outer wavy layer of the cell wall, the lipopolysaccharide layer, was a possible area for contraction, this layer was extracted for analysis. This material, obtained by phenol extraction, proved to be

lipopolysaccharide by finding that it consisted of KDO, heptose, and a lipid containing glucosamine (Table 7). The purity of the extracted lipopolysaccharide was thought to be relatively high since only a few amino acids could be found in any of the lipopolysaccharide preparations.

To date, there has been no chemical investigation of the lipopolysaccharide layer of gliding bacteria. Therefore, comparisons can only be drawn between gram-negative non-gliders and Vitreoscilla sp. strain UM3. Comparative chemical analysis of gram-negative endotoxins is summarized in Table 15. One immediately notes the sporatic information available and the different results obtained with different extraction procedures. In toto, the Vitreoscilla lipopolysaccharide contains 20% total carbohydrate which in most cases is lower than the carbohydrate values obtained for most other lipopolysaccharides. The heptose content of Vitreoscilla lipopolysaccharide runs, on the average, 12% which is in good agreement with other hot phenol-water extracted endotoxins, although the Salmonella are much lower, as were lipopolysaccharides extracted by other procedures (Anacker, et al., 1964; Nowotny, et al., 1963; and Alaupovic, et al., 1966). The Vitreoscilla lipopolysaccharide does however, contain less KDO than most endotoxins examined, which indicates its polyheptose backbone is probably composed of a longer heptose chain, since it is generally accepted that the backbone is connected to the lipid A via one mole of KDO. The lipid content reported for other genera range from 8.6% for Serratia (Table 15) to 60% in Viellonella (Hageage, personal communication). Vitreoscilla sp. strain UM3 lies

TABLE 15

Comparative Chemical Data for the Lipopolysaccharide of some Gram-Negative Bacteria

Organism & Method	Carbo-hydrate	Glucose	Galactose	Heptose	KDO	Hexos-amine	Pi	Lipid
E. coli HPW	34.5	12.5	9.9	11.0	4.8	9.5		
E. coli 0111B4 CAP	48.0	+	+			15.0	2.0	
E. coli K-12 HPW	19.7					2.0		
E. coli K-12 TCA	11.8					1.4		
E. coli 0113 TCA	43.0			1.6	0.77	11.0	1.6	
E. coli 0113 AE	36.0			1.7	0.79	14.0	0.88	
Sal. typhosa 0901 HPW	25.0					5.3		9.6
Sal. typhosa 0901 TCA	13.3					7.3		19.4
Sal. typhimurium HPW		4.8	20.8	7.1	4.0	5.9		
Sal. paratyphi HPW		6.9	13.6	4.2	4.1	5.7		

TABLE 15 (Continued)

Organism & Method	Carbohydrate	Glucose	Galactose	Heptose	KDO	Hexosamine	Pi	Lipid
S. marcescens HPW	21.8					4.4		8.6
S. marcescens TCA	34.2					4.0		15.6
X. campestis HPW		0.9	24.5				1.9	
Vit. UM3 HPW	22.4	4.42	2.85	9.95	0.36	7.23	1.28	25.0
Vit. UNH-L HPW	18.55	4.06	2.59	12.68	0.25	7.35	1.07	
Vit. LCI HPW	19.55	5.02	2.39	12.95	0.40	7.05	1.22	
Vit. stercoraria HPW	19.17	4.61	1.57	12.03	0.32	7.17	1.15	

approximately in the middle showing 25% lipid from the phenol-water extraction. Hexosamines also varied from 1.4% to 14% and once again the Vitreoscilla are found to lie midway at 7%.

A structural consideration of Vitreoscilla sp. strain UM3's lipopolysaccharide is possible if one makes a few assumptions. It is generally agreed that the backbone of the lipopolysaccharide contains a heptose, phosphate, and KDO (Osborn, 1966; Rothfield, et al., 1966; Weiner, et al., 1966). In E. coli and Salmonella species, the backbone is linked to the one mole of KDO which is connected to the lipid portion of the molecule (Rothfield, et al., 1966; Weiner, et al., 1966). This means one must have a 1:1 molar ratio of heptose to phosphate in order to diagram a polyheptose-phosphate backbone structure. Upon examination of the phosphate contained in the entire lipopolysaccharide molecule of Vitreoscilla sp. strain UM3, as well as the isolated lipid A portion, it became apparent that the polysaccharide portion of the molecule did not possess a 1:1 ratio but a 2:1 heptose-phosphate ratio. We thus concluded, that the backbone is probably similar in structure, containing heptose and phosphate, but that the arrangement was different. In order to hold to a heptose and phosphate backbone, one must assume a $(\text{Heptose-heptose-phosphate})_n$ polymeric backbone, i.e., one heptose linked to its neighboring heptose molecule which is linked via a phosphate to the next heptose, containing approximately 18 to 20 heptoses connected to one mole of KDO. This, then gives a backbone of alternating glycosidic and phosphodiester linked heptoses. There is precedence for such a structure. Burton

FIGURE 20.

