VIGDAHL, Roger Leon, 1935-
CHEMISTRY AND TOXICITY OF LIPID A FROM VIBRIO FETUS LIPOPOLYSACCHARIDE.

Iowa State University, Ph.D., 1967
Biochemistry

CHEMISTRY AND TOXICITY OF LIPID A FROM *VIBRIO FETUS* LIPOPOLYSACCHARIDE

by

Roger Leon Vigdahl

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Ames, Iowa

1967

TABLE OF CONTENTS

INTRODUCTION

Vibrio fetus is the causative microorganism of Vibriosis, an infection of the reproductive organs of ruminants. This disease is common in cattle and sheep and the Gram-negative organism has been found in humans. Vibriosis often results in infertility and abortion and much recent research has investigated the characteristic pathologic lesions.

Serological tests have been satisfactory in identifying different isolates; however, little is known about the chemistry of this organism's antigenic components. Of the eight formal reports dealing with the isolation of antigens, three have undertaken the study of their gross chemical composition. More data concerning the chemical composition and structure of both cell wall and protoplasmic antigens of Vibrio fetus might be helpful in ascertaining the factor or factors responsible for its virulence.

A preliminary comparison (120) of cell wall antigens by immunodiffusion experiments has revealed the presence of one common antigen from eight strains of Vibrio fetus. Chemical composition studies of this antigen, extracted and purified from three strains of Vibrio fetus, have shown it to be a high molecular weight, complex lipopolysaccharide exhibiting typical endotoxic manifestations in mice: i.e. fever and paralysis followed by eventual death from concentrations of 200-250 micrograms of lipopolysaccharide per mouse (120).

In the literature the term "endotoxin" is often equated with lipopolysaccharide antigens as the principal toxic material of the cell wall in many pathogenic and nonpathogenic bacteria. Many endotoxin preparations may contain protein and nucleic acid, depending upon the method of isolation

and the purification techniques employed. In this thesis, lipopolysaccharide will refer to chemically purified, toxic preparations which are protein and nucleic acid free.

The lipid moiety of lipopolysaccharides was designated lipid A by Westphal (126) to distinguish this firmly-bound lipid from a second lipid, lipid B, loosely bound to the complex.

Lipid A was first implicated in the toxicity of lipopolysaccharides by Lüderitz and Westphal (127) who found about one-tenth the toxicity of the intact complex with purified lipid A preparations. However, controversy exists and debate is frequent over the portion of the lipopolysaccharide complex responsible for its toxic properties. More data on the biological properties as well as the chemistry and structure of lipid A from a wide variety of pathogenic Gram-negative bacteria would help to clarify the relationship between lipid A and the toxic and pyrogenic activities of lipopolysaccharides.

The relationship between lipopolysaccharide, lipid A, and the virulence of Vibrio fetus is unknown. In our previous studies (120), no correlation was found between the virulence of various strains of Vibrio fetus and their respective lipopolysaccharides or lipid A components. Studies conducted on pathogenic and nonpathogenic strains of Brucella abortus (4) have also revealed about the same level of toxicity in lipopolysaccharides extracted from both types.

The study of lipid A from Vibrio fetus lipopolysaccharide was undertaken with the following objectives:

1. Determining the chemical composition of lipid A obtained from purified lipopolysaccharide.

2. Determining the chemical structure consistent with composition.

3. Determining the type of chemical linkage between lipid A and polysaccharide moieties.

4. Determining the relationship of lipid A to the toxic properties of the lipopolysaccharide.

LITERATURE REVIEW

Review of *Vibrio fetus* Antigens

In 1918 Smith and his associates (106) isolated a comma-shaped organism from cases of bovine abortion. It was named *Vibrio fetus* by Smith and Taylor in 1919 (107). Park (84) in 1961 questioned whether the organism should be classified as a *Vibrio* or as a *Spirillum*. He insisted that for inclusion in the *Vibrio* group an organism should form curved, S-shaped, and spiral forms, and some spheroplasts; it should also produce catalase, require some oxygen, and be generally benefited by extra carbon dioxide. On these criteria, *Vibrio fetus* qualifies as a vibrio; but the nonpathogenic vibrios of bovine origin that produce only curved forms, that do not form spheroplasts, that grow anaerobically as well as aerobically, and that are catalase-negative, should, according to Park, be excluded from the genus.

Vibrio fetus is an actively motile, curved, nonspore-forming rod with a single polar flagella. It is Gram-negative and varies from 1.5 to 5.0 microns in length and from 0.2 to 0.3 microns in width (53). It is microaerophilic with optimal growth in an atmosphere containing 6% oxygen (63). For initial isolation, media containing blood or serum are normally used. Once the organism becomes established, cultures can be maintained on other media such as thiol and albimi agar.

Stegena (110) proved in 1949 that the bull is the primary source of infection and that bovine genital vibriosis was a venereal disease. Infection is at first acute and gradually assumes a chronic form.

The antigenic structure of Vibrio fetus is still somewhat obscure. Blakemore and Gledhill (11) in 1946 obtained serological evidence for the existence of type specific O antigens and for considerable overlapping among the H antigens in cultures of Vibrio fetus. They classified freshly isolated catalase-positive strains into one or the other of two antigenic types, either by somatic agglutination or by complement fixation. Type one appears to occur mainly in cattle and type two mainly in sheep.

Various fractionation techniques have been tried in order to separate Vibrio fetus antigenic types. During the last fifteen years, fractionation attempts have been made using phenol-water, saline, and acid solutions as reagents, with varying degrees of success. Gallut (29) attempted to separate antigenic types by chemical fractionation in 1952. Utilizing a 95% phenol extraction he succeeded in separating a carbohydrate and a protein fraction from Vibrio fetus cultures. Immunochemical methods revealed that the polysaccharide fraction corresponds to the type specific O antigen and that the protein fraction was common to nine of the ten Vibrio fetus isolates studied.

Mitcherlich and Liesz (67) in 1957 extracted an antigenic substance with phenol from catalase-positive and catalase-negative Vibrio fetus cells. On the basis of complement fixation titers obtained against Vibrio fetus antiserums, the authors confirmed the earlier work of Gallut -- that the organism possessed two antigenic fractions. Another antigen was postulated as characteristic of the catalase-negative vibrios, as no cross reaction existed between the antigens of catalase-positive vibrios and antiserum prepared against catalase-negative vibrios. In the same study,

a slight immunochemical affinity between type two strains of Vibrio fetus and strains of Brucella abortus and Trichomonas fetus was reported (67).

Vibrio fetus cultures of ovine and bovine origin were extracted with saline and phenol by Robertstad and Morrison (97). Using the Oakley-Fulthorpe modification of the gel precipitin technique, three separate antigen groups were found. Cultures which comprised groups I and II each possessed a single antigen in their saline and phenol fractions and the antigens from groups I and II were unrelated. The isolates in group III possessed multiple antigens which reacted with antiserums prepared against all the cultures. No significant differences were observed between the phenol and saline extracts used as antigens and no antigenic pattern was found associated with the source of isolation.

Antigenic substances have also been isolated from broth cultures of twenty-one Vibrio fetus strains by Nageswararao and Blobel (70). In this study chemical methods were not used to fractionate or extract Vibrio fetus intracellular antigens. Isolation of antigenic substances from the broth culture filtrates was accomplished by acid precipitation and was undertaken to provide data which would be of significant use in the serological diagnosis of vibriosis. However, differences were noted between the antigenic composition of the acid precipitable materials and the respective homologous cell extracts lyzed by ultrasonic oscillation. Immunochemical data suggested that, at least in some vibrio cultures, antigenic composition may be separated into two types: cellular antigens and soluble antigens. Chemical assays of the acid precipitable material and the homologous cell extracts for protein and carbohydrate content gave evidence of a difference

in the protein to carbohydrate ratio, thus indicating, the authors felt, that the acid precipitable material was not cellular components freed by lysis of the vibrios, but represented separate antigenic material.

A heat stable, water soluble substance (termed "HS"), partly polysaccharide in nature, was isolated from smooth Vibrio fetus cells of bovine origin by Ristic and Brandly (96). Its carbohydrates yielded only pentoses on hydrolysis and the fraction contained 14% nitrogen and 6.5% phosphorus. Immunologic reactivity was demonstrated by the production of agglutinating antibody in the rabbit and guinea pig. The "HS" fraction was not, however, examined for homogeneity by chemical or physical criteria.

Winter (132) disrupted Vibrio fetus cells ultrasonically and by means of phenol extraction and analyzed the soluble products. He isolated a toxic fraction which he presumed to be an endotoxin and which was lethal for mice. A small concentration of this material also caused skin reactions in rabbits. A heat stable precipitinogen found in the ultrasonic extracts was isolated but not purified. It was considered to be identical to the polysaccharide portion of the endotoxin based upon immunoprecipitin lines formed in agar gel.

On the basis of agar precipitin and indirect hemagglutination reactions of the ultrasonic extracts, Winter (131) has extended the knowledge of antigenic relationships in Vibrio fetus. Experiments comparing thirty-two strains from widely varying sources have led Winter to conclude that an antigenic relationship exists among all strains of Vibrio fetus. Recently, a purified lipopolysaccharide, representing the antigenically and biologically active portion of the endotoxin, was isolated from a strain

of Vibrio fetus var. intestinalis (133). The lipopolysaccharide had a lethal effect in mice and produced a biphasic febrile response and the generalized Schwartzman reaction in rabbits. Chemical analyses revealed a composition similar to other lipopolysaccharide antigens, except for the absence of pentose sugars and an exceptionally high yield of lipid A (28%). In addition, small amounts of nucleic acid and 2% protein were present in the preparations. Dennis (21) isolated a toxic lipopolysaccharide antigen from Vibrio fetus using the phenol-water extraction method developed in 1954 by Westphal (129). The lipopolysaccharide was antigenic, toxic, and pyrogenic in mice and rabbits. The toxicity was greatly enhanced by a short period of heating. Chemical analysis was confined to absorption spectra to determine nucleic acid contamination and to examination of the sample for the presence of an aldoheptose component which, it was thought, might be an antigenic determinant of the molecule.

We have recently conducted studies on the lipopolysaccharide antigen isolated from three strains of Vibrio fetus with 50% phenol extraction (120). Chemical composition analyses showed the antigens to contain pentose, hexose, and heptose sugars, the amino sugar glucosamine, five long chain fatty acids, four amino acids, and several unidentified components.

Studies conducted on the endotoxic properties of the three lipopolysaccharides demonstrated they were toxic in small doses in mice (200-250 µg). The polysaccharide and lipid A moieties were not tested extensively because limited amounts were available, but both were nontoxic at the 200-250 µg/mouse level in the single determinations that were conducted.

This survey of the literature suggests that there need to be more quantitative chemical studies performed on Vibrio fetus antigens. To date, investigation has been limited primarily to serologic studies aimed at elucidation of the overall antigenic pattern of the organism and to development of in vivo diagnostic tests. With the exception of the endotoxin isolated by Dennis (21) and by Winter (133), no attempt has been made to purify and chemically analyze Vibrio fetus lipopolysaccharide antigen. This study has been undertaken with chemical purification and analysis of the lipid A moiety as one of the prime objectives.

Review of Lipopolysaccharide Antigens

Andre Boivin (14) first extracted endotoxins from bacteria over three decades ago. He found a large molecular complex composed of protein, lipid and polysaccharide fractions. Endotoxins have continued to intrigue the chemist since this time. The molecular basis for their biological action seems always on the verge of discovery but somehow just eludes detection. Furthermore, arguments over artifact versus the real thing and fractionation versus contamination are so frequent and so vigorous when analytic methodology is discussed that they seem to be achieving a kind of cyclic immortality.

Lipopolysaccharides possess a range of biological activity (117, 10). They can affect structure and function of numerous organs and cells; change tissue and blood levels of many enzymes; modify carbohydrate, fat and protein metabolism; raise or lower body temperature; increase or decrease resistance to bacterial and viral infections and other noxious stimuli

(including themselves); cause hemorrhage and increase coagulation of blood; modify hemodynamics in every accessible anatomical site; cause or prevent shock; modify gastric secretion; destroy tumors; and disrupt the activity of several endocrine glands. They have been suggested repeatedly in the literature as a possible key to understanding the peculiar and distinguishing features of the diseases produced by Gram-negative bacteria.

Because of this wide range of biological activities, the lipopolysaccharides are of extraordinary interest; at the same time, when the ultimate aim is the correlation of chemical structure with biological function, such multiple effects inevitably increase the difficulty of characterizing them. The methods employed to extract and purify the complexes also affect, to a certain extent, their chemical composition. Degradative procedures have been developed, however, which allow separation of the complexes into lipid, carbohydrate, and (often) peptide moieties.

Lipopolysaccharides have been prepared from Gram-negative bacteria by a variety of methods (27). The best known are those involving extraction of bacilli with trichloroacetic acid, ethylene glycol, phenol-water, ether-water (125), and most recently with dimethyl sulfoxide (1). All of these procedures extract complexes of protein, lipid and polysaccharide but in varying proportions dependent upon the type of reagent used. They all evoke the spectrum of characteristic endotoxin reactions mentioned previously, but differ widely as regards their potency on a weight basis, and particularly as regards their capacity to produce lethal effects (27).

An early method of preparation of the lipopolysaccharide was that of Raistrick and Topley (88). In this procedure, a suspension of bacteria was subjected to autolysis in the presence of toluene. Trypsin was added to the autolysate and the products of digestion were fractionated with alcohol to yield a lipopolysaccharide.

A milder procedure was developed by Morgan (69) to prepare the bacterial complexes. Repeated extractions of bacterial cells with diethylene glycol yielded strongly antigenic material from smooth strains of Shigella dysenteriae. The product was obtained in yields of six to seven percent of the original dry weight of bacteria. Morgan described this material as forty-eight percent specific polysaccharide, containing bound protein and lipid. Jesaitis (45) reported, however, that the efficacy of diethylene glycol as an extracting solvent depended on the bacterial source and that specific yields of the lipopolysaccharide complex from Shigella flexneri were very low. When a fifty percent aqueous pyridine solvent was substituted, better yields were obtained, although the product was more contaminated with nucleic acids and inert polysaccharides. Jesaitis characterized the material prepared with this solvent as a lipopolysaccharide-protein complex.

Westphal et al. (127) developed procedures using aqueous phenol to prepare lipopolysaccharide complexes which yield somewhat different products, depending on the temperature at which the extraction is carried out. When Escherichia coli organisms were extracted in the cold with forty-five percent aqueous phenol, a preparation which Westphal characterized as an active glycoprotein was obtained in the aqueous phase; when the same

extraction was performed at 65°C, carbohydrate and nucleic acids were found in the aqueous phase, with protein remaining in the phenol phase. In either case, phospholipid was removed from the complex. A lipid component, which Westphal termed lipid A, remained attached to the polysaccharide (128); the phospholipid which was removed from the complex was loosely bound, biologically inert lipid, termed lipid B, often associated with complexes obtained using milder extraction methods.

In 1958 Tauber and Russell (115) modified Westphal's procedure slightly using a twenty percent acetone solution of the aqueous phase as the starting material for purification procedures. For the present study, lipopolysaccharides have been prepared from Vibrio fetus by this method and by the dimethyl sulfoxide procedure of Adams (1).

Purified lipopolysaccharide preparations exhibit high toxic activity as water-soluble colloids. By short-acid hydrolysis, endotoxic lipopolysaccharides may be dissociated into polysaccharide, lipid A and free fatty acids (128, 81). The lipid-free polysaccharide component of endotoxins constitutes the O antigen of each bacterial species. A large number of polysaccharides have been analyzed (46) and the specific polysaccharides of one serogroup were always found to be built up on the same series of sugar constituents. These highly branched polysaccharides are sometimes composed of as many as six different monosaccharides: hexosamine, heptoses, hexoses, pentoses, 6-deoxyhexoses, and 3,6-dideoxyhexoses (46, 129).

The idea that the polysaccharide portion is composed of two parts, core and side chain, was first proposed by Westphal and his co-workers (129) as a result of extensive qualitative analyses of lipopolysaccharides from

a number of Salmonella strains, including rough mutants. It was found that, although the sugar composition varies greatly from species to species, all the strains examined contained at least glucose, galactose, heptose and glucosamine. The glucosamine, however, was thought to be present in the lipid rather than the polysaccharide portion. Also, they found that the lipopolysaccharide of rough mutants is reduced in composition, in the sense that it contains only the aforementioned four sugars, however complex the composition of smooth polysaccharides may be. On the basis of these facts, Westphal and his co-workers suggested that the polysaccharides of rough mutants might represent the central core portion of the more complex polysaccharide from the wild-type smooth strains, and the structure of the core might be the same in all Salmonella lipopolysaccharides. They also suggested that rough mutants might have lost the ability to make the side chains or to transfer them to the core structure, and thus only the core structure remains attached to lipid A.

Since Westphal's initial work, other investigators have continued to elucidate both core and side chain structure and biosynthesis. Horecker (37), working with rough mutant strains of Salmonella and using radioactive labeling techniques, found evidence for the structure of core polysaccharide as a polysphosphoheptose chain to which are attached alternate glucose and galactose residues. Lüderitz (59) reported observations favoring the assumption that O-specific polysaccharides from Salmonella minnesota and Salmonella ruiru contain a common basal core to which side chains are attached. He proved the side chains are composed of repeating oligosaccharide units which carry the serological O-specificities. Mutants

defective in the synthesis of side chains contained the basal lipopolysaccharide, and mutants with a deeper defect synthesized incomplete basal lipopolysaccharides.

In some bacteria the role of 3,6-dideoxyhexoses as immunologically determinant end groups has been confirmed by the synthesis of artificial antigens (60). Colitose (3-deoxy-L-fucose), the determinant terminal sugar in the endotoxin antigen of Escherichia coli strain 0111, was chemically coupled to an inert protein carrier (serum albumin). Injection of the artificial colitose-protein complex led to the production of antibodies which not only reacted with the artificial colitose antigen, but also cross reacted with Escherichia coli 0111 and serologically related strains.

A complete survey of the published findings concerning lipopolysaccharide complexes was not attempted here, particularly in regard to their biological effects, since it is beyond the scope of this investigation. Among those who have reviewed the numerous studies in this area are Westphal (124, 126), Lederer (56), and Westphal and Lüderitz (128). Also available is a recent monograph on the molecular biology of lipopolysaccharides (78) and a book on the proceedings of a 1965 symposium on bacterial endotoxins (54).

Review of Lipid A

The lipid A component is prepared from the chloroform extract of the hydrolyzed lipopolysaccharide. The amount of lipid A present depends on the source of the complex: Westphal's summary data for several Gram-negative

organisms show that lipid A forms between ten and twenty-six percent of the lipopolysaccharide complex.

The chemistry of lipid A is incompletely known, but its main constituents are D-glucosamine phosphoric acid ester and long-chain fatty acids, including a large proportion of hydroxy fatty acids (126, 81, 102).

Ikawa et al. (38) have investigated a lipopolysaccharide complex from Escherichia coli which caused hemorrhage of tumor tissue. This complex contained nearly twenty-five percent bound lipid. A number of components in the lipid were identified: 2-hydroxymyristic, lauric, myristic and palmitic acids (39); glucosamine, ethanolamine, and aspartic acid (40); and a long-chain diamine base, the structure of which was established as 4,5-diamino-N-eicosane (40), and which was named necrosamine. Necrosamine has not been found in the lipid A components of other bacterial lipopolysaccharides.

Purified lipid A prepared from Escherichia coli, strain 08, by Westphal et al. (130) contained nearly nineteen percent hexosamine, nitrogen and phosphorous in a ratio of approximately three to one, and long-chain fatty acids. The preparation was obtained in only 0.5% yield, based on the dry weight of the cells, indicating one major problem encountered in the study of lipid A: obtaining a sufficient quantity of purified material for chemical and biological studies.

Burton and Carter (17) purified and characterized the lipid A component of Escherichia coli, strain B4 0111, in 1964. Their analytical data are consistent with a structure containing two glucosamine molecules, one phosphate, three to four acetyl groups, and five long-chain fatty acids.

From these data they have tentatively proposed a structure for lipid A in which the basic unit consists of two glycosidically linked, fully acylated glucosamine molecules to which the fatty acids are held in ester linkages.

Previously, Nowotny (77) investigated the structure of purified lipid A from several Salmonella strains. The basic structure was identified as a poly-D-glucosamine-phosphate chain in which the glucosamine is esterified with fatty acids. Nowotny also found evidence for small amounts of amino acids and thus proposed that a peptide component attached to the poly-D-glucosamine might serve as a linkage between the lipid and polysaccharide moieties.

Evidence is accumulating, however, that 2-keto-3-deoxyoctonoate (KDO) may function as the linking group between lipid and polysaccharide. This keto acid was first discovered by Heath (36) as a component of Escherichia coli lipopolysaccharide. Osborn (83) presented data suggesting that the polysaccharide itself consists of heptose phosphate chains terminating in KDO which is at the reducing end of the chains and which forms the linkage between the polysaccharide chains and lipid A. Horecker (37) conducted studies on the structure of the polysaccharide in Salmonella typhimurium lipopolysaccharide and has found similar evidence for the function of KDO. His hypothesis suggests the reducing end is linked to the lipid through either the hydroxyl groups of glucosamine or β-hydroxymyristic acid.

Identification of the products obtained by mild hydrochloric acid hydrolysis of Salmonella lipid A preparations revealed equimolar amounts of 6-phospho-D-glucosamine, 4-phospho-D-glucosamine, and D-glucosamine (77). From these data Nowotny postulates a tentative structure in which the

D-glucosamine units are linked through phosphodiester bridges instead of glycosidically.

In previous studies with thin-layer chromatography on silica gel, Kasai (48) revealed the presence of at least eight to ten components in lipid A preparations. Relative amounts of these component lipids varied with bacterial species, cultural conditions, and method of isolation. The major difference noted at this time was the quantitative relationship of fatty acids bound to each component.

Recently, Kasai (48) has extended this work in a comparison of the chemical and biological properties of thin-layer chromatography components isolated and purified from Escherichia coli and several strains of Bordetella. Definite chemical qualitative differences were found between the lipid A components of these two bacterial species and differences in contents, and compositions were obtained from the same culture of Escherichia coli. Investigations of the biological activities of these lipid components suggested that the quantitative and qualitative differences have a marked influence on the biological activities of their respective lipopolysaccharides. In general, higher toxicity and Schwartzman activity was obtained from lipopolysaccharides rich in the lipid A components with the highest R_f values on thin-layer chromatography. Also, the multifunctional activities of the lipopolysaccharides were lost when the lipid moiety was dissociated from the complex and strong toxic or Schwartzman activity could not be reproduced by either component alone. Kasai concludes that the structure of lipid A must have some influence on these activities through formation of a macromolecular complex with the polysaccharide.

Review of the Toxicity and Virulence of Endotoxins and Their Chemical Derivatives

Controversy exists and debate has been frequent and partisan over the portion of the endotoxin complex which is responsible for its toxic properties. The lipid, polysaccharide, and protein materials associated with these complexes have all been suggested as responsible for toxicity. While evidence is slowly accumulating indicating that lipid A is intimately involved, it is still an unsettled question.

As early as 1955, Landy et al. (55) found that it was possible to remove virtually all protein from the endotoxin complex without reducing its biologic potency. This finding was reaffirmed by Ribi (95) and it is now generally accepted that the protein moiety does not function in toxicity. However, Jenkin and Rowley (44) have isolated a toxic protein from Inaba and Ogawa strains of Vibrio cholera which accounted for the major portion of toxicity of the whole organism. Immunological and chemical data suggest that this is the toxic protein derived from the endotoxin complex. The lipopolysaccharides from these organisms were relatively nontoxic when compared with lipopolysaccharides from other Gram-negative bacteria, although they were similar in gross chemical composition, i.e. containing pentose, hexose and heptose sugars, hexosamine and 20% lipid.

Haskins et al. (34) have reported extensive alterations in lipopolysaccharide from Salmonella enteritidis which undergoes treatment with 0.1 N acetic acid, the method of isolating lipid A. A striking reduction in the toxicity occurred within minutes after exposure and continued progressively

until, after 60-90 minutes, virtually all of the original activity had been abolished. On the basis of this evidence, toxicity was attributed to large complexes, while smaller units were found to be inactive. Ribi et al. (94) also suggest that reactivity might be dependent upon macromolecular properties; that is, a complex of critically large size is essential and the destructive effect of acid represents a simple depolymerization. An alternate possibility suggested by Oroszlan and Mora (82) was that the major toxic properties were attributable to some small moiety whose attachment to a macromolecular carrier is essential for activity.

In an attempt to evaluate this hypothesis, Beer et al. (8) have recently conducted a study of particle sizes and shapes in relationship to lipopolysaccharide toxicity. Their lipopolysaccharide preparations from Escherichia coli were fractionated by means of sucrose-density gradient centrifugation followed by dialysis against weak alkali solutions to dissociate the large polydisperse aggregates into smaller particles. This dissociation was found to be accompanied by a marked increase in toxicity and the isolation of slender, pilli-like rods (8) which gave the highest, if not all lethal activity. The authors speculate that these rods may be polymerized from different, less toxic subunits or, as Ribi suggested (94), may constitute only an efficient carrier for a hypothetical low molecular weight toxin.

It is conceivable from these data, and that of Nowotny et al. (80) showing marked heterogenity of purified lipopolysaccharide preparations, that only a few among many kinds of lipopolysaccharide-protein particles are toxic. In that event, the study of the chemical composition of

lipopolysaccharides by classical chemical methods may not yield much information about the toxic particle.

Nowotny (79) recently published a critical evaluation of the need for a large size complex to express toxicity and presented preliminary results of his own group (76), indicating that long chain carboxylic acids esterify to hydroxyl groups of carbohydrate in the lipopolysaccharides, and that these O-acyl groups are involved in the toxic effects.

Continuing these studies, Tripodi and Nowotny (119) examined the kinetics of alkaline hydrolysis and its effects on lipopolysaccharides in order to determine the smallest building block which would still retain full potency. Alkaline degradation of the average particle size from nine million to three million occurred during the first hour of hydrolysis, with no affect on the toxic property of the lipopolysaccharide preparation. In three to six hours, when detoxification became evident, only an eight percent decrease in the light scattering ratio of the particles occurred resulting in nontoxic particle of about one million molecular weight. Also, the particle size determinations of other treated lipopolysaccharide preparations showed no direct correlation between size and lethal potency. Fatty acids, however, could be shown to be split from the preparation during alkaline detoxification, a result which correlates with Nowotny's previous data on the essential nature of O-acyalated long-chain aliphatic compounds (76).

