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HOMAYOUN ROSTAM-ABADI

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University of New Hampshire

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LECTINS FROM THE HORSESHOE CRAB, *LIMULUS POLYPHEMUS*,
REACTIVE WITH BACTERIAL LIPOPOLYSACCHARIDE

BY

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B.S., Wayne State University, 1973
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A DISSERTATION

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in
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REACTIVE WITH BACTERIAL LIPOPOLYSACCHARIDE

by
HOMAYOUN ROSTAM-ABADI

University of New Hampshire, May, 1980

The Limulus serum is capable of precipitating various components of the lipopolysaccharide molecule from R mutants of Salmonella minnesota. Precipitation-inhibition studies using 2-keto-3-deoxyoctonate (KDO) have supported the hypothesis that this acidic residue is the immunodeterminant group in the core region of the lipopolysaccharide molecule. Limulus also contains a lectin which interacts with the lipid A region of lipopolysaccharide; the proposed binding site in this region is the glucosamine backbone of the lipid A molecule.

A sialic acid-binding lectin from the serum of the horseshoe crab, Limulus polyphemus, was isolated by preparative polyacrylamide slab gel electrophoresis and by ion-exchange chromatography on a column of diethylaminoethyl (DEAE)-Sephadex. The purified lectin precipitates the lipopolysaccharide molecules containing the KDO residue, and agglutinates chicken erythrocytes. This lectin does not bind to the glucosamine backbone of the lipid A. Hemagglutination and hemagglutination-inhibition studies, using untreated chicken erythrocytes and chicken erythrocytes coated with glycolipid from the Re mutant of S. minnesota indicated that the isolated lectin reacts with
both N-acetylneuraminic acid and KDO. Cross-inhibition studies suggest that the same combining site on the lectin is involved in these reactions.
CHAPTER I

INTRODUCTION

The hemolymphs of many invertebrates contain glycoproteins capable of agglutinating a variety of cell types (66). They function by binding specifically to polysaccharides on the surface of cells, and thus provide a tool for study of cell surface structures (11). Although the physiological importance of these agglutinins is not yet understood, they may serve in a host defense capacity similar to that of vertebrate antibodies. They agglutinate a variety of heterologous (12) cells but do not agglutinate homologous cell types. Thus a role of "self recognition" can be suggested.

Hemagglutinin from Limulus polyphemus

Noguchi (50) was the first to describe the hemagglutinin in the serum of Limulus polyphemus. Subsequently Cohen and co-workers (10, 7, 11) using erythrocytes as the agglutinogen described some basis parameters of this lectin, and postulated a receptor site containing N-acetyl neuraminic acid (NANA) as the site of attachment of the Limulus hemagglutinin (12). Later Cohen et al. (9) reported greater agglutination of human leukemic than normal lymphocytes by Limulus serum.

Two approaches have been used to purify Limulus agglutinins. The first approach is a strictly biophysical one, which is based upon the charge, size, and conformation of the molecule.
This approach was first used by Marchalonis and Edelman (45) who combined a series of steps in which hemocyanin was removed by ultracentrifugation, and the remaining clear fluid was separated using starch block electrophoresis. That portion containing hemagglutinating activity was further fractionated by gel filtration.

Finstad et al. (20, 21), using preparative ultracentrifugation and zone electrophoresis, also isolated a hemagglutinin from the serum of Limulus polyphemus. It was capable of agglutinating horse, sheep, and rabbit erythrocytes.

Further work on the sialic acid-binding lectin in Limulus was carried out by Roche and Monsigny (62) using combined gel filtration and ion-exchange chromatography. The homogeneity of the purified lectin was demonstrated by immunoelectrophoresis and polyacrylamide disc gel electrophoresis; however, in isoelectric focusing, three sharp bands appeared in polyacrylamide gels.

The second major approach to the isolation of the agglutinin utilizes affinity chromatography. Nowak and Barondes (51), using horse red blood cells as an affinity adsorbent, isolated a Limulus agglutinin with a structure identical to that recovered using strictly biophysical methods. Hemagglutination of horse erythrocytes was inhibited by NANA and D-glucuronic acid, and the latter was used to elute the agglutinin from the erythrocytes.

Oppenheim (53) first isolated the hemagglutinin in Limulus serum by affinity chromatography using bovine submaxillary mucin, a substance known to be rich in sialic acid residues,
as the ligand. A 1500-fold increase in activity was observed in a sodium citrate eluate. The agglutinin was shown to have no activity against desialated bovine submaxillary mucin, and was homogeneous by polyacrylamide gel electrophoresis.

More recently Roche and Monsigny (63) described the isolation of a sialic acid-binding lectin (limulin III) from the serum of *Limulus* using ion-exchange chromatography combined with affinity chromatography on immobilized submaxillary mucin. Limulin III was shown to be homogeneous by crossed immunoelectrophoresis using rabbit antisera against the active fraction. A purification factor of 250,000 was obtained which is much higher than that obtained using other purification methods; i.e. 20-fold (45), 60-fold (62), 1500-fold (53).

The structural characterization of the *Limulus* hemagglutinin has provided information which has served as a model for the study of invertebrate lectins. Marchalonis and Edelman (45) first characterized the *Limulus* agglutinin using various physico-chemical techniques. They concluded that the hemagglutinin is a protein of relatively low electrophoretic mobility (acidic) which comprises 2-5% of the total hemolymph protein. The intact molecule has a sedimentation coefficient of $S_{20,W}^0$ 13.5 S and changes in pH alone are sufficient to reduce the sedimentation coefficient to 4 S. The intact molecule has a mass of 400,000 daltons and contains subunits of an approximate molecular weight of 20-25,000 daltons. The molecular weight of the subunits was determined by reduction and alkylation of the hemagglutinin in 8 M urea followed by gel electrophoresis in acid urea. These subunits are arranged
as trimers in subunit structure, and the intact molecule is comprised of 6 of these subunits. The association of subunits is noncovalent; such forces as hydrophobic interactions, electrostatic interactions and hydrogen bonds are responsible for stabilization of the intact hemagglutinin molecule.

Based on electron microscopic examination (19) and hydrodynamic studies, these same authors proposed that the hemagglutinin has a ring shape structure, and is composed of eighteen subunits each with a sedimentation coefficient of 1.5 S (22,500 daltons). Furthermore, based on an amino acid analysis of the purified lectin it was concluded that Limulus lectin and vertebrate antibodies are unrelated in evolutionary development.

A subsequent report by Finstad et al. (20, 21) confirmed the general make-up of the Limulus hemagglutinin. In addition, they showed that the lectin possesses a carbohydrate content of 24%, contains intra- rather than inter-chain disulfide bonds, and is predominantly composed of glutamic acid, aspartic acid, leucine, and glycine. The conformation of the Limulus agglutinin was also studied through the examination of its circular dichroic spectra in both the near and far ultraviolet (UV) range. Spectral analysis in the far UV range of the native molecule in the presence of Ca\(^{++}\) revealed a conformation similar to a beta sheet.

Similar results on the physicochemical properties of the Limulus hemagglutinin has been reported by Roche and Monsigny (62, 44). According to these authors, limulin is an acidic protein. Its isoelectric point of 5 is consistent with
a relative preponderance of acidic amino acids. The content of carbohydrate is less than 4% with 5 glucosamines per 1,000 residues. The molecular weight calculated from the diffusion constant and the sedimentation coefficient ($S_{20,W}^{13.9}$) was 340,000 daltons. Behavior of the hemagglutinin in polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate and mercaptoethanol suggested an average subunit molecular weight of 29,000. Similar results were obtained by Nowak and Barondes (51); however, they reported a molecular weight of 460,000 daltons with a subunit molecular weight of 22,000.