A Proposed Structural View of Vitreoscilla sp. Strain UM3's Lipopolysaccharide.

F-GA
GA-F
P
F-GA
(Hep)2-P-(Hep)2-P-(Hep)2-P-(Hep)2-P-(Hep)2-P-(Hep)-P-KDO-GA-F
P
GA-F
F-GA
P
GA-F
F-GA
---Glu-Gal-Glu-Uronic-Uronic
---Glu-Gal-Glu-Uronic-Uronic
---Glu-Gal-Glu-Uronic-Uronic
---GLU-Gal-Glu-Uronic-Uronic
BACKBONE
CORE
LIPID A

and Carter (1964) described an alternating glycosidic-phosphodiester linkage for the glucosamine in the lipid A backbone of Salmonella typhimurium.

The side chain polysaccharide of the backbone has also been worked out for E. coli and Salmonella typhimurium. The type structural arrangement, a glucose-galactose-glucose-glucosamine polymer, was reported by Osborn et al. (1964) for the Salmonella and by Weiner et al. (1966) for E. coli K12, which Weiner claimed was modified by the addition of colitose. The polysaccharide portion of Vitreoscilla sp. strain UM3 contained only glucose, galactose, and uronic acid. In addition to the heptose and KDO, no glucosamine could be correlated with the polysaccharide portion since the moles of glucosamine found in the isolated lipopolysaccharide were the same as that found in the isolated lipid A fraction. We believe that all the glucosamine is incorporated into the lipid A fraction. The molar ratio of glucose to galactose is 2:1 which corresponds to the molar ratios of other investigated core structures (Osborn, et al., 1964; Nowotny, 1961; Weiner, et al., 1966).

Uronic acid has been reported as being present in the lipopolysaccharides of most organisms investigated, however, it has never been incorporated into their structures. Since Vitreoscilla sp. strain UM3 contains as much uronic acid as glucose, it is felt that it plays an important role in the lipopolysaccharide structure. The uronic acid detected is associated with the polysaccharide portion and, at least in part, may take the place of glucosamine. In the final

analysis, we believe the polysaccharide side chain to contain glucose, galactose, and uronic acid.

Since the monosaccharide units found are the same as those found in other lipopolysaccharides, it is believed that the lipopolysaccharide of Vitreoscilla sp. strain UM3 may be arranged in approximately the same order. All core polysaccharides studied to date show their chain of monosaccharides extending from the heptose of the heptose-phosphate backbone, in the order of glucose-galactose-glucose-glucosamine. Vitreoscilla sp. strain UM3 contains these sugars with the exception of the glucosamine and the addition of uronic acid. It may be that uronic acid, in this case, replaces the glucosamine and a possible structural view may be glucose-galactose-glucose-uronic acid-uronic acid. Since we are dealing with a structural arrangement without the aid of enzymatic mutant analysis, we must admit that these sugars may be arranged in a completely different manner. There are numerous combinations possible when arranging three different units in a linear arrangement not to mention the possibilities that some may be arranged as a side chain so that a structure such as galactose-glucose-uronic acid-glucose with another mole of uronic acid attached to the first glucose, could also be a possibility. There also remains the possibility that the uronic acid may act as a single antigenic side chain as found for colitose in E. coli K12 by Weiner, et al. (1966).

The lipid A portion, thought to be connected to the polysaccharide core via the KDO, seems very similar to that

proposed by Burton and Carter (1964) for Salmonella typhimurium, which is in contrast to Nowotny's (1961) polyglucosamine-phosphate structure. A molar ratio of 2:1 glucosamine to phosphate was obtained for Vitreoscilla sp. strain UM3 lipid A. These results indicated that the structural arrangement of the lipid A could not possibly be a glucosamine-phosphate polymer, but rather an alternating glycosidic-phosphodiester linkage as proposed by Burton and Carter.

Taking into consideration the above results and discussion, we feel that one of the possible structural arrangements for Vitreoscilla sp. strain UM3's lipopolysaccharide may correspond to the arrangement outlined in Figure 19. We recognize the fact that there are many arrangements possible from the given data, but it is felt that this is one of the better possibilities, taking into consideration other structural views put forward in recent years. The question of the exact structure of Vitreoscilla lipopolysaccharide can not be answered till mutants are isolated blocked at particular synthetic steps and the biosynthesis of its lipopolysaccharide correlated with the quantitative chemistry presented herein.

