AGE-DEPENDENT DETERMINANTS OF BACTERIAL ADHESION IN THE *RHIZOBIUM*-CLOVER SYMBIOSIS

By

Maria R. Urbano

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Microbiology and Public Health

1980

ABSTRACT

AGE-DEPENDENT DETERMINANTS OF BACTERIAL ADHESION
IN THE *RHIZOBIUM*-CLOVER SYMBIOSIS

By

Maria R. Urbano

The goal of this research is to determine how culture age affects the selective adherence of *Rhizobium trifolii* to the root hairs of its host, clover. This is a portion of a long-range goal to determine the biochemical basis of host specificity in this nitrogen-fixing symbiosis. Broth cultures of *R. trifolii* 0403 in early stationary phase have a greater affinity than cultures in exponential phase for clover root hairs. Antiserum to purified lipopolysaccharide (LPS) from cells in early stationary phase was adsorbed with cells in exponential phase to remove antibodies against LPS determinants which were conserved during this shift in growth phase. This adsorbed antiserum detected a unique antigenic determinant on the surface of cells in early stationary phase of bacterial agglutination, passive hemagglutination, immunofluorescence, and enzyme linked immunosorbent assay. This unique antigenic determinant is exposed on cells for only brief periods as they leave lag phase and again as they enter stationary phase. Analyses of the LPS isolated from cells in exponential and early stationary phases of growth indicated a change in ratio of four monosaccharides, a decrease in O-acetyl and an increase in O-pyruvyl non-carbohydrate substitutions, increases

in apparent molecular weight, and the ability of the LPS from cells in early stationary phase to combine with the clover lectin, trifoliin. Immune Fab fragments isolated from antibodies in the adsorbed antiserum blocked the agglutination of cells in early stationary phase with trifoliin and blocked the attachment of these cells to clover root hairs. These unique determinants are important to the attachment of R. trifolii in early stationary phase to the roots of its host, clover.

ACKNOWLEDGEMENTS

I wish to express sincere appreciation to my advisor, Dr. Frank B. Dazzo, for his guidance and encouragement in my research and the preparation of this thesis. Thanks are due to the other members of my guidance committee, Drs. Walter Esselman and Robert Brubaker. A special thanks to Dr. Robert Moon for his helpful advice and encouragement.

The suggestions, support, and assistance of my friends and co-workers, Estelle Hrabak, Dennis Reinecke, and Carol Glaubiger was instrumental in helping me to complete this project. To them, I express sincere gratitude and love.

Most importantly, I would like to express my love and appreciation to my parents who have been a major influence in my life and have helped to make all my educational experiences possible.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The selectivity of bacterial attachment is a major underlying basis for tissue, organ, and host tropisms (35,70) in a variety of microorganisms. In the nitrogen-fixing Rhizobium-clover symbiosis, the bacteria selectively adhere to root hairs of the host prior to specific infection of these specialized epidermal cells. Immunochemical (23) and genetic (4) studies suggest that this selective adherence may be initiated by a specific cross-bridging of antigenically related saccharide determinants on the surface of the bacterium and the cell wall of the host by a multivalent, host-coded lectin called trifoliin.

The ability of R. trifolii to adhere selectively to clover root hairs is influenced by conditions that affect the saccharide receptor on the bacterium and the accumulation of trifoliin on the surface of the host root. The saccharide sequence which binds trifoliin is not constitutive on the surface of R. trifolii 0403 in batch culture; but rather its detection on cells is at a minimum during their balanced growth in exponential phase and shifts to a maximum for a brief period as the culture enters early stationary phase (27). Similar observations on the influence of culture age have been made on the specific interaction between R. japonicum 3I1b123 and soybean lectin (3). The purpose of this research is to examine the structure of one of the trifoliin-binding polysaccharides from R. trifolii 0403, namely, the LPS, in early

stationary phase, and determine how it differs from the LPS isolated from cells at exponential phase. Identification of differences in structure of the corresponding polysaccharides should yield important clues to the molecular nature of the trifoliin-binding site.