Kaplan et al. (35) have determined the amino acid sequence of limulin. The first observation concerning the primary structure of limulin is that the protein yielded a unique sequence with no indication of heterogeneity, either at the amino-terminus or in the interior of the protein sequenced to date. This is true in spite of the fact that the isolated protein exhibits a mild degree of heterogeneity on polyacrylamide gels. Characterization of the amino-terminus indicated the absence of histidine and alanine within the first 50 amino-terminal residues and the presence of eight prolines within the first 30 amino-terminal residues suggesting a beta configuration (or coil) for that part of the molecule. The region from residue 13 to 19 is particularly rich in proline and also in serine which may represent the point of attachment of carbohydrates to the protein component of limulin. The protein contains one intrachain disulfide bond, and no homologies are apparent between limulin and antibodies of higher mammals or other plant or animal lectins. The carboxy-terminal segment
of the molecule shows a marked tendency to self-aggregate, a phenomenon that could be related to the association of the identical subunits \textit{in vivo}.

The hemagglutination activity of \textit{Limulus} hemagglutinin, unlike that shown by hemagglutinating antibodies is enhanced by the presence of divalent cations. Marchalonis and Edelman (45) reported a ten-fold to thousand-fold increase in the titers of \textit{Limulus} agglutinin in the presence of calcium chloride. This effect could be reversed by the addition of citrate. They suggested that bound calcium stabilizes the native structure of the protein, because in the presence of 0.1 M-citrate the sedimentation coefficient of the molecule dropped from 13.5 S to 10.6 S.

The role of calcium in determining and stabilizing the overall structure of \textit{Limulus} agglutinin was also examined by Finstad et al. (20). They showed that the removal of calcium by treatment of the purified agglutinin with EDTA was accompanied by a partial dissociation of the 13.6 S molecule into polydisperse subunits that ranged in sedimentation coefficient from 2.5 S to 6.0 S. Readdition of calcium to the EDTA-inactivated agglutinin resulted in a reassociation of the subunits to the 13.6 S reconstituted molecule and the complete restoration of full erythrocyte-agglutinating activity.

Similar results were obtained by other investigators (11, 62, 51, 53), who reported an increase in the hemagglutinin activity of \textit{Limulus} agglutinin with the addition of 0.1 M calcium chloride. Magnesium ion also increases the agglutinating activity of the lectin, whereas lithium, potassium, and sodium ions decrease it (11).
Other activities in Limulus hemolymph

In Limulus hemolymph there exists besides a naturally occurring agglutinin directed against NANA receptors, a precipitin reactive with galactogen from Helix pomatia (73), and a mannose-containing substance which can be detected by a specific reaction with, e.g., the plant lectin Concanavalin-A. The galactogen of Helix pomatia is a branched polysaccharide consisting of 90% D-galactose, and 10% L-galactose. D-galactose has been shown not to inhibit the precipitation reaction. However, a mixture of D- and L-galactose could inhibit precipitation of the Helix pomatia extract with Limulus hemolymph fluid.

Cohen et al. (8) have investigated an anti-galactan precipitin in Limulus polyphemus hemolymph with a specificity against O-Beta-D-galactopyranosido-(1-3)-D-galactose. This disaccharide occurs in some galactans of snail origin, and is referred to as digalactobiose II. The precipitation reaction appears to be stereo-specific because no reaction was observed when digalactobiose I (O-Beta-D-galactopyranosido-(1-6)-D-galactoside) was used as the antigen.

It has been shown (64) that limulin is capable of stimulating about 50% of human peripheral T lymphocytes, and this mitogenic effect could be inhibited by prior treatment of the cells with neuraminidase. The authors proposed that the lymphocyte stimulation is triggered by binding of limulin to a glyco-conjugate bearing NANA-GalNac- or NANA-Gal-GlcNac-Man in a terminal nonreducing position.
The reactivity of the sialic acid binding lectin (limulin III) from *Limulus polyphemus* with different glyco-substances devoid of sialic acid has been reported by Vaith et al. (72). Sugar analysis showed the presence of glucuronic acid besides neutral sugars in all of these materials. Although the biological activity of limulin is not known, the authors suggested that it may be involved in the agglutination of a bacteria by *Limulus* hemolymph.

The interaction of limulin with membrane glycoproteins has been reported by Fletcher et al. (22). They demonstrated that sialic acid-containing glycoproteins from a variety of mammalian cell membranes could inhibit the agglutination of horse erythrocytes by limulin. The authors proposed that although limulin has not been widely exploited for this purpose, it is a very useful tool for study and purification of membrane glycoproteins.

Recent evidence indicates that cell-free *Limulus* hemolymph is capable of agglutinating a variety of microorganisms (58, 59). Using both direct agglutination and agglutination-adsorption studies, Pistole (59) has demonstrated that the deep core mutant of *Salmonella minnesota* (Re mutant) was capable of removing the agglutinating activity directed against other Gram-negative bacteria belonging to this species. Preliminary evidence suggests that 2-keto-3-deoxy octonate (KDO) may act as the immunodeterminant residue in the core region of *Enterobacteriaceae*.

A bacterial agglutinin has been isolated by Gilbride and Pistole (26) from the hemolymph of *Limulus* with activity
against *Staphylococcus aureus*. The lectin was shown to bind galactose residues, and requires Ca$^{++}$ for biological activity. The agglutinin was isolated by affinity chromatography on Sepharose 4B, and has a molecular weight of approximately 200,000 daltons. The molecule showed five distinguishable subunits when electrophoresed in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. This lectin showed no activity against *Salmonella minnesota* or chicken erythrocytes.

**Lectins from Other Invertebrates**

Naturally occurring agglutinins from a number of invertebrates possess specificity for microorganisms which are commonly encountered in their habitat. Hemolymph and other body fluids from invertebrate species may also contain hemagglutinins, some with specificity for human blood-group antigens. Such materials have been useful in elucidating the molecular structure of various bacterial and blood group antigens. In fact, Springer et al. (70) have shown that receptor molecules responsible for antigenic specificity on the erythrocyte membrane are also found on the cell surface of many bacteria.

Hammarstrom et al. (30) have purified a blood-group-A-reactive hemagglutinin from *Helix pomatia* which precipitates lipopolysaccharide from the rough mutants of *Salmonella typhimurium*. Inhibition studies using blood-group oligosaccharides suggested a common binding site on the agglutinin for blood-group-A-substance and polysaccharide receptor on *Salmonella* mutants. They have suggested that the activity of
Helix pomatia hemagglutinin is directed toward D-galactose which is present in the core region of Ra and Rb chemotypes and absent in the more highly deficient mutants, Rc through Re, which are non-reactive with this lectin. Indeed others, Ishiyama et al. (34) have shown that substances with alpha(1-6) glycosyl or galactosyl residues are reactive with this lectin.

Kohler et al. (36) and Ottensooser et al. (55) have reported that extracts from the albumin gland of Helix pomatia consistently agglutinate group C Streptococci. A second agglutinin from Helix pomatia directed against the alpha-N-acetyl-D-glucosamine residue in teichoic acid from Staphylococcus aureus has also been reported by Hammarstrom et al. (29).

Recently Shimizu et al. (67) investigated the multiple lectins in the hemolymph of the Japanese horseshoe crab Tachypleus tridentatus. They isolated four distinct hemagglutinins by affinity chromatography using bovine submaxillary mucin conjugated to CNBr-activated Sepharose and N-acetyl-D-glactosamine conjugated to epoxy-activated Sepharose 6B. Two of the four lectins were shown to be homogeneous by polyacrylamide disc gel electrophoresis, and calcium chloride increased the specific activity of three of the four lectins. The activity of the lectins were not affected by addition of magnesium chloride. Using inhibition studies the hemagglutinins have a stronger affinity for sugars bound to proteins than for monosaccharides.