Electron Microscopy

The electron microscopy of isolated lipopolysaccharide has showed generally two structures. The first and most common type is a spherical structure as reported by Bladen and Mergenhagen (1964) for Veillonella. This same structural type has been reported for E. coli (Work, Knox, and Vesk, 1966), Salmonella typhimurium (Rothfield, et al., 1966), Serratia

marcescens (Rothfield, et al., 1966; Ikawa and Forest, personal communication), and a species of Flexibacter (Hageage, 1967). The second morphological type of lipopolysaccharide has the form of long strands which may or may not contain periodic blebs forming a bead-like appearance. This type of morphological structure has been observed by Taylor et al. (1966) for the extracellular lipopolysaccharide of E. coli grown under lysine limiting conditions, as well as for the lipopolysaccharide of Ferrobacillus ferooxidans (Korczynski, et al., 1967). Bladen has noted a similar lipopolysaccharide structure in E. coli and in Nesseria menningitidis (personal communication). The phenol extracted, purified lipopolysaccharide from Vitreoscilla showed both morphological forms. Negative staining (Figure 14) shows the oval or circular structures occasionally found while Figure 15 shows the second structure common to all Vitreoscilla preparations. Comparative observations showed the stringy form was more closely related to those of N. meningiditis (Hageage, personal communication).

In mixtures of lipopolysaccharide and phosphatidylethanolamine, Rothfield and Horne (1967) reported continuity between recognizable lipopolysaccharide spheroids and phospholipid leaflets. This suggested to them a direct insertion of lipopolysaccharide into the phospholipid leaflet. They stated that this suggests a tentative model, in which the native cell envelope contains lipopolysaccharide and phospholipid molecules in a common leaflet structure. In the case of the second lipopolysaccharide structure observed for Vitreoscilla, it may

be that the LPS is extracted closer to its "native" state (the state in which it exists while in association with the living organisms) and that this state is related to Rothfield and Horne's artifical common leaflet structure.

It is possible that there are differences in polar and non-polar forces between those lipopolysaccharides exhibiting the stringy-like structure and those showing predominately round A structures. Those extracted as stringy-like material may possess polar forces similar to those produced artificially by Rothfield and Horne (1967). In areas where the bonding is weak, spheroids may be produced by cleavage and recombination of the broken ends. Figure 17 shows an A particle in direct association with the stringy-like material and this looks extremely like that produced artifically with LPS and phospholipid by Rothfield and Horne (1967). It may be that the ribbon like structure is close to the type structure (native) seen when the lipopolysaccharide is in association with the cell envelope. Supporting this contention is the fact that the LPS found to be extruded from E. coli into its surrounding growth medium appears to have the same predominating structure (stringy-like material) as the LPS from the Vitreoscilla. It may be that the harsh chemicals used for extraction in many cases is enough to cause the weaker links in the LPS to break and round up thus producing the normally found A particle. This may be one explanation for the two morphological varieties of lipopolysaccharides found and why both varieties are present in homogeneous preparations of Vitreoscilla lipopolysaccharide.

SUMMARY AND CONCLUSION

The cell envelopes of the gliding bacterium Vitreoscilla were found to possess major chemical differences when compared to non-gliders. The amino sugars, i.e. glucosamine and muramic acid, were present but in very low concentration indicating that there may be less mucopeptide associated with the organism than with non-gliders. This may be the basis for the observed flexibility of the cell and would be necessary for the gliding action if a contractile mechanism initiated from the plasma membrane. The amino sugar content seems to be a consistent chemical factor differentiating gram-negative gliders from non-gliders. The second apparent difference lies in the high content of lipid displayed by the Vitreoscilla cell envelopes while to date no structural or functional purpose can be attributed to the lipid.

The only monosaccharides found in the envelopes were glucose, galactose, and a heptose plus the amino sugars previously mentioned. The amino acids also showed no qualitative differences from gram-negative non-gliding bacteria. A spectrochemical analysis showed nothing extraordinary except for a slightly elevated amount of aluminum. The results obtained could not be related to any type of gliding mechanism.

One would expect that any proposed contractile mechanism would reside in some protein fraction of the cell envelope. The total protein varied between strains of

Vitreoscilla while the types of amino acids found remained constant. The data, therefore, could not be related to a contractile mechanism either qualitatively or quantitatively. One might approach the problem by isolating large peptides from the cell envelopes and comparing these peptides to those of the actinmyosin type known to cause contraction in higher organisms. These peptides could also be examined for their ability to contract when in the presence of an energy source such as ATP and/or ADP.

The outer layer of the cell envelope, the lipopolysaccharide, was also examined chemically and does not seem to possess components that could be related to a contractile mechanism of locomotion. However, the lipopolysaccharide isolated from Vitreoscilla sp. strain UM3 did show some peculiarities. Glucosamine was absent from the polysaccharide portion of the molecules core while all other lipopolysaccharides reported in the literature seem to possess glucosamine in this area as well as in the backbone structure of the lipid A portion. Total phosphate present in the endotoxin was also low. In the polysaccharide portion the molar ratio of heptose to phosphate was 2:1, thus precluding the usual heptose-phosphate backbone. The lipid A portion also had a 2:1 ratio of glucosamine to phosphate and may be related to Burton and Carter's (1964) suggested structure for the lipid A of Salmonella typhimurium. The last feature was the high content of uronic acid found in the polysaccharide portion which was present in the same concentration as glucose.