LITERATURE REVIEW

The genus Rhizobium contains those species of bacteria able to form nodules on the roots of a member of the plant family Leguminosae (34). In the nodules, atmospheric nitrogen gas is reduced into ammonia which is biologically available to the plant host. Many legumes are of agricultural and economic importance as food and forage crops. Nitrogen fixed within the root nodule is a readily available, inexpensive "fertilizer" which legumes can use for growth.

Rhizobia are gram negative rods occurring singly or in pairs. They are generally motile and often have prominent intracellular granules of poly-B-hydroxybutyrate. Rhizobia grow best at 25-30°C on chemically defined media. Rhizobia are classified as either "fast growers" with a mean generation time of 2-4 hours, e.g., R. trifolii, or "slow growers" with a mean generation time of 6-8 hours, e.g., R. japonicum. Differentiation of species of Rhizobium is based upon host specificity, e.g., R. trifolii infects clover plants only while R. japonicum infects soybean plants only (34).

Species of Rhizobium infect, nodulate, and enter into a nitrogen fixing symbiosis with the roots of leguminous plants (19). The infection process consists of several essential events which precede active nitrogen fixation in the Rhizobium-legume symbiosis. These include mutual host-symbiont recognition of the Rhizobium species on the legume

rhizoplane, specific rhizobial adherence to differentiated epidermal cells called root hairs, root hair curling, shepherd's crook formation, root hair infection, root nodulation, and transformation of vegetative bacteria into enlarged pleiomorphic bacteroids which fix nitrogen (52). Infectiveness is defined as the ability to form infection threads in root hairs. The high degree of rhizobial specificity for its hosts is displayed prior to the formation of root hair infection threads (48). The development of a unique "interface" between the microbe and plant root may be required for recognition. Lectins are proteins or glycoproteins that having carbohydrate binding specificity that are found on root hairs of legumes, they may be an integral part of the initial Rhizobium-legume "interface".

Hypotheses based on experimental evidence supporting the involvement of lectins in the recognition process have been proposed. The lectin-recognition hypothesis states that recognition at infection sites involves the binding of specific lectins to unique carbohydrates found only on the surface of the appropriate rhizobial symbiont (8). The basic working hypothesis states that host specificity involves an interaction of complementary macromolecules on the surfaces of each symbiont. This hypothesis has been supported by several studies. Bohlool and Schmidt (8) introduced the possible role of lectins in host specificity by showing that a fluorescein isothiocyanate-labeled soybean lectin bound only to strains of R. japonicum capable of nodulating soybean. Bauer et al. (2) have confirmed these observations with tritiated soybean lectin and have shown that the binding of R. japonicum is reversible by N-acetylgalactosamine, a potent specific inhibitor of

soybean lectin binding. Wolpert and Albersheim (73) reported similar lectin-binding specificities with rhizobial LPS preparations but reversible binding studies were not included in their report. Serological studies using hyperimmune antisera obtained by a persistent immunization schedule have shown antigenic differences between infective and related noninfective strains of R. trifolii, suggesting that specific macromolecules may be involved in infection (24). Further studies showed that the surfaces of infective R. trifolii and clover root hairs shared a unique antigen which was immunochemically cross-reactive indicating structural relatedness (25). This cross-reactive antigen (CRA) contains receptors that bind to the clover lectin, trifoliin, which has been purified from seeds and roots and partially characterized (28).

Trifoliin activity can be measured by its ability to agglutinate R. trifolii. This assay has been refined by attention to critical variables so that a 100-fold increase in sensitivity over original attempts has been achieved (23). One of the major variables, as is also seen with the R. japonicum-soybean system (3) was the age of the culture of cells.