General Structure of the Lipopolysaccharide

The cell wall lipopolysaccharides (LPS) of Gram-negative bacteria are acidic amphipathic macromolecules (Fig. 1).
Fig. 1. General structure of the lipopolysaccharide molecule.
A. Major regions of the lipopolysaccharide molecule.
C. Proposed structure of a lipid A unit from S. minnesota (Reprinted from same).
Fig. 1
They contain a heteropolysaccharide, the O-specific chain, which is linked to a central heteropolysaccharide, the core, which in turn is linked to an acylated oligosaccharide substituted with fatty acids termed lipid A. LPS being anchored on the outer membrane of Gram-negative bacteria through the lipid A component and thus occupying an exposed position, plays a significant role in the interaction of bacteria with the host during infection because of their unique biological properties.

LPS is the source of the endotoxic activity of Gram-negative bacteria and is responsible for many physiopathological reactions expressed during infection. Most of these activities reside in the lipid A moiety. Lipid A exhibits a high affinity for cell membranes, proteins, and lipids, and this property is assumed to initiate biological reactions, including lethal toxicity, pyrogenicity, complement inactivation, adjuvanticity, and B lymphocyte mitogenicity. It has long been known that LPS also represents the heat-stable main antigens of Gram-negative bacteria, the so-called Q antigens. Their structure and composition determine the serological specificity of the bacterial cells, and this forms the basis of serological classifications for *Salmonella*, the Kauffmann-White scheme.

Structural investigation of the core region of *Salmonella* LPS became possible when it was recognized that R-mutants are blocked in the biosynthesis of their LPS (23). Two main groups of R-mutants can be distinguished: first, Ra mutants, often with a mutation in the rfb locus, which are defective in O-side chain biosynthesis and therefore, contain the complete
core (N-acetyl-D-glucosamine, D-glucose, D-galactose, L-glycero-D-mannoheptose, and KDO) linked to lipid A via KDO; and second, Rb to Re mutants, often with a mutation in the rfa locus, which are defective in core biosynthesis, and represent intermediates in LPS biosynthesis. The lipopolysaccharides from these mutants are serologically distinct, with each terminal sugar residue representing the immunodominant sugar unit.

In contrast to the great variation in composition and structure of O-specific chains of different Gram-negative species, the core region is similar for larger groups of bacteria. There is serological and analytical evidence for a common core structure in Salmonella species (43). Five distinct core structures have so far been identified in Enterobacteriaceae which are shared by Escherichia coli, Shigella, Arizona, and Citrobacter. The core structure in lipopolysaccharide from Gram-negative bacteria other than Enterobacteriaceae may, however, be quite different. This is based on the finding that the core region of such LPS may lack heptose or KDO, or both. In Vibrio cholerae, fructose has been found, which may replace KDO as a link between the core and lipid A (23).

KDO was initially shown to be a component of the LPS of Enterobacteriaceae by Heath et al. (33) and Osborn (54). The study of LPS structure of mutant Gram-negative bacteria demonstrated to these investigators that KDO occupies the innermost position in the polysaccharide complex, and provides the point of attachment to the Lipid-A moiety which is embedded
in the outer membrane of these organisms. KDO is present in virtually all strains of the Enterobacteriaceae, although absent in some other Gram-negative organisms (23), e.g. Pseudomonas pseudotuberculosis, Bacteroides fragilis, Bacteroides melaninogenicus (48).

In lipopolysaccharides from the Enterobacteriaceae, as well as other Gram-negative bacteria, KDO represents the link between the polysaccharide and the lipid A component of the molecule. Structural investigation has revealed that the lipid A of Salmonella lipopolysaccharide consists of a backbone of beta(1-6)-linked D-glucosamine disaccharide which is substituted at positions 1 and 4 by phosphate residues, and at position 3 by the KDO component of the core polysaccharide (43). The other hydroxyl and the amino groups of the backbone are acetylated by long chain fatty acids, of which lauric, myristic, palmitic and beta-hydroxy-myristic acid predominate.

Studies carried out in other laboratories have shown that B(1-6) linked glucosamine residues also form the lipid A backbone of other lipopolysaccharides including Serratia marcescens, Selenomonas ruminantium, and Shigella sonnei, Pseudomonas aeruginosa, and Pseudomonas alcaligenes (43).

Hase et al. (32) have developed a new method for isolation and analysis of the lipid A backbone of smooth and rough form lipopolysaccharide. The product, which represents the reduced glucosamine disaccharide (open chain) was further studied using combined gas-liquid chromatography-mass spectrometry. With the aid of these methods and the enzyme N-B-acetylglucosaminidase, an investigation of the lipid A backbone
of lipopolysaccharide from *Salmonella minnesota* (smooth and rough chemotypes), *E. coli*, *Shigella flexneri*, *Xanthomonas enterocolitica*, and *Fusobacterium nucleatum* was performed. In all cases, lipid A was made up, as in *Salmonella*, of a B(1–6) linked D-glucosamine disaccharide, which carries one phosphate group linked to the reducing glucosamine residue (23).

Analysis of the glycolipid from *Salmonella minnesota* Re mutants indicates that, in *Salmonella* lipid A, 4-amino-arabinose is linked to ester-linked phosphate groups of the backbone. In Re mutants of *Salmonella minnesota* only 10–20% of the phosphate groups are substituted by this amino sugar, representing another example of structural heterogeneity within the lipid A component of lipopolysaccharide (23). Current investigation suggests that phosphate or pyrophosphate bridges are present in lipid A, but further work is needed to elucidate their quantity, location, and significance.

Lipid A contains long chained non-hydroxlated as well as hydroxylated fatty acids (C10–C18). They are responsible for the hydrophobic properties of lipid A and seem to play a role in the endotoxic activity, since their removal leads to preparations with reduced endotoxic activities. The fatty acids are bound to the lipid A backbone through ester and amide linkages. Depending upon the source of lipid A, various kinds of ester-bound fatty acids may be present (43). In contrast to the variety of ester-bound fatty acids found in lipid A, only one type is involved in amide linkages, namely beta-hydroxy fatty acids. In most bacterial lipopolysaccharides 55–75% of the total fatty acids are beta-hydroxy acids.
Phosphoethanolamine and ethanolamine are frequently encountered in lipid A. In the neutral lipid A precursors, phosphoethanolamine is linked to the phosphate group, which is bound to C₁ of the glucosamine backbone. Polyamines, as well as cations (Na⁺, K⁺, Ca²⁺, Mg²⁺) which are present in small but significant amounts in lipid A, can be removed from lipopolysaccharide by high voltage paper electrophoresis, ion-exchange chromatography or more efficiently by electrodialysis (23).

Peptides and phospholipids (lipid B) are present only in minor amounts (0.1%) in phenol-chloroform-petroleum ether extracts of lipopolysaccharide. They possibly are complexed to lipid A through hydrophobic or ionic interactions.
Bacterial Cultivation

R mutants of S. minnesota (obtained from S. Schlecht, Max-Planck Institute Fur Immunbiologie, Freiburg, West Germany) grown on blood agar plates were checked for purity using colonial appearance. Rough colonies were inoculated onto triple sugar iron agar and those which showed typical reactions were selected for further cultivation. S. minnesota mutant strains of the following chemotypes were used: Rc, Rd1, Re. They were grown in trypticase soy broth in Fernbach flasks at 37°C with constant shaking for 24 hr.