These experiments indicate that while no significant changes in particle mass occur during alkaline detoxification of lipopolysaccharides (119), large dissymetry alterations became apparent and these can be interpreted

as reflecting conformational changes in the endotoxic particles during detoxification. This suggests that a specific steric arrangement of functional groups is required for lethality, rather than a critical particle size.

A variety of techniques have been used to chemically modify lipopolysaccharides in an attempt to isolate particular linkages essential in expressing toxicity. Among the earliest attempts was the work of Neter et al. (73) who investigated the effects of heat, sodium hydroxide and periodate oxidation upon toxicity, pyrogenicity and the ability of enterobacterial lipopolysaccharides to modify erythrocytes so they would adsorb homologous antibody. Treatment with heat or mild sodium hydroxide for short periods of time was found to increase their capacity to modify erythrocytes without altering their antigenicity. A marked loss in toxicity and pyrogenicity was noted with both treatments. In contrast, the toxicity and pyrogenicity of the periodate-treated preparation remained in tact while antigenicity was markedly altered. The polysaccharide moiety was shown to be disrupted by periodate treatment, with a C-C cleavage of every fourth sugar residue resulting in two titratable aldehyde groups per cleavage (73).

Freedman (26) and Sultzer and Freedman (113) have described the acetylation of lipopolysaccharides and the resultant dissociation of in vivo biological effects. Acetylation resulted in loss of original lethal potency for mice and rabbits, pyrogenicity in rabbits, and in induction of tolerance in both species. The detoxification found after acetylation was completely reversible by mild saponification, indicating introduction

of O-acetyl groups had occurred. Thus, both acetylation (26) and deacylation (76) of native endotoxin preparations have resulted in their detoxification.

Treatment of Serratia marcescens TCA extracted lipopolysaccharide with boron trifluoride, potassium methylate and pyridinium formate reduced the toxicity, pyrogenicity and Schwartzman activity in mice and rabbits (46). Antigenicity and hypotensive action on the other hand appeared to be only slightly diminished. The nature of the linkage disrupted by these reagents is unknown. Investigations utilizing well defined chemical substances as models possessing some of the linkages known to be present in lipopolysaccharides are being undertaken by Nowotny (79). Preliminary data have revealed that the removal of short O-acyl linkages is a characteristic common to all three reagents.

Noll and Braude (74) investigated the effects of chemical reduction on the toxicity of Escherichia coli lipopolysaccharide extracted with TCA. They prepared nontoxic and nonpyrogenic lipopolysaccharide of high immunogenic potency by reduction with lithium aluminum hydride. This treatment reductively cleaves fatty acid ester bonds and thus affects lipid A directly without concomitant damage to the polysaccharide chain. By observation of the infrared spectra of the reduced product, the authors reported no extensive fragmentation occurs during reduction and conclude that a single molecular configuration is responsible for the toxic manifestations of lipopolysaccharide.

Fukushi et al. (28) have sharply criticized these experiments on the basis that infrared spectra are not an adequate method of assaying changes

in linkages with materials as large and complex as lipopolysaccharides. In attempting to duplicate exactly the work of Noll and Braude (74) using Escherichia coli extracted with TCA, they achieved no success in preparing the lithium aluminum hydride reduction product. They obtained instead a ten percent yield of a pure unphosphorylated polysaccharide which was neither antigenic, pyrogenic or toxic. Also, they report no correlation between fatty acid ester content, as estimated from infrared spectra, and toxic properties of the lipopolysaccharide.

Kim and Watson (51) have conducted a very interesting experiment in which they inactivated the lethal and pyrogenic activities of lipopolysaccharides by treatment with the enzyme papain. Papain is known to split peptide, ester and amide linkages, a fact which may suggest the primary toxicity is closely associated with N-acyl or O-acyl groups of lipid A.

Westphal et al. (127) believe lipid A is involved in toxicity. They have prepared an artificial lipoprotein of which only lipid A was of bacterial origin; this synthetic molecule was pyrogenic and toxic whereas the polysaccharides derived from the lipopolysaccharide were inactive. Ribi and Landy (93) have challenged Westphal's data on the basis that his lipoprotein preparations were not entirely free of polysaccharide and thus may have contained residual amounts of lipopolysaccharide. They have presented evidence (94), indicating toxicity of lipopolysaccharides can be attributed to the polysaccharide moiety. Their lipopolysaccharides, prepared from Salmonella strains by aqueous-ether extraction, produced material containing a very reduced content of lipid A without impairment of toxic or antigenic activity. Furthermore, parallel bioassays of lipid A preparations

and the lipopolysaccharides from which they had been derived showed that the lipids possessed less than 1% of the potency of intact lipopolysaccharide.

Nakano (71) has supported the conclusion of Westphal et al. (127) that the polysaccharide moiety is not directly related to toxicity, by testing lipopolysaccharides derived from mutants of Salmonella typhimurium defective in uridine diphospho-galactose-4-epimerase. These mutants contain only glucose in their cell wall and are entirely lacking in galactose, rhamnose, mannose and tyvelose, all of which are regarded as the usual constituents of cell wall from wild type cells. Lipopolysaccharide from these epimer-less mutants showed the same degree of toxicity as the wild type despite the lack of these sugars.

In direct contrast to Nakano's (71) results, Kessel et al. (50) reported Salmonella typhimurium mutants, with known differences in the composition of their cell wall polysaccharides, whose lethality for mice proved to be correlated with the complexity of the polysaccharide. For example, no toxic effects were encountered with lipopolysaccharide from heptose deficient mutants and relatively low toxicity characterized preparations from mutants that contained only a heptose-phosphate backbone or from mutants with heptose-phosphate backbone plus glucose. The authors have suggested, in view of these results, that susceptible cells may differ as a function of the polysaccharide constitution of the lipopolysaccharide used and that lipid A does not function as the primary determinant in toxic reactions.

It is evident from this review that while the chemical basis of toxicity in lipopolysaccharides has not been fully elucidated, progress toward this

goal has been achieved. Investigations aimed at correlating toxicity or content of lipopolysaccharides with the virulence of their respective bacteria have not been as successful.

Although several attempts have been made to correlate the virulence of Gram-negative bacteria with their lipopolysaccharide content or composition, no direct relationship has been discovered. Studies have been conducted on Brucella abortus (4, 109), gonococci and meningococci organisms (105, 2, 16), and Salmonella pullorum (18) comparing lipopolysaccharides extracted from virulent and avirulent strains of these organisms. Very slight differences were found in the toxicity of equal amounts of lipopolysaccharide regardless of the virulence of the strain. Baker et al. (4) have also indicated that nutritional and metabolic differences between strains of brucellae of high and low virulence directly affect their ability to multiply intracellularly and consequently are related to virulence.

In our own laboratory, previous studies (120) conducted with three strains of Vibrio fetus differing markedly in virulence for cattle showed no differences in the toxicity of their respective lipopolysaccharides. There have been cases reported, however, in which lipopolysaccharide has induced abortions: Escherichia coli lipopolysaccharide in doses as low as 5 micrograms was shown to produce abortion in 85 percent of pregnant mice (92). The aborting effect of lipopolysaccharide probably does not occur simply as a result of making the mother quite ill. Zahl and Bjerknes (134) were able to abort pregnant mice without any associated mortality. In addition, Thomas (116, 117) has shown that the lethal and abortive effects of lipopolysaccharide can be dissociated by using cortisone which protects against toxicity but not against the abortive reaction.

Despite the fact that no direct relationship has, as yet, been found between toxicity and virulence, it is possible that the disease state associated with Vibriosis leading to abortion and death of the fetus could be caused by release of endotoxin during the acute stage of infection. The physiological mechanisms involved in endotoxic-induced abortions in mice are currently under study by Kass (49) who has also suggested that endotoxin complexes might be one cause of premature births and abortion in human beings (49).

EXPERIMENTAL METHODS

The variety of procedures presented here represent experiments incident to the achievement of the objectives of this work. This section is divided into six parts. Part I is concerned with the general methods used in this study. Part II pertains to the isolation and purification of lipopolysaccharide from Vibrio fetus. Part III deals with the isolation and purification of lipid A from the purified lipopolysaccharide and parts IV and V deal with the chemical and structural studies conducted on purified lipid A. Part VI concludes this section with the experimental procedures used in evaluating the toxicity of lipid A and lipopolysaccharide preparations.

An outline of the general experimental pattern of this study appears below. Detailed discussion of the techniques follows the outline.

I. General Methods

- A. Paper chromatography
- B. Infrared spectrophotometry
- C. Melting point determinations
- D. Thin-layer chromatography

II. Isolation and Purification of Lipopolysaccharide

- A. Growth, harvest and storage of Vibrio fetus cells
- B. Phenol-water extraction
- C. Acetone-NaCl precipitation
- D. Differential centrifugation
- E. Chemical analysis of fractions obtained by purification

III. Isolation and Purification of Lipid A

- A. Acid hydrolysis of lipopolysaccharide with 0.1 N formic acid
- B. Chloroform extraction of lipid A from the hydrolysate
- C. Acetone extraction of chloroform soluble crude lipid A
- D. Column chromatography of crude lipid A on organic Sephadex and silicic acid

E. Qualitative examination of the fractions from silicic acid chromatography

1. IR spectrophotometry
2. Paper chromatography of acid hydrolysates
3. Thin-layer chromatography

IV. Chemical Studies on Lipid A

A. Analytical data

1. Solubility and melting point
2. Nitrogen and phosphorous content
3. Total fatty acid content
4. Qualitative and quantitative hexosamine analysis
5. High voltage electrophoresis (fingerprinting technique)
6. Analysis for fatty acid ester and amide
7. Test for reducing sugar

B. Analyses of fatty acid components

1. Preparation of methyl esters
2. Isolation of hydroxy fatty acid esters
3. TLC of fatty acid esters
4. Vapor phase chromatography of fatty acid esters
5. Qualitative vinyl ether determination
6. Iodination and bromination

V. Qualitative Structural Studies on Lipid A

A. Evaluation of the linkage between lipid and polysaccharide moieties

1. Evaluation of a possible peptide linkage by mild alkaline degradation
2. Analysis for 2-keto-3-deoxyoctonoate (KDO)

B. Determination of the linkage between glucosamine residues

1. Alkaline degradation studies
2. Sodium borohydride reduction studies

VI. Toxicity Studies of Lipopolysaccharide and Lipid A

A. Assay method for toxicity
B. Preparation of chemically altered lipopolysaccharide

1. Papain-treated lipopolysaccharide

2. Periodate-treated lipopolysaccharide
3. Sodium hydroxide-treated lipopolysaccharide

C. Preparation of an artificial lipoprotein

General Methods

I. Paper chromatography

A. Chromatographic spray reagents

1. Ninhydrin (12) - Ninhydrin spray reagent containing 0.3% ninhydrin in ethanol[1] was used for all determinations. The spots were usually developed without heating.

2. Periodate (12) -

Solution 1: 2% sodium metaperiodate.

Solution 2: 1% potassium permanganate in 2% sodium carbonate.

Four volumes of solution 1 were mixed with one volume of solution 2 immediately before use. Each chromatogram was allowed to develop for 10 to 15 minutes, then washed with tap water and air dried. The spots appeared as tan spots against a white background.

3. Acid aniline phthalate (12) - To 100 ml. of water-saturated n-butanol, 1.66 g. phthalic acid and 0.93 g. aniline were added. This solution was used to spray the chromatograms which were then air dried and heated at 110°C for 5 minutes. The spots which appeared are reddish to light brown in color.

---

[1]Sigma Chemical Company, St. Louis, Mo.

4. Hanes-Isherwood spray (12) -

Perchloric acid (60%)	5 ml.
Hydrochloric acid (1 N)	10 ml.
Ammonium molybdate (4%)	25 ml.
Water	60 ml.

The thoroughly dried chromatogram was sprayed with this solution using an all glass sprayer. The chromatogram was air dried, then exposed to ultraviolet light. Organic phosphates gave blue spots, while inorganic phosphate gave a light green coloration.

5. Elson-Morgan reagents (85) -

a. Acetylacetone reagent

Solution 1: 0.5 ml. of acetylacetone dissolved in 50 ml. of n-butanol.

Solution 2: 50% (w/v) aqueous KOH (5 ml.) in 20 ml. absolute ethanol. Solution 2 (0.5 ml.) was added to solution 1 (10 ml.) just before using. The reagent was not stable.

b. p-Dimethylaminobenzaldehyde reagent

One gram of p-dimethylaminobenzaldehyde was dissolved in 30 ml. of absolute ethanol and 30 ml. of concentrated HCl. This stock solution was diluted 1:3 (v/v) with n-butanol just before using.

The dried chromatogram was sprayed with reagent 1, air dried, and heated for 5 minutes at 105°C. The chromatogram was then sprayed with reagent 2, air dried, and returned to the oven for 1 to 3 minutes. Under these conditions, free hexosamines gave a cherry red color, while N-acetylated hexosamines gave a strong purple color.

B. Development of chromatograms For most chromatography, Whatman No. 1 filter paper sheets were used. When specified, borate impregnated paper, as prepared by the following procedure, was substituted: To 12 g. sodium tetraborate and 7 ml. 6 N HCl, add 200 ml. distilled water. Dip sheets of Whatman No. 1 filter paper in the solution, blot the sheets to remove excess solution, and air dry.

II. Infrared spectroscopy

Infrared spectra were obtained using a Beckman IR9 double beam spectrophotometer. Samples were prepared in spectrophotometric grade chloroform.[1]

III. Melting point determinations

The melting point apparatus[2] employed consisted of a polarizing microscope equipped with a glass covered, electrically heated stage and a thermometer calibrated in degrees Centigrade. The melting point of a lipid sample was considered to be the point at which a clear melt of the substance was rapidly produced.

IV. Thin-layer chromatography

Samples of crude lipid A, fractions of lipid A from column chromatography, and preparations of fatty acid esters from lipid A were analyzed

---

[1]Spectrar chloroform, Mallinckrodt Chemical Works, St. Louis, Mo.

[2]Fisher-Johns melting point apparatus, Fisher Scientific Company, Pittsburgh, Penn.

by thin-layer chromatography. Silica gel HR[1] and MN-Kiselgel G-HR[2] were used as adsorbants; thin layers (0.5 mm. thick) were prepared with 0.02 N sodium acetate adjusted to pH 8.0 and used without activation.

Solvent systems most commonly employed included the following:

1. Chloroform:methanol:water, 65:25:4 (v/v)
2. Methylene chloride:methanol:water, 7:3:0.4 (v/v)
3. Trichloroethylene:methanol:water, 7:3:0.4 (v/v)
4. Hexane:ethyl ether, 85:15 (v/v) - fatty acid esters

The chromatographs were developed at 20 to 25°C for 30 to 45 minutes and the spots detected with Rhodamine B (7), a general lipid reagent; 2,6-dichloroquinone-chlorimide-borax (86), a phosphate ester spray; bromothymol blue in dilute NaOH, for fatty acid esters; and by heating for 30 minutes after spraying with equal volumes of 20% ammonium sulfate and 20% ammonium hydrogen sulfate (135). Unless otherwise described, 10 to 20 µg./5 µl. of the chloroform solutions of each lipid fraction were applied on to the silica gel or Kiselgel layer.

Preparation and Purification of Lipopolysaccharide

I. Source of bacteria

The bacteria used in this study were strain 925 of *Vibrio fetus* furnished by J. H. Bryner[3] from stock cultures maintained at the National Animal Disease Laboratory.

---

[1]Silica gel HR, Brinkman Instruments, Inc., Westbury, L.I., New York.

[2]MN-Kiselgel G-HR, Brinkman Instruments, Inc., Westbury, L.I., New York.

[3]J. H. Bryner, U. S. Department of Agriculture, National Animal Disease Laboratory, Ames, Iowa.

Approximately one-half of the cells were grown in Manclark and Pickett's (63) liquid culture media in 5 liter quantities using the New Brunswick Fermentor.[1] The cell crops, harvested at maximum growth as determined by turbidity readings, were centrifuged at 5,000 xg for 20 minutes to sediment the cells. After the supernatant fluid was poured off, the cells were washed twice with 0.85% (w/v) sodium chloride (physiological saline), centrifuged at 5,000 xg, and frozen until use.

The remainder of the cells used in this study were grown by Fort Dodge Laboratories, Inc. from the same strain and using the same media. They were shipped to us in the frozen state; cells from each shipment of bacteria were subjected to the Gram stain (31), and examined under the oil immersion microscope and with the electron microscope. In each case, Gram-negative Vibrio were the only cells observed. Considerable breakage of the cells was also observed, probably caused by freezing and thawing.

II. Isolation of lipopolysaccharide

Vibrio fetus cells were extracted by the phenol-water extraction method, as first described by Westphal (128) and as modified by Tauber and Russell (115). The procedure is summarized in Figure 1. To each volume of cells, washed twice with three volumes of 0.85% sodium chloride, were added two volumes of redistilled phenol and one volume of distilled water. The mixture was blended for eight minutes and cooled to 20°C. After centrifugation for 10 minutes at 3,000 xg the mixture separated into two phases

---

[1]New Brunswick Fermentor Model FS-307, New Brunswick Scientific Company, Inc., New Brunswick, New Jersey.

and a fluffy, white interface; the clear upper phase and interface were siphoned off and the lower phase, containing proteins and peptide material, was extracted with distilled water and centrifuged again. The upper phases and interface material were combined and centrifuged again for 20 minutes at 3,000 xg. The combined supernatants were dialyzed against tap water for two to three days and against distilled water for one day.

To isolate lipopolysaccharide, sodium chloride (1 mg./1 ml.) and two volumes of cold acetone were added to the dialyzed supernatant. The precipitate was removed by low speed centrifugation, dissolved in distilled water, and centrifuged at 3,800 xg. The sediment was discarded and 0.2 volumes of cold acetone were added to the opalescent supernatant. The mixture was centrifuged for one hour in a Servall type SS1 centrifuge[1] at 35,000 xg. The supernatant, presumed to contain mainly nucleic acids, was discarded. The sediment was dissolved in distilled water and centrifuged again for 1 hour at 35,000 xg. The resultant sediment was dissolved in distilled water and lyophilized. This material, similar to that isolated by Tauber and Russell (115), was presumed to be the crude lipopolysaccharide. Portions of the crude lipopolysaccharide were removed and saved for initial biological assays. Some purification was achieved by adding distilled water at 90°C to the crude material in small portions until a final concentration of 2 mg./ml. was obtained. The lipopolysaccharide was again centrifuged for 1 hour at 35,000 xg, and the sediment was dissolved in distilled water and lyophilized. A white, fluffy,

---

[1]Servall Type SS1 Centrifuge, Ivan Sorvall, Inc., Norwalk, Connecticut.

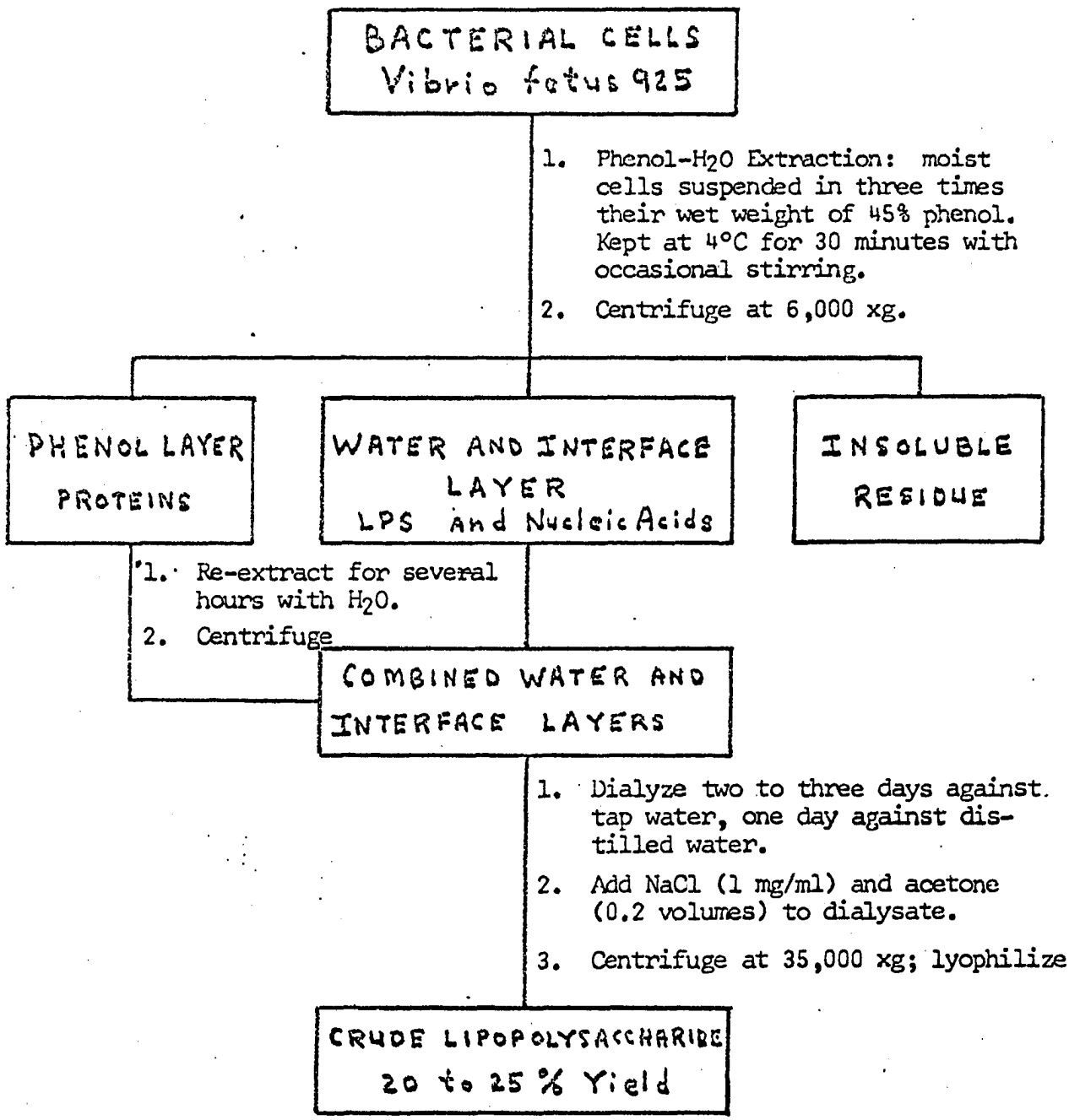


Figure 1. Preparation of crude lipopolysaccharide.

hygroscopic powder was obtained. It resembled the product obtained by Tauber and Russell (115) from Escherichia coli using this procedure and was presumed to be crude lipopolysaccharide, based upon analyses for nitrogen, phosphorous, total carbohydrate and lipid (Table I).

III. Routine purification of crude lipopolysaccharide

A combination of low speed centrifugation and high speed centrifugation from 1 N NaCl solution has been found to give a lipopolysaccharide fraction which is free of nucleic acids and peptides (fraction IV), a fraction of high nucleic acid content (fraction II), and two peptide fractions (fractions I and III). This procedure is outlined in Figure 2. Analytical data for each of the fractions prepared in a typical run are given in Table I; ultraviolet absorption spectra for the different fractions are given in graphs 1a and 1b.

The purified lipopolysaccharide obtained in fraction IV was used as the starting material for preparation of lipid A. Fraction II appears to be largely nucleic acid, while nucleic acid contamination has been removed from fraction IV: the purified lipopolysaccharide. Fractions I and III, of high nitrogen content, did not have marked ultraviolet absorption, suggesting that these fractions were largely peptide contaminants. Paper chromatography of the acid hydrolysates (6 N HCl, 6 hours at 100°C) of the different fractions confirmed this suggestion.

Preparation and Purification of Lipid A

I. Hydrolysis and extraction of crude lipid

A. Preliminary experiments Lipid A can be split from lipopolysaccharide preparations with different types of acidic hydrolysis.