Dazzo and Hubbell (26) have extended the lectin-recognition hypothesis to explain early recognition (pre-infection) events. According to this hypothesis, trifoliin recognizes unique saccharide receptors on both R. trifolii and clover and acts as a cross-bridge, connecting them to form the correct molecular interfacial structure that allows for the specific adsorption of bacteria to the root hair. The cross-bridging model is a modification of an earlier hypothesis by

Hamblin and Kent (37) and Bohlool and Schmidt (8) who proposed the role of lectin in adsorption and specific recognition of rhizobia on the root surface. In addition, it adds two other components necessary for specificity determination: the unique CRA present on both symbionts which bind the multivalent lectin.

Much experimental evidence has been provided to support this hypothesis. The CRA is present only on strains of Rhizobium which infect white clover (25), and only these strains are agglutinated by trifoliin (28). Clover root hairs preferentially adsorb infective R. trifolii (26) and its CRA (21). The receptor sites on clover roots match the distribution of trifoliin on clover root surfaces which bind the CRA (28). These receptor sites accumulate at root hair tips and decrease in amount towards the root hair base (28). In contrast, the CRA is uniformly distributed on epidermal surfaces in the root hair zone of the seedling root (23). Trifoliin is multivalent in binding and agglutinating R. trifolii (21,25,28). The sugar 2-deoxyglucose specifically inhibits the agglutination of R. trifolii by trifoliin and anti-clover root antibody (21,25). Two-deoxyglucose inhibits the binding of R. trifolii or its capsular polysaccharide to clover root hairs (21,26) and specifically elutes trifoliin from intact clover roots (21). As another system for comparison, 2-deoxyglucose does not inhibit the adsorption of R. meliloti or its capsular polysaccharide to alfalfa root hairs (21,26). Intergeneric transformation studies indicate that genes controlling the synthesis of the surface determinants of R. trifolii which bind trifoliin and anti-clover root antibody co-transform with a frequency of 100%, suggesting they are the same (4). Rhizobium cells

that have been pre-coated with their corresponding lectin have an increased ability to adsorb to the root surface of their host (26,66). The CRA related to R. trifolii is unique to the host, clover, and is not detectable immunochemically on the non-host root surfaces of alfalfa or joint vetch (23).

There are several mechanisms of rhizobial attachment to clover root hairs as is seen using quantitative microscopy (18,20) and electron microscopy (18,23). There exists non-specific mechanisms of attachment that allow all rhizobia to attach at low background levels. In addition, there is a specific mechanism of attachment for R. trifolii cells. The latter mechanism is specifically inhibited by 2-deoxyglucose suggesting that trifoliin may be involved. These values for specific rhizobial attachment are significantly higher at the 99.5% confidence level (26). The primary bacterial attachment stage in this symbiosis (phase 1 adherence) as demonstrated by transmission electron microscopy consisted of the fibrillar capsule of R. trifolii in contact with electron dense globular particles on the surface of the fibrillar clover root hair cell wall (25). A similar situation has been found with the primary attachment phase of R. japonicum to soybean root hairs (1). Other mechanisms involved in firmly anchoring the bacteria to the root hair surface (phase 2 adherence) may go into effect during later pre-infection stages (after 12 hours incubation) following specific recognition (18). These mechanisms may include anchoring to the root surface by extracellular cellulose microfibrils made by rhizobia (55).

Further evidence for the lectin recognition hypothesis is seen with competition experiments which have shown that trifoliin and cross-reactive anti-clover root antibodies bind to the same or similar overlapping determinants on the surface of R. trifolii. The exposure of CRA is necessary for attachment of the bacteria to clover root hairs (23). Monovalent Fab fragments from IgG of anti-clover root anti-serum contain the antigen-combining sites that can bind specifically to cell surface antigens but they cannot cross-bridge antigens on neighboring cells to cause agglutination (62). Therefore, Fab fragments of IgG against the clover root CRA can bind to and block the CRA on the R. trifolii surface. These Fab fragments have been shown to specifically block the ability of R. trifolii to bind to clover root hairs (23). In both experiments, Fab fragments from normal pre-immune rabbit IgG had no effect demonstrating the specificity of the reaction with immune Fab fragments against CRA. Also, Azotobacter vinelandii hybrids carrying the clover root CRA receptor as a result of intergeneric transformation with DNA from R. trifolii (4) bound in high numbers specifically to clover root hair tips (23).