Glycolipid Extraction Procedure

A mixture containing liquid phenol (9 grams dry phenol plus 1.1 ml distilled water), chloroform and petroleum ether (boiling point 40-60°C) in a volume ratio of 2:5:8, respectively, was used. Glycolipid was isolated from the rough strains with this extraction mixture at 4°C using the method of Galanos et al. (24). Each glycolipid was purified by repeated ultracentrifugation at 100,000 xg for 4 hr. The purified material was lyophilized and stored under vacuum at room temperature until used.

Hydrolysis of Glycolipids with Mild Acid

The lipid A from the respective glycolipids was removed by 1% acetic acid (pH 3.4) at 100°C for 1.5 hr. After removal
of lipid A by centrifugation at 3,000 xg for 10 min, the water-soluble portion was further purified by ultracentrifugation at 100,000 xg for 4 hr to remove intact glycolipid. The remaining fraction consisted of a mixture of the Rc oligosaccharide (heptose fraction), KDO-phosphoethanolamine (KDO-P-EtN), and KDO.

**Purification of Rc Oligosaccharide**

Rc oligosaccharide was separated from free KDO and KDO-P-EtN using high-voltage paper electrophoresis (15). The slowest moving fraction, identified as the Rc oligosaccharide, was eluted from the paper, lyophilized, and stored at -15°C. Rc core was hydrolyzed in 2 N HCl (100°C, 1 hr) and the hydrolysate was subjected to paper chromatography (15), to confirm the presence of heptose and KDO.

**Hydroxylaminolysis**

Fifteen mg of lipid A was treated with 3 ml of 2% hydroxylamine in 4% NaOH in ethanol at 68°C for 6 min, a modification of a technique originally described by Snyder and Stephens (69). The liberated fatty acids in the form of hydroxamates were found in the supernatant fluid. The precipitate was washed exhaustively with small fractions of alkaline hydroxylamine, and recovered each time by centrifugation (10 min, 300 xg, 10°C). The precipitate was resuspended in a small volume of standard diluent (CaCl2, 0.01 M, NaCl 0.15 M) pH 2 and extracted three times with chloroform. The chloroform-insoluble fraction was dried in a vacuum desiccator to remove hydroxylamine, and lyophilized after dissolving in standard diluent. Both chloroform-soluble and insoluble fractions were subjected to
paper chromatography in 1-propanol-water (3:1), and stained with ammonium silver nitrate and ninhydrin. Glucosamine and chitobiose served as standards.

**Liberation of Fatty Acids from the Glycolipid**

Three mg of Rd glycolipid was heated at 100°C for 24 hrs in 4 N HCl in a sealed tube. The hydrolysates were extracted exhaustively with chloroform-methanol (2:1). The combined chloroform extracts were dried under vacuum. The chloroform-insoluble fraction was lyophilized and tested for the presence of glucosamine residue by the ninhydrin spray method (52).

**Modification of Fatty Acids**

Fatty acids were methylated in the presence of BCl₃. The dried fatty acids (1 mg) were dissolved in 5 ml of methanol. The mixture was exposed to BCl₃ in an esterification apparatus, and refluxed at 90°C for 10 min. The methylated fatty acids were concentrated in vacuo and the residue was dissolved in methylene chloride for gas-liquid chromatographic analysis (69).

**Solubilization of Lipid-A**

Lipid A prepared from Re glycolipid by acetic acid hydrolysis was checked for the absence of KDO and heptose. Ten mg of lipid A in distilled water (5 ml) was solubilized by the addition of triethylamine (25-35 ul). This solution was mixed with a solution of bovine serum albumin (10 mg) in water (5 ml). The resulting mixture was dried in a rotary evaporator (25). The complex was redissolved in standard
diluent (pH 7.4) and lyophilized. Stock solutions were prepared by dissolving the complex in standard diluent.

**Authentic Fatty Acids**

Beta-hydroxy myristic acid and other saturated and unsaturated fatty acid methyl esters (1%) used as standards for gas-liquid chromatography were obtained from M. Ikawa (Department of Biochemistry, University of New Hampshire, Durham, N. H.).

**Paper Electrophoresis and Paper Chromatography**

Paper electrophoresis was carried out in pyridine-acetic acid-water (100-40-860 v/v/v, pH 5.3). Approximately 50 ul of sample were applied to a 6 X 57 cm chromatography-grade paper (Whatman #1), and the material was electrophoresed at 45V/cm for 1½ hr (15). Ascending paper chromatography was performed on Whatman #1 paper in 1-propanol-water (3:1).

**Gas-Liquid Chromatography**

Gas-liquid chromatography was carried out in a Barber Colman Selectra System series 5000 with flame ionization detector on a column packing of 12% diethylene glycol succinate on 80/90 mesh Anachrom A (1/4" X 6') at 210°C. The carrier gas was nitrogen.

**Colorimetric Determinations**

Throughout these studies KDO was identified using the thiobarbituric acid spray method; glucosamine in acid hydrolysates (4 N HCl, 24 hr, 100°C) by ninhydrin; and heptose with \( \text{H}_2\text{SO}_4/\text{cysteine} \) as described by Nowotny (52). Phosphate was
detected by the spray technique of Hanes and Isherwood as described by Nowotny.

**Qualitative Precipitin Test (Ring-Test)**

Test antigens were suspended in high purity water (Milli-Q system, Millipore; 18 megaohm) or standard diluent. Precipitation was carried out in 4 mm diameter test tubes using 0.2 ml each of Ag and whole *Limulus* hemolymph or purified fractions. After 3 to 18 h at room temperature, the test was scored qualitatively.

**Preparation of LPS Immunoadsorbents**

M-R595 (Re) glycolipid was insolubilized according to the following (17): 25% glutaraldehyde was added to a 1% solution of Re glycolipid in 0.1 M phosphate buffer, pH 7.0, to obtain a 2% final concentration. An equal volume of saturated, neutralized ammonium sulfate was then added, and the mixture was stirred for one hour at room temperature. The precipitate was collected by centrifugation at 4500 rpm for 20 min and washed with distilled water until free of sulfate ions, and finally suspended in 0.1 M phosphate-buffered saline, pH 7.2, containing merthiolate 1:1000. The immunoadsorbent was stored at 4°C when not in use. Adsorption was carried out by mixing 1 ml of *L. polyphemus* whole serum with the immunoadsorbent. The suspension was gently stirred for 30 min at room temperature. The adsorbed serum was separated by centrifugation at 4500 rpm for 15 min and the supernate was tested for the absence of precipitin to Re glycolipid.
Coupling of Re Glycolipid to Chicken Erythrocytes

Coupling was performed according to Neter as described by Nowotny (52). A 5 mg preparation of Salmonella minnesota Re glycolipid in 5 ml saline was immersed in a boiling water bath for 2 h. After cooling to room temperature (25°C) this material was added to 2 ml of packed chicken erythrocytes which had previously been washed three times in saline (1000xg 5 min). The mixture was then incubated at 37°C for 60 min, and centrifuged at 1000xg for 5 min. The cells were suspended at a concentration by volume of 8% in 0.1 M phosphate-buffered saline pH 7.2. An equal volume of 3% formalin in 0.1 M phosphate-buffered saline pH 7.2 was added, and the mixture was incubated at 37°C for 16 h with moderate shaking. The cells were then washed 4 times in 5 volumes of saline per packed cell volume, and stored at 4°C as a 10% suspension in saline. Formalinization was performed according to Nowak and Barondes (51) to prolong the usefulness of Re-coated chicken erythrocytes.