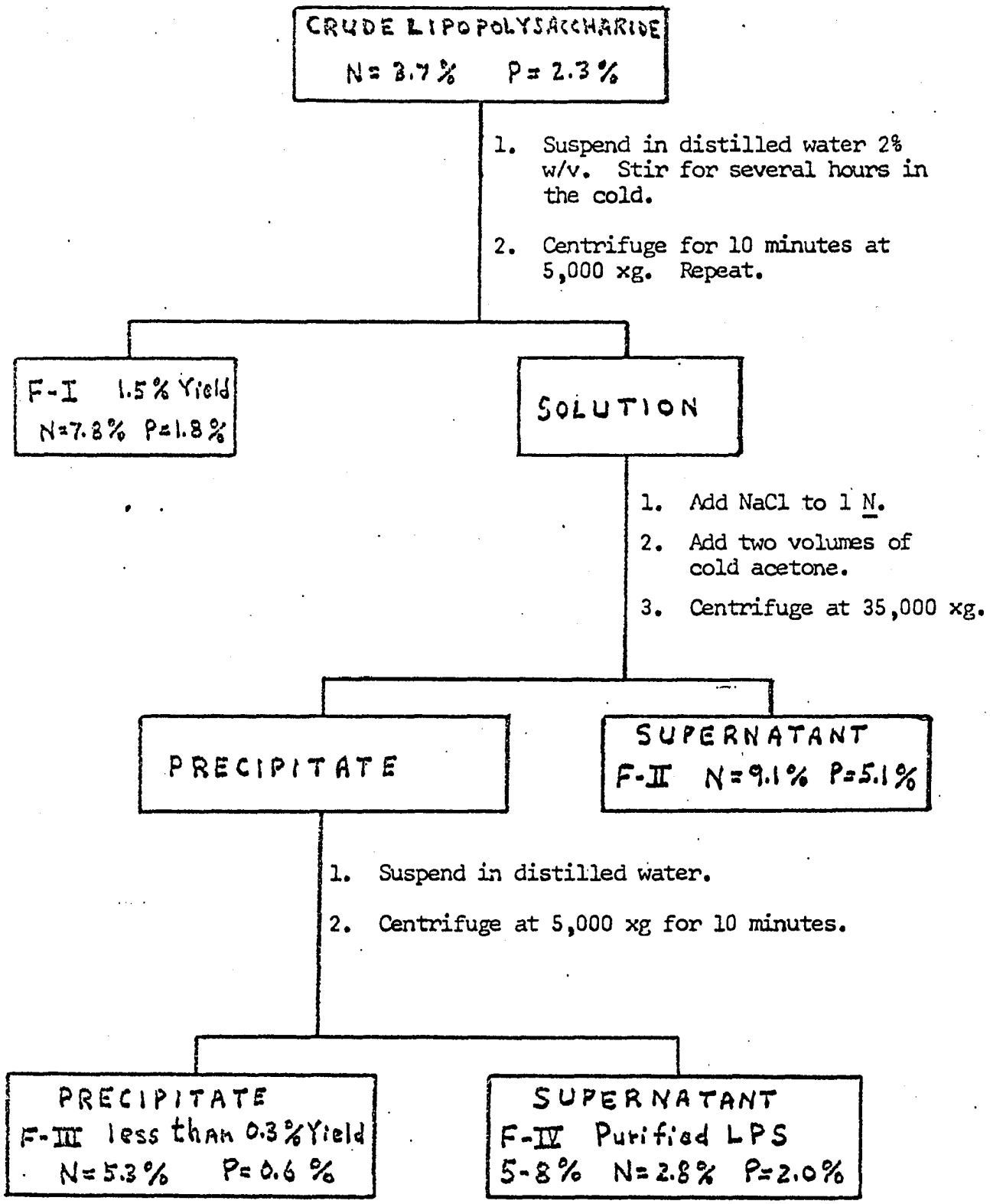
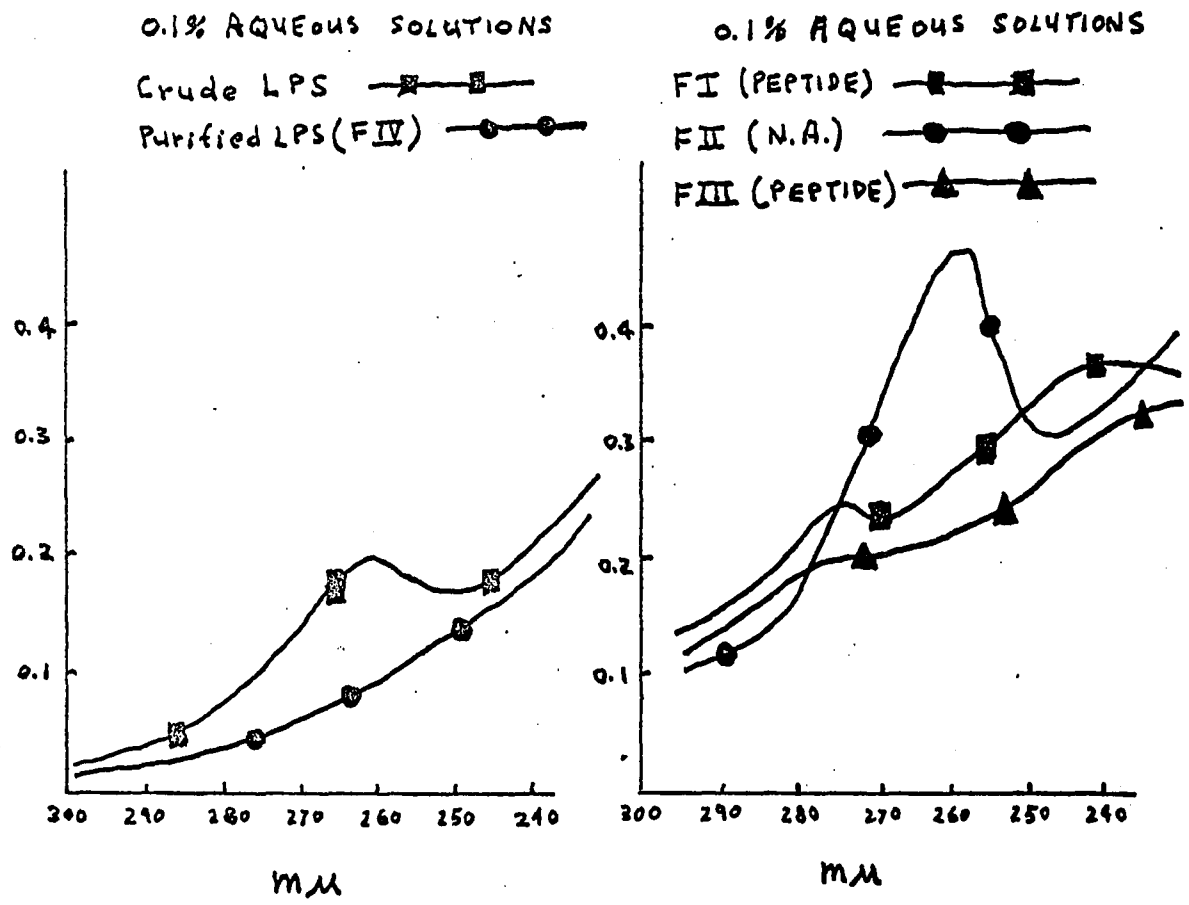


Figure 2. Purification of crude lipopolysaccharide.

Table I. Analytical data for fractions prepared during the routine purification of crude lipopolysaccharide

	N %	P %	Sugar %	Lipid %
Crude lipopolysaccharide	3.7	2.3	48	9.3
F-I	7.8	1.8	----	----
F-II	9.1	5.1	----	----
F-III	5.3	0.6	----	----
F-IV	2.8	2.0	64.7	21



Graphs 1a and 1b. Ultraviolet absorption spectra of fractions prepared during the routine purification of lipopolysaccharide.

Boivin et al. (15) first used 0.2 N acetic acid at 100°C to precipitate lipid A from endotoxin complexes in 1933. After this first report of the isolation of a biologically active lipid from endotoxins, several other workers described the cleavage of endotoxins into a haptenic polysaccharide and a nitrogen-containing phospholipid (125, 30, 87).

In addition, all methods described not only split lipids from other parts of the endotoxin complex but liberate and precipitate 40 to 60% of its long chain fatty acid content during hydrolysis. This fact shows that these methods not only liberate bound lipids but partially destroy them as well. Our preliminary experiments were aimed at determining the hydrolysis conditions which would give a product with the highest yield of bound lipid and the lowest percentage of free fatty acids in the crude lipid mixture, thus, showing less degradation of the lipopolysaccharide components.

Samples of purified Vibrio fetus lipopolysaccharide (100 mg.) were refluxed at 100°C for 30 minutes using 0.1 N and 1.0 N hydrochloric acid, 0.2 N acetic acid, and 0.1 N formic acid. The crude lipid A mixtures were extracted with chloroform, dried, and weighed in tared beakers. Free fatty acids were then extracted with acetone and aliquots of the crude mixtures were analyzed for carbohydrate content.

The crude lipid A derived from both hydrochloric acid hydrolysates contained high amounts of free fatty acid, a condition that indicates extensive degradation occurs using these reagents. Hydrolysis with 0.2 N acetic acid gave a slightly greater yield of crude lipid and 2% less free fatty acid content than did hydrolysis with 0.1 N formic acid. The results obtained are shown in Table II.

Table II. Comparisons of crude lipid A obtained by different hydrolytic methods

Hydrolysis for 30 minutes with	Crude lipid A yield %	Analysis of crude lipid A	
		Free fatty acid content %	Anthrone positive carbohydrate %
0.1 N HCl	12	44	4.8
1.0 N HCl	14	52	3.9
0.2 N Acetic acid	21	14	7.5
0.1 N Formic acid	18	16	2.8

The data of Table II also show the anthrone positive carbohydrate content of the crude lipid A obtained by different hydrolytic methods. The relatively low carbohydrate percentage of the lipids indicates that these treatments removed most, but not all, of the polysaccharides. Hydrolysis with 0.2 N acetic acid was the least effective from this point of view.

On the basis of yield, amount of degradation, and carbohydrate content found in each of the lipid preparations, hydrolysis with 0.1 N formic acid was the hydrolytic method giving the optimum preparation.

B. Routine extraction of crude lipid A. Purified Vibrio fetus lipopolysaccharide (1.0 g.) was added, with stirring, to 100 ml. of 0.1 N formic acid which was heated to 85°. The mixture was heated under refluxing conditions for 30 minutes, then cooled in an ice bath. The reaction mixture was milky in appearance, with white particles in suspension. Chloroform, in five 50 ml. portions, was used to extract the mixture. The combined chloroform extracts were washed three times with 50 ml. portions of distilled water, then dried in vacuo to a final weight of 180 mg. (18%).

The acidic layer was subjected to 15 minutes additional hydrolysis, after which an average of 22.4 mg. of additional material was released by chloroform. The acidic layer was concentrated and its acidity increased to 0.5 N formic acid. Hydrolysis for 2 hours at this normality released very small amounts of additional lipid, generally less than 3 mg. On the basis of this experiment, the hydrolysis conditions were changed slightly in that the time of hydrolysis was increased to 45 minutes.

II. Acetone fractionation

The chloroform soluble extract from the lipopolysaccharide was extracted three times with hot acetone in 15 ml. portions. The acetone insoluble lipid was collected on a filter, dissolved in a minimum amount of chloroform, and precipitated with three volumes of acetone. The precipitate was again collected on a filter, dried and weighed to give acetone insoluble lipid A. The acetone soluble lipid when dried in vacuo accounted for 8 to 10% of the crude extract.

III. Column chromatography of lipid A

A. Chromatography on organic Sephadex Using high flow rates a column of 50 x 1.5 cm. was packed with Sephadex LH-20[1] in chloroform. This was done because the gel has a lower specific gravity than chloroform and tends to float in the solvent at slower rates. Once packed, the gel was secured in place with a teflon plunger.

Acetone insoluble lipid A was applied to the column and eluted with chloroform at a flow rate of 1 ml./min. A typical separation gave two

---

[1]Sephadex LH-20, Pharmacia Fine Chemicals, Inc., New York, New York.

fractions: the first eluted just after the hold-up volume and constituted 94 to 98% of the lipid samples; the second fraction was eluted in the 30-40 ml. aliquot after the first fraction. Both fractions were examined qualitatively by infrared spectroscopy. Fraction I gave an absorption typical of lipid A spectra obtained in our previous studies (120); fraction II shows a series of sharp, progression peaks between 1380 cm^{-1} and 1180 cm^{-1}, considered typical of fatty acid absorption.

B. Silicic acid chromatography Lipid A (fraction I, 503 mg.) from the Sephadex column was applied in chloroform (10 ml.) to a column prepared from silicic acid:celite (4:1) by the method of Rouser et al. (100). This method consisted of washing successively with methanol, chloroform:methanol (1:1), three volumes of chloroform and one volume of methanol under gentle suction from a water pump. The material was dried, then activated by heating for 12 hours at 110°C before it was used to prepare the column.

After application of the sample, the column was eluted with 100 ml. of $CHCl_3$, then successive 200 ml. volumes of $CHCl_3$:MeOH in the proportions 9:1, 8:2 and 7:3, respectively. Finally, 200 ml. of 95% aqueous MeOH was used to strip the column. Just before each eluant was changed to the next one of increased polarity, 5 ml. of the eluting solvent was collected in a separate vessel, dried and weighed, to check the completeness of elution with that solvent. The results of chromatography are summarized in Table III.

Of the lipid applied to the column, 395.2 mg. was eluted by $CHCl_3$:MeOH (9:1). This fraction was applied to another silicic acid:celite column and chromatographed in an identical manner. In this case 367 mg. (93%) was eluted by $CHCl_3$:MeOH (9:1).

Table III. Column chromatography of lipid A (silicic acid:celite)

Fraction	Eluant $CHCl_3$:MeOH	Volume of eluant, ml.	Weight of fraction, mg.	Percent
1	100%	100	4.8	0.9
2	9:1	200	395.2	79
3	8:2	200	34.1	6.8
4	7:3	200	18.8	3.8
5	95% aqueous	200	27.0	5.4
	Total recovery 95.9%			

A second aliquot of fraction I (304 mg.) from organic Sephadex chromatography was chromatographed on silicic acid:celite. The elution pattern was similar to the first column: 234 mg. (77%) was eluted by $CHCl_3$:MeOH (9:1) and rechromatographed to give 222 mg. (95%) of fraction II.

IV. Qualitative examination of the fractions from silicic acid chromatography

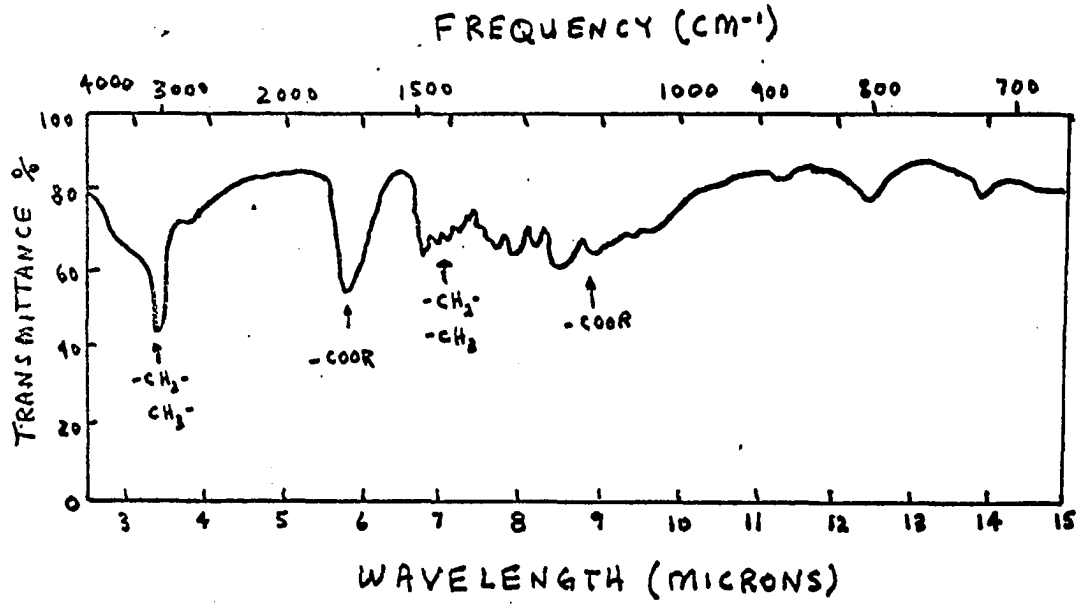
A. Infrared spectra The infrared spectrum of fraction I from a typical column chromatogram shows marked carboxyl absorption at 1705 cm^{-1} and a series of progression peaks between 1380 cm^{-1} and 1180 cm^{-1}, typical of fatty acid absorption. The spectra of fractions II, III and IV reflect a progressive decrease in the ratio of carboxylester absorption at about 1735 cm^{-1} to the acid amide absorption at 1650 cm^{-1}. The spectrum of fraction IV shows an additional weak absorption at 1270 cm^{-1}, an absorption assigned to the phosphate group. The infrared spectra of fractions I and II are reproduced in Figure 3 and the assignments of structure are summarized below.

Position of Peak	Assignment (9, 5, 6)
Broad 3100-3600 cm^{-1}	-NH-, possibly OH
2850, 2920	$-CH_2-$, $-CH_3$
1720	-COOR
1655, 1545	-CONH-
1465, 1380	CH_3-, $-CH_2-$
Broad 1150-1180	-COOR
Broad 855	α-Pyranose form

B. Paper chromatography Approximately 10 mg. samples of each of the lipid fractions were transferred to 2.5 ml. vials; 1.0 ml. 6 N HCl was added to each sample, and the vials were sealed. The hydrolysis mixtures were heated at 100°C for 6 hours. Following hydrolysis, the acidic solutions were clarified by centrifugation, then evaporated to dryness in a vacuum desiccator over NaOH pellets to remove most of the strong acid. Suitable dilutions of the samples with distilled water were made before applying the samples to Whatman No. 1 filter paper for chromatography. The chromatograms were developed in isopropanol:acetic acid:water, 3:1:1 (v/v), ascending.

The water soluble components in the acid hydrolysates were detected, following chromatography, with ninhydrin, periodate, acid aniline phthalate and Elson-Morgan spray reagents. Glucosamine, after treatment with the same acidic conditions, was chromatographed as a standard and glycerol was also used as a chromatographic standard. The results of these chromatograms are summarized in Figures 4 and 5.

FRACTION I



FRACTION II

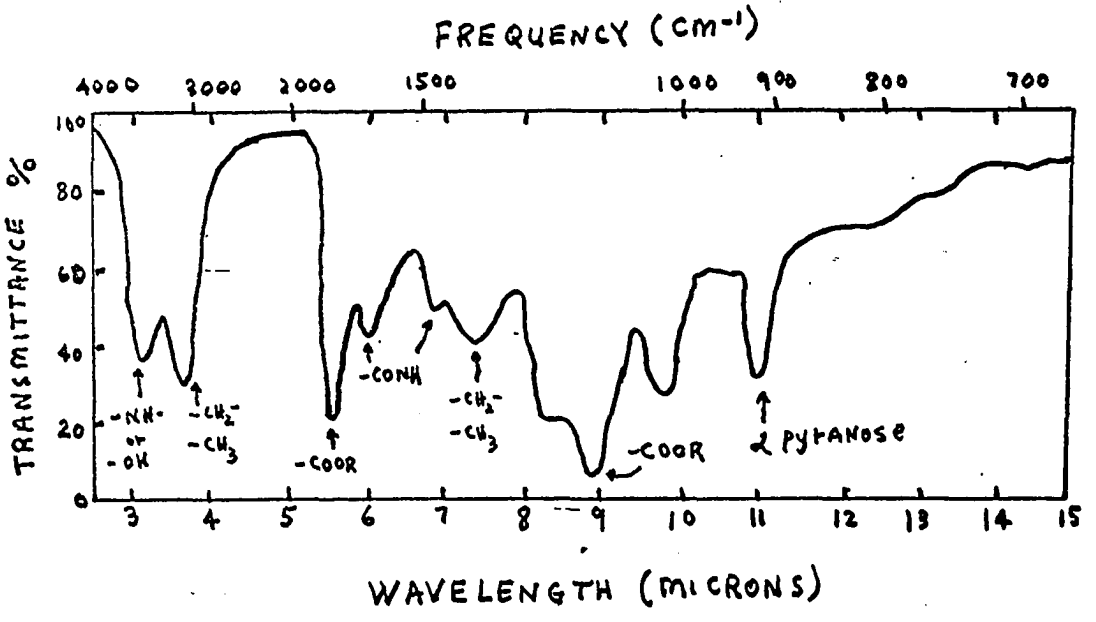
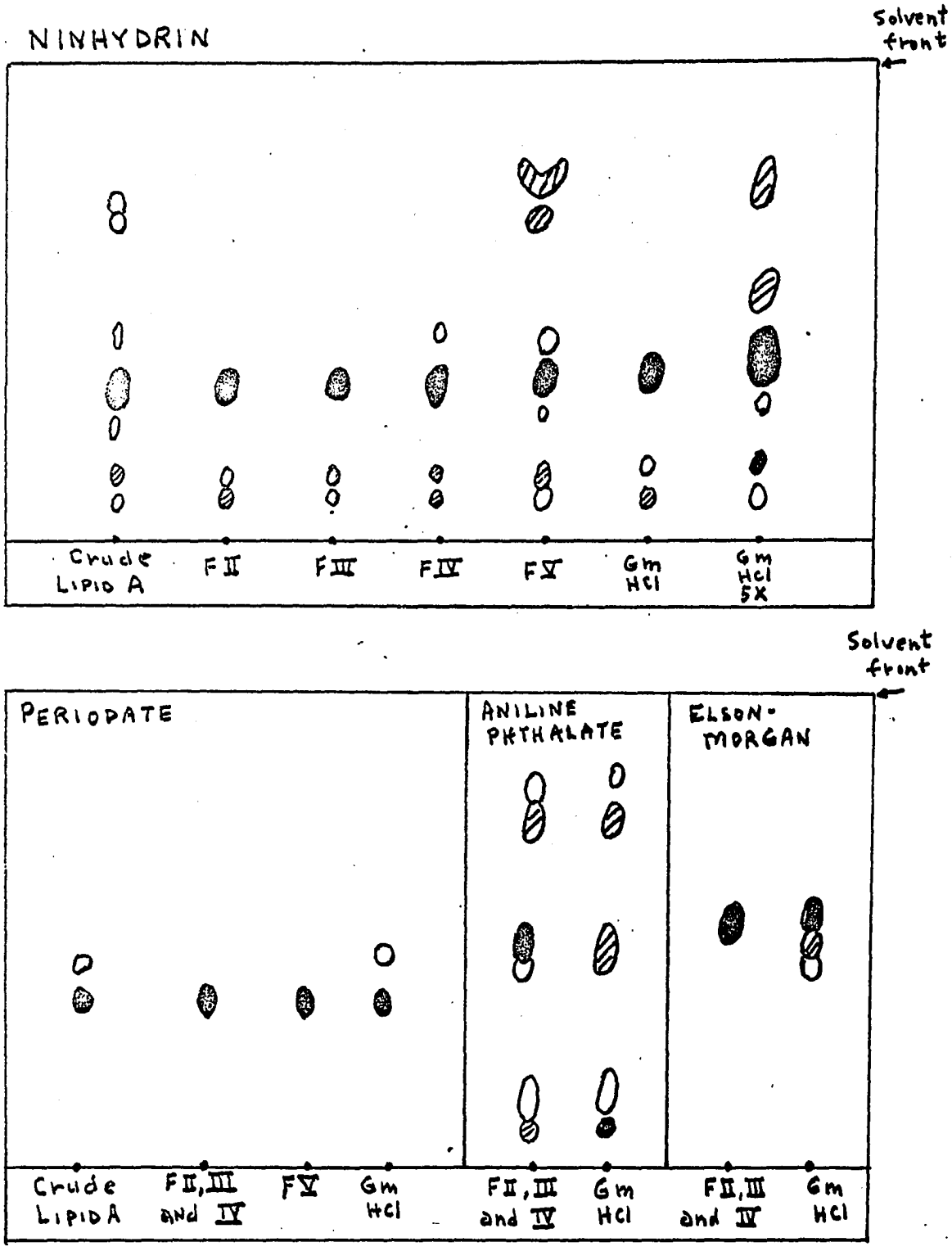


Figure 3. Infrared spectra of the lipid fractions I and II obtained from silicic acid column chromatography.



Figures 4 and 5. Paper chromatography of lipid fractions from silicic acid chromatography.

C. Thin-layer chromatography Fractions I through V were analyzed for homogeneity on Silica gel HR and Kiselgel absorbants as described in section IV under General Methods, page 31. Fractions I and II moved as single components in all solvent systems tested. Fraction I, less than 1% of the crude lipid, moved in all cases with the $CHCl_3$:MeOH solvent front and probably comprises free fatty acids as indicated by the infrared spectra. Fraction II, 75-77% of the crude lipid A, was detected by ultraviolet light and lipid and phosphate spray reagents as one spot with R_f values between 0.5 and 0.7 depending upon the solvent system used.

Fractions III, IV and V were not separable in any of the solvent systems tested. They gave a tailing or smearing effect for the most part, with occasional spotting of the bulk of a sample at R_f values lower than that of fraction II.

Chemical Studies on Lipid A

I. General properties

Upon chromatography of the acetone insoluble lipid from Sephadex fraction I on silicic acid, as described in the previous section, most of the lipid was obtained in fraction II. This fraction was rechromatographed on silicic acid; the lipid in fraction II from this second chromatographic column was termed lipid A-II.

A-II is a clear, light tan, waxy solid, m.p. 205-208°C. Of the common laboratory solvents, only chloroform and pyridine dissolve the lipid completely; it is insoluble in hot absolute ethanol, ether, methanol, acetone, benzene, and dimethylsulfoxide.

II. Partial chemical characterization

A. Analytical data

1. Nitrogen - The modified Kjeldahl microprocedure for samples of 10-100 µg. was used to determine nitrogen.

2. Phosphorous - Phosphorous was determined colorometrically with N-phenyl-p-phenylenediamine reagent according to the procedure of Dryer et al. (22). All samples were read at 350 mµ on the Beckman Model DB spectrophotometer. Potassium dihydrogen phosphate standards covering the range of the determination (0.2-10 µg.) were run concurrently.

3. Total fatty acid content - A-II (25.2 mg.) was hydrolyzed with 6 N HCl (0.1 ml./2 mg. lipid) for 6 hours. The reaction mixture was extracted three times with chloroform. The chloroform extracts were washed with distilled water and dried under reduced pressure. The dried extracts were completely soluble in acetone. The yield from A-II was 15.6 mg. (62.4%).

4. Qualitative hexosamine determination - Routine paper chromatography of the acid hydrolysates of A-II (6 N HCl, for 6 hours at 100°C) was carried out using isopropanol:acetic acid:water, 3:1:1 (v/v), ascending. The chromatogram was sprayed with Elson-Morgan reagents, which revealed the presence of one cherry red spot at the same R_f as acid-treated glucosamine. To distinguish between the hexosamines, glucosamine, galactosamine and mannosamine, acid hydrolysates of A-II were acetylated according to Crumpton's modification (19) of the method of Roseman and Ludowieg (99). To 1 ml. of a solution containing approximately 2 mg. of amino sugar was added 1.5 ml. of 0.5% $NaHCO_3$ solution and, immediately, two drops of acetic anhydride. The mixture was shaken and allowed to stand at room temperature

for 24 hours. Sodium ions were removed by passage through a small column of Dowex -50 resin (H^+ form); the eluate was dried *in vacuo*, then redissolved to give a 0.1% solution.

The acetylated acid hydrolysates were chromatographed on borate-treated paper in n-butanol:pyridine:water, 6:4:3 (v/v), descending. It has been reported (112) that under these conditions, N-acetyl galactosamine and N-acetyl mannosamine move together at approximately half the R_f of N-acetyl glucosamine. Samples of glucosamine and galactosamine were acetylated and chromatographed with A-II. After development and drying, the chromatogram was sprayed with the Elson-Morgan reagents. The results are represented in Figure 6.