Recently, it has been shown that genetic elements important to surface polysaccharides and consequent symbiont recognition are encoded on plasmids of Rhizobium (57). The report that a fast-growing strain of R. trifolii has all the necessary genes to enable effective soybean nodulation has been retracted (58).

Solheim has proposed a similar model of rhizobial adsorption based on the production of a "curling factor" (66) and the effect of Vicia lectin on rhizobial attachment to Vicia roots (67). According to this

model, the root hair cell wall has a "stabilizing factor" (presumably a lectin) which is secreted from the root and binds to a "curling factor" on the surface of Rhizobium. These bacteria with a "stabilizing factor" on their surface then have a strong affinity for attachment to the root. The basic difference in the models of Solheim and that of Dazzo and Hubbell is whether the lectin is released or remains bound to the root surface. The presence of trifoliin in isotonic washings of root hairs and homologous rhizobial agglutination in hydroponic culture suggests that some trifoliin is released. Trifoliin can be detected on both the root hair surface (28) as well as in aseptically collected clover root exudate (Hrabak and Dazzo, manuscript in preparation). Thus, both models may be operative; the released lectin model may act as a salvage mechanism for eluted lectin.

The nature of the carbohydrate lectin receptor on the surface of Rhizobium is an important factor in hypotheses concerning the recognition process between rhizobia and their legume hosts. Lectin receptors have been identified as capsular polysaccharide (25), lipopolysaccharide (73), and glycan (61). The omnipresence of a small, neutral polysaccharide in the capsule suggests that it normally associates with the acidic polysaccharide, perhaps to act as a "glue" to perpetuate the capsular structure on the bacterial cell surface (23). Zevenhuisen et al. (75) has shown that this glucan is made of unbranched chains of β-1,2 linked glucose residues forming cyclic structures. It is bound to the cell wall. He suggests that this β-1,2 glucan may be involved in masking receptors on LPS that are important for specific host recognition. R. trifolii has a very prominent fibrillar capsule surrounding the cell

which stains with ruthenium red (polycationic electron dense stain) and can be seen in electron photomicrographs (23). Almost all encapsulated cells are immunofluorescent when incubated with trifoliin and anti-clover root antiserum as compared to unencapsulated cells which are mostly nonfluorescent. The same observation was made with R. japonicum and soybean lectin (1,3).

Kamberger (46) has recently shown both host-specific LPS-lectin interactions (R. meliloti-alfalfa) and specific extracellular polysaccharide-lectin interactions (R. japonicum-soybean). In agreement with the results of Dazzo and Hubbell (25), these have all been characterized as surface, acidic heteropolysaccharides (12,25).

In a recent study (23), the capsule of R. trifolii 0403 was extracted from cells at early stationary phase and fractionated by ion-exchange and gel filtration chromatography yielding three polysaccharides. The major polysaccharide was an acidic heteropolysaccharide lacking components of the R core (KDO, heptose) and endotoxic lipid A. The second polysaccharide had properties consistent with LPS. The third polysaccharide was minor and was smaller in size than the second polysaccharide. It contained saccharides not present in the other two polysaccharides and was non-antigenic.

Both trifoliin and anti-clover root antibody reacted through 2-deoxyglucose reversible binding with the first two polysaccharides; no binding to the third polysaccharide was observed (23). Both polysaccharides that reacted with trifoliin carried antigenic determinants in common but were electrophoretically distinguishable molecules. Electrophoretic mobility in SDS-polyacrylamide gels suggested that the

first polysaccharide was larger than the second but acid hydrolysates of both polysaccharides yielded the same paper chromatograms when developed with alkaline silver nitrate. These data support the hypothesis that the major polysaccharide in the capsule at this culture age is an acidic heteropolysaccharide which immunochemically conforms to the same O-antigen found in the LPS preparation isolated from cells of this age. Its larger size and absence of heptose suggests that this major polysaccharide in the capsule is not a partial degradation product of LPS.