Hemagglutination and its Inhibition

Hemagglutination was performed as follows: Two fold serial dilution of the proteins were made in 25 ul of 0.1 M NaCl and 0.1 M CaCl₂, and 25 ul of a 3% suspension of chicken-Re or washed chicken erythrocytes were added. Hemagglutination was performed in microtitration plates (Microtiter, Cooke Engineering Co., Alexandria, Va.). After 1 h at room temperature, the degree of agglutination was assessed on a scale of 0 to 4+, and the least amount of protein required to cause complete 4+ agglutination of the cells was taken as one unit.
The inhibition of hemagglutination was carried out as follows: Different concentrations of various inhibitors (25 ul) were added to a solution (25 ul) of lectin with an agglutination activity of four or eight units. A two-fold serial dilution of the inhibitor was then made in 25 ul of four or eight units of lectin in the adjacent wells. After incubation (1 h) at room temperature, a 3% suspension of chicken-Re or chicken erythrocytes was added and agglutination was scored as above. The inhibition activity was determined by the concentration of inhibitor needed to inhibit 4 units of the lectin. The following carbohydrates were tested: N-acetyl neuraminic acid, 2-keto-3-deoxy-octonate (KDO), core oligosaccharide (heptose fraction) of Salmonella minnesota Rc mutant. Heparin was also tested to rule out nonspecific reaction of the ionic substances with the lectin.

**Protein Concentration**

Protein concentrations were determined by the method of Lowry et al. (42), and by the Bio-Rad protein assay of Bradford (6) as described in the Bio-Rad protein assay bulletin (4). Bovine serum albumin was used as the standard.

Protein profiles were also made using absorbance at 280 nm on a Bausch and Lomb Spectronic 600 spectrophotometer (Bausch and Lomb, Rochester, N. Y.).

**Column Chromatography**

A K16/100 jacketed chromatography column and a R15/16 eluant reservoir (Pharmacia Fine Chemicals, Uppsala, Sweden) were employed in all chromatography experiments, unless otherwise
specified. Throughout these studies gel chromatography was performed at 5°C.

**Purification of Limulin**

Limulin was purified by a modification of the method of Roche and Monsigny (62). Hemolymph was obtained from *Limulus polyphemus* during the summer mating season. The serum was separated from cellular material by filtration through cheese cloth and low speed centrifugation (1200xg, 30 min). The serum was then subjected to ultracentrifugation on a Beckman Model L2-65B, using a Ti-60 rotor. Ultracentrifugation was carried out at 5°C for 6 h at 100,000xg. The clear supernatant was dialysed against 0.1 M NaCl, 0.01 M CaCl₂, 0.05 M Tris-HCl buffer pH 8.5 at 5°C. The material was then subjected to ion-exchange chromatography.

**DEAE-Sephadex (A-25) Ion-Exchange Chromatography**

The active material from the previous step was applied directly without concentration onto a column (5 x 30 cm) of DEAE-Sephadex (A-25) equilibrated with 0.1 M NaCl, 0.01 M CaCl₂, 0.05 M Tris-HCl pH 8.5 at 5°C. The material was eluted with the same buffer at a flow rate of 30 ml/h, and fractions of 8 ml were collected. The protein peak with chicken erythrocyte agglutinating, and lipopolysaccharide-precipitating activity was pooled, and aliquots frozen at -20°C.

**Polyacrylamide Disc Gel Electrophoresis**

The discontinuous Tris-HCl buffer system of Davis (14), using a 7% separating gel at pH 8.6 (Tris-HCl 0.378 M), and a
3% stacking gel at pH 6.7 (Tris-HCl 0.056 M) was used. Electrophoresis was performed in a Canalco model 1200 unit (Canalco, Rockville, MD.), using 0.384 M Tris-glycine as electrode buffer (pH 8.3). Two hundred and fifty micrograms of the supernate obtained following ultracentrifugation of the hemolymph (100,000xg, 6 h), 50 ug of limulin, and 20 ug of band three (see text) were electrophoressed using the above system. Electrophoresis was performed at 2mA per gel until the proteins entered the separating gel (30 min), the current was then increased to 4mA per tube until the bromphenol blue reached 5 mm from the bottom of the tube (0.5 x 8 cmm). The gels were fixed and stained according to the procedure of Laemli (37), and destained using Canalco Quick Gel Destainer model 1801.

Limulin was isolated by preparative vertical slab electrophoresis using a Hoefer model PS 101 power supply (Hoefer Scientific Instruments, San Francisco, California). The stacking gel consisted of 3% polyacrylamide pH 6.7 at 5°C, and the separating gel consisted of 4% polyacrylamide at pH 8.6 as described by Davis (14). Three ml of the supernatant of 25 mg of protein, were loaded on the gel. The electrode buffer consisted of Tris-glycine pH 8.3 at 5°C. The gels were electrophoressed at 5°C, using 20 mA until the tracking dye (bromphenol blue) entered the stacking gel. The current was increased to 35 mA until the tracking dye reached 1 cm from the bottom of the slab. The location of protein bands was determined by staining a small segment of the separating gel in hot stain (100°C, 15 min). The gels were stained according to the procedure of Laemli (37). The gel was destained using
Canalco Quick Gel Destainer model 1801. The limulin was isolated by cutting out the appropriate protein band, and eluting the proteins from the gel in 1 ml of Tris (0.05 M), NaCl (0.1 M), and CaCl\(_2\) (0.1 M) pH 8.5 at 5°C.
CHAPTER III

RESULTS

The Analysis of Degraded Glycolipids

Glycolipids from various mutants of *S. minnesota* were extracted as described by Galanos (22). After acid hydrolysis (1% acetic acid, 100°C, 1.5 h), the degraded glycolipids from the Rc mutant were analyzed for their sugar constituents, using high voltage paper electrophoresis. The hydrolysate consisted of three fractions, as previously reported by Droge (15). The KDO fraction, having the fastest electrophoretic mobility, was free of heptose and glucose and stained intensively with thio-barbituric acid. This fraction represents a mixture of free KDO and KDO which has been altered in an unknown way by the action of acetic acid. A second fraction, representing KDO-phosphorylethanolamine, stained with ammonium silver nitrate, thio-barbituric acid, and ninhydrin, indicating the presence of ethanolamine bound to KDO. The heptose-containing fraction exhibited the least electrophoretic mobility and stained with thio-barbituric acid and ammonium silver nitrate, but not with ninhydrin. The presence of heptose was confirmed by analysis of the component sugars and comparing their mobility with authentic heptose.

Purity of this heptose-containing fraction was further investigated using gas-liquid chromatography. Chromatographic analysis of this fraction revealed no detectable fatty acids.
and, since this fraction did not stain with ninhydrin, glucosamine was also absent from the purified material.

Lipid A, recovered from acid-hydrolyzed glycolipid, was treated with hydroxylamine to cleave fatty acids from the glucosamine backbone. Gas chromatographic analysis of the chloroform-insoluble residue from this treatment indicated that more than 99% of all fatty acids, including amide-linked beta-hydroxy-myristic acid, were removed. Examination of this same fraction by paper chromatography showed that it had a mobility comparable to the glucosamine disaccharide, chitobiose, and that it stained with ninhydrin.

**Purification of Limulin by Preparative Slab Gel Electrophoresis**

Limulin was isolated from the colorless supernate obtained from the ultracentrifugation of *Limulus* serum, by preparative electrophoresis on 4% polyacrylamide gels at pH 8.3 using Tris-glycine buffer. The purification scheme is shown in Table 1. The specific activity of the purified lectin increased 181-fold (see Table 1). The recovered protein appeared homogeneous on 7% polyacrylamide gels at pH 8.3 using Tris-glycine as the running buffer (Fig. 3A).