The problem of gliding locomotion is still far from a solution. There still remains the four proposed theories and no one to say that each one may fit some specific organism or that any other conjured proposal does not have the same merit. Elucidation of the gliding phenomenon must be met by the combined efforts of electron microscopy, chemical identification, and enzymology. If it is a structural component, electron microscopist should be able to find it, it should be isolated and chemically defined, and finally its priming energy source identified. It is not until more interest is developed towards gliding bacteria, more organisms tested in the above manner, and this information combined that the mechanism of bacterial gliding will be clear.

BIBLIOGRAPHY

1. Adams, G. A., and S. M. Martin. 1967. Cellular polysaccharides of Serratia marcescens. Cand. J. Biochem. 45:471-491.

2. Adanson, M. 1767. Un mouvement particulier decouvert dane une plante appellee Tremella. Mem. Acad. Sci. Paris. 564-572.

3. Alaupovic, P., A. A. Olson, and J. Tsang. 1966. Studies on the characterization of lipopolysaccharides from two strains of Serratia marcescens. Ann. N. Y. Acad. Sci. 133:546-565.

4. Albertsson, P. A. 1958. Particle fractionation in liquid two phase systems. The composition of some phase systems and the behavior of some model particles in them. Application to the isolation of cell walls from microorganisms. Biochim. Biophys. ACTA. 27:378-395.

5. Alderton, G., W. H. Ward, and H. L. Fevold. 1945. Isolation of lysozyme and related enzymes. J. Biol. Chem. 157:43-49.

6. Anacker, R. L., R. A. Finkelstein, W. T. Haskins, M. Landy, K. C. Milner, E. Ribi, and P. W. Stashak. 1964. Origin and properties of naturally occurring hapten from Escherichia coli. J. Bacteriol. 88:1705-1720.

7. Asbell, M. A., and R. G. Eagon. 1966a. The role of multivalent cations in the organization and structure of the bacterial cell wall. Biochem. Biophys. Res. Commun. 22:664-671.

8. Asbell, M. A., and R. G. Eagon. 1966b. Role of multivalent cations in the organization, structure, and assembly of the cell wall of Pseudomonas aeruginosa. J. Bacteriol. 92:380-387.

9. Barkulis, S. S., and M. F. Jones. 1957. Studies of Streptococcal cell walls. I. Isolation, chemical composition, and preparation of M. protein. J. Bacteriol. 74:207-216.

10. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. J. Biol. Chem. 234:466-468.

11. Bisset, K. A. 1950. The Cytology and Life History of Bacteria. E. and S. Livingston, Ltd., Edinburg, Scotland.

12. Bitter, T., and R. Ewins. 1961. A modified carbazole reaction for uronic acids. Biochem. J. 81:43P.

13. Bladen, H. A., and S. E. Mergenhagen. 1964. Ultrastructure of Veillonella and morphological correlation of an outer membrane with particles associated with endotoxic activity. J. Bacteriol. 88:1482-1492.

14. Bladen, H. A., H. Gewurz, and S. E. Mergenhagen. 1967. Interactions of the complement system with the surface and endotoxic lipopolysaccharide of Veillonella alcalescens. J. Exp. Med. 125:767-786.

15. Bolle, A., and E. Kellenberger. 1958. Comportement de la paroi bacterienne en presence de detergents, en particulier le lauryl-sulfate de sodium. Pharm. ACTA Helv. 33:379-390.

16. Bradley, D. E., and C. A. Dewar. 1966. Structure of Phage-like objects associated with non-induced bacteriocinogenic bacteria. J. Gen. Microbiol. 45:399-408.

17. Brown, A. D. 1961. The peripheral structure of gram-negative bacteria. I. Cell wall protein and the action of a lytic enzyme system. Biochim. Biophys. ACTA 48: 352-361.

18. Brown, A. D. 1962. The peripheral structure of gram-negative bacteria. III. Effects of cations on proteolytic degradation of the cell envelope of a marine pseudomonad. Biochim. Biophys. ACTA. 62:132-144.

19. Brown, A. D. 1964. Aspects of bacterial response to the ionic environment. Bacteriol. Rev. 28:296-329.

20. Buchanan, R. E. 1925. General systematic bacteriology. Vol. 1. Williams and Wilkins, Baltimore, Md.

21. Burchfield, H. P., and E. E. Storrs. 1962. Biochemical applications of gas chromatography. Academic Press Inc. New York.

22. Burkholder, P. R. 1934. Movement in the Cyanophyceae. Quart. Rev. Biol. 9:438-459.

23. Burton, H. J., and H. E. Carter. 1964. Purification and characterization of the lipid A component of the lipopolysaccharide from Escherichia coli. Biochem. 3:411-418.

24. Campling, J. D., and D. A. Nixon. 1954. The inositol content of foetal blood and foetal fluids. J. Physiol. 126:71-80.