Under certain conditions, capsular acidic polysaccharides can be transferred to the core-lipid A and replace the neutral O-antigen polysaccharide in LPS (42,43). It is also possible, as suggested by Humphrey and Vincent (41), that the LPS and capsular polysaccharide of some Rhizobium strains may be similar antigenically. In gel diffusion studies, R. trifolii strain TA1 polysaccharide antigens formed two precipitin bands with homologous whole cell antiserum. These two bands became the same when the major antigen was disaggregated by SDS, suggesting that preparation consisted of two antigenically similar polysaccharides that differed in their states of aggregation.

Most important to be considered was the finding that although sugar component differences were found between corresponding acidic heteropolysaccharides from two distinct R. trifolii strains, the determinants which bound trifoliin and anti-clover root antiserum were conserved (23). The structural diversity of LPS and exopolysaccharides of Rhizobium is enormous (11,30), and therefore it is of utmost importance to distinguish important saccharide constituents which may function in

recognition from structures on the same molecule which are unimportant to this process.

LPS has been considered as one of the important lectin binding polysaccharides. The many steps of LPS biosynthesis have been described for Salmonella and E. coli (60). The O-antigen chain is assembled by a series of reactions involving a C_{55}-polyisoprenoid-monophosphate carrier and membrane bound glycosyltransferases. Sugars are transferred by specific transferases from their activated sugar nucleotides into oligosaccharide units of O-antigen covalently attached to an antigen carrier polyisoprenoid lipid (ACL). These units are then polymerized by a polymerase with the side chain being lengthened by one repeating unit at each growing step. The final O-antigen chain is transferred by a translocase onto the R core which becomes anchored to the lipid A on the outer envelope of the Gram negative bacterium. Other enzymes (e.g., methylases, acetylases) then transfer additional substituents (e.g., O-acetyl groups from acetyl coenzyme A) onto the O-specific chain after the LPS is completed. The highly polymerized non-LPS polysaccharides are probably synthesized by the same mechanism (42,43). The lipid A- and heptose-free polysaccharide of identical O-antigen composition is believed to accumulate when control of the O-antigen polymerase by the translocase is not functioning (42,43).

A specific interaction between the O-antigen containing LPS of R. japonicum, R. leguminosarum, R. phaseoli, and R. spp. and lectins purified from their respective legume hosts has been demonstrated (73). In this study, purified lectins were bound to agarose for use in affinity chromatography and the respective LPS was passed through the

lectin column. In each case, the lipopolysaccharide interacted with the lectin of its symbiont but not with other lectins. This study suggests that the LPS is important as a lectin binding polysaccharide. In each case, though, only 25-35% of the symbiont-specific LPS actually bound to the affinity column. A possible explanation for this small percentage is the fact that rhizobia were harvested in late exponential phase. The age of the culture is important in determining lectin-polysaccharide interactions. At this growth stage, only a small percentage (20%) of R. trifolii 0403 cells bind the unique host lectin (27). This age effect occurs with other rhizobial species as well (3).

The LPS of R. leguminosarum, R. phaseoli, and R. trifolii have been further purified and partially characterized (12). Purified lipopolysaccharides extracted from rhizobia harvested in late exponential phase were partially characterized by gas chromatography and mass spectrometry of the alditol acetate derivatives of these polysaccharides. These results show that LPS from different species as well as strains within a species have unique sugar compositions. This structural diversity revealed by the composition analyses was confirmed by immunochemical studies of the immunodominant antigen.

Included in this study was a structural analysis of R. trifolii 0403 LPS harvested from late exponential phase. Mannose; galactose; glucose; glucosamine; uronic acids; KDO; fucose; 2-O-methyl-6-deoxyhexose; heptose; and N-methyl-3-amino-3,6-dideoxyhexose were reported. Quinovosamine was not reported. The authors suggest that the data presented in this paper demonstrate that rhizobial LPS are structurally diverse and that there is no obvious correlation between LPS structure

and nodulation specificities. They are also careful to point out that these data do not rule out the possibility that structural regions in the LPS other than the immunodominant sites could be involved in determining the specificity of Rhizobium-legume interactions as is indicated in this thesis.