The acetylated acid hydrolysate of A-II was also subjected to the fingerprinting technique of Ingram (41), as suggested by Salton (101), who reported a separation of N-acetyl muramic acid from N-acetyl glucosamine under these conditions. Glucosamine, N-acetyl glucosamine, N-acetyl muramic acid and N-acetyl-6-phosphoglucosamine were used as standards. 6-Phosphoglucosamine, reported as a constituent of lipid A from *Escherichia coli* (77), was prepared by the method of Jourdian and Roseman (47) and acetylated as described before. A-II and the standard preparations were chromatographed on Whatman No. 3 mm filter paper in isopropanol:acetic acid:water, 3:1:1 (v/v) for 11 hours, dried, and sprayed with pyridine: acetic acid:water, 10:0.4:90 (v/v) prior to electrophoresis. The moist paper was immediately placed in the high voltage electrophorator[1] and

---

[1]High Voltage Electrophorator, Model D, Gibson Medical Electronics Co., Middleton, Wisconsin.

electrophoresed for 2 hours at 2,000 volts, 150-200 milliamperes. The sheets of paper were removed, dried at room temperature overnight and sprayed with the Elson-Morgan reagents. Only one spot was observed in the acid hydrolysate of A-II as represented in Figure 7.

5. Quantitative glucosamine determination - The method of Elson and Morgan (24), as modified by Rondle and Morgan (98), was used to determine the amount of glucosamine present in lipid A-II. The test samples (10-15 mg.) were first hydrolyzed with 6 N HCl for 6 hours. The hydrolysates were diluted to 2 N acid, then applied to small columns of Dowex-50 (200 to 400 mesh, H^+ form), as described by Boas (13). Each column was eluted first with 10 ml. of distilled water, then with 10 ml. of 2 N HCl; the acidic eluate containing the glucosamine to be measured was collected in a 10 ml. volumetric flask.

The water eluate contained neutral sugars; since any glucosamine which was still phosphorylated would also be eluted in this fraction, an aliquot of this eluate was tested for the presence of glucosamine. The hydrolysis of glucosamine-phosphate was apparently complete under the conditions used, however, since no glucosamine was detected in this fraction from hydrolyzed samples. Because of the importance of complete hydrolysis to the quantitative measurement of glucosamine, this observation was confirmed by subjecting the acid hydrolysate of A-II to high voltage electrophoresis, together with standard solutions of glucosamine and glucosamine-6-phosphate. The glucosamine-6-phosphate was prepared in our laboratory by the method of Jourdian and Roseman (47). The conditions for electrophoresis were the same as those described previously for N-acetylated hexosamines, but omitting the chromatography in this case. After

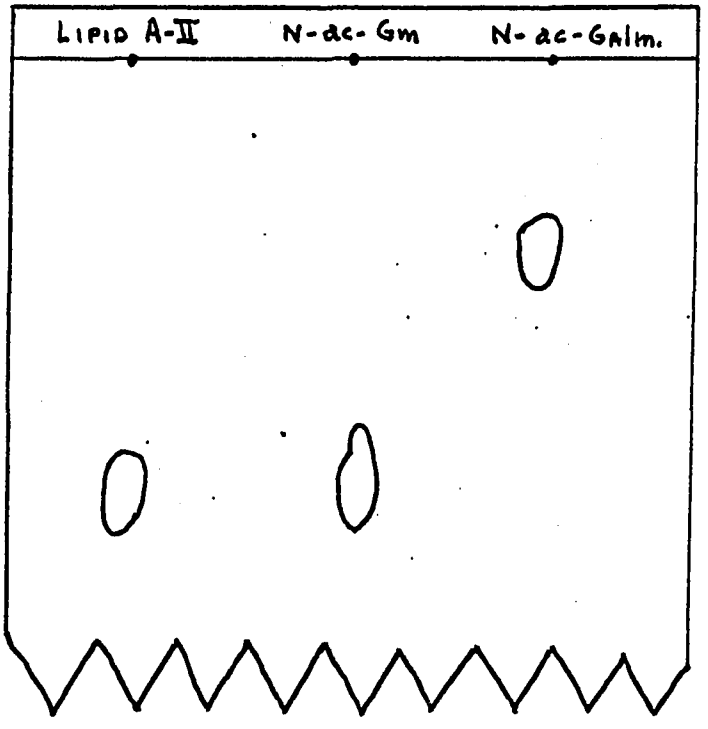


Figure 6. Paper chromatography of acetylated hexosamines on borate treated paper.

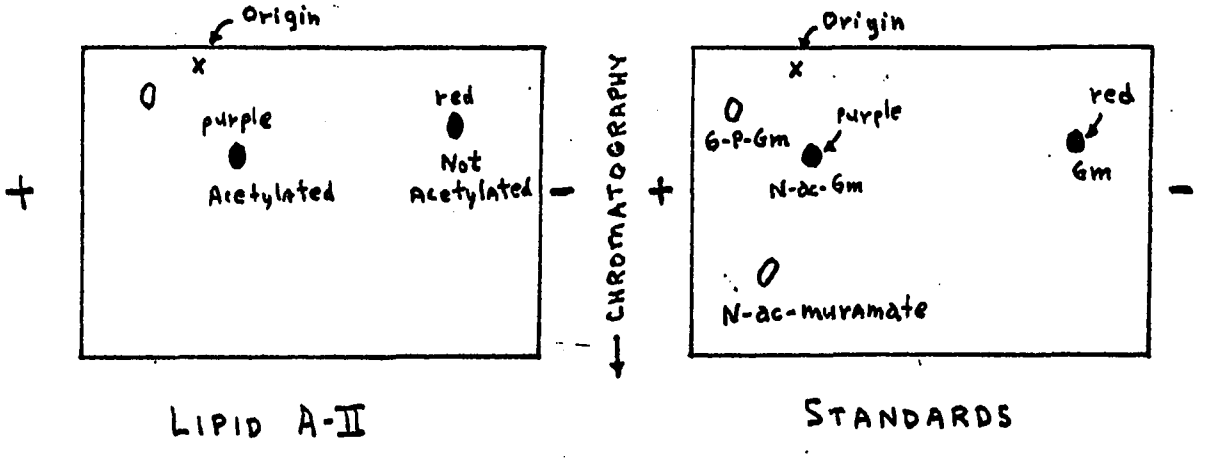
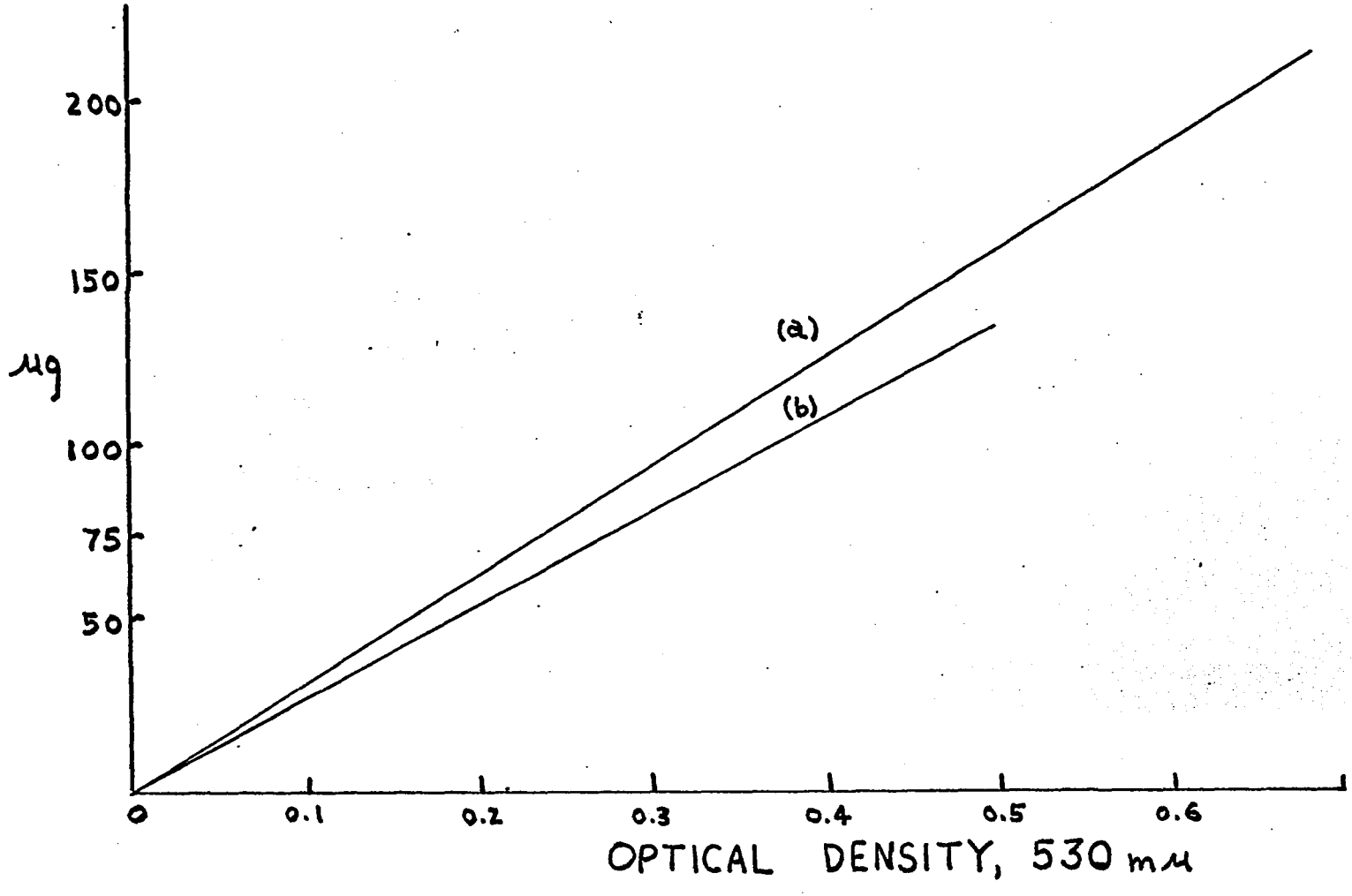


Figure 7. Fingerprinting (paper chromatography followed by high voltage electrophoresis) of acetylated hexosamines.

electrophoresis the paper strip was air dried, then sprayed with the Hanes-Isherwood reagent. Only the light green spot due to inorganic phosphate was observed from the acid hydrolysate of A-II; glucosamine-6-phosphate moved 4 cm. toward the anode under these conditions, while glucosamine moved 16 cm. toward the cathode.

The glucosamine samples to be measured, 0.5 to 1.0 ml. aliquots of the acidic eluates from the Dowex-50 columns, were transferred to 15 ml. test tubes. One drop phenophthalein solution was added to each sample; 4 N NaOH was added dropwise until the solutions were pink; and 0.3 N HCl was then added dropwise to discharge the color. One milliliter of Elson-Morgan reagent A was added to each solution and mixed well by shaking. Each tube was covered with a loose stopper and heated for 20 minutes in a boiling water bath. After cooling to room temperature, 5 ml. of absolute ethanol was added to each solution, then 1 ml. of reagent B. The contents were thoroughly mixed and warmed for 10 minutes at 65-70°C to liberate CO_2. After cooling to room temperature, the final columes were adjusted to 10 ml. and the contents again mixed thoroughly. The intensity of the color was measured at 530 mμ using a Beckman DB spectrophotometer.

This procedure was first applied to standard solutions for glucosamine which were: a) untreated, and b) subjected to 6 N HCl for 6 hours. The results are summarized in the standard curve found in graph 2, page 53. The optical density readings obtained illustrate the variations usually observed for duplicate samples and the range of glucosamine content (20 to 100 μg.) which is most satisfactorily determined. The results show that some destruction of glucosamine occurs during acid hydrolysis. In this case



Graph 2. Quantitative estimation of glucosamine. (a) Untreated glucosamine standard. (b) Glucosamine subjected to 6 N HCl for 6 hours prior to analysis.

the ratio of the reading for untreated glucosamine to that for treated glucosamine is 1.23. In a typical determination of unknown samples, both treated and untreated standards are included in order that this ratio may be used to correct the readings of the test solutions for the destruction of glucosamine.

The error inherent in this method includes weighing errors for samples which are 5 to 10 mg. in size, volumetric errors including pipetting and adjustment of the final volume of the test solutions, and the losses due to hydrolysis. This last source of error is probably the most serious, despite the controls with standard solutions. The destruction of fully acylated or glycosidically linked glucosamine may not be comparable to that observed for free glucosamine. It is estimated that this determination involves a relative error of ± 15%.

6. Determination of fatty acid ester (FAE) and fatty acid amide (FAA) - To determine the fatty acid ester (FAE) and fatty acid amide (FAA) content of A-II, the spectrophotometric method of Snyder and Stevens (108) was used. This procedure was modified for endotoxins and lipid A by Tauber (114) and for simultaneous determination of fatty acid amide by Haskins (33). These modifications were incorporated into the determination of FAE and FAA in A-II.

The alkaline hydroxylamine and ferric perchlorate reagents were prepared as directed by Snyder and Stevens (108). Recrystallized tripalmitin served as the standard for the fatty acid ester determination; its homogeneity was ascertained by thin-layer chromatography on silica gel prior to use. Chromatographically pure N-acetyl glucosamine was used as the fatty acid amide standard.

Samples of A-II and the standard preparations containing 1 to 5 microequivalents of fatty acid ester and amide were placed in 16 x 150 mm. screw-capped culture tubes; 2 ml. of freshly prepared alkaline hydroxylamine reagent was added, and the tubes were tightly sealed with teflon-lined caps. The samples, with reagent blank, were placed in a water bath at 67°C for five hours. At the expiration of the incubation period the tubes were examined for loss in volume resulting from a leaky closure. Such samples gave an abnormal color reaction and were discarded. The tubes were removed and cooled to room temperature, and 5 ml. of ferric perchlorate reagent added to each. After 30 minutes the purple color was read in a 1 cm. cuvette in a Beckman B spectrophotometer at 530 mμ. Standard curves, prepared from tripalmitin and N-acetylglucosamine, when plotted as equivalents of acid vs. absorbance were straight lines coincident over the range of 1 to 5 microequivalents. To determine the amide-bound fatty acids (FAA) in relation to the ester-bound fatty acids (FAE) a second set of samples was analyzed by the procedure of Tauber (114) to obtain the FAE content. Subtraction then gave the FAA content.

7. Test for reducing sugar - Lipid A-II was dissolved in pyridine and applied to Whatman No. 1 filter paper. Standard solutions of sucrose, N-acetyl glucosamine, and glucosamine were also applied to the paper. A 0.5% solution of triphenyltetrazolium chloride in pyridine was used to spray the paper. After drying, the paper was sprayed with 0.5 N KOH in 95% ethanol, then heated over steam. No spot was observed for sucrose; N-acetyl glucosamine gave a deep purple-red color, and glucosamine gave a

cherry red color. The paper surrounding the spots was light pink in color. Lipid A-II, when tested in this manner, gave a deep purple colored spot.

B. Analysis of component fatty acids

1. Preparation of methyl esters - Fractions A-II, A-III, A-IV and A-V from fractionation of crude lipid A on silicic acid were subjected to alkaline hydrolysis, acidified, and extracted with acetone. These fractions were also chloroform soluble and their infrared spectra in chloroform were typical of that for fatty acids. The samples were subjected to boron trifluoride catalyzed methanolysis according to the method of Metcalf and Schmitz (65).

To 102 mg. of A-II was added 3 ml. of BF_3 reagent in a 20 x 150 mm. test tube. The mixture was boiled on a steam bath for 2 minutes. The boiled mixture was transferred to a separatory funnel with 30 ml. of petroleum ether (reagent grade) and 20 ml. of water added. The mixture was shaken vigorously. The petroleum ether layer was filtered through paper into a 50 ml. beaker and evaporated over a 60°C water bath. The methyl esters were then taken up in hexane prior to use. Smaller samples of A-III, A-IV and A-V were similarly prepared.

2. Isolation of hydroxy fatty acid esters of A-II - An aliquot of the methyl ester preparation of lipid A-II (51 mg.) was applied to a small column of silicic acid. Initially, methylene chloride was used to elute the nonhydroxy fatty acid esters; then methylene chloride containing 3% methanol was used to elute hydroxy fatty acid esters. Under these conditions, Scheuerbrandt and Bloch (103) have reported the efficient separation of hydroxy fatty acid esters from saturated and unsaturated esters of

fatty acids. The isolated hydroxy fatty acid esters (18 mg., 37%) and nonhydroxy fatty acid esters (30 mg., 63%) were analyzed separately by gas and thin-layer chromatography.

3. Thin-layer chromatography of fatty acid esters - The application of thin-layer chromatography for separation of methyl esters of fatty acids is described by Viroque and Holman (121) who reported separations according to chain length as well as degree of unsaturation and polarity.

Methyl esters of the fatty acids from fractions A-II to V and the hydroxy and nonhydroxy fatty acid esters of A-II separated by adsorption column chromatography were analyzed by thin-layer chromatography on silica gel G. The TLC plates were developed with a mixture of hexane:ethyl ether (85:15). The proportion of ethyl ether in the mixture was raised to 40% to give improved resolution of the polar hydroxy esters when this sample was chromatographed.

4. Vapor phase chromatography - The samples of methyl esters described in the preceding section were subjected to vapor phase chromatography using the Microtek GC 2000 MF gas chromatograph.[1] Two 3-foot U-shaped columns were used: one packed with diethylene glycol succinate and the second with apiezon. Fatty acid methyl esters were identified by co-chromatography with authentic samples.[2] There was difficulty in identifying ß-hydroxymyristic acid because no reference standard was available. Tentative identification was made based upon a comparison of relative

---

[1]Microtek GC 2000 MF Gas Chromatograph, Microtek Instruments, Inc., Baton Rouge, Louisiana.

[2]Fatty acid methyl ester standards, Applied Science Laboratories, State College, Pennsylvania.

retention times in this experiment to those obtained by Nesbitt and Lennarz (78) on fatty acids from lipopolysaccharide isolated from Escherichia coli. The same conditions were used in both experiments.

5. Possible presence of a neutral component -

a. Qualitative vinyl ether determination - The method described by Norton (75) was applied to samples of A-II. However, a reference standard, such as plasmalogen, was not available. The lipid was spotted on Whatman No. 1 filter paper, which was then immersed for 30 seconds in 1% aqueous $HgCl_2$. Excess $HgCl_2$ was removed with five washes in 1% NaCl and five washes in distilled water. The presence of Hg in the lipid spot was detected by immersing the sample for 2 minutes in a 0.1% solution of diphenylcarbohydrazide in 70% ethanol, which is 0.1 N with respect to KOH. After washing thoroughly in water to remove excess diphenylcarbohydrazide reagent, the appearance of a deep purple spot of Hg salt against an unstained background constitutes a positive test. This test was not clearly positive for any of the A-II samples, after 30 seconds immersion in $HgCl_2$; when the samples were treated in exactly the same manner, except for increasing the time of exposure to $HgCl_2$ to 1 minute, a strong positive test was observed for all samples.

b. Attempted iodination, bromonation - The method of iodination described by Rapport et al. (89, 90), which is a modification of the method of Siggia and Edsberg (104), was applied to A-II. In this method the lipid sample (requiring about 2 μmoles of I_2) is dissolved in $CHCl_3$: MeOH, 2:1 (0.6 ml.). Methanol (0.8 ml.) is added to give a clear or finely turbid solution. The tube is stoppered and the sample chilled to 0° in

an ice water bath. Then 0.20 ml. of standard I_2 solution (0.05 N) is added and the mixture agitated vigorously for 2 minutes. The mixture is then returned to the ice bath for 12 to 15 minutes.

Excess I_2 is titrated with aqueous 0.1 N $Na_2S_2O_3$, using an ultramicroburette. The titration is conducted with the tip of the burette below the fluid surface; a rapid stream of N_2 bubbling through the mixture insures mixing. The end point, determined with good lighting against a white background, is taken as the disappearance of the yellow I_2 color. Samples were determined in triplicate, with the first sample used to establish the endpoint. The pH of the solution should be between 2 and 7.

When this method was applied to A-II, an apparent I_2 uptake of 0.044 μmoles/mg. was measured. The precipitation of A-II during titration of the excess I_2 made detection of the endpoint uncertain.

Bromination, according to the method of Trappe (118), was attempted but the lipid sample was completely insoluble under the reaction conditions.

Qualitative Structural Studies of Lipid A

I. Determination of the linkage between lipid and polysaccharide moieties

A. Evaluation of a possible peptide linkage To investigate the possibility of a peptide linkage between lipid A and the polysaccharide moiety, intact lipopolysaccharide was subjected to mild alkaline hydrolysis, which would be expected to cleave ester bonds, but leave intact amide and glycosidic bonds.

Lipopolysaccharide (150 mg.) was suspended in 15 ml. of distilled water and stirred for 30 minutes at room temperature. To this suspension was added 1.5 ml. of 2 N NaOH; the mixture was stirred for 24 hours at temperatures between 35° and 40°C. Considerable clearing of the suspension was noted as base was added; after 24 hours, the lipopolysaccharide solution was completely clear. The solution was chilled in an ice bath, acidified to pH 1 with 2 N HCl, and extracted four times with 20 ml. portions of $CHCl_3$. The $CHCl_3$ extract was washed three times with distilled water, then evaporated to dryness in vacuo to a weight of 11.2 mg. (7.5%, N = 0.10%, P = 0.06%). The infrared spectrum of this extract showed strong carboxyl absorption at 1700 cm^{-1}, as well as weak amide absorption at 1650 cm^{-1} and 1545 cm^{-1}.

The washes of the $CHCl_3$ extract were concentrated and added to the aqueous solution, the pH of which was adjusted to 7 with 0.5 N NaOH. The aqueous solution was dialyzed overnight at 4°C against 500 ml. of distilled water. The inner solution was dialyzed against two more changes of deionized water, then lyophilized to give 123.8 mg. (82.6%, N = 2.0%, P = 2.8%). The first dialysate was concentrated almost to dryness. The major portion of the dialysate, as well as that of the $CHCl_3$ extract, was applied to Whatman No. 1 filter paper and chromatographed in isopropanol:acetic acid:water. The dried chromatograms were sprayed with ninhydrin, periodate, and Elson-Morgan reagents.

B. Evaluation of a possible linkage involving KDO (2-keto-3-deoxyoctonoate

Osborn (83) presented evidence that the polysaccharide from Salmonella typhimurium consists of heptose phosphate chains terminating

in the eight carbon keto acid, 2-keto-3-deoxyoctonoate at the reducing end of the chains. This molecule, Horecker suggests (37), forms the linkage to the lipid through either the hydroxyl groups of glucosamine or 2-hydroxymyristic acid. KDO has been found in several lipopolysaccharide preparations (37, 91, 83) and in lipid A by Kasai (48) and Nowotny (80).

Preparations of lipopolysaccharide and lipid A-II from Vibrio fetus were analyzed for quantitative and qualitative content of KDO. Samples of a commercial Escherichia coli lipopolysaccharide preparation,[1] known to contain KDO, were also examined. A standard preparation of KDO, which had been chemically synthesized according to the method of Heath (35), was kindly provided by Dr. Paul Rebers, National Animal Disease Laboratory.

Qualitative examination of lipopolysaccharide and lipid A-II preparations for KDO were made by paper chromatography. Samples were hydrolyzed (1 N HCl for 60 minutes) prior to chromatography. It has been reported (91) that, under these conditions, the maximum amount of thiobarbituric acid reactive substances is formed from lipopolysaccharides.

The samples and standard were spotted on Whatman No. 1 paper and developed in pyridine:butanol:water (4:6:3) for 8 hours. After drying, the chromatograms were sprayed with thiobarbituric acid spray reagent developed by Warren (122) for detection of deoxy sugars and sialic aicds. After drying, the chromatograms were heated at 100°C for 5 minutes; red spots, which also give off a red fluorescence under ultraviolet light, were produced by the thiobarbituric acid-KDO coupled product. The

---

[1] Escherichia coli lipopolysaccharide, Strain 0111:B4, Difco Laboratories, Detroit, Michigan.

chromatograms revealed the presence of spots at the same R_f value as KDO in Vibrio fetus lipopolysaccharide and lipid A-II and in Escherichia coli lipopolysaccharide. Two additional spots, which are probably deoxyhexoses released from the polysaccharide moieties, were observed for both lipopolysaccharide preparations.

The thiobarbituric acid procedure of Aminoff (3) was used to quantitatively assay the preparations. Samples of lipopolysaccharide and lipid A-II were hydrolyzed (2 N HCl, 1.5 hours) prior to analysis. Aliquots of each sample, blank and standard (containing 5-40 µg. of KDO) in 0.5 ml. water, were treated with 0.25 ml. of periodate reagent (25 mmoles periodic acid in 0.125 N H_2SO_4) for 30 minutes in a water bath at 37°C. The excess of periodate was then reduced with 0.2 ml. of 2% sodium arsenite. As soon as the yellow color of the liberated iodine disappeared (1-2 minutes), 2 ml. of a 0.1 M solution of thiobarbituric acid was added and the test sample covered and heated in a boiling water bath for 7.5 minutes. The colored solutions were then cooled in ice water and shaken with 5 ml. of butanol containing 5% (v/v) 12 N HCl. The separation of the two phases was facilitated by a short, rapid centrifuging; and the absorption curves of the periodate-thiobarbituric acid products were read in the visible range (320 mµ-700 mµ) using the Beckman DB spectrophotometer. The absorption intensity of 549 mµ is directly proportional to the concentration of KDO and the standard preparations gave a straight line plot at this wavelength.

II. Determination of the linkage between glucosamine residues in lipid A

A. Alkaline hydrolysis of lipid A Lipid A-II (170 mg.) was suspended in 17 ml. of 1.00 N NaOH in methanol. The reaction vessel was

connected with an Erlenmeyer flask containing 10 ml. of borate indicator solution to trap any NH_3 evolved. The mixture was stirred at room temperature for 4 days. N_2 was then bubbled through the mixture to force NH_3 into the trap; titration of the indicator solution in the trap with N/70 HCl showed that 0.013 meq. of nitrogen (6.2% of the nitrogen originally present in A-II) was evolved as NH_3.

Extraction of the alkaline solution with hexane gave 8.3 mg. hexane soluble material (4%). The infrared spectrum of this fraction showed amide, but no ester absorption.

The alkaline solution was chilled, then titrated to the phenophthalein endpoint with 1.00 N HCl. The saponification equivalent calculated from this titration is of little significance, since the evolution of ammonia indicates consumption of alkali other than that required for hydrolysis of fatty acid esters.