**Purification of Limulin by DEAE-Sephadex Chromatography**

Limulin was purified by ion-exchange chromatography on a DEAE-Sephadex A-25 column (Fig. 2). The starting material was the colorless supernate following ultracentrifugation of *Limulus* serum. The purification scheme is shown in Table 2. Limulin was obtained with a 53-fold purification, as determined
Table 1. Purification of Limulim from *Limulus* Serum by PAGE*

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume ml</th>
<th>Total Protein Conc (mg/ml)</th>
<th>HA** Activity</th>
<th>Specific Activity</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultracentrifugation</td>
<td>3</td>
<td>25</td>
<td>48</td>
<td>1.92</td>
<td>1</td>
</tr>
<tr>
<td>(100,000xg, 2 h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAGE</td>
<td>1</td>
<td>0.184</td>
<td>64</td>
<td>347</td>
<td>181</td>
</tr>
</tbody>
</table>

* Polyacrylamide disc gel electrophoresis

**Total hemagglutinin activity

The specific activity is expressed as the ratio of the total hemagglutination activity per mg of protein.
Fig. 2. Purification of Limulus hemagglutinin by DEAE-Sephadex chromatography. 50 ml of supernatant fluid (100,000xg, 6 h) from Limulus serum was dialyzed against 0.05 M Tris-HCl, 0.01 M CaCl, 0.1 M NaCl pH 8.5 at 5°C, and then passed through a column (5 x 40 cm) of DEAE-Sephadex equilibrated and developed with the same buffer at 5°C. Fractions of 8 ml at a flow rate of 30 ml/hr were collected.
Table 2. Purification of Limulin from Limulus Serum by Ion-exchange Chromatography

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Volume ml</th>
<th>Total Protein Conc (mg/ml)</th>
<th>HA* Activity</th>
<th>Specific Activity</th>
<th>Fold Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultracentrifugation (100,000xg, 6h)</td>
<td>50</td>
<td>100</td>
<td>4000</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>Dialysis**</td>
<td>50</td>
<td>100</td>
<td>4000</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Sephadex(A-25)</td>
<td>60</td>
<td>36</td>
<td>76800</td>
<td>2133</td>
<td>53</td>
</tr>
</tbody>
</table>

* Total hemagglutinin activity
** 0.05 M Tris, 0.1 M NaCl, 0.01 M CaCl₂ pH 8.5 at 5°C

The specific activity is expressed as the ratio of the total hemagglutination activity per mg of protein.
by assays of its biological activity. This preparation of limulin was heterogeneous upon electrophoresis in 7% polyacrylamide gel in Tris-glycine at pH 8.3 (Fig. 3C).

Electrophoresis of Whole Serum and Adsorbed Fractions

The ultracentrifuged (100,000xg, 6 h) fraction of Limulus serum was electrophoresed as indicated in the Materials and Methods. This fraction was resolved into 12 distinct bands, as shown in Fig. 3D. Adsorption of this Limulus serum fraction with insolubilized Re glycolipid prior to electrophoresis resulted in deletion of the four top bands (Fig. 3B). These proteins, which exhibited a relatively slow electrophoretic mobility, were labeled the limulin pool, while the lower eight bands comprised the hemocyanin pool. The bands which were removed by adsorption with Re glycolipid corresponded in mobility to those seen in the purified limulin preparations (Fig. 3A and 3C).

Qualitative Precipitation Tests

Qualitative analysis of precipitation reactions clearly demonstrated that Re and Rc glycolipids equally precipitated Limulus serum (see Table 3). The precipitation appeared to be specific in that neither melibiose nor cellobiose in high concentrations (12 mg/ml) exhibited any reaction with Limulus serum. Chitobiose, a disaccharide of glucosamine obtained by partial hydrolysis of chitin, weakly precipitated Limulus serum. Glucosamine monosaccharide, even in high concentrations (5 mg/ml) did not precipitate whole serum. The Rc oligosaccharide precipitated these fractions while the core mixture, composed
Fig. 3. Polyacrylamide disc gel electrophoresis (7%) in Tris-glycine pH 8.3. A. Eighteen ug of limulin isolated by preparative slab gel electrophoresis. B. Two hundred and fifty ug of Limulus serum adsorbed with Re-glycolipid. C. Fifty ug of limulin isolated by DEAE-Sephadex chromatography. D. Two hundred and fifty ug of clear ultracentrifuged (100,000xg, 2 hr) fraction of *Limulus* serum. Electrophoresis was performed at 2 mA per tube for 30 min and 4 mA per tube for 115 min.
Table 3. Qualitative Precipitin Tests Using Whole Limulus Serum and Various Components of Lipopolysaccharide

<table>
<thead>
<tr>
<th>Test substance</th>
<th>Conc. (mg/ml)</th>
<th>Whole serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re glycolipid</td>
<td>1</td>
<td>3+</td>
</tr>
<tr>
<td>Rc glycolipid</td>
<td>1</td>
<td>3+</td>
</tr>
<tr>
<td>Rc oligosaccharide*</td>
<td>3</td>
<td>3+</td>
</tr>
<tr>
<td>NH$_2$OH-treated Lipid A**</td>
<td>3</td>
<td>3+</td>
</tr>
<tr>
<td>Chitobiose</td>
<td>2</td>
<td>1+</td>
</tr>
<tr>
<td>Melibiose</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Glucosamine</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Rc core mixture***</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>BSA</td>
<td>5</td>
<td>+/-</td>
</tr>
<tr>
<td>BSA-Lipid A</td>
<td>1</td>
<td>4+</td>
</tr>
<tr>
<td>Diluent (CaCl$_2$-NaCl)</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Heptose Fraction

** Hydroxylamine treated lipid A

*** Rc core mixture contains Rc oligosaccharide, KDO-P-Etn, and KDO.
of the Rc oligosaccharide, KDO-P-Etn, and KDO, did not precipitate Limulus serum. Bovine serum albumin (BSA)-solubilized lipid A also formed a strong precipitin line with Limulus serum.

The precipitating activity of limulin isolated by ion-exchange chromatography is presented in Table 4. Limulin precipitated Re glycolipid very strongly, but, in contrast to the Limulus serum, exhibited no activity with the glucosamine backbone of lipid A. No precipitating activity was observed against solubilized lipid A or lipopolysaccharide from Bacteroides melaninogenicus, which is devoid of KDO in its core polysaccharide (48). Heparin, even in high concentrations, did not precipitate the lectin.

Hemagglutination and Hemagglutination-Inhibition Studies

The hemagglutinating activity of limulin obtained by preparative electrophoresis is shown in Table 5. Limulin exhibited a higher activity against chicken erythrocytes coated with Re glycolipid as compared to the uncoated chicken red cells. The hemagglutinating activity of the purified lectin increased 8-fold, and that of the partially purified serum fraction (100,000xg, 2 h), 4-fold, when chicken erythrocytes were coated with the KDO-containing material.