Extracellular polysaccharides have been considered as playing a role in host symbiont recognition. Structural studies (44) of the extracellular polysaccharide from R. meliloti have shown that this polysaccharide has pyruvic acid ketalically linked to the 4 and 6 carbon positions of a terminal β-D-glucopyranosyl group. An octasaccharide repeating unit structure was proposed for this extracellular polysaccharide. This octasaccharide is the largest repeating unit that has been demonstrated for any bacterial polysaccharide.

The same research group has investigated the structure of the extracellular polysaccharide of R. trifolii strain U226 and a heptasaccharide repeating unit was proposed (45). The polysaccharide was shown to contain O-acetyl groups linked to C-2 and C-3 of 4-O-substituted-D-glucopyranosyl chain residues and D-galactopyranosyl groups but that one-third of the "repeating units" lack the terminal pyruvylated D-galactopyranosyl groups (45). Two-deoxyglucose residues were not found in this polysaccharide. Janssen et al. (45) also suggested that observed differences in the composition between polysaccharides elaborated by various strains of R. trifolii, R. leguminosarum, and R. phaseoli were due to previously imperfect analytical methods rather than to significant differences in structures of the polysaccharide. It is difficult to evaluate these data since the polysaccharide

preparation that was used was not purified to homogeneity (5), and the protocol for fractionation of the culture fluid would have yielded a mixture of the polysaccharides produced by this microorganism. This fraction may represent a combination of both capsular and lipopolysaccharide. In addition, polysaccharides were harvested from the broth after four days of growth which would correspond to late stationary phase accompanied by cell lysis. Structural constituents important to recognition are not available on the polysaccharides when the culture has advanced to this late stage as was found in this research.

Zevenhuisen (74) isolated a small polysaccharide (3000 daltons) from R. trifolii which contained D-glucose, D-galactose, D-glucuronic acid, pyruvic acid, and O-acetyl groups in the molar ratio 5:1:2:2:3. Bishop et al. (13) found the same structural groups in another strain of R. trifolii in the proportions 6:1:1.4:2:2 which were in good agreement with the data of Zevenhuisen. They have tentatively proposed an octasaccharide repeating unit for the structure of R. trifolii polysaccharide. Somme et al. (68) found similar molar proportions in an extracellular polysaccharide from other strains of R. trifolii.

Dudman and Heidelberger (31) found that although the polysaccharides of five strains of R. trifolii contained glucose, galactose, and glucuronic acid in the same relative proportions, as judged by chromatography, they displayed little serological cross-reactivity. Both acetyl and pyruvyl groups were found in the polysaccharide of all strains of R. trifolii and R. meliloti studied. Loss of pyruvyl and acetyl groups abolished serological activity in R. trifolii strain TA1. The sequence of glycosidic linkages, as well as the non-carbohydrate

substitutions, are immunochemically important. Thus, despite similarities in sugar constituents, the polysaccharides or R. trifolii may become immunochemically distinct based on the presence and position of non-carbohydrate constituents.

Other studies concerning the nature of the carbohydrate lectin receptor have been done using mutant strains of rhizobia. Recently, detailed analyses of nodulating and related non-nodulating mutant strains of Rhizobium have revealed structural changes in their LPS which may be important to their nodulation capabilities (51,62). An R. leguminosarum mutant strain (EXO-1) that does not nodulate peas had only 27% of its total LPS mass as anthrone-reactive carbohydrate whereas the wild type R. leguminosarum has 63% of its total LPS mass as anthrone-reactive carbohydrate (63). The mutant strain was selected to produce small, non-gummy colonies. The glycosyl and antigenic composition of the O-antigen of the mutant and wild type strains did not seem to be different. The author's interpretation of these data was that the mutant strain had reduced its production of extracellular polysaccharide and not of LPS, and therefore the extracellular polysaccharide was important in nodulation (63). No explanation was offered for the large difference in carbohydrate contents of the LPS from the wild type and mutant strains. The author has since retracted these interpretations orally.