The neutralized solution was extracted five times with $CHCl_3$ to yield 107 mg. (63%) $CHCl_3$ soluble material, the infrared spectrum of which was typical of that for fatty acids. The pH of the reaction mixture was adjusted to 2 again and extracted with $CHCl_3$ to yield 10.6 mg. (6.2%) $CHCl_3$ soluble material. The infrared spectrum of this fraction shows both ester and amide absorption.

The methanolic solution was neutralized and dried under reduced pressure. The residue contained salts and a fraction which was not soluble in acid, but dissolved in 2 N NaOH to form a yellow colored solution. This fraction was not obtained entirely free of salt. Its N to P ratio was 2.6. An infrared spectrum was obtained which showed amide absorption at 1650 cm^{-1} to 1555 cm^{-1}, and no ester absorption.

B. Sodium borohydride reduction Lipid A-II (250 mg.) was suspended in 5 ml. of absolute ethanol by stirring for 2 hours at temperatures up to 70°C. $NaBH_4$ (100 mg.) in 3 ml. absolute ethanol was added drop by drop with stirring. The reaction mixture was stirred continually for three days at room temperature, occasionally heating to 70°C. The ethanol was removed under reduced pressure. The product was suspended in 10 ml. of water, then chilled in an ice bath. The pH of the solution was adjusted to 1 by the dropwise addition of 2 N HCl. The acidified suspension was extracted five times with $CHCl_3$, to give 107 mg. (42.8%) of an oily $CHCl_3$ soluble material (fraction I). The infrared spectrum of this fraction, obtained in $CHCl_3$, showed medium hydroxyl absorption at 3500 cm^{-1} and strong ester absorption at 1730 cm^{-1}.

The aqueous extract contained a considerable amount of suspended material. The suspension was clarified at 10,000 xg and the supernatant carefully removed. The pH of the supernatant was adjusted to 7.6 with 0.5 N NaOH; the solution was taken to dryness under reduced pressure, then dried overnight in a vacuum desiccator. The residue was stirred with 30 to 50 ml. of MeOH and several drops of concentrated HCl was added. MeOH was then removed in vacuo; the methanolic-HCl treatment was repeated four times.

The residue was added to 10 ml. of water, which was filtered to give water soluble fraction II (2.8 mg., 1.1%) and water insoluble fraction III (78 mg., 31%). The infrared spectrum of fraction III showed weak, broad ester absorption between 1700 and 1750 cm^{-1}, as well as medium amide absorption at 1645 cm^{-1} and 1555 cm^{-1}.

The pellet from centrifugation was suspended in a minimum amount of water and centrifuged again at 10,000 xg. After removal of the water wash, the pellet, fraction IV, was dried in a vacuum desiccator overnight to give 40.7 mg. (24.3%). The infrared spectrum of this fraction showed no ester absorption, and marked amide absorption at 1645 cm^{-1} and 1555 cm^{-1}. This fraction was completely insoluble in water, $CHCl_3$, acid or alkali, dissolving only in pyridine.

Fractions II and III were slightly ninhydrin positive, but gave negative tests with the triphenyltetrazolium chloride reagent. Fraction IV gave a positive test with the latter reagent, but was ninhydrin negative.

Fractions I-IV were analyzed for nitrogen, phosphorous and glucosamine. Only small samples (approximately 5 mg.) were available for the nitrogen and phosphorous determinations, and in most cases, duplicates were not possible. Fraction II was diluted to 25 ml. and 5 ml. aliquots taken for the determination of nitrogen and phosphorous. The results of these analyses appear in the Results and Discussion section.

Toxicity Studies of Lipopolysaccharide and Lipid A Preparations

I. Method of assay for toxicity

The toxicity of the preparations used in this study was evaluated by assay in 14-day-old male mice. Ten mice were used at each dose level and the ratio of number of survivors versus the number inoculated was noted after 36 hours. Inoculations were given after lipopolysaccharide and polysaccharide fractions were dispersed in sterile saline solutions and

lipid preparations were dispersed in 5% solutions of Tween 80,[1] a complex mixture of polyethylene ethers. All of the doses were given intravenously in 0.1 ml. quantities with appropriate controls. The endpoint of each preparation was considered to be that dose which was lethal for 50% of the experimental animals (LD_{50}).

II. Preparation of chemically altered lipopolysaccharides

A. Papain-treated lipopolysaccharide Purified Vibrio fetus lipopolysaccharide was treated by the method of Kim and Watson (51) with twice crystallized preparations of papain.[2] Because papain acts only in the reduced state, it was possible to include an additional control involving nonactivated papain. The experiment, therefore, included lipopolysaccharide plus activated papain, lipopolysaccharide plus nonactivated papain, and lipopolysaccharide plus reduced buffer. The experiments were run as follows: (a) Activated papain. 10 ml. of lipopolysaccharide (10 mg./ml.) was mixed with 90 ml. of reduced buffer solution containing 100 µg./ml. of twice crystallized papain; this buffer contained 0.04 M phosphate at pH 7.0, NaCl 0.15 M, L-cysteine HCl 0.005 M, and ethylene-diamine-tetraacetic acid, tetrasodium salt (EDTA) 0.001 M. (b) Nonactivated papain. 10 ml. of lipopolysaccharide solution (10 mg./ml.) was mixed with 90 ml. of non-reduced buffered solution containing 100 µg./ml. of papain; this buffered solution did not contain the cysteine HCl and EDTA. (c) Reduced buffer. 10 ml. of lipopolysaccharide (10 mg./ml.) was added to 90 ml. of reduced

---

[1]Tween 80, Nutritional Biochemical Corp., Cleveland, Ohio.

[2]Papain, Worthington Biochemical Corporation, Freehold, New Jersey.

buffered saline; this was the same as (a) without the addition of papain. Incubation was at 65°C for 16 hours. Any remaining enzyme was destroyed by heating in a boiling water bath for 2 minutes. The preparations were then dialyzed against distilled water, lyophilized, and stored at 4°C until used.

B. Periodate-treated lipopolysaccharide The periodate-treated Vibrio fetus lipopolysaccharide was prepared as described by Neter et al. (73): 200 mg. samples of lipopolysaccharide were dissolved in 160 ml. of distilled water; 20 ml. of sodium acetate buffer, pH 5.0 and 20 ml. of 0.1 N $NaIO_4$ solution were added. The material was kept in the dark for 6.5 hours at room temperature and then dialyzed against distilled water, concentrated in vacuo, and lyophilized. Control samples were prepared in an identical manner, omitting the periodate. Preparations of Escherichia coli endotoxin, obtained from Difco Laboratories, were also treated with periodate and tested for toxicity along with the purified Vibrio fetus lipopolysaccharide.

C. Sodium hydroxide-treated lipopolysaccharide The lipopolysaccharide of Vibrio fetus was treated with sodium hydroxide according to the procedure of MacPherson (61): 50 mg. of the purified antigen in 10 ml. saline solution was mixed with 45 ml. of 0.25 N NaOH; the mixture was kept in a water bath at 37°C for 3 hours and then neutralized with 45 ml. of 0.25 N HCl. The solution was then dialyzed, lyophilized, and stored at 4°C until used.

III. Preparation of an artificial lipoprotein complex

An artificial lipoprotein complex was prepared from Vibrio fetus lipopolysaccharide and a nontoxic, nonbacterial protein (bovine albumin[1]). Twice crystallized bovine albumin (200 mg.) and purified lipopolysaccharide (50 mg.) were dispersed in 75 ml. of distilled water. The pH was adjusted to 8.5 with 0.5 NaOH and the solution stirred at room temperature for 3 hours. The solution became less turbid during this period and assumed an opalescent appearance.

After adjusting the pH of the solution to 6.0 with 0.5 N HCl, an attempt was made to precipitate the protein by the addition of 5% TCA. Albumin was not thrown out of solution. Acetic acid was then added to a concentration of 1% (v/v) and the solution was placed in a boiling water bath for 1.5 hours in an attempt to split off and degrade the polysaccharide portion of the LPS-protein complex. During the hydrolysis, the solution lost its opalescence and a precipitate formed. This observation is similar to that noted during the isolation of lipid A using 0.1 N formic acid hydrolysis. The precipitate was centrifuged from the solution and saved for carbohydrate and lipid analyses. The solution was then dialyzed and lyophilized yielding 205 mg. of a white, fluffy hygroscopic material readily soluble in water.

Aliquots of the precipitate formed during hydrolysis (31.6 mg.) were used to determine total carbohydrate content by the method of Dubois et al. (23) using glucose as the standard. By this method the carbohydrate content of the precipitate was 90.5% while that of the lyophilized artificial

---

[1]Bovine albumin, Mann Research Laboratories, New York, N.Y.

complex averaged 1.8% on five samples, varying from 1.4% to 2.8%. No lipid could be extracted from the hydrolysis precipitate and this material was not soluble in water. The lyophilized artificial complex was stored at 4°C until used in the toxicity studies.

RESULTS AND DISCUSSION

Preparation of Lipopolysaccharide

I. Extraction of the complex

On the basis of preliminary experiments, the phenol-water extraction method of Westphal (128), as modified by Tauber and Russell (115), produced a preparation with the next to highest yield and lowest amount of protein and DNA contamination. Extraction with dimethyl sulfoxide gave a higher yield than with phenol-water, but this lipopolysaccharide preparation also contained a higher content of DNA (5 to 8%) relative to the phenol-water extracted lipopolysaccharide (1-2%). Protein and DNA contamination was also found to be higher in preparations extracted with pyridine-formic acid, aqueous ether, saline and water. These preparations all gave about the same yield as extraction with phenol-water.

From 50 g. aliquots of cells extracted routinely with phenol-water, an average of 21.8% crude lipopolysaccharide was obtained, based on the dry weight of the cells.

II. Purification of the complex

Crude lipopolysaccharide, prepared routinely by the aqueous-phenol extraction, was contaminated with nucleic acid, as indicated by its ultraviolet absorption spectrum, and by peptide components, as indicated by paper chromatography. To develop an adequate purification procedure, experiments were directed toward removing one or both of these contaminants.

High speed centrifugation, used in conjunction with sodium chloride and acetone precipitation, formed the basis for the routine purification of

crude lipopolysaccharide. This procedure, outlined in Figure 2, page 37, gave a white, fluffy, hygroscopic powder in yields of 5 to 8% based upon weight of the crude starting material. The purified preparations, readily soluble in water, were subjected to analysis for nucleic acid contamination by ultraviolet absorbance at 260 and 280 mμ and by analysis for deoxyribonucleic acid using the method of Webb and Levy (123) in which a specific purple colored complex is formed with DNA and read at 560 mμ. The 280 and 260 mμ absorbencies were 0.01 and 0.02 optical density units as contrasted with 0.8 and 0.9 obtained with the crude lipopolysaccharide. Analysis for DNA content indicated the presence of less than 1% DNA in the purified preparation.

The supernatant obtained after ultracentrifugation of the purified lipopolysaccharide was examined for a second lower molecular weight lipoplysaccharide, reported by Burton and Carter (17) and others (125, 20) to be present after isolation of the initial lipopolysaccharide. Only nucleic acid was found in this fraction as determined by chemical analysis for DNA, carbohydrate, and lipid. Results of immunodiffusion of the material against immune serum prepared from Vibrio fetus lipopolysaccharide were negative.

Preparation and Purification of Lipid A

I. Hydrolysis and extraction of crude lipid A

To obtain lipid A, free of polysaccharide, the purified lipopolysaccharides were subjected to mild acid hydrolysis; a one percent solution of the material in 0.1 N formic acid was heated under refluxing conditions for forty-five minutes. Preliminary experiments, using a number of hydrolytic reagents, indicated that 0.1 N formic acid gave the optimum results

in terms of yield and minimum degradation. Chloroform soluble extracts of 18 to 20% were obtained from the purified lipopolysaccharide.

II. Acetone fractionation

The chloroform soluble lipids were extracted repeatedly with hot acetone. Fractionation was completed by dissolving the acetone insoluble residue in a minimum volume of chloroform, then precipitating with acetone. In this manner free fatty acids were removed from acetone insoluble lipid A. In a typical preparation, 91% of the chloroform extract of the lipopolysaccharide was acetone insoluble; lipid A, therefore, comprises 16 to 18% of the purified lipopolysaccharide.

III. Column chromatography of crude lipid A

A. Organic Sephadex chromatography As with Sephadex preparations of the G series used with aqueous solvents, separations on organic Sephadex LH-20 are based mainly on molecular dimensions. To take advantage of this sieving effect, acetone insoluble lipid A was chromatographed to remove any remaining free fatty acids or other small molecular weight lipids, e.g. triglycerides, which may have been carried through the purification and lipid extraction procedures.

The major fraction of the material placed on the column (94-98%) eluted shortly after the holdup volume; a second minor fraction was eluted between 30 and 40 ml. later. The major fraction gave a typical infrared absorption spectrum for Vibrio fetus lipid A, as obtained in our previous studies (120), while the minor fraction showed a series of peaks typical of fatty acids. The major fraction was fractionated by silicic acid column

chromatography and the minor fraction was saved for esterification and gas chromatography analysis.

B. Silicic acid chromatography Lipid A from the Sephadex column was chromatographed on silicic acid to take advantage of two basic forms of interaction between lipids and silicic acid. Hydrogen bonding may occur through the Si=O, Si-O-Si and Si-OH groups of silicic acid. Electrostatic bonding may occur through proton transfer of the Si-OH group with proton acceptor groups (phosphate, sulfate and amino) of ionic lipids.

The lipid was dissolved in chloroform and applied to a column prepared from a mixture of silicic acid and celite (4:1) which had been activated by heating for twelve hours at 110°C. The column was eluted with successive portions of chloroform (100%), chloroform:methanol in the proportions 9:1, 8:2 and 7:3, respectively, and finally with 95% aqueous MeOH. The results of the chromatography are summarized in Table III, page 40, in the Experimental Methods section. Fraction II, the material eluted by chloroform methanol 9:1, constitutes 79% of the material eluted from the column. When fraction II was rechromatographed, more than ninety percent of the lipid applied was eluted by chloroform methanol (9:1).

IV. Qualitative examination of the silicic acid chromatography fractions

The major portion of the lipid applied to silicic acid was eluted in fraction II and is considered to be purified lipid A. It will be discussed below in the section on chemical analysis of lipid A-II.

The other fractions illustrate the type of impurities which can be removed by silicic acid chromatography. The infrared spectrum of fraction I shows that this fraction is chiefly fatty acids. Fractions III and IV

are qualitatively similar in composition to fraction II, as indicated by infrared spectra and by paper chromatography of the acid hydrolysates of these fractions. The difference between fraction II and either fraction III or IV is possibly one of fatty acid substitution as will be discussed below. The infrared spectra of fractions I and II are shown in Figure 3, page 42.

Evidence was obtained from paper chromatography of the acid hydrolysates that purified lipid A-II does not contain amino acids or glycerol. Nowotny (77) has speculated that amino acids could form a peptide linkage between lipid A and the polysaccharide moiety, and has found amino acids in lipid A isolated from Escherichia coli. As reported previously (120) four amino acids were identified in preparations of lipopolysaccharide from three strains of Vibrio fetus, including strain 925, the strain used in this study.

Fraction V was shown by hydrolysis studies to contain all of the ninhydrin positive components, other than glucosamine, present in crude lipid A. Fractions II and III on acid hydrolysis yielded one predominant ninhydrin positive component, which was also periodate and Elson-Morgan positive, as well as a limited number of less intense ninhydrin positive spots; this series of spots is also observed when glucosamine alone is chromatographed after treatment under the same hydrolytic conditions. These observations do not exclude the possibility that amino acids are present in lipid A; further discussion of this point is included in the section on chemical studies of lipid A-II below. The qualitative evidence indicates only that glucosamine can account for all the ninhydrin positive spots

observed on chromatograms of purified lipid A-II. No glycerol was detected in the acid hydrolysates of any of the lipid fractions.

Using a variety of solvent systems, thin-layer chromatography of the fractions gave evidence that fraction II is homogeneous; some slight tailing was observed, however, in one or two instances. Fractions III, IV and V were not separable into distinct spots in any of the systems tested. The tailing or smearing effect given by these fractions probably indicates their increased polar nature or possibly the attachment of several different fatty acids in various proportions to the core structure. Kasai (48) has shown a progressive decrease in R_f values on thin-layer chromatography of fractions from silicic acid chromatography of Escherichia coli lipid A; this decrease correlated to their elution from the column and hence their increasing polarity.

Results of Chemical Studies on Lipid A-II

I. General properties

The purified lipid A component (A-II) from the lipopolysaccharide is a clear, light brown, waxy solid which melts at 205°-208°C. The lipid is soluble in chloroform and pyridine; in other common laboratory solvents, such as methanol, ether, absolute ethanol, benzene, acetone and dimethyl sulfoxide, lipid A-II is completely insoluble.

II. Partial chemical characterization

A. Analytical data The analytical data for lipid A-II are summarized below:

N (%)	P (%)	N/P	Nonvolatile fatty acids (%)	Glucosamine (%)
1.76	1.96	1.99	62-65	20.3

FAE equivalents/mg.	FAA equivalents/mg.
1.6	1.3

Nonvolatile fatty acids consisted of the chloroform soluble material resulting from vigorous acidic hydrolysis of the lipid sample. More suitable photometric methods, such as that of Rapport and Lerner (90), for the determination of esterified fatty acids on a small scale were not applicable because of the solubility properties of the lipids.

A nitrogen to phosphorous ratio of 2 to 1 was found for lipid A-II as indicated in the table. The fatty acid ester and fatty acid amide values are expressed as equivalents of ester/mg. of lipid A-II. If a value for the molecular weight of lipid A (1700) as found by Burton and Carter (17) were assumed for lipid A-II from Vibrio fetus, a value of 4.93 equivalents of ester/mole can be calculated. Of this amount, 2.83 equivalents of FAE and 2.10 equivalents of FAA per mole of A-II are found.

Glucosamine was determined qualitatively and quantitatively as described in the Experimental Procedures section. The value of 20.3% is an average of five determinations which varied from 19.8% to 20.6%.

The glucosamine values for lipid A-II account for the nitrogen in the two samples within the limits of error of the determination.

Glucosamine	
Found (%)	Calculated from N (%)
20.3	22.5

This observation confirms two earlier results: that the purified lipids are free of amino acids, as indicated by paper chromatography; and that the hexosamine present in the lipids is glucosamine. In the first case, the results of the hexosamine determination, when combined with the almost exact ratio of 2 to 1 for nitrogen to phosphorous, are not consistent with the presence of any amino acid. In the second case, the original identification of the hexosamine in the lipids as glucosamine was made on the basis of paper chromatography. Paper chromatography is admittedly not completely satisfactory for positive identification; the possibility always exists that another substance, perhaps present in lower concentrations, may escape detection. In addition to paper chromatography, the high voltage fingerprinting technique of Ingram (41) as applied to amino sugars by Salton (101) also confirms the presence of only glucosamine in lipid A-II.

The quantitative data also confirm the identification of glucosamine. Strange (111) has reported that when galactosamine is determined by the Rondle and Morgan procedure, it gives eighty-three percent of the value which glucosamine gives under the same conditions. Muramic acid gives only thirty percent, while mannosamine gives a value of sixty-nine percent that of glucosamine. If part or all of the hexosamine present in lipid A were any of these sugars, rather than glucosamine, the hexosamine values found by the Rondle and Morgan procedure would be significantly lower than those obtained. The nitrogen content of the lipids does not allow the presence of more hexosamine than found. For example, if the hexosamine were mannosamine, the observed value of 20% would represent 29% hexosamine, corresponding to a nitrogen content of 2.26%. On the basis of the data and

because of the limits of error in the hexosamine determination, it is possible that half of the hexosamine is glucosamine, half galactosamine. However, the chromatographic methods would have detected galactosamine if present in amounts equal to glucosamine.

Since the preparation of lipid A from lipopolysaccharide depends upon acidic hydrolysis of the latter, one possibility for the linkage of lipid to polysaccharide is a glycosidic bond between the hexosamine in lipid A and the terminal sugar of the polysaccharide. In this case, the lipid should have a reducing end group. When the lipid fractions from the silicic acid chromatography were dissolved in pyridine and tested with the modified triphenyl tetrazolium chloride reagent (64), a deep purple-red color was observed for fractions II, III and IV, thus indicating they are reducing. The result for fraction V was uncertain; fraction I was negative, supporting previous data that this fraction is composed of fatty acids.

B. Component fatty acids The fatty acids present in lipid A-II and the minor fractions from silicic acid chromatography - I, III, IV, and V - were examined qualitatively. The methyl esters of the fatty acids, released from the lipids by alkaline hydrolysis, were prepared using methanolic boron trifluoride and were subjected to vapor phase and thin-layer chromatography. Part of the fatty acid ester preparation from lipid A-II was chromatographed on silicic acid to separate hydroxy and nonhydroxy fatty acid esters. The hydroxy fatty acid ester fraction constituted 27% of the lipid applied to the column while the nonhydroxy fatty acid ester fraction accounted for 65%.

1. Examination of methyl esters by vapor phase chromatography - The esters from A-II gave five predominant peaks corresponding to the methyl esters of myristic, palmitic, palmitoleic, oleic and β-hydroxy-myristic acids, respectively, and three minor, unidentified peaks. Because a reference standard was not available, the methyl ester of β-hydroxy-myristic acid was tentatively identified by comparison of the relative retention time obtained by Nesbitt and Lennarz (72) for their reference standard. A rough approximation of the relative amounts of the fatty acids present was made by measuring the area under each of the chromatographic peaks and calculating its percent of the total area observed. Such an estimation showed that 19% of the fatty acid from A-II is β-hydroxy-myristic acid, that approximately 58% are saturated fatty acids, that 16% is unsaturated fatty acid, and that the unidentified peaks amount to 7.4%. The vapor phase chromatography data are summarized in Table IV. Figure 8 shows the chromatography of A-II on the polar (DEGS) column.

The minor fractions obtained during silicic acid chromatography of the lipids may differ from purified lipid A-II in fatty acid substitution. To investigate this possibility, a comparison was made of the relative fatty acid content of fractions III and IV obtained during silicic acid chromatography. No qualitative differences were noted; however, fraction III does contain approximately 5% more hydroxy fatty acid than fraction II, based upon the measurement of peak areas. Such a difference may explain why a somewhat more polar solvent (chloroform:methanol, 8:2) was required to elute fraction III than that which was sufficient for elution of fraction II. Another possibility is that the minor fractions are less fully

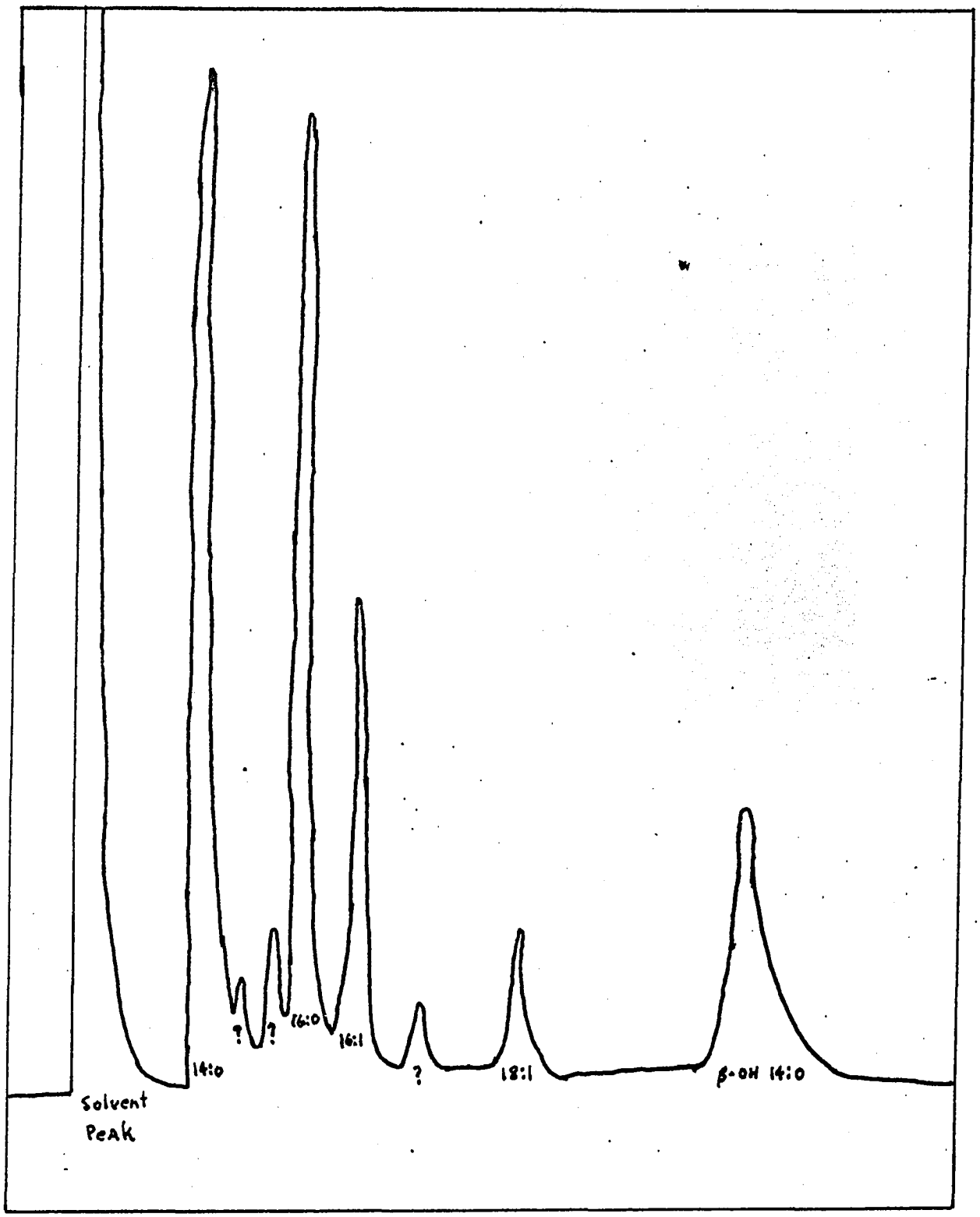


Figure 8. Vapor phase chromatography of the fatty acid methyl esters from fraction A-II. Chromatographed on diethylene glycol succinate absorbent.