25. Carlson, K. J., and R. G. Eagon. 1966. Lysozyme sensitivity of the cell wall of Pseudomonas aeruginosa: Further evidence for the role of the non-peptideoglycan components in the cell wall rigidity. Cand. J. Microbiol. 12:105-108.

26. Christian, J. H. B., and M. Ingram. 1959. Lysis of Vibrio costicolus by osmotic shock. J. Gen. Microbiol. 20:32-42.

27. Cohn, F. 1875. Untersuchumgen uber bakterien. II Heft. 3 Beitr. Biol. Pflanz. 1:141-208.

28. Collins, F. M. 1963. Composition of cell walls of ageing Pseudomonas aeruginosa and Salmonella bethesda. J. Gen. Microbiol. 34:379-388.

29. Collins, F. M. 1964. Cell wall composition of a marine Cytophaga. Aust. J. Exp. Biol. Med. Sci. 42:263-265.

30. Costerton, J. W. F., R. G. E. Murray, and C. F. Robinow. 1961. Observations on the mobility and the structure of Vitreoscilla. Cand. J. Microbiol. 7:329-339.

31. Cummins, C. S. 1956. The chemical composition of the bacterial cell wall. Intern. Rev. Cytol. 5:25-50.

32. Cummins, C. S., and P. Harris. 1958. Studies on the cell-wall composition and taxonomy of Actinomycetales and related groups. J. Gen. Microbiol. 18:173-189.

33. Dankert, M., A. Wright, W. S. Kelley, and P. W. Robbins. 1966. Isolation, purification, and properties of the lipid-linked intermediates of O-antigen biosynthesis. Arch. Biochem. Biophys. 116:425-435.

34. Dawson, I. M. 1949. Cited from, Miles, A. A. 1949. The status of some arguments about the bacterial surface. Soc. Gen. Microbiol. Symp. 1:119.

35. Dische, Z. 1953. Qualitative and quantitative colorimetric determination of heptoses. J. Biol. Chem. 204:983.

36. Drum, R. W., and J. T. Hopkins. 1966. Diatom locomotion: An explanation. Protoplasm, Bd. LXII:1-33.

37. Eagon, R. G., G. P. Simmons, and K. J. Carson. 1965. Evidence for the presence of ash and divalent metals in the cell wall of Pseudomonas aeruginosa. Cand. J. Microbiol. 11:1041-1042.

38. Folch, J., M. Lees, and G. H. S. Stanley. 1957. A simple method for the Isolation and Purification of Total Lipides from Animal Tissues. J. Biol. Chem. 226:497-509.

39. Folin, F. A., and L. W. Malmrose. 1929. Cited from Umbriet, Burris, and Stauffer. 1959. Manometric techniques. Burgress Publishing Company. New York. 231-239.

40. Foster, J. W., R. M. Cowan, and T. A. Magg. 1962. Rupture of bacteria by explosive decompression. J. Bacteriol. 83:330-334.

41. Fukushi, K., R. L. Anacker, W. T. Haskins, M. Landy, K. C. Milner, and E. Ribi. 1964. Extraction and purification of endotoxin from Enterobacteriaceae: A comparison of selected methods and sources. J. Bacteriol. 87:391-400.

42. Ghatak, S., V. Verma, and S. B. Misra. 1966. Monosaccharide constituents of the walls of H-901, O-901, and Vi-1 strains of Salmonella typhosa. Die Naturwissenschaften 15:384-385.

43. Gilby, A. R., A. V. Few, and K. McQuillen. 1958. The chemical composition of the protoplast membrane of Micrococcus lysodeikticus. Biochim. Biophys. ACTA 29:21.

44. Glauert, A. M. 1962. The fine structure of bacteria. Brit. Med. Bull. 18:245-250.

45. Graf, W. 1965. Bewegungsorgellen bei Myxobakterien. Arch. Hyg. (Berlin) 149:518-526.

46. Hageage, Jr., G. J. 1963. A cytological study of gliding microorganisms. Thesis, University of Maryland, Ph.D. Microbiology.

47. Hageage, Jr., G. J. 1967. Observations on the fine structure and cell surface of Flexibacter species. Bacteriol. Proc. pg. 25.

48. Heath, E. C., and M. A. Ghalambor. 1963. 2-keto-3-deoxy-octonate, a constituent of cell wall lipopolysaccharide preparations obtained from Escherichia coli. Biochem. Biophys. Res. Comm. 10:340-345.

49. Heath, E. C., R. M. Mayer, R. D. Edstrom, and C. A. Beaudreau. 1966. Structure and biosynthesis of the cell wall lipopolysaccharide of Escherichia coli. Ann. N. Y. Acad. Sci. 133:315-333.

50. Herzberg, M. and J. H. Green. 1964. Composition and characteristics of cell walls of smooth strains of Salmonella typhimurium and derived rough varients. J. Gen. Microbiol. 35:421-436.