Another possible explanation is that the phenotype of this mutant strain is due to a genetic defect of the polymerase which adds repeating oligosaccharide units onto ACL to form the long repeating O-antigen of the LPS (20). If the O-antigen were not polymerized, the percentage of

total LPS that would be anthrone reactive carbohydrate would be very small. If the O-antigen must be of a certain minimum length in order to be important to attachment and nodulation, then the mutant strain would not be able to nodulate its host.

Another analysis of nodulating and non-nodulating mutant strains of R. japonicum showed that the antigenic difference on their cell surface residues in the O-antigens (51). Discrete silver-nitrate reactive components are missing in acid hydrolysates of LPS and acidic heteropolysaccharides from the R. japonicum non-nodulating mutant strains. These results suggest that the mutant strains may lack the ability to add components to the nascent O-antigen chain or modify them after LPS assembly is complete. Therefore, several modifications of intact LPS may explain the inability of the Rhizobium mutant strains to nodulate their legume hosts.

Regulation of the recognition process between symbionts is perhaps the most important aspect of this symbiosis and is just beginning to be understood. It has been known since 1864 that fixed nitrogen interferes with the development of the Rhizobium-legume symbiosis in nature. Dazzo and Brill (22) have developed an immunocytofluorimetric assay to study the effect of fixed nitrogen on recognition processes between rhizobia and their hosts. This assay, which is very specific for trifoliin on clover, allows quantitation of the effect of NO_3^- or NH_4^+ on the levels of trifoliin present on the seedling root epidermis. Intact root surfaces are incubated with anti-trifoliin antiserum and examined by immunofluorescence. The fluorescent light intensity is then measured (as photovolts/mm^2) (28). As the concentration of NO_3^- or NH_4^+ was

increased in the rooting medium, immunologically detectable levels of trifoliin and the specific binding of R. trifolii to root hairs decreased in parallel. Counter ion controls showed conclusively that the inhibitory effects were due specifically to the fixed nitrogen ions. These experiments support the hypothesis that trifoliin is important to binding R. trifolii to clover root hairs.

The above data support the hypothesis that the selective ability of R. trifolii to bind to clover root hairs is influenced by conditions that affect the quantity of trifoliin on the host root surface and the lectin receptor on the surface of the bacterium. Another important supportive study showed that under certain growth conditions, the transient appearance of trifoliin receptors on R. trifolii may influence the ability of bacteria to attach to clover root hairs (27). Cells grown on agar plates of defined media showed the greatest amount of agglutination by trifoliin when they were grown for five days. When the bacteria were grown in broth culture, antigenic determinants cross-reactive with clover roots were present only on cells for brief periods as they left lag phase and again as they entered early stationary phase. Only a small percentage of cells cross-reacted antigenically with clover roots when the culture was in exponential phase. R. trifolii attached to clover roots in greatest numbers when harvested from broth cultures at early stationary phase or plate cultures grown for five days as predicted based on their optimal reactivity with trifoliin. These studies suggest why many anomalous lectin-binding results in other systems have been obtained, e.g., Chen and Phillips (14); Law and Strijdom (47) and also indicate that the regulation of these surface lectin receptors may be

very complex.

The infectivity of Agrobacterium tumefaciens, a gram negative bacterium which incites crown gall tumors in various plant hosts, displays a similar biphasic binding curve as is found for trifoliin receptors on R. trifolii. Attachment to host tissue is necessary for infection to occur. Agrobacterium attaches to its host in the greatest numbers during lag and early stationary phases of growth. Little attachment occurs during the exponential phase of growth (Lippincott, J. A., unpublished observation). The infection of white clover root hairs is maximal when the standardized inoculum of R. trifolii NA30 is taken from early stationary phase (54). In the R. japonicum-soybean system, Bauer et al. (3) have observed a transient appearance of the receptor on R. japonicum that specifically binds soybean lectin. Most strains bound soybean lectin in greatest numbers during early and mid-log phases of growth but one strain accumulated the lectin receptors as cells entered early stationary phase. As cultural aging occurs, the galactose residues important to the binding of the capsular polysaccharide to the lectin become methylated in the 4 position. As methylation occurs, lectin binding activity decreases (3).