Table IV. The fatty acid composition of lipid A fractions A-II, A-III, A-IV and A-V from silicic acid column chromatography

Fatty acid	Percent of total fraction*			
	A-II	A-III	A-IV	A-V
Myristic	31.5	34.8	24.6	22.4
Unknown #1	1.8	2.8	2.9	0.8
Unknown #2	3.0	3.1	4.8	0.7
Palmitoleic	10.3	11.4	17.7	12.0
Palmitic	26.1	15.8	17.6	13.7
Unknown #3	2.6	1.2	2.8	2.6
Oleic	5.8	6.7	7.9	11.7
β-Hydroxymyristic	19.0	24.1	21.7	15.2
Unknown #4	----	----	----	7.2
Unknown #5	----	----	----	10.9

Summary: Fatty acids of A-II

Saturated fatty acids 58%

Unsaturated fatty acids 16%

Hydroxy fatty acid 19%

Unidentified 7.4%

*These values represent an approximation of the relative amounts of each fatty acid present in the sample. To arrive at these values each peak was measured and its percent of the total area of the chromatogram calculated.

acetylated than fraction II; comparison of the infrared spectra shows less ester absorption relative to amide for these fractions than for fraction II. With more hydroxy substituents free in fractions III and IV than in fraction II, more polar solvents would be required to elute these fractions from silicic acid.

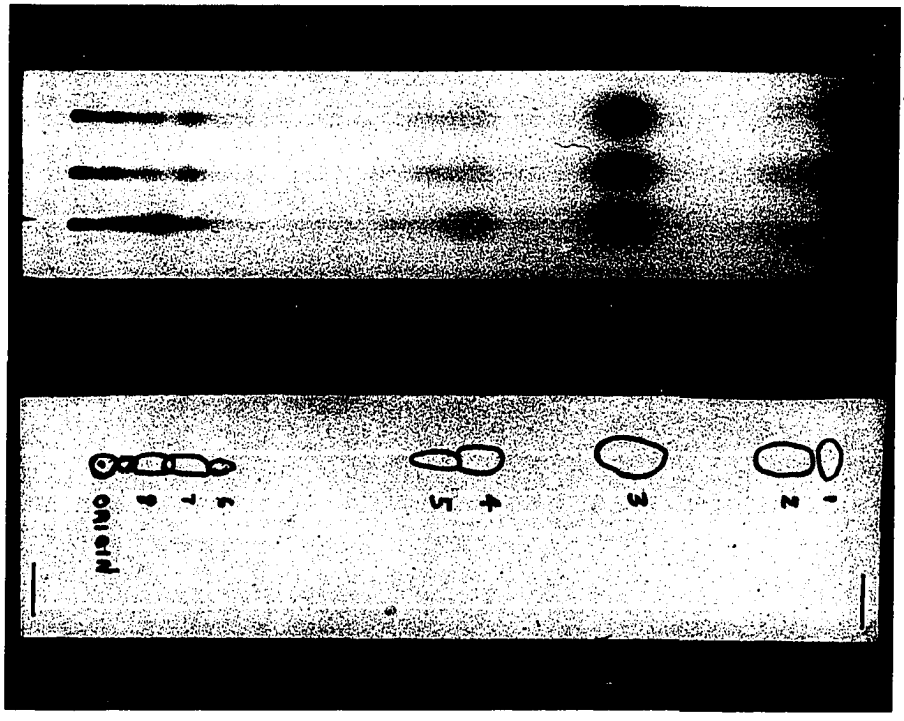
The hydroxy fatty acid ester fraction (obtained by chromatography of methyl esters from A-II on silicic acid) was subjected to vapor phase chromatography to determine if hydroxy fatty acids other than β-hydroxymyristic acid were present. The results, obtained under programmed temperature conditions, indicate the presence of only β-hydroxymyristate.

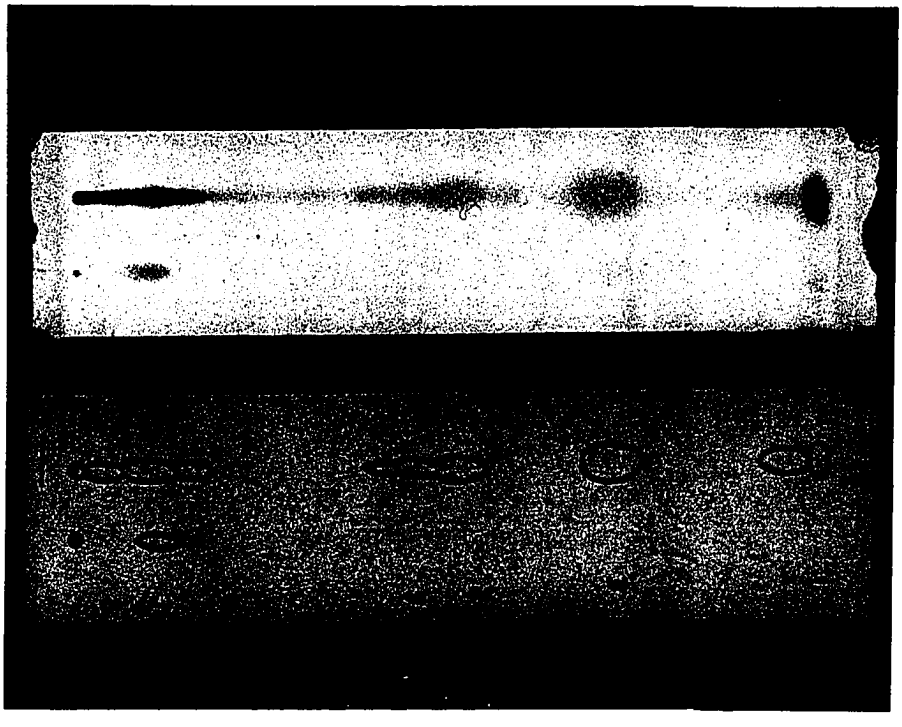
2. Examination of methyl esters on thin-layer chromatography - The methyl esters of fatty acids from fractions A-I, A-II and A-III were chromatographed on thin layers of silica gel. Eight components were observed in each fraction. The results shown in Plate 1 confirm the findings of vapor phase chromatography of the same preparations. Four of the spots were identified, with the aid of reference standards, as myristic, palmitic, palmitoleic and oleic acids. A reference standard for β-hydroxymyristic acid was not available. However, by chromatography with the preparation obtained from separation of hydroxy from nonhydroxy fatty acid esters of A-II, the spot was tentatively identified as β-hydroxymyristate. This result is shown in Plate 2 and it also confirms the result obtained by vapor phase chromatography - the presence of only one hydroxy fatty acid.

3. The possible presence of a neutral component - The unidentified peaks observed during the chromatography of the fatty acids from the lipids could be unsaturated fatty acids or aldehydes, compounds whose retention

Plate 1. Thin-layer chromatography of fatty acid methyl esters from fractions A-I, A-II, and A-III on silica gel.

Plate 2. Thin-layer chromatography of fatty acid methyl esters from fraction A-II and the hydroxy fatty acid fraction of A-II.


origin
+
-



times correspond to those observed for the unidentified peaks when chromatographed under similar conditions.

A qualitative test for vinyl ether, a possible source of aldehyde upon acid hydrolysis, was applied to lipid A-II according to the method of Norton (75). This test depends on the addition of mercuric chloride to the reactive double bond. It is specific for the vinyl ether linkage unless the reaction is allowed to proceed too long; in which case double bonds also begin to react with the reagent. In this test, the lipid samples were faintly positive after reaction with mercuric chloride for the thirty seconds prescribed by Norton, and gave a very positive test after one minute. An authentic reference standard, such as plasmologen, was not available for the determination; therefore, a positive test was determined by a deep purple spot against an unstained background as described by Norton (75).

Another reaction, more specific for the double bond of the vinyl ether, is the addition of iodine. Iodination of A-II, under conditions similar to those of Rapport et al. (89, 90), was attempted with unreliable results: precipitation of the lipid during titration of the reaction mixture made detection of the end point uncertain. However, the vinyl ether linkage is improbable because the linkage is stable to base, while the components giving rise to the minor peaks observed during vapor phase chromatography were released by alkaline hydrolysis.

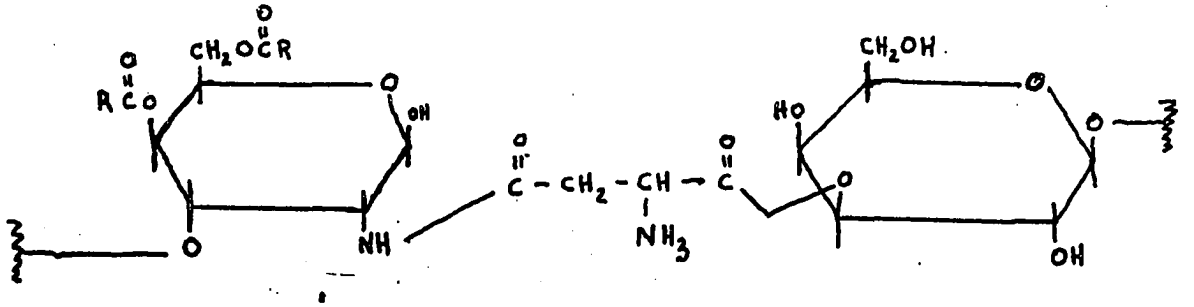
The alkaline lability of the unidentified compounds is puzzling in any case, if these compounds are in fact aldehydes. Acetals, for example, should be stable to base. The alternative explanation, that these peaks represent unsaturated fatty acids, was not fully investigated; an attempt

to measure unsaturation present in A-II by bromination was unsuccessful because a satisfactory solvent system for this reaction, in which the lipid was either soluble or finely suspended, was not found. However, the components giving rise to the unidentified peaks are present in such minor amounts as to suggest that they are artifacts. Chemical degradation of the β-hydroxymyristic acid by dilute alkali is certainly a possibility.

Results of Structural Studies on Lipid A

I. Determination of the linkage between lipid and polysaccharide moieties

A. Evaluation of a possible peptide linkage During our previous studies of Vibrio fetus lipopolysaccharide (120), strain 925, we detected the presence of four amino acids: aspartic acid, glutamic acid, alanine and phenylalanine. Paper chromatography of acid hydrolysates of lipopolysaccharide in this study confirmed the presence of the same four amino acids. This result raises the question whether they are contaminants carried through the purification procedure or whether they serve a function in the structure of the intact lipopolysaccharide. The limited spectrum of amino acids observed, with half of them bifunctional, suggested the possibility that amino acids may link lipid A to polysaccharide. One such linkage is illustrated below, with aspartic acid serving to join the glucosamine of lipid A to the polysaccharide moiety through an amide-ester linkage.



Glutamic acid could function similarly, or a peptide composed of all four amino acids could link lipid and polysaccharide. One alternate possibility is that amino acids may function, in ester bonds, as links between chains of the lipopolysaccharide: possibly between hydroxyl groups of sugar residues or between hydroxy fatty acids of separate chains.

To investigate the possibility of an amino acid link between lipid A and the polysaccharide, intact lipopolysaccharide was subjected to mild alkaline hydrolysis which would be expected to cleave ester bonds but would leave intact amide and glycosidic bonds. The hydrolysis was carried out with 0.2 N NaOH for 24 hours at 37°C; the solution was then chilled and carefully acidified before extraction with chloroform. Because the hydrolysis of fatty acid esters would certainly have changed the solubility properties of lipid A, the aqueous solution, after chloroform extraction and neutralization, was dialyzed and the dialysate examined for the presence of glucosamine and amino acids.

More than 80% of the lipopolysaccharide was recovered in non-dialysable form. From 5 to 12% was liberated as chloroform soluble material; its infrared spectrum showed strong carboxyl absorption, no amide absorption and absorption typical of that for fatty acids. Analysis of this fraction showed a nitrogen content of 0.10% and negligible phosphorous content. The dialysate contained several ninhydrin positive components, including the four amino acids. None of these components, however, gave a typical color with the Elson-Morgan reagents or with periodate, thus indicating that glucosamine was not released during this hydrolysis. These results are represented in Figure 9.

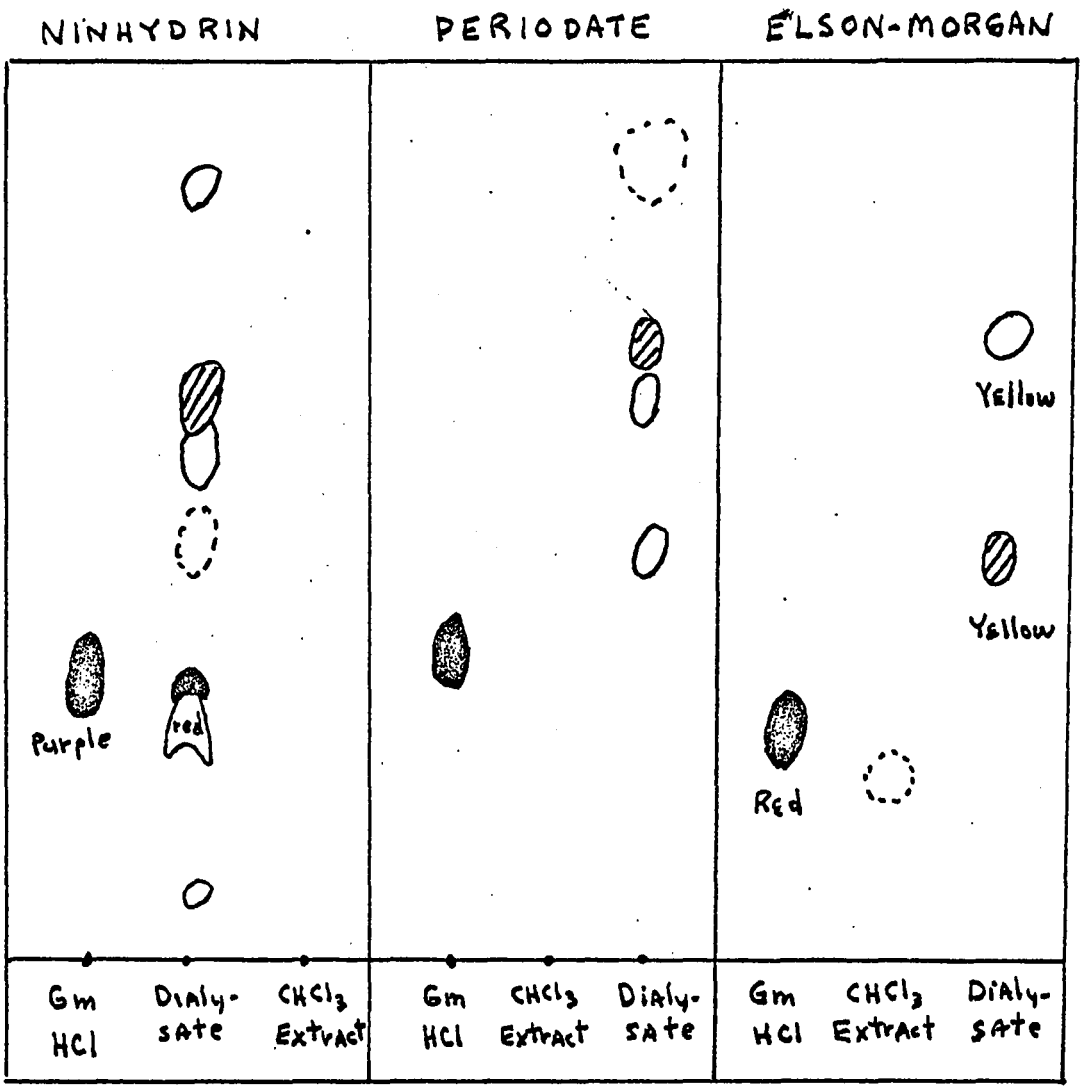


Figure 9. Paper chromatography of the products of mild alkaline hydrolysis of purified lipopolysaccharide.

The results of the experiment indicate that amino acids do not link lipid to polysaccharide in Vibrio fetus lipopolysaccharide, a possibility suggested by Nowotny (77) for Escherichia coli lipopolysaccharide; however, these acids are present either as esters or as contaminants which have been carried through the purification process.

B. Analysis of lipopolysaccharide and lipid A for KDO (2-keto-3-deoxyoctonoate) The keto acid, 2-keto-3-deoxyoctonoate (KDO) was first discovered in lipopolysaccharide of Escherichia coli by Heath (36). Osborn (83) presented evidence suggesting that the polysaccharide moiety consists of heptose phosphate chains terminating in KDO, which, at the reducing end of these chains, functions as the lipid-polysaccharide linkage group. Similar evidence for the function of KDO has been found in Salmonella typhimurium lipopolysaccharides (37), and Kasai (48) isolated lipid bound KDO by mild acid hydrolysis from lipopolysaccharide of Escherichia coli. All of these studies point to KDO as the terminal component of the polysaccharide to which the lipid A moiety is attached. On the assumption that a similar structural link may be present in Vibrio fetus lipopolysaccharide, both lipid A-II and the purified lipopolysaccharide were assayed for KDO.

Qualitative analysis by paper chromatography revealed the presence of one spot for lipid A-II with the same R_f value as authentic KDO. Both lipopolysaccharides gave three spots, one of which was identified as KDO. The other two components are probably deoxyhexoses, which are known constituents of the polysaccharide moieties of a variety of lipopolysaccharides. The results of qualitative analysis for KDO are represented in Figure 10.

The sample preparations of lipid A-II and lipopolysaccharides were also assayed for KDO by the periodate-thiobarbituric acid reaction of Aminoff (3). The absorption curves obtained for the standard lipid A-II and LPS preparations are shown in Graph 3. The absorption peaks at 549 mμ, which corresponds to that obtained with the standard, are presumptive evidence for the presence of KDO in both lipid A-II and the intact lipopolysaccharide. An additional peak at 452 mμ was observed for the lipopolysaccharide from Escherichia coli which was not found in the Vibrio fetus preparation. This peak is probably a KDO derivative, perhaps KDO-heptose-phosphate, which forms a similar complex with thiobarbituric acid. Other deoxyhexoses not present in the Vibrio fetus lipopolysaccharide could also account for this absorption. Based upon analysis in triplicate for KDO, 1.4% of the lipopolysaccharide and 0.20% of the lipid A-II consists of KDO. The result obtained for the lipopolysaccharide is probably high since the deoxyhexoses found in the lipopolysaccharides (paper chromatography) are also being measured in this reaction.

The fact that KDO was found in both lipid A-II and lipopolysaccharide suggests that this residue may function as the linking group between lipid and polysaccharide. Since no conclusive structural analysis of heptose-phosphate-KDO-lipid A derivatives was attempted, the evidence remains presumptive only.

II. Determination of the linkage between glucosamine residues

A. Possible structures for lipid A The essential features of the structure of lipid A with which this study is concerned are the linkages between lipid and polysaccharide (discussed previously) and between

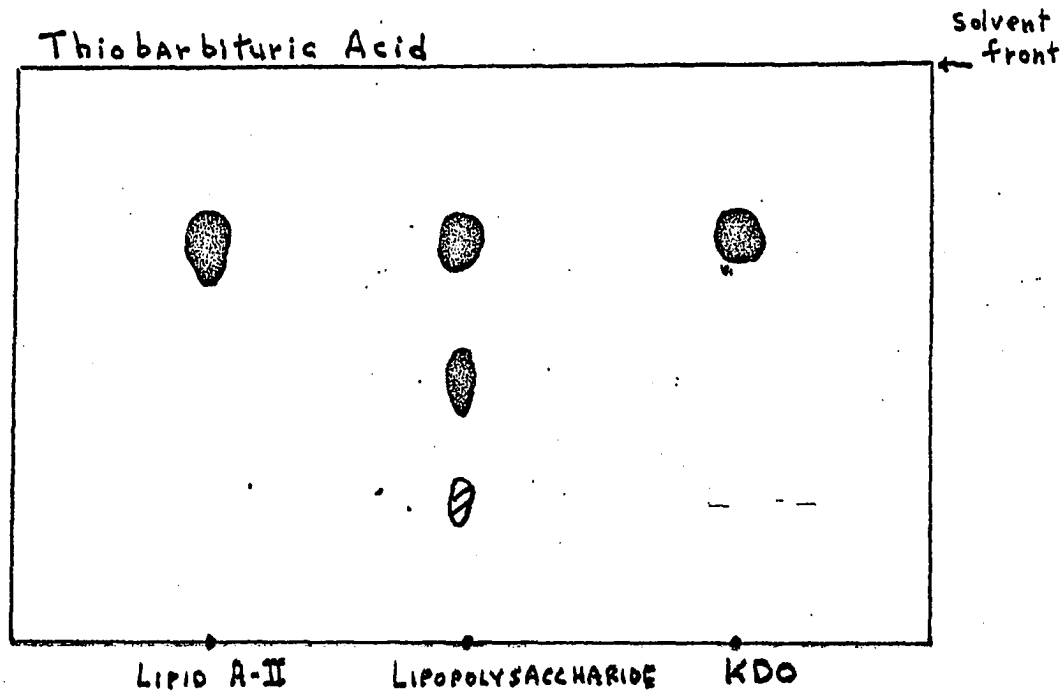
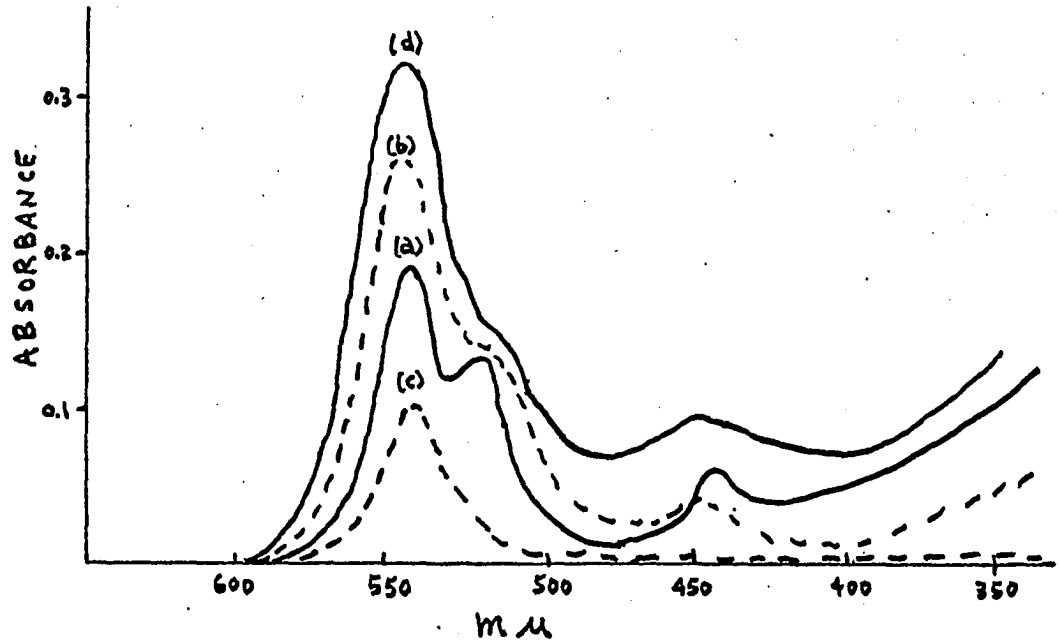
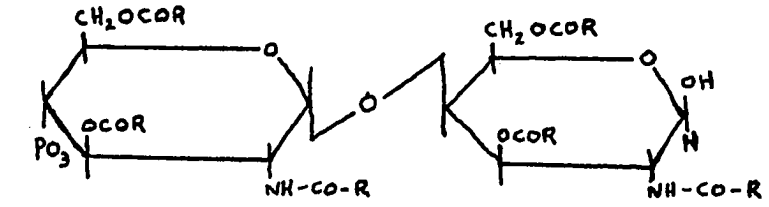


Figure 10. Paper chromatography of the acidic hydrolysates of lipid A-II and lipopolysaccharide for detection of KDO.

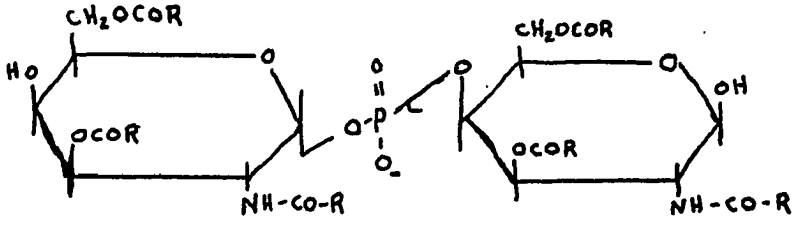


Graph 3. Absorption curves of the products of the periodate-thiobarbituric acid reaction. (a) Escherichia coli LPS, (b) Vibrio fetus LPS, (c) Lipid A-II, (d) KDO standard.

glucosamine molecules. Equally possible are a glycosidic bond and a phosphate diester as illustrated by the following structures:



Glycosidic Linkage



Phosphodiester Linkage

In both of these structures, R represents the possible locations of acetyl or long-chain fatty acids. Acetyl groups may also be esterified with the hydroxyl groups of β-hydroxymyristic acid.

A molecular weight based on either of these unit structures has been calculated. Table V compares this molecular weight, as well as the calculated percent of each component present, with the analytical data found for A-II.

Table V. Analytical data for lipid A-II

	Calculated	Found
Molecular weight	1680	-----
Fatty acids (5)	68%	62-65%
Nitrogen	1.67%	1.76%
Phosphorous	1.85%	1.96%
Glucosamine	21%	20.3%

Table V (Continued)

	Calculated	Found
FAE, equivalents/mole	4.0[1] or 5.0[2]	2.8[4]
FAA, equivalents/mole	2.0[3]	2.1[4]

[1]Based upon glycosidic linkage.

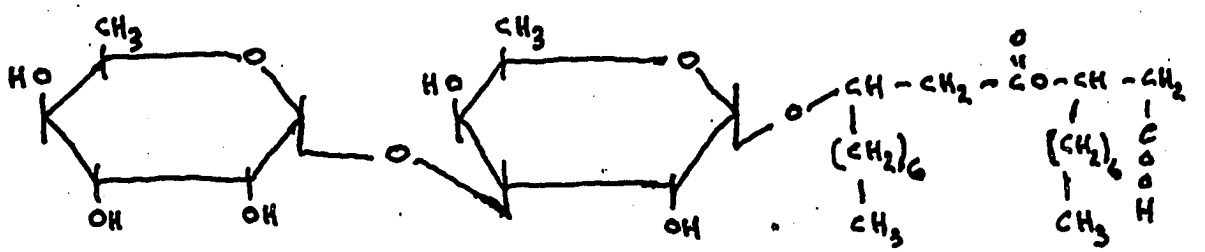
[2]Based upon phosphodiester linkage.