The hemagglutination activity of limulin isolated by ion-exchange chromatography is shown in Table 6. The lectin exhibited a 20-fold increase in activity against chicken erythrocytes, and a 14-fold increase in titer against passively coated chicken red blood cells as compared to preparations obtained by electrophoresis. As with the lectin purified by electrophoresis, this limulin preparation also demonstrated a higher
Table 4. Qualitative Precipitation Tests Using Limulin Purified by Ion-exchange Chromatography and Various Components of LPS

<table>
<thead>
<tr>
<th>Test Substance</th>
<th>Concentration (mg/ml)</th>
<th>Limulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Re-Glycolipid</td>
<td>1</td>
<td>4+</td>
</tr>
<tr>
<td>NH₂OH-Treated Lipid A*</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>BSA-Lipid A**</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bacteroides LPS***</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Heparin</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* Hydroxylamine treated lipid A

** Lipid A solubilized by conjugation to bovine serum albumin

*** Lipopolysaccharide extracted from Bacteroides melaninogenicus
Table 5. Hemagglutination Activity of Limulin Purified by PAGE*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chicken RBC (Titer$^{-1}$)</th>
<th>Re-Chicken RBC**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (100,000xg, 2 h)</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td>Limulin</td>
<td>64</td>
<td>512</td>
</tr>
</tbody>
</table>

* Preparative polyacrylamide disc gel electrophoresis

** Glycolipid extracted from the Re mutants of *Salmonella minnesota*
Table 6. Hemagglutination Activity of Limulin Purified by Ion-exchange Chromatography

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chicken RBC (Titer$^{-1}$)</th>
<th>Re-Chicken RBC*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum (100,000xg, 6 h)</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>Dialyzed Serum**</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>Limulin</td>
<td>1280</td>
<td>7120</td>
</tr>
</tbody>
</table>

* Glycolipid extracted from the Re mutant of *Salmonella minnesota*

** 0.05 M Tris, 0.1 M NaCl, 0.01 M CaCl$_2$, pH 8.5 at 5°C
activity against chicken cells coated with Re glycolipid as compared to the uncoated erythrocytes.

Additional studies were conducted using the monosaccharides KDO and NANA to block the hemagglutinating activity of the limulin preparations. The agglutinating activity of four units of limulin, purified by electrophoresis, was completely inhibited by 100 mM KDO (Fig. 4) and 100 mM NANA (Fig. 5), when reacted with untreated erythrocytes. Both acidic sugars exhibited similar inhibition activity against unmodified chicken erythrocytes.

In comparable studies using limulin purified by ion-exchange chromatography, 40 mM NANA (Fig. 6) and 50 mM KDO (Fig. 7) were required to inhibit completely agglutination of untreated chicken erythrocytes. The agglutination of Re-coated red cells by limulin was inhibited by 250 ug of the heptose-containing fraction obtained from the RC glycolipid (Fig. 8) and by 12.5 mM KDO (Fig. 9).
Fig. 4. Inhibition of hemagglutination of chicken erythrocytes (3%) by limulin (4 units) isolated by preparative electrophoresis using various concentrations of 2-keto-3-deoxyoctonate.
Figure 4
Fig. 5. Inhibition of hemagglutination of chicken erythrocytes (3%) by limulin (4 units) isolated by preparative electrophoresis using various concentrations of N-acetyl neuraminic acid.
Fig. 6. Inhibition of hemagglutination of chicken erythrocytes by 4 units of limulin (DEAE-Sephadex eluate) using various concentrations of N-acetyl neuraminic acid (NANA).
Fig. 7. Inhibition of hemagglutination of chicken erythrocytes by 4 units of limulin (DEAE-Sephadex eluate) using various concentrations of 2-keto-3-deoxyoctonate (KDO).
Figure 7

% Inhibition

mm KDO

12.5 25 50 100

6.25 12.5 25 50
Fig. 8. Inhibition of hemagglutination of Re-coated chicken erythrocytes by 4 units of limulin (DEAE-Sephadex eluate) using various concentrations of Rc oligosaccharide "heptose fraction".
Figure 8
Fig. 9. Inhibition of hemagglutination of Re-coated chicken erythrocytes by 4 units of limulin (DEAE-Sephadex eluate) using various concentrations of 2-keto-3-deoxyoctonate (KDO).
CHAPTER IV

DISCUSSION

Procedures used in this study were designed to isolate the various components of the LPS molecule to determine which portion was reactive with the lectin from *L. polyphemus*. In most cases the protocols employed were used without modification and the results obtained paralleled those reported in the literature. A notable exception was the treatment of isolated lipid A by alkaline hydroxylamine. Although designed to cleave fatty acids from the glucosamine backbone, this procedure has been reported to be ineffective in hydrolyzing amide-linked beta-hydroxy myristic acid from the saccharide core (69). Thus, the expected products of hydroxylaminolysis were the glucosamine backbone containing beta-hydroxy myristic acid and free fatty acids. In fact, the glucosamine backbone was devoid of all fatty acids. In view of the fact that the precipitate obtained after this procedure was further exposed to hydroxylamine during several centrifugation steps, it appears that longer treatment of lipid A with this reagent can remove the normally resistant amide-linked fatty acids, as well as the ester-linked ones, from the glucosamine core.

Serological studies of isolated fractions of *S. minnesota* glycolipids indicate that two separate sites on the glycolipid react with components of *Limulus* serum. The first of these can be demonstrated using fractions obtained from the core saccharide region of the glycolipid molecule. Since both Re and
Rc glycolipids precipitate Limulus serum equally well, the one sugar common to both, KDO, is the likely binding site for the serum lectin. Further evidence for the role of KDO in this reaction was obtained by comparing the precipitation reaction of the saccharide mixture obtained following acid hydrolysis of the Rc glycolipid, and the isolated Rc core oligosaccharide. The Rc core oligosaccharide, consisting of Glc-(Hep)₃-KDO, precipitates with Limulus serum, while the mixture of hydrolysis products, containing this fraction plus free KDO and KDO-P-EtN, does not. It is likely that in this latter case the monosaccharides are acting as haptene inhibitors of the precipitation reaction.

A second site of reactivity with Limulus serum is found on the glucosamine backbone of lipid A. This material was shown to be free of both fatty acids and KDO. Its mobility on paper chromatography was comparable to chitobiose suggesting that the glucosamine units were linked as disaccharides. Subsequent studies using the purified lectin limulin revealed no evidence of reactivity with this lipid A backbone indicating that the reactive material present in whole serum is distinct from this lectin. It is possible that the material responsible for binding to glucosamine residues is the lectin recovered earlier in our laboratory with reactivity for Staphylococcus aureus (26).

The lectin limulin was recovered from Limulus serum by two separate procedures. Purification by preparative electrophoresis in polyacrylamide resulted in a protein which appeared as a single band following electrophoresis in 7% polyacrylamide
at pH 8.3. The specific activity of the lectin isolated in this fashion increased 181 times compared to the starting material. The latter suggests the presence of an inhibitor in whole serum, which was removed during purification.

Lectin purified by ion-exchange chromatography appeared heterogeneous upon analysis by polyacrylamide gel electrophoresis. These results are consistent with those of Roche and Monsigny (62) who developed the basic protocol and who recovered three closely related proteins called limulin I, II, and III. In the present study the multiple bands seen on analytical electrophoresis were all eliminated when Limulus serum was preincubated with Re glycolipid, strongly suggesting that they are isolectins (62), that is, proteins with the same reactive sites but slightly different overall compositions.

Whole Limulus serum and more recently purified limulin have been shown to react specifically with NANA-containing ligands (12, 7, 45, 51, 53, 62). Limulin obtained in the present study also has this activity as shown by the ability of this lectin to agglutinate chicken erythrocytes, a rich source of glycoproteins with terminal sialic acid residues (18). That the ligand involved was NANA was shown by the specific blocking of this agglutination reaction by free NANA.

These same purified lectins also reacted with KDO. Erythrocytes coated with Re glycolipid were readily agglutinated by these lectins. Furthermore, free KDO was an effective blocking agent for this reaction. The results of cross-inhibition studies where NANA was used to block KDO-mediated agglutination and free KDO, to block agglutination of unmodified
chicken erythrocytes, confirm that KDO and NANA compete for binding on the lectin molecules.