Recent reports (53) have shown that capsular polysaccharides extracted from two strains of R. japonicum contain galactose, a sugar known to bind soybean lectin. The proportion of galactose in the capsular polysaccharide in stationary phase cultures is one-third to one-sixth of the quantity found in log phase cultures. In these strains, lectin binds only to cells from log phase cultures. Also, the ability of capsular polysaccharide to inhibit binding of the soybean

lectin to affinity beads and to lectin-binding cells fell as the 4-O-methyl galactose content increased. These data help confirm the hypothesis that galactose in the capsular polysaccharide of R. japonicum is important to the binding of lectin to the bacteria. The binding of highly purified, ferritin conjugated soybean lectin to the cell surface of R. japonicum was examined by whole mount, thin section, and freeze-etch electron microscopy. The conjugated lectin bound to the capsular material on the outer surface of the bacterium. N-acetyl-D-galactosamine at 5 mM fully prevented the binding of the lectin to the capsular material (10).

New reports from Mort and Bauer (in press) have shown that both capsular and extracellular polysaccharides of R. japonicum (3I1b138 and 110) contain approximately constant amounts of mannosyl, glucosyl, and galacturonosyl residues in a molar ratio of 1:2:1. Varying amounts of galactosyl and 4-O-methyl galactosyl residues are dependent on culture age. The total of galactose plus 4-O-methyl galactose, however, is constant suggesting that the 4-O-methyl galactose residues arise by methylation of galactose residues in the polysaccharides. In addition, there is a 50% reduction in the percentage of cells which are encapsulated as cultures mature from early to late log phase. Only encapsulated cells can bind lectin. The combination of capsular composition change and loss of encapsulation may explain the loss of lectin binding capacity during culture growth in these two strains of R. japonicum.

The effect of culture age on the modification of lectin receptors may influence the behavior of different rhizobial strains in the soil ecosystem. The one strain of R. japonicum (3I1b123) that accumulates

the lectin receptor during early stationary phase (3) has been shown to be the most frequently found serogroup in soybean nodules from many areas of the central United States (17).

Studies on other gram negative and gram positive bacteria have shown that polysaccharide antigens on microbial surfaces undergo structural modification during shifts in growth phase. The somatic O-antigen of Salmonella LPS has a stronger expression in stationary phase than in exponential phase as a result of an increase in length of the polysaccharide (15). In continuous culture, the length of the Salmonella O antigen is inversely related to the dilution rate of the culture (64). Cultures growing at slower rates have longer O antigens. The relative amount of glucose in the cell wall polysaccharide of cariogenic Streptococcus mutans increases as the culture advances to stationary phase (6). Streptococcus pyogenes, a pathogen which attaches to human pharyngeal mucosal surfaces, is more adhesive to epithelial cells while in early stationary phase than in other growth phases due to increased expression of a virulence-related surface antigenic protein (32).

MATERIAL AND METHODS

Rhizobial Culture

Rhizobium trifolii 0403 was obtained from P. S. Nutman and maintained on BIII agar slants (27). Cells were either grown on freshly poured agar plates or in broth at 30°C with rotary shaking.

Purification of Polysaccharide

Cells in exponential and early stationary phases of growth were harvested with a Sharples contrifuge and kept frozen until extracted. These phases of growth correspond to 50 and 90 units, respectively, as determined by a Klett-Summerson colorimeter using a 660 nm red filter (23). Rhizobial polysaccharides were extracted according to Carlson *et al.* (12) as outlined in Figure 1. Thirty to