[3]Based upon either glycosidic or phosphodiester linkage.

[4]Based upon a calculated molecular weight of 1680.

This comparison of the data suggests that the core structure of lipid A is composed of two fully acylated glucosamine molecules. Changes in the position of the glycosidic bond or of the phosphodiester, respectively, allow a number of variations of the two basic possibilities of linkage. A glycosidic bond may join one glucosamine molecule to the 3-, 4-, or 6-position of the second glucosamine molecule. The unusual stability toward acid hydrolysis of the glycosides of glucosamine and of N-acetyl glucosamine, as compared with the glycosides of glucose, has long been recognized (42, 68, 25). The mild acidic conditions used in the preparation of lipid A (0.1 N formic acid for 45 minutes at 100°C) would not be expected to degrade much of the glycosidic linkage, if the glucosamine units in lipid A are joined in this manner. Also possible is a glycosidic bond between a glucosamine molecule and the hydroxyl substituent of β-hydroxymyristic acid, the carboxyl function of which could form either an ester or an amide with a second glucosamine molecule. Such a possibility

is suggested by the structure of a glycolipid produced by Pseudomonas aeruginosa (43), in which rhamnose and β-hydroxy decanoic acid are joined glycosidically. The following structure has been proposed for this glycolipid:



This compound also shows considerable resistance to acid hydrolysis (43).

In the case of a phosphate diester, the linking may be between the 4-position of one glucosamine molecule and the 3-, 4-, or 6-position of a second glucosamine molecule. Linkage may also occur between the 1-position of a glucosamine molecule and the 4-position of another; or the 4-position of one glucosamine molecule may be linked with the hydroxyl function of β-hydroxymyristic acid, a structure which again could form either an ester or an amide with a second glucosamine molecule. The primary, and probably the secondary, phosphate esters show enough resistance to acid hydrolysis (62) to have survived the conditions of preparation of lipid A. Using 6-phosphoglucosamine only 1-2% of the phosphorous was released after 30 minutes hydrolysis at 100° in 1 N HCl. A linkage involving the 1-position, however, is not likely because N-acetyl glucosamine 1-phosphate, as shown by Leloir and Cardini (57), is unstable to 1 N HCl at 37°C and is hydrolyzed in 10 minutes in 1 N sulfuric acid under refluxing conditions (58).

To distinguish between the two basic possibilities of linkage within lipid A, two chemical approaches are especially applicable. The first of these, alkaline degradation under mild conditions, should preserve glycosidic linkages, while causing degradation of carboxyl esters and possibly of phosphate diesters, though some degradation of the reducing amino sugar can also be expected.

The second approach, sodium borohydride reduction of the lipid, allows conversion of all the free carbohydrate aldehyde to alcohol, with possibly some concomitant reduction of the esters. Characterization of the products following acid hydrolysis of the reduced lipid should distinguish between glycosidically linked structures and those in which both glucosamine molecules are reducing.

B. Alkaline hydrolysis of lipid A When lipid A-II was hydrolyzed under mild conditions (1 N NaOH in MeOH at room temperature for 4 days), 63% was recovered as long-chain fatty acids; ammonia amounting to 6.2% of the nitrogen originally present in the reaction mixture was also released; 10.4% was insoluble in water and chloroform, and soluble in pyridine; about 6% was recovered as unchanged lipid A.

The evolution of ammonia indicates some hydrolysis of amides, possibly due to the duration of the reaction: the hydrolysis was prolonged because of the insolubility of the initial lipid in methanol. The result of the triphenyltetrazolium chloride test - that lipid A is reducing - is substantiated by the release of ammonia, since reducing amino sugars evolve considerable ammonia under the same conditions.

The amount of fatty acid released is comparable to the total non-volatile fatty acid content of lipid A (60-65%). About one-third were hydroxy fatty acids as shown by vapor phase chromatography. These results imply that hydroxy fatty acids are not glycosidically bound in lipid A; since some amide bonds were cleaved, this experiment does not give information on the relative amounts of N-acyl and N-acetyl substitution.

The moiety of limited solubility (only soluble in pyridine) was found to contain a nitrogen to phosphorous ratio of 2.6. Though insoluble in water, this material dissolved in 2 N NaOH to give a deep yellow colored solution. This fraction exhibited strong amide and no ester absorption in the infrared and is considered to be the degraded glucosamine core of lipid A.

C. Sodium borohydride reduction Samples of A-II were subjected to sodium borohydride reduction in absolute ethanol. After 72 hours reaction at room temperature, with occasional heating to 70°C, the mixture was a clear orange solution. Four distinct fractions were obtained from the solution: (1) a chloroform soluble oil (42.8%), (2) a water soluble fraction (1.1%), (3) a water insoluble fraction (31%), and (4) a fraction only soluble in pyridine (24.3%).

Analytical data, including nitrogen, phosphorous and glucosamine content, were obtained where possible for each of the fractions. These data are summarized together with calculations based upon them in Table VI. (These data were obtained on small samples and, in most cases, were single

determinations only. Such limiatations must be kept in mind when considering the implications of the data.)

Table VI. Products of reduction of A-II

Fraction	N	P	N/P	Total N (%)	Glucosamine %	GM found / GM calcu.
1	0.02	0.03	2	1.2	--	----
2	----	----	---	----	--	----
3	1.93	2.10	2.1	34	13	52.9
4	2.8	3.1	1.9	25	16	45.0

In Table VI the percent total nitrogen is the amount of the nitrogen - originally present in the reaction mixture - which is found in the respective fractions. The calculated glucosamine value is based on nitrogen content.

Fraction 1 showed considerable hydroxyl and marked ester absorption in the infrared, while little or no amide absorption was apparent. The presence of low amounts of nitrogen in this fraction is possibly due to some degradation of the chief products of the reductions.

In view of the limited water solubility of fraction 3, fraction 2 and 3 are probably similar material differing slightly in fatty acid substitution. These fractions when tested with the triphenyltetrazolium chloride reagent gave a negative test, indicating complete reduction. They were also slightly ninhydrin positive, the result of some hydrolysis of amide which probably occurred during the removal of borate from the

reduction products (repeated evaporation of acidic methanol at room temperature). Fraction 2 was too small (2.8 mg.) to allow glucosamine determination, but such determination is obtained for fraction 3. When the amount of glucosamine found in fraction 3 is compared with the possible glucosamine content calculated from the percent nitrogen in these fractions, it is apparent that approximately half of the amino sugar is still present as glucosamine, while the other half is presumably glucosaminol since the latter does not react with the Elson-Morgan reagents. Fractions 2 and 3 represent about 35% of the total nitrogen originally present in the reaction mixtures. Their limited water solubility, compared with the insolubility of fraction 4, indicates that the relationship between these fractions and fraction 4 is probably one of fatty acid substitution, in particular, amide substitution. The fact that fractions 2 and 3 are slightly ninhydrin positive suggests N-acetyl substitution.

Fraction 4, accounting for 25% of the total nitrogen, is the main nonfatty acid product of the reduction. This fraction exhibited only amide absorption in the infrared. When tested with the triphenyltetrazolium chloride reagent, fraction 4 was negative. The ratio of glucosamine, calculated from the percent nitrogen in this fraction, to that actually found indicates that 45%, or approximately half, of the expected glucosamine is present. The remaining 55%, as with fraction 3, is presumably present as glucosaminol. A satisfactory solvent system was not found for the separation of acid hydrolysates of N-acetyl glucosamine and N-acetyl glucosaminol. Therefore, analysis of these fractions by chromatography on borate-treated paper proved to be unsatisfactory.

The results of the reduction and alkaline degradation studies of lipid A (performed on a semi-micro scale) clearly indicate that two glucosamine molecules are linked glycosidically, since after complete reduction approximately half the amino sugar present is still glucosamine. The apparent stability of the reduced unit to alkaline hydrolysis, if confirmed by isolation and characterization of the disaccharide, would indicate that the glycoside joins two glucosamine molecules without involving a hydroxy fatty acid; either an amide or an ester of the latter with a second glucosamine molecule should cleave under the conditions of alkaline hydrolysis.

A phosphate diester linkage does not seem likely because two glucosamine molecules so joined should be completely reduced to glucosaminol, in which case a nonreducing unit should contain no glucosamine. Such a unit should also cleave under alkaline conditions.

Results and Discussion of the Toxicity Studies on Lipopolysaccharide, Lipid A and Lipoalbumin Preparations

One of the purposes of this investigation was to obtain information on the relationship of lipid A to the toxic effects of lipopolysaccharide antigen, detected in our previous studies (120) with this strain. In addition to assaying the toxicity of purified and crude lipid A directly, an artificial lipoprotein was prepared and assayed. It was composed of lipid A and a nonbacterial, nontoxic protein. In this manner, the lipid moiety was assayed for its toxic effect in the absence of the polysaccharide moiety.

The intact lipopolysaccharide complex was also subjected to chemical alterations, using mild sodium hydroxide and the enzyme papain, in an attempt to split the fatty acid ester linkages of lipid A. In addition, lipopolysaccharide was oxidized with periodate, a procedure effecting changes only in the polysaccharide moiety. These preparations were then assayed for loss of toxicity relative to intact lipopolysaccharide.

I. Preparation of chemically altered lipopolysaccharide and lipid A

The artificial lipoprotein was prepared by coupling lipopolysaccharide to twice crystallized bovine albumin forming a complex which in turn could be split with dilute acid into degraded polysaccharide and artificial lipoalbumin. This procedure is shown schematically in Figure 11. The coupling was accomplished at pH 8.0 and the solution was then acidified to pH 6.0. The addition of 5% TCA at this point did not throw albumin out of solution as would be the expected if it were free in solution. So it can be argued that there is some artificial complex formation. If this protein, lipid and polysaccharide complex (which may be taken as a model of the whole endotoxic complex) is treated with dilute acetic acid, then the polysaccharide is split off in the degraded state, and an artificial lipoprotein containing only 1.4 to 2.8% carbohydrate is obtained. The preparation, freely soluble in saline, was tested for toxicity in concentrations up to 1200 µg/mouse.

Treatment of lipopolysaccharide with NaOH resulted in degradation of the lipid. Fatty acids and a ninhydrin positive substance, identified as hexosamine, were released by mild alkaline hydrolysis (0.25 N NaOH, 3 hours at 37°C). The reaction product still contained lipid which could be

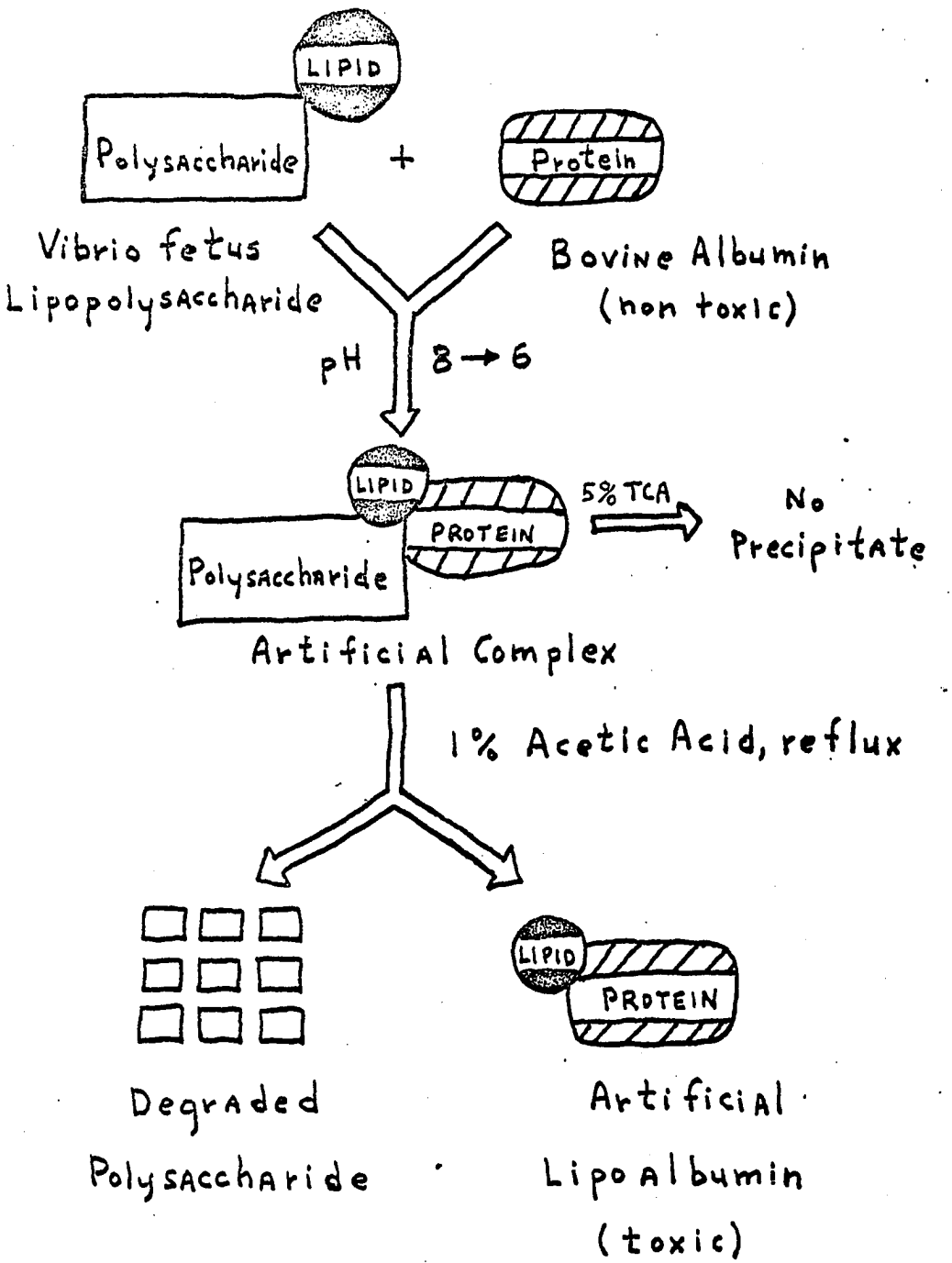


Figure 11. Schematic diagram illustrating the preparation of artificial lipoprotein complex.

liberated and precipitated with 0.1 N formic acid. Treatment with NaOH appears, therefore, to change the lipid qualitatively by splitting off fatty acid residues. To ascertain the effects of NaOH degradation, samples of the NaOH-treated lipopolysaccharide were tested for toxicity.

Treatment of intact lipopolysaccharide with the enzyme papain did not result in release of measurable fatty acid residue. This enzyme, capable of splitting both ester and amide bonds (52) in proteins, apparently does not function in like manner toward the long chain fatty acid esters and amides linked to glucosamine in lipid A. Despite the apparent lack of chemical alteration, a moderate loss of toxicity was observed for the papain preparation, a result which will be discussed in the next section.

Periodate oxidation of lipopolysaccharide caused a complete loss of serological O antigen specificity as shown by immunodiffusion tests. It also resulted in oxidative cleavage of sugar units as determined by titration of free aldehyde groups. Since the oxidized polysaccharide component remains chemically bound to the complex, the molecular weight remains essentially unchanged. There is no apparent effect of periodate treatment on the lipid A component; neither fatty acids or glucosamine were released. Any observed loss of toxicity from this preparation should therefore be attributable to changes in the polysaccharide moiety.

II. Toxicity of the preparations

The toxicity of the lipopolysaccharide, lipid A and polysaccharide moieties and artificial lipoprotein were determined in male mice. Three days after the inoculation the number of surviving animals was determined

and the LD_{50} (lethal dose for 50% of the animals) was calculated according to the method of Miller and Tainter (66). In Table VII are recorded the mean LD_{50} values obtained in these experiments.

The results shown in Table VII are summarized and discussed below with respect to each preparation.

A. Mild sodium hydroxide treatment resulted in the complete loss of lethal effects for preparations of the lipopolysaccharide tested in concentrations up to 800 µg/mouse. At 1000 µg, death occurred in 10% of the animals tested. Above this concentration the sample was not completely soluble and was not tested further.

B. Treatment of lipopolysaccharide with active papain resulted in an LD_{50} of 400 µg as compared with 300 µg for the controls and 267 µg for untreated lipopolysaccharide. Interestingly, the control preparations gave an LD_{50} 33 µg higher than untreated lipopolysaccharide. This could be attributed to the effect of the EDTA present in the reduced buffer. Gray and Wilkinson (32) have recently examined the effect of EDTA on the cell walls of several Gram-negative bacteria; they report that EDTA exerted a potent bactericidal action against these organisms by solubilizing the phosphorous and carbohydrate components of the cell walls, thus destroying their structural integrity. Similar effects could account for the lower toxicity of the control preparations, as well as exerting an effect on the active papain-treated lipopolysaccharide.

C. Periodate-treated lipopolysaccharide was nontoxic in concentrations up to 1000 µg while the periodate control gave the same LD_{50} as untreated lipopolysaccharide. As discussed in the previous section, periodate

Table VII. Toxicity of lipopolysaccharide, lipid and artificial lipoprotein preparations

Preparation	LD_{50} (μg/mouse)
LPS-Untreated	267
Endotoxin-*Escherichia coli*	375
LPS-Dimethylsulfoxide prep.	Nontoxic at 1000 μg
LPS-NaOH treated	Nontoxic at 800 μg; 10% deaths at 1000 μg
LPS-NaOH control	267
LPS-Papain (active)	400
LPS-Papain (inactive)	300
LPS-Papain (buffer only)	300
LPS-Periodate	Nontoxic at 1000 μg
LPS-Periodate control	267
Polysaccharide moiety	Nontoxic at 3000 μg
Lipoalbumin	1,067
Crude lipid A	5,900
Fraction A-II	5,900

	2.5 mg dose	Deaths / # Tested	5.0 mg dose	Deaths / # Tested
Fraction A-III		2/4		3/3
Fraction A-IV		2/3		3/3
Fraction A-V		3/4		3/3

treatment results in cleavage of sugar residues without apparent loss of the oxidized polysaccharide components from the complex, and it also results in loss of serological O antigen specificity. Structurally intact polysaccharide appears, therefore, to be necessary for expression of *in vivo* toxicity. The oxidation of a certain portion of the sugar residues may prevent the polysaccharide from functioning as an effective water soluble carrier for the lipid A moiety or from binding to sites on susceptible cell surfaces. An alternate explanation could be that toxicity is dependent upon a specific conformation of the polysaccharide chains. The destruction of such a conformation by periodate oxidation would result in an inactive preparation.

D. The polysaccharide moiety, recovered after mild acid hydrolysis and precipitation of lipid A, was nontoxic in concentrations up to 3000 μg. Similar results were found in our previous studies (120) with *Vibrio fetus* lipopolysaccharides.

E. The artificial lipoalbumin was toxic at a relatively high concentration - an LD_{50} of 1067 μg compared to 267 μg for the untreated lipopolysaccharide. Thus, our best preparation of the lipoprotein was of the order of one-fourth the activity of the original material, whereas the degraded polysaccharide is quite inactive. From these data it can be argued that the lipid component is the most important moiety for toxic activity, while the polysaccharide functions as a water-solubilizing carrier, the function of which may be taken over by some otherwise biologically inert protein.

F. As the results of Table VII indicate, lipid A, while still toxic, is not nearly as potent a toxin as the entire lipopolysaccharide complex.

The LD_{50} value of 5900 µg for both crude and purified lipid A is 22 times higher than that of the lipopolysaccharide. The limited solubility of lipid A may have an effect on its toxicity; Westphal (128) found a correlation between toxicity and the dispersion of lipid A in water: ultrasonicating preparations of lipid A from Escherichia coli increased by five-fold the toxicity of these preparations.

It is interesting to note that purification of lipid A did not alter its toxicity. Apparently lipid A, to exert maximum toxic effect, must be bound to a larger complex such as polysaccharide or protein. Whether the polysaccharide functions simply as a water soluble carrier or exerts an active effect, such as attachment of the complex to cell surfaces, is open to question. It can, however, be argued from results in Table VII that lipid A is in some way essential to the toxicity of Vibrio fetus lipopolysaccharide.

III. Possible roles of lipid A in toxicity of lipopolysaccharide

A. A direct role The observations concerning the toxicity of Vibrio fetus lipopolysaccharide suggest that the toxic effects are probably not associated with the polysaccharide and antigenic side chains, but seem to be related to their O-acetylated constituents. The loss of ester and amide bound fatty acids correlates with the loss of toxicity of the lipopolysaccharide. This could mean that a certain unique fatty acid or lipid is present which is toxic by itself and which represents the actual toxophore group. Our results do not tend to confirm this idea, although as mentioned previously, the limited solubility of lipid A in aqueous media may prevent its adequate testing. It must also be mentioned that it has

never been possible to obtain 100% recovery of the lipopolysaccharide by totaling all identified compounds. This indicates that a certain percentage of unknown or unidentified compounds exist in most lipopolysaccharide preparations.

B. Indirect roles

1. It is possible that lipid A may function as a stabilizer for a specific "toxic conformation": a specific steric configuration which is responsible for eliciting some toxic effects. Once these fatty acids are removed, the preparation, although still retaining the actual chemical groups responsible for toxicity, does not have the proper steric configuration necessary for eliciting the reactions. Perhaps this can best be explained by an unfolding or uncoiling of the particles.

2. Fatty acids of lipid A might afford a considerable stability toward the endotoxin-target cell interaction through apolar bonding with lipophillic components of the target cell. Without these fatty acids only a loose association might ensue, which would allow the lipopolysaccharide to be more easily dissociated from the target cell.

3. Fatty acids might provide a nonpolar envelope which inhibits catabolic hydrophillic enzyme systems from degrading or eliminating lipopolysaccharide.

4. Lipid A may provide, through mediation of their fatty acids, a passage through lipophillic membranes where they gain access to target areas within the cells.

In light of the chemical and biological heterogeneity of lipopolysaccharide preparations, the possibility of finding a specific chemical

component responsible for all the toxic manifestations may be fairly remote. The toxicity may be produced by the cumulative effects of a series of reactions, mediated by a complex array of both physically and chemically distinct components, in which lipid A is an essential constituent.

SUMMARY

In our previous studies (120), preliminary immunodiffusion experiments indicated the presence of an antigen common to the cell wall material of several strains of Vibrio fetus. This antigen was isolated and chemically characterized as a lipopolysaccharide.

In this study, a series of experiments were undertaken to determine the chemical composition and toxicity of the lipid moiety of lipopolysaccharide extracted from Vibrio fetus.

Lipopolysaccharide from virulent strain 925 of Vibrio fetus was prepared by extraction of the cells with 45% aqueous phenol and purified using acetone-sodium chloride precipitation in conjunction with ultracentrifugation. The purified product was devoid of protein and nucleic acid contaminants as shown by ultraviolet absorption spectra and chemical analyses for protein and nucleic acid.

After a comparative study of the yield and amount of degradation obtained using different hydrolytic methods, 0.1 N formic acid was chosen as the hydrolytic method for the preparation of lipid A from the purified lipopolysaccharide. The extraction mixture, containing free fatty acids and crude lipid A, was purified by column chromatography on Sephadex and silicic acid. These steps resulted in the recovery of 79% of the crude mixture.

Purified lipid A was characterized chemically using qualitative and quantitative procedures. Fatty acids, constituting 60-63% of lipid A, were extracted with acetone following alkaline degradation. Their methyl esters were prepared and analyzed by thin-layer and vapor phase chromatography. Five long-chain fatty acids were identified in the following amounts:

myristic (31.5%), palmitic (26.1%), palmitoleic (10.3%), oleic (5.8%), and ß-hydroxymyristic (19.0%). Three to four unidentified components, constituting 7% of the total, were also noted on both thin-layer and gas chromatograms.

The core structure of lipid A was analyzed by paper chromatography techniques and high voltage electrophoresis of acid hydrolysates. Polyglucosamine containing 1.96% phosphorous was identified as the core material. Quantitative analysis revealed that glucosamine constitutes 20.3% of the lipid A moiety. This value accounts for all the nitrogen content of the lipid within the limits of error of the determination. Analysis of lipid A for fatty acid ester and amide content indicates that the hydroxyl groups of glucosamine are fully acylated with long chain fatty acids or acetyl groups.

Structural studies on lipid A using sodium borohydride reduction and alkaline degradation have shown that glucosamine molecules in the core structure are linked glycosidically. Approximately equal quantities of glucosamine and glucosaminol, accounting for all the nitrogen present, were recovered upon reduction with sodium borohydride, whereas alkaline degradation did not extensively alter the lipid A core. The bifunctional nature of the amino acids, aspartic and glutamic, found in the intact lipopolysaccharide suggested that a peptide component could serve as a linkage between lipid and polysaccharide moieties. An experiment using 0.2 N sodium hydroxide under mild conditions, designed to split such a linkage, did not result in degradation of the lipopolysaccharide into lipid and polysaccharide components.

The component linking lipid and polysaccharide in other Gram-negative organisms, 2-keto-3-deoxyoctonoate, was also identified in lipid A from Vibrio fetus lipopolysaccharide by paper chromatography. Quantitative analysis indicated approximately 1% 2-keto-3-deoxyoctonoate in lipopolysaccharide and 0.2% in lipid A.

Chemically modified preparations of lipopolysaccharide from Vibrio fetus were tested for toxicity in mice. Lipopolysaccharide, when modified by treatment with 0.2 N sodium hydroxide or degraded with papain, resulted in a loss of fatty acids and small amounts of glucosamine. Complete loss of toxicity occurred in the sodium hydroxide treated lipopolysaccharide and also in lipopolysaccharide partially oxidized with 0.1 N periodate. Papain treatment resulted in a moderate loss of toxicity.