Qualitative precipitation tests confirmed the specificity of the isolated lectin. While Re glycolipid was readily precipitated by limulin, the lipid A portion was unreactive. Lipopolysaccharide from B. melaninogenicus, which lacks KDO (48), was likewise unreactive. Furthermore, since heparin does not precipitate with limulin, it appears that nonspecific ionic interactions do not play a role in these interactions.

In addition to the sialic acid-binding lectin reported in Limulus polyphemus (7, 12, 45, 51, 53, 62), lectins with reactivity against N-acetyl neuraminic acid have been found in the lobster Homarus americanus (28), the pacific oyster Crassostrea gigas (31), the snail Helix pomatia (73), and the prickly lettuce plant Lactuca scariola (76). Recently a number of steric similarities between the methyl beta-D-glycoside of KDO, and sialic acid (NANA) methyl alpha-D-glycosides have been established, which extend beyond their both being 2-keto-3-deoxy acids, and which could possibly have some biological significance (3). The spatial similarities (Fig. 10) of these acidic sugars extend to the carbons of their exocyclic chains in addition to their pyranose ring carbons, because of proposed hydrogen bonding between their axial carboxylate groups and the hydroxyl groups on C-8 of both molecules (3). On the basis of the evidence with the Limulus lectin, it is probable that the sialic acid-binding lectins reported in plants and other invertebrates also react with KDO.
Fig. 10. Comparative steric arrangement of the carboxylate form of the molecules of methyl-D-3-deoxy-manno-octulosonic acid (upper) and methyl-alpha-D-N-acetyl-neuraminic acid (lower) (reprinted from Reference 3).
Fig. 10
In addition to limulin, other lectins with reactivity against lipopolysaccharide include snail A-hemagglutinin from *Helix pomatia* (28), concanavalin A (74, 27, 16, 49, 1, 75), phytohemagglutinin (74, 1, 75), soy bean agglutinin (74, 1, 75), and potato lectin (65). Studies have shown that the precipitation of *Salmonella typhimurium* LPS by snail A-hemagglutinin is inhibited by N-acetyl-D-galactosamine (30), and the concanavalin A precipitation of lipopolysaccharide by alpha-methyl mannose (49) and glucose (16). Similarly the erythroagglutination of concanavalin A, phytohemagglutinin, soy bean agglutinin was inhibited by alkaline-treated lipopolysaccharide (74). From these and other studies (66) on the binding specificities of these lectins, it is unlikely that any of them react with the KDO portion of the LPS molecule.

The lectins of legumes have been shown to react specifically with the O-antigen-containing lipopolysaccharides of their rhizobia symbionts (1, 5). It has been suggested that plant lectins may play a role in the specificity of the rhizobium-legume root nodule symbiosis (5). It is possible that plant lectins are enzymes (glycosyl transferases) which degrade the lipopolysaccharides of the symbiont rhizobia (1). The legume lectin may play an analogous role to vertebrate antibodies in the natural immune system of plants in combating diseases resulting from bacterial or fungal infections in their natural habitat.

In addition to the serum glycoproteins with activity against LPS (58, 59), *Limulus* hemolymph contains circulating cells, amoebocytes, which release materials capable of reacting
with bacterial endotoxin (68, 2, 38, 39, 40). The detection of endotoxin using a lysate prepared from these amoebocytes differs from the lectin-mediated reactions in that the kinetics of the reaction between amoebocytes lysate (coagulogen) and endotoxin are consistent with the concept that an enzymatic system mediates the conversion of the cell-derived protein in to gel by endotoxin (38, 39, 40). Therefore, there appears to be two separate systems in *Limulus* by which the organism can specifically recognize and interact with bacterial lipopolysaccharide. These mechanisms may have developed in the course of evolution as complementary systems to combat the bacterial disease of *Limulus polyphemus*.

Besides a natural hemagglutinin with specificity against N-acetyl neuraminic acid (7, 12, 45, 51, 53, 62) and KDO, a galactose-binding lectin has been identified (73, 8) and purified (26) from the hemocyanin fraction of *Limulus* hemolymph. These lectins appear to differ in molecular weight, electrophoretic mobility, and binding specificity. The demonstration of separate lectins with different binding specificities in *Limulus* as well as in other invertebrates (28) provides evidence for the hypothesis (13) that invertebrate agglutinins function as recognition factors for a variety of foreign substances leading to their phagocytosis and detoxification. The agglutinins would, in this respect, be functionally analogous to vertebrate antibodies.

The *Limulus* agglutinins are probably involved in the phagocytosis of microorganisms by the amoebocytes (60). Phagocytosis of Gram-negative bacteria by *Limulus* amoebocytes was
observed when washed amoebocytes and bacteria were incubated with a source of agglutinin *in vitro* (60). There was no phagocytosis of bacteria in experiments where washed amoebocytes were incubated without agglutinin present. These results are in accord with studies in cray fish (47), sea hare (57), snail (61), oyster (71), and lobster (28) which demonstrate that agglutinins enhance phagocytosis *in vitro*. The ability of invertebrate agglutinins to function as opsonins is additional evidence for their proposed role in defense in invertebrates, and is another way in which agglutinins may be functionally analogous to vertebrate antibodies.

A simple model for self-non-self-discrimination in invertebrates has recently been proposed by Parish (56). The model assumes that the agglutinins (recognition factors) are composed of some of the glycosyl transferases which the invertebrates use to synthesize their own complex carbohydrates. Each sugar residue is attached by a different glycosyl transferase which has specificity for the sugar acceptor. These transferases act as the subunits of the recognition factors, are secreted by hemocytes into the hemolymph, and randomly associate into hexamers. The recognition factor is formed by polymerization of the transferases which is initiated by an additional protein for which an acceptor site is located on hemocyte surface. This protein imparts cytophilic and opsonic properties to the recognition factors. There are only five basic recognition units in this model; the fact that they polymerize in a random manner into hexamers enables them to react in a multipoint fashion with foreign substances.
This model has several important theoretical implications. First, it ensures that self-reactive recognition factors are not generated. Second, due to multipoint binding, a comparatively wide range of foreign antigenic specificities can be recognized. Third, there would be no memory component in this form of immune recognition. It is conceivable, however, that foreign substances may activate the hemocytes to multiply and secrete the recognition factors more rapidly.

In both plants and animals, glycosyl transferases have been shown to have a role in cell-cell recognition and cell-glycoprotein interactions (56). These findings support the possibility that glycosyl transferases have developed into a primitive protective system against infectious agents.
REFERENCES


### APPENDIX

#### ABBREVIATIONS USED

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AraN</td>
<td>4-Aminoarabinose</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin (fraction IV)</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
</tr>
<tr>
<td>EtN</td>
<td>Ethanolamine</td>
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<tr>
<td>Glc</td>
<td>Glucose</td>
</tr>
<tr>
<td>GlcN</td>
<td>Glucosamine</td>
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<tr>
<td>Hep</td>
<td>Heptose</td>
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<tr>
<td>KDO</td>
<td>2-keto-3-deoxyoctonate</td>
</tr>
<tr>
<td>KDO-P-EtN</td>
<td>KDO-phosphoethanolamine</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>NANA</td>
<td>N-acetylneuraminic acid</td>
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<td>PAGE</td>
<td>Polyacrylamide disc gel electrophoresis</td>
</tr>
<tr>
<td>Rc</td>
<td>Mutant of <em>Salmonella minnesota</em></td>
</tr>
<tr>
<td>Re</td>
<td>Mutant of <em>Salmonella minnesota</em></td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol; Tris(hydroxymethyl) aminomethane</td>
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