51. Horowitz, H. N., M. Ikawa, and M. Fling. 1950. Interference by sugars and amino acids in the Elson-Morgan method for hexosamine. Arch. Biochem. 25:226-229.

52. Horowitz, H. N., and G. W. Beadle. 1943. A microbiological method for the determination of choline by use of a mutant of Neurospora. J. Biol. Chem. 150:325-333.

53. Ikawa, M., and E. E. Snell. 1960. Cell wall composition of lactic acid bacteria. J. Biol. Chem. 235:1376-1382.

54. Jesaitis, M. A., and W. F. Goebel. 1952. The chemical and anti-viral properties of the somatic antigen of phage II. Shigella sonnei. J. Exp. Med. 96:409-424.

55. Johnson, F. H., and F. R. Baker. 1947. The Electron and Light Microscopy of Beggiatoa. J. Comp. Cell Physiol. 30:131-146.

56. Kasai, N. 1966. Chemical studies on the lipid component of endotoxin, with special emphasis on its relation to biological activities. Ann. N. Y. Acad. Sci. 133:486-507.

57. Kasai, N., and A. Yamano. 1964. Studies on the lipids of endotoxins. Thin layer chromatography of lipid fractions. Japan J. Exp. Med. 34:239-344.

58. Kates, M. 1964. Bacterial lipids. Adv. Lipid Res. 2: 17-90.

59. Kauffman, F., O. Luderitz, H. Stierlin, and O. Westphal. 1960. Zur chemie der O-antigene von Enterobacteriaceae. I Analyse der zuckerbausteine von Salmonella-O-antigenen. Zentr. Bakteriol. Parasitenk. Abt. I. Orig. 178:442-458.

60. Kellenberger, E., and A. Ryter. 1958. Cell wall and cytoplasmic membrane of Escherichia coli. J. Biophys. Biochem. Cytol. 4:323-326.

61. King, H. K., and H. Alexander. 1948. The mechanical destruction of bacteria. J. Gen. Microbiol. 2:315-324.

62. Korczynski, M. S., A. Agate, D. G. Lundgren, and D. J. Ellar. 1967. Inhibition of iron oxidation and removal of lipopolysaccharide structure in Ferrobacillus ferooxidans. Bacteriol. Proc. pg. 25.

63. Landsteiner, K. 1945. The specificity of serological reactions. Harvard Univ. Press, Cambridge.

64. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

65. Luderitz, O., and O. Westphal. 1952. Uber bakterielle reizstoffe. II Mitt. Qualitative und quantitative papierchromatographische bestimmung der zuckerbausteine eines hoch gereenigton polysaccharide aus colibakterien. Z. Naturforsch. 7b:548-554.

66. Luderitz, O., A. M. Staub, and O. Westphal. 1966. Immunochemistry of O and R antigens of Salmonella and related Enterobacteriaceae. Bacteriol. Rev. 30:192-255.

67. Marr, A. G., and T. Kaneshiro. 1960. Lipids of the cell envelope of Azotobacter vinlandii. Bacteriol. Proc. p. 63.

68. Martin, H. H., ad H. Frank. 1962. Die mucopeptid-grundstruktur in der zellwand gram-negativer bakterien. Zentr. Bakteriol. Parasitenk. Abt. I, Orig. 1, 184:306-311.

69. Mason, D. J., and D. Powelson. 1958. The cell wall of Myxococcus xanthus. Biochim. Biophys. ACTA. 29:1-7.

70. McQuillen, K. 1958. Lysis resulting from metabolic disturbance. J. Gen. Microbiol. 18:498-512.

71. Mergenhagen, S. E., H. A. Bladen, and K. C. Hsu. 1966. Electron microscopic localization of endotoxic lipo-polysaccharide in gram-negative organisms. Ann. N. Y. Acad. Sci. 133:279-291.

72. Morgan, W. T. J., and S. M. Partridge. 1941. Studies in immunochemistry. 6. The use of phenol and of alkali in the degradation of antigenic material isolated from Bacterium dysenteriae (Shiga). Biochem. J. 35:1140-1163.

73. Morgan, W. T. J., and S. M. Partridge. 1942. An examination of the O antigenic complex of Bacterium typhosum. Brit. J. Exp. Pathol. 23:151-165.

74. Morgan, W. T. J., and W. F. Goebel. 1945. Taken from a review by Luderitz, O., A. M. Staub, and O. Westphal. 1966. Bacteriol. Reviews. 30:192.

75. Mukerjee, H., and J. Sri Ram. 1964. Paper chromatographic separation of glucosamine and galactosamine. Anal. Biochem. 8:393.

76. Murray, R. G. E. 1962. Fine structure and taxonomy of bacteria. Symp. Soc. Gen. Microbiol. 12:119-144.

77. Murray, R. G. E. 1963. On the cell wall structure of Spirillum serpens. Cand. J. Microbiol. 9:381-392.

78. Murray, R. G. E., and A. Birch-Anderson. 1963. Specialized structure in the region of the flagella tuft in Spirillum serpens. Cand. J. Microbiol. 9:393-401.