The toxicity of the lipid A and polysaccharide moieties was also tested in mice. The polysaccharide was nontoxic, while lipid A resulted in an LD_{50} of 5900 µg compared with 260 µg for the native lipopolysaccharide. An artificial lipoprotein which was prepared from lipopolysaccharide and bovine albumin - a nontoxic, nonbacterial protein - was one-fourth as toxic as the native lipopolysaccharide. This preparation contained only 1-2% residual carbohydrate and all of the lipid from the lipopolysaccharide preparative material. The toxicity results indicate the polysaccharide moiety may function as a water soluble carrier for lipid A, which is involved either directly or indirectly in the toxic manifestation. Whether the toxic lipopolysaccharide from Vibrio fetus is the major factor in the virulence of this organism remains to be evaluated.

LITERATURE CITED

1. Adams, G. A. Extraction of lipopolysaccharides from Gram-negative bacteria with dimethyl sulfoxide. Canadian Journal of Biochemistry 45:422-426. 1967.

2. Algire, G. H., Legallais, F. G. and Park, H. D. Comparison of virulent and avirulent gonococci and meningococci organisms. Journal of the National Cancer Institute 8:53. 1947.

3. Aminoff, D. Methods for the quantitative estimation of N-acetylneramic acid and their application to hydrolysates of sialomucoids. Biochemical Journal 81:384-391. 1961.

4. Baker, P. J., Wyly, M. V. and Wilson, J. B. Factors related to virulence in smooth strains of Brucella abortus. Bacteriological Proceedings, Report of the 64th annual meeting, New York, 1964 official report, p. 83. 1964.

5. Barker, S. A., Bourne, E. J., Stacey, M. and Whiffen, D. H. Infrared spectra of carbohydrates. Part I. Some derivatives of D-glucopyranose. Journal of the Chemical Society of London 1954:171-176. 1954.

6. _____, _____ and Whiffen, D. H. Methods of Biochemical Analysis. Volume 3. Interscience Publishers, Inc., New York, N.Y. 1956.

7. Battaile, J., Dunning, R. L. and Loomis, W. D. Biosynthesis of terpenes. I. Chromatography of peppermint oil terpenes. Biochimica et Biophysica Acta 51:538-544. 1961.

8. Beer, H. and Braude, A. I. A study of particle sizes, shapes and toxicities in a Boivin type endotoxic preparation. Annals of the New York Academy of Sciences 133:450-475. 1966.

9. Bellamy, L. J. The Infra-red Spectra of Complex Molecules. Second edition. John Wiley and Sons, Inc., New York, N.Y. 1958.

10. Bennett, I. L. and Cluff, L. C. Bacterial pyrogens. Pharmacological Reviews 9:427-475. 1957.

11. Blakemore, F. and Gledhill, A. W. Studies on vibrionic infection of sheep. I. The agglutination test as a means of diagnosis. II. The antigenic relationship between strains of Vibrio fetus. Journal of Comparative Pathology 56:69-77. 1946.

12. Block, R. J., Durrum, E. C. and Zweig, G. A Manual of Paper Chromatography and Paper Electrophoresis. Second edition. Academic Press, Inc., New York, N.Y. 1958.

13. Boas, N. F. Method for the determination of hexosamines in tissues. Journal of Biological Chemistry 204:553-563. 1953.

14. Boivin, A., Mesrobeanu, I. and Mesrobeanu, L. Extraction d'un complexe toxique et antigenique a partir du Bacille d'Aertrycke. Comptes Rendus des Seances Societe Biologia 114:307-310. 1933.

15. _____ and Mesrobeanu, L. Recherches sur les antigens somatiques et sur les endotoxines des bacteries. 1. Considerations generales et expose des techniques utilises. Revue d'Immunologie 1:533-569. 1935.

16. Boor, A. K. and Miller, P. A carbohydrate-lipid fraction of gonococcus and meningococcus. Journal of Infectious Diseases 75:45-57. 1944.

17. Burton, A. and Carter, H. Purification and characterization of the lipid A component of the lipopolysaccharide from Escherichia coli. Biochemistry 3:411-418. 1964.

18. Cameron, J. A., Holtman, D. F. and Jefferies, C. D. The association of virulence with endotoxin in Salmonella pullorum. Journal of Infectious Diseases 106:159-161. 1960.

19. Crumpton, M. J. Identification of amino sugars. Biochemical Journal 72:479-485. 1959.

20. Davies, D. A. L. A specific polysaccharide of Pasteurella pestis. Biochemical Journal 63:105-112. 1956.

21. Dennis, S. M. Isolation of a lipopolysaccharide from Vibrio fetus. Nature 183:186-187. 1959.

22. Dryer, R. L., Thomas, A. R. and Routh, J. I. The determination of phosphorous and phosphatase with N-phenyl-p-phenylene-diamine. Journal of Biological Chemistry 225:117-133. 1956.

23. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. Colorometric method for determination of sugars and related substances. Analytical Chemistry 28:350-357. 1956.

24. Elson, L. A. and Morgan, W. T. J. A colorimetric method for the determination of glucosamine and chondrosamine. Biochemical Journal 27: 1824-1828. 1933.

25. Foster, A. B. and Horton, D. Aspects of the chemistry of the amino sugars. Advances in Carbohydrate Chemistry 14:214-279. 1959.

26. Freedman, H. H. and Sultzer, B. M. Dissociation of the biological properties of bacterial endotoxin by chemical modification of the molecule. Journal of Experimental Medicine 116:929-943. 1962.

27. Fukushi, K., Anacker, R. L., Haskins, W. T., Landy, M., Milner, K. C. and Ribi, E. Extraction and purification of endotoxin from enterobacteriaceae: a comparison of selected methods and sources. Journal of Bacteriology 87:391-400. 1964.

28. _____, _____, _____, _____, _____ and _____. Reaction of Escherichia coli endotoxin with lithium aluminum hydride. Proceedings of the Society for Experimental Biology and Medicine 115: 133-144. 1964.

29. Gallut, J. Recherches immunochimiques sur Vibrio fetus. II. Fractionement chimique des corps microbiens et etude serologique de deux fractions antigeniques. Annales Institute Pasteur 83:455-463. 1952.

30. Gardell, S. Separation of mucopolysaccharides on a cellulose column. Acta Chemica Scandinavica 11:668-675. 1957.

31. Gram, C. Fortschritte der Medizin 2:185. 1884. Original not available; cited in Salle, A. J., Fundamental Principals of Bacteriology. Fourth edition. McGraw-Hill Book Company, Inc., New York, N.Y. 1954.

32. Gray, G. W. and Wilkinson, S. G. The effect of ethylene diaminetetraacetic acid on the cell walls of some Gram-negative bacteria. Journal of General Microbiology 39:385-399. 1965.

33. Haskins, W. T. Spectrophotometric determination of fatty acid amides in lipids. Analytical Chemistry 33:1445-1446. 1961.

34. _____, Landy, M., Milner, K. C. and Ribi, E. Biological properties of parent endotoxins and lipoid fractions with a kinetic study of acid hydrolyzed endotoxin. Journal of Experimental Medicine 114: 665-684. 1961.

35. Heath, E. C. Preparation of 3-deoxyoctulosonate. Methods of Enzymology 8:134-136. 1966.

36. _____ and Ghalambor, M. R. 2-Keto-3-deoxyoctunoate, a constituent of cell wall lipopolysaccharide preparations obtained from Escherichia coli. Biochemistry and Biophysics Research Communications 10:340. 1963.

37. Horecker, B. L. Structure and biosynthesis of core polysaccharide. In Landy, M. and Braun, W., eds. Bacterial endotoxins. pp. 72-82. Princeton University Press, Princeton, N.J. 1964.

38. Ikawa, M., Koepfli, J. B., Mudd, S. G. and Niemann, C. An agent from Escherichia coli causing hemorrhage and regression of experimental mouse tumor. III. The component fatty acids of the phospholipide moiety. Journal of the American Chemical Society 75:1035-1039. 1953.

39. Ikawa, M., Koepfli, J. B., Mudd, S. G. and Niemann, C. An agent from Escherichia coli causing hemmorhage and regression of an experimental mouse tumor. IV. Some nitrogenous components of the phospholipide moiety. Journal of the American Chemical Society 75:3439-3442. 1953.

40. _____ and Niemann, C. The synthesis of 4,5-diamino-N-eicosane and of 2-methyl-3,4-diamino-N-nonadecane. Journal of the American Chemical Society 75:6314-6315. 1953.

41. Ingram, V. M. Abnormal human haemoglobins. I. The comparison of normal and sickle-cell haemoglobins by "fingerprinting". Biochimica et Biophysica Acta 28:539-545. 1958.

42. Irvine, J. C. and Hynd, A. The conversion of D-glucosamine into D-glucose. Journal of the Chemical Society 101:1128-1146. 1912.

43. Jarvis, F. G. and Johnson, M. J. A glyco-lipide produced by Pseudomonas aeruginosa. Journal of the American Chemical Society 71: 4124-4126. 1949.

44. Jenkin, C. R. and Rowley, D. Toxic proteins from Vibrio cholera and water Vibrios which are lethal for mice. Journal of General Microbiology 21:191-200. 1959.

45. Jesaitis, M. A. and Goebel, W. F. The chemical and antiviral properties of the somatic antigen of phase II Shigella sounei. Journal of Experimental Medicine 96:409-424. 1952.

46. Johnson, A. G. and Nowotny, A. Relationship of structure to function in bacterial O antigens. III. Biological properties of endotoxins. Journal of Bacteriology 87:809-814. 1964.

47. Jourdian, G. W. and Roseman, S. D-Glucosamine 6-phosphoric acid and D-mannosamine phosphoric acid. Biochemical Preparations 9:44-47. 1962.

48. Kasai, N. Chemical studies on the lipid component of endotoxin, with special emphasis on its relation to biological activities. Annals of the New York Academy of Sciences 133:486-507. 1966.

49. Kass, E. H. Effect of corticosteroids and of hormones of pregnancy on the lethal action of bacterial endotoxin. Annals of the New York Academy of Sciences 88:107-115. 1960.

50. Kessel, R. W. I., Freedman, H. H. and Braun, W. Relationship of polysaccharide content to some biological properties of endotoxins from mutants of Salmonella typhimurium. Journal of Bacteriology 92: 592-596. 1966.

51. Kim, Y. B. and Watson, D. W. Inactivation of Gram-negative bacterial endotoxin by papain. Society for Experimental Biology and Medicine 115:140-142. 1964.

52. Kimmel, J. R. and Smith, E. L. The properties of papain. Advances in Enzymology 19:267-334. 1957.

53. Laing, L. A. Vibrio fetus infection of cattle. Food and Agricultural Studies No. 51. 1960.

54. Landy, M. and Braun, W. Bacterial Endotoxins. Rutgers University Press, New Brunswick, N.J. 1964.

55. _____, Johnson, A. G., Webster, M. E. and Sagin, J. F. Studies on the O antigen of Salmonella typhus. II. Immunological properties of the purified antigen. Journal of Immunology 74:466-478. 1955.

56. Lederer, E. Physiologic effects of endotoxins from Gram-negative bacterial organisms. Colloquim. Society for Physiological Chemistry 119:67-73. 1958.

57. Leloir, L. F. and Cardini, C. E. Enzymes acting on glucosamine phosphates. Biochimica et Biophysica Acta 20:33-42. 1956.

58. Lohmann, K. The chemistry and metabolism of the compounds of phosphorus. Annual Reviews of Biochemistry 7:125-142. 1938.

59. Lüderitz, O. Structural relationships of Salmonella O and R antigens. Annals of the New York Academy of Sciences 133:349-375. 1966.

60. _____, Staub, A. M., Stirm, S. and Westphal, O. Colitase (3-desoxy-L-fucose), ein neur zucker-baus-tein mit immunspezifischer funktion in endotoxinen (lipopolysacchariden) einger Gram-negative bakterien. Biochemie Zeitschrift 330:193-210. 1958.

61. MacPherson, I. A., Wilinson, J. A. and Swain, R. H. A. The effect of Klebsiella aerogenes and Klebsiella cloacae polysaccharides on haemagglutination by the influenza viruses. British Journal of Experimental Pathology 34:603-615. 1953.

62. Maley, F. and Lardy, H. A. Phosphoric esters of biological importance. VI. The synthesis of D-glucosamine-6-phosphate and N-acetyl-D-glucosamine-6-phosphate. Journal of the American Chemical Society 78: 1393-1397. 1956.

63. Manclark, C. R. and Pickett, M. Quantitative production of Vibrio fetus cells. Journal of Bacteriology 79:752-753. 1960.

64. Mattson, A. N. and Jensen, C. O. Colorimetric determination of reducing sugars with triphenyltetrazolium chloride. Analytical Chemistry 22:182-185. 1950.

65. Metcalf, L. R. and Schmitz, A. A. The rapid preparation of fatty acid esters for gas chromatographic analysis. Analytical Chemistry 33:363-364. 1961.

66. Miller, L. C. and Tainter, M. L. Estimation of the ED_{50} and its error by means of logarithmic-probit graph paper. Proceedings of the Society for Experimental Biology and Medicine 57:261-264. 1944.

67. Mitcherlich, E. and Liesz, B. Die serologische differenzierung von Vibrio fetus - Stammen. Deutsche Tierärztliche Wochenschrift 65: 2-5. 1958.

68. Moggridge, R. C. G. and Neuberger, A. J. Methylglucosaminide: its structure, and the kinetics of its hydrolysis by acids. Journal of the Chemical Society 1938:745-750. 1938.

69. Morgan, W. T. J. Studies in immuno-chemistry. II. The isolation and properties of a specific antigen from B. Dysenteriae. Biochemical Journal 31:2003-2021. 1937.

70. Nageswararao, G. and Blobel, A. Antigenic substances isolated from filtrates of Vibrio fetus broth cultures. American Journal of Veterinary Research 24:1313-1320. 1963.

71. Nakano, M. Mutants of Salmonella with unusually low toxicity for mice. Nature 196:1118-1119. 1962.

72. Nesbitt, J. A. and Lennarz, W. J. Comparison of lipids and lipopolysaccharide from the bacillary and L forms of Proteus P18. Journal of Bacteriology 80:1020-1025. 1965.

73. Neter, E., Westphal, O., Lüderitz, O., Gorzynski, E. and Eichenberger, E. Studies of enterobacterial lipopolysaccharides. Effects of heat and chemicals on erythrocytes modifying antigenic, toxic and pyrogenic properties. Journal of Immunology 76:377-385. 1956.

74. Noll, H. and Braude, A. Preparation and biological properties of a chemically modified Escherichia coli endotoxin of high immunogenic potency and low toxicity. Journal of Clinical Investigation 40: 1935-1951. 1961.

75. Norton, W. T. Reaction of mercuric chloride with plasmologen. Nature 184:1144-1145. 1959.

76. Nowotny, A. Chemical detoxification of bacterial endotoxins. In Landy, M. and Braun, W., eds. Bacterial Endotoxins. pp. 29-38, Rutgers University Press, New Brunswick, N.J. 1964.

77. _____. Chemical structure of a phosphomucolipid and its occurrence in some strains of Salmonella. Journal of the American Chemical Society 83:501-503. 1961.

78. Nowotny, A. Molecular biology of Gram-negative bacterial lipopolysaccharides. Annals of the New York Academy of Sciences 133:277-786. 1966.

79. _____. Relation of chemical structure to pathogenic activity of endotoxins. In Mills, L. C. and Moyer, J. H. eds. Shock and Hypertension. pp. 32-48, Grune and Stratton Co., New York, N.Y. 1965.

80. _____, Cundy, K. R., Neale, N. L., Nowotny, A. M., Radvany, R., Thomas, S. P. and Tripodi, D. J. Relation of structure to function in bacterial O-antigens. IV. Fractionation of the components. Annals of the New York Academy of Sciences 133:586-603. 1966.

81. _____, Lüderitz, O. and Westphal, O. Rundfilter-chromatographie langkettiger fettsauren bei der analyse bakteieller lipopolysaccharide. Biochemie Zeitschrift 330:47-54. 1958.

82. Oroszlan, S. I. and Mora, D. T. Dissociation and reconstitution of an endotoxin. Biochemical and Biophysical Research Communications 12:345-349. 1963.

83. Osborn, M. J. Studies on the Gram negative cell wall. I. Evidence for the role of 2-keto-3-deoxyoctonoate in the lipopolysaccharide of Salmonella typhimurium. Proceedings of the National Academy of Sciences 50:499. 1963.

84. Park, R. W. A. A note on the systematic position of Vibrio fetus. Journal of Applied Bacteriology 24:23-26. 1961.

85. Partridge, S. M. Filter-paper partition chromatography of sugars. Biochemical Journal 42:238-248. 1948.

86. Peereboom, J. W. C. The analysis of plasticizers of micro-adsorption chromatography. Journal of Chromatography 4:323. 1960.

87. Peterson, E. A. and Sober, H. A. Chromatography of proteins. I. Cellulose ion-exchange adsorbents. Journal of the American Chemical Society 78:751-755. 1956.

88. Raistrick, H. and Topley, W. W. C. Immunizing fractions isolated from Bact. aertrycke. British Journal of Experimental Pathology 15: 113. 1934.

89. Rapport, M. M. and Franzl, R. E. The structure of plasmologens. III. The nature and significance of the aldehydogenic linkage. Journal of Neurochemistry 1:303-310. 1957.

89. _____ and Lerner, B. The structure of plasmologens. IV. Lipids in normal and neoplastic tissues of man and in normal tissues of rabbit and rat. Biochimica et Biophysica Acta 33:319-325. 1959.

91. Rebers, P. A., Heddleston, K. L. and Rhodes, K. R. Isolation from Pasteurella multicida of a lipopolysaccharide antigen with immunizing and toxic properties. Journal of Bacteriology 93:7-14. 1967.

92. Reider, R. F. and Thomas, L. Studies on the mechanism involved in the production of abortion by endotoxin. Journal of Immunology 84:189-193. 1960.

93. Ribi, E., Haskins, W. T., Landy, M., and Milner, K. C. Preparation and host reaction properties of endotoxin with low content of nitrogen and lipids. Journal of Experimental Medicine 114:647-664. 1961.

94. _____, _____, _____ and _____. Symposium on bacterial endotoxins. I. Relationship of chemical composition to biological activity. Bacteriological Reviews 25:427-436. 1961.

95. _____, Hoyer, B. H., Milner, K. C., Perrine, T. D., Larsen, C. L. and Goode, G. Physical and chemical analysis of endotoxins from Salmonella enteritidis. Journal of Immunology 84:32-47. 1960.

96. Ristic, M. and Brandly, C. A. Characterization of Vibrio fetus antigens. I. Chemical properties and serological activities of a soluble antigen. American Journal of Veterinary Research 20:148-153. 1959.

97. Robertstad, G. W. and Morrison, S. M. An in vitro study of extracted antigens of Vibrio fetus isolates. American Journal of Veterinary Research 21:327-329. 1961.

98. Rondle, C. J. M. and Morgan, W. T. J. The determination of glucosamine and galactosamine. Biochemical Journal 61:586-589. 1955.

99. Roseman, S. and Ludoweig, J. N-acetylation of the hexosamines. Journal of the American Chemical Society 76:301-302. 1954.

100. Rouser, G., O'Brien, J. and Heller, D. The separation of phosphotidyl ethanolamine and phosphotidyl serine by column chromatography. Journal of the American Oil Chemists Society 38:14-19. 1961.

101. Salton, M. R. J. An improved method for the detection of N-acetyl-amino sugars on paper chromatograms. Biochimica et Biophysica Acta 34:308-312. 1959.

102. Schenker, E. Neuere methoden der präparactiven organischen chemie. IV. Anwendung von komplexen borhydriden und von diboran in der organischen chemie. Angewandte Chemie 73:81-85. 1961.

103. Scheuerbrandt, G. and Bloch, K. Unsaturated fatty acids in microorganisms. Journal of Biological Chemistry 237:2064-2068. 1962.

104. Siggia, S. and Edsberg, R. L. Iodometric determination of vinyl alkyl ethers. Analytical Chemistry 20:762-763. 1948.

105. Smith, R. T. and Thomas, L. Influence of age upon response to Meningococcal endotoxin in rabbits. Proceedings of the Society for Experimental Biology and Medicine 86: 806-809. 1954.

106. Smith, T. Spirilla associated with diseases of the fetal membranes in cattle (infectious abortion). Journal of Experimental Medicine 28:701-719. 1918.

107. _____ and Taylor, M. S. Some morphological and biological characters of the spirilla (Vibrio fetus, N. Sp.) associated with disease of the fetal membranes in cattle. Journal of Experimental Medicine 30:299-311. 1919.

108. Snyder, F. and Stevens, N. A simplified spectrophotometric determination of ester groups in lipids. Biochimica et Biophysica Acta 34:244-245. 1959.

109. Spink, W. W. and Anderson, D. Experimental studies of the significance of endotoxin in the pathogenesis of brucellosis. Journal of Clinical Investigation 33:540-548. 1954.

110. Stegena, T. and Terpstra, J. I. Over Vibrio foetus infecties bijhet rund en enzootishe steriliteit. Tijdschrift voor Diergeneeskude Deel 74:239-269. 1949.

111. Strange, R. E. Glucosamine values of muramic acid and other amino sugars by the Elson and Morgan method. Nature 187:38-40. 1960.

112. Stromiger, J. L. Enzymic transfer of pyruvate to uridine diphosphoacetylglucosamine. Biochimica et Biophysica Acta 30:645-646. 1958.

113. Sultzer, B. M. and Freedman, H. The stimulation of nonspecific host resistence to infection by chemically modified endotoxin. Journal of Experimental Medicine 116:943-956. 1962.

114. Tauber, H. Fatty acid ester groups in lipopolysaccharide endotoxins. Federation Proceedings 19:245. 1960.

115. _____ and Russell, H. General method for the isolation of endotoxins; study of lipid and polysaccharide moieties. Experimental Medicine and Surgery 19:161-170. 1961.

116. Thomas, L. Physiologic and pathologic alterations produced by endotoxins of Gram negative bacteria. Archives of Internal Medicine 101:452-461. 1958.

117. _____. The physiological disturbances produced by endotoxins. Annual Review of Physiology 16:467-472. 1954.

118. Trappe, W. Modifikation der jodzahlbestimmung-methode nach H.P. Kaufmann für kleine und kleinste fettmengen. Biochemie Zeitschrift 296:180-185. 1938.

119. Tripodi, D. and Nowotny, A. Relation of structure to function in bacterial O-antigens. V. Nature of active sites in endotoxic lipopolysaccharides of Serratia marcescens. Annals of the New York Academy of Sciences 133:604-621. 1961.

120. Vigdahl, R. L. Isolation and chemical composition of antigens from Vibrio fetus. Unpublished M.S. thesis. Library, Iowa State University, Ames, Iowa. 1965.

121. Viroque, E. and Holman, R. T. Quantitative estimation of esters by thin layer chromatography. Journal of the American Oil Chemists Society 39:63-66. 1962.

122. Warren, L. Thiobarbituric acid spray reagent for deoxy sugars and sialic acids. Nature 186:237. 1960.

123. Webb, E. C. and Levy, A. L. A sensitive method for the determination of DNA in tissues and microorganisms. Journal of Biological Chemistry 213:107-117. 1955.

124. Westphal, O. Die struktur die antigene und das wesen der immunologischenspezifitat. Naturwissenschaften 46:50-54. 1959.

125. _____. Polysaccharides in Biology 2:115-144. Josiah Macey Jr. Foundation, New York, N.Y. 1957.

126. _____. Recentes recherches sur la chimie et al biologie des endotoxines des bacteries a Gram negatif. Annales Institute Pasteur 98:789-813. 1960.

127. Westphal, O., Hammer, D., Lüderitz, O., Nowotny, A., Eichenberger, E. and Goebel, F. Die inaktivierung der endotoxischen lipoidkomponente (lipoid A) bakterieller lipopolysaccharide durch pferdeserum. Zeitschrift fur Naturforschung 136:572-580. 1958.

128. _____ and Lüderitz, O. Chemische erforschung von lipopolysacchariden gramnegativer bacterien. Angewandte Chemie 66:407-412. 1954.

129. Westphal, O. and Lüderitz, O. 3-6-didesoxyhexosen chemie und biologie. Angewandte Chemie 72:881-887. 1960.

130. _____, Nowotny, O., Lüderitz, O., Hurni, H., Eichenberger, E. and Schönholzer, G. Die bedeutung der lipoid-komponente (lipoid A) für die biologischen wirkungen bakterieller endotoxine. Pharmaceutia Acta Helvetiae 33:401-411. 1958.

131. Winter, A. J. An antigenic analysis of Vibrio fetus. I. Properties of soluble extracts of the organism. Journal of Veterinary Research 23:150-158. 1962.

132. _____. An antigenic analysis of Vibrio fetus. II. Further characterization of the precipitating antigens. Journal of Veterinary Research 24:193-198. 1963.

133. _____. An antigenic analysis of Vibrio fetus. III. Chemical, biologic and antigenic properties of the endotoxin. Journal of Veterinary Research 27:653-658. 1966.

134. Zahl, P. A. and Bjerknes, C. Induction of decidua-placental hemorrhage in mice by the endotoxins of certain Gram negative bacteria. Proceedings of the Society for Experimental Biology and Medicine 54: 329-338. 1943.

135. Ziminski, T. and Borowski, E. A new spray reagent replacing sulphuric acid in thin layer chromatography. Journal of Chromatography 23: 480-482. 1966.

ACKNOWLEDGMENTS

The following persons have generously contributed to the completion of this work and the writing of the manuscript and the author would like to express his sincere thanks and appreciation:

To Dr. Richard Keeler, for his suggestion of the problem.

To Mr. Alfred Ritchie, whose assistance in correcting the manuscript and suggestions during the research and writing were indispensable.

To Dr. Walter Hearn, for his encouragement and advice throughout graduate school.

To Dr. George Engstrom and Dr. Paul Rebers for their valuable suggestions concerning the problems encountered during completion of this work.

To Dr. Milton Allison, Dr. Phillip O'Berry, Mr. Robert Patterson, Mr. John DeLance and Mr. John Foley, for technical assistance in carrying out the numerous experiments involved in this work.

To Mr. Dale Ross, for reading and correcting the manuscript and to Mrs. Annette Bates for typing both the rough draft and final manuscript.

To Diane Y. Vigdahl, the author's wife, for the many sacrifices and adjustments required during the completion of the work and manuscript and for her continuous encouragement. Without her help the work would not have been completed nor this manuscript finished. To her the author is forever indebted.