79. Nikaido, H., and K. Nikaido. 1965. Biosynthesis of cell wall polysaccharide in mutant strains of Salmonella. IV. Synthesis of S-specific side chains. Biochem. Biophys. Res. Comm. 19:332-342.

80. Nowotny, A. 1961. Chemical structure of a phosphomucolipid and its occurrence in some strains of Salmonella. J. Am. Chem. Soc. 83:501-503.

81. Nowotny, A. 1963. Relation of structure to function in bacterial O antigens. II. Fractionation of lipids present in Boivin-type endotoxin of Serratia marcescens. J. Bacteriol. 85:427-435.

82. Nowotny, A. M., S. Thomas, O. S. Duron, and A. Nowotny. 1963. Relation of structure to function in bacterial O antigens. I. Isolation methods. J. Bacteriol. 85: 418-426.

83. Osborn, M. J. 1963. Studies on the gram-negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonate in the lipopolysaccharide of Salmonella typhimurium. Proc. Natl. Acad. Sci. 50:499-506.

84. Osborn, M. J. 1966. Biosynthesis and structure of the core region of the lipopolysaccharide in Salmonella typhimurium. Ann. N. Y. Acad. Sci. 133:375-383.

85. Osborn, M. J., S. M. Rosen, L. Rothfield, L. D. Zeleznick, and B. L. Horecker. 1964. Lipopolysaccharide of the gram-negative cell wall. Biosynthesis of a complex heteropolysaccharide occurs by successive addition of specific sugar residues. Sci. 145:783-789.

86. Pelczar, Hansen, and Konetzka. 1961. Quantitative bacterial physiology laboratory experiments. Burgess Publishing Co., New York. pg. 110.

87. Perry, M. B., and G. A. Adams. 1967. Characterization of 3-deoxy-D-Manno octulosonic acid as a component of the cell wall lipoglycan from Escherichia coli (2101-R). Biochem. Biophys. Res. Comm. 26:417-422.

88. Primosigh, J., H. Pelxer, D. Maass, and D. Weidel. 1961. Chemical characterization of mucopeptides released from the E. coli cell walls by enzymic action. Biochim. Biophys. ACTA. 46:68-80.

89. Pringsheim, E. G. 1949. The relationship between bacteria and Myxophyceae. Bacteriol. Rev. 13:47-98.

90. Pringsheim, E. G. 1951. The Vitreoscillaceae: A family of colorless, gliding, filamentous organisms. J. Gen. Microbiol. 5:124-149.

91. Raff, R. A., and R. W. Wheat. 1966. Occurrence of a 3-amino sugar in the cell wall of Citribacter freundii 8090. Biochim. Biophys. ACTA. 127:271-273.

92. Rapin, A. M. C., and H. Mayer. 1966. Complex polysaccharides in different strains of Escherichia coli K-12. Ann. N. Y. Acad. Sci. 133:425-437.

93. Reichenbach, H. 1965. Rapidosomen bei Myxobacterien. Arch. Mikrobiol. 50:246-255.

94. Ribi, E., K. C. Milner, and T. D. Perrine. 1959. Endotoxic and antigenic fractions from the cell wall of Salmonella enteritidis. Methods for separation and some biologic activities. J. Immunol. 82:75-84.

95. Ribi, E., T. Perrine, R. List, W. Brown, and G. Goode. 1959. Use of pressure cell to prepare cell walls from Mycobacteria. Pro. Soc. Exp. Biol. Med. 100:647-651.

96. Ribi, E., W. T. Haskins, M. Landy, and K. C. Milner. 1961. Preparation and host-reactive properties of endotoxins with low content of nitrogen and lipid. J. Exp. Med. 114:647-663.

97. Ribi, E., W. T. Haskins, M. Landy, and K. C. Milner. 1961. Symposium on bacterial endotoxins. I. Relationship of chemical composition to biological activity. Bacteriol. Rev. 25:427-436.

98. Roberson, B. S., and J. H. Schwab. 1960. Studies on the preparation of bacterial cell walls and criteria of homogeneity. Biochim. Biophys. ACTA. 44:436-444.

99. Robrish, S. A., and A. G. Marr. 1962. Location of enzymes in Azotobacter agiles. J. Bacteriol. 83:158-168.

100. Rothfield, L., and R. W. Horne. 1967. Reassociation of purified lipopolysaccharide and phospholipid of the bacterial cell envelope: Electron microscopic and monolayer studies. J. Bacteriol. 93:1705-1721.

101. Rothfield, L., and M. Takeshita. 1966. The role of phospholipid in the biosynthesis of cell wall lipopolysaccharide in Salmonella typhimurium. Ann. N. Y. Acad. Sci. 133:384-390.

102. Rothfield, L., M. Takeshita, M. Pearlman, and R. W. Horne. 1966. Role of phospholipid in the enzymatic synthesis of the bacterial cell envelope. Fed. Proc. 25:1495-1502.

103. Salton, M. R. J. 1953. Cell structure and the enzymatic lysis of bacteria. J. Gen. Microbiol. 9:512-523.

104. Salton, M. R. J. 1953. Studies on the bacterial cell wall. IV. The composition of the cell walls of some gram-positive and gram-negative bacteria. Biochim. Biophys. ACTA. 10:512-523.

105. Salton, M. R. J. 1956. Bacterial Anatomy: Bacterial cell walls. Soc. Gen. Microbiol. Symp. 6:81-110.

106. Salton, M. R. J. 1958. The lysis of Micro-organism by lysozyme and related enzymes. J. Gen. Microbiol. 18: 481-490.

107. Salton, M. R. J. 1964. The bacterial cell wall. Elsevier Publishing Company, Amsterdam.

108. Salton, M. R. J., and R. W. Horne. 1951. Studies of bacterial cell walls. II. Methods of preparation and some properties of cell walls. Biochim. Biophys. ACTA. 7:177-126.

109. Sutherland, I. W., and J. F. Wilkinson. 1966. The composition of lipopolysaccharides of Klebsiella aerogenes and Aerobacter cloacae. Biochim. Biophys. ACTA. 117: 261-263.

110. Taylor, A., K. W. Knox, and E. Work. 1966. Chemical and biological properties of an extracellular lipopolysaccharide from Escherichia coli grown under lysine-limiting conditions. Biochem. J. 99:53-61.

111. Vincenzi, L. 1887. Hoppe-Seyl. Z., 11:181, taken from Salton, M. R. J. 1964. The bacterial cell wall. Elsevier Publishing Company, Amsterdam.

112. Wardlaw, A. C. 1964. Endotoxin and complement substrate. Taken from Landy, M., and W. Braun. Bacterial endotoxins. Proc. Symp. Inst. Microbiol., Rutgers, the State Univ., Quinn and Boden Pub. Co., Rahway, N. J.

113. Weibull, C. 1953. The isolation of protoplasts from Bacillus megaterium by controlled treatment with lysozyme. J. Bacteriol. 66:688.

114. Weibull, C. 1960. Movement. In I. C. Gunsalus and R. Y. Stanier. The Bacteria, Vol. I. Academic Press, Inc., New York. 153-205.

115. Weibull, C. B., and L. Bergstrom. 1958. The chemical nature of the cytoplasmic membrane and cell wall of Bacillus megatherium, strain M. Biochim. Biophys. ACTA. 30:340-351.

116. Weidel, W. 1961. Mucopolymers of the cell wall of Escherichia coli B. Bull. Soc. Chem. Biol. 62:1833-1835.

117. Weidel, W., H. Frank, and H. H. Martin. 1960. The rigid layer of the cell wall of Escherichia coli, strain B. J. Gen. Microbiol. 22:158-166.

118. Weidel, W. and J. Primosigh. 1958. Biochemical parallels between lysis by virulent phage and lysis by penicillin. J. Gen. Microbiol. 18:513-517.

119. Weiner, I. M., T. Higuchi, M. J. Osborn, and B. L. Horecher. 1966. Biosynthesis of O-antigen in Salmonella typhimurium. Ann. N. Y. Acad. Sci. 133:391-404.

120. Weissbach, A., and J. Hurwitz. 1959. The formation of 2-keto-3-deoxyheptonic acid in extracts of Escherichia coli B. J. Biol. Chem. 234:704-705.

121. Welsch, M. 1958. Lysis by agents of microbial origin. J. Gen. Microbiol. 18:491-497.

122. Westphal, O., and O. Luderitz. 1954. Chemische erforshung von lipopolysacchariden gramnegativer bakterien. Angew. Chem. 66:407-417.

123. White, P. B. 1928. Further notes on spontaneous agglutination of bacteria. J. Pathol. Bacteriol. 31:423-433.

124. White, P. B. 1929. Notes on intestinal bacilli with special reference to smooth and rough races. J. Pathol. Bacteriol. 32:85-94.

125. White, D., and M. Dworkin. 1967. Murein of Myxococcus xanthus. Bacteriol. Proc. 38.

126. Work, E. 1949. Chromatographic investigations of amino acids from micro-organisms. I. The amino acids of Corynebacterium diphtheria. Biochim. Biophys. ACTA. 3: 400-411.

127. Work, E. 1951. The isolation of ae-Diaminopimelic acid from Corynebacterium diphtheria and Mycobacterium tuberculosis. Biochem. J. 49:17-25.

128. Work, E., K. W. Knox, and M. Vesk. 1966. The chemistry and electron microscopy of an extracellular lipopolysaccharide from Escherichia coli. Ann. N. Y. Acad. Sci. 133:438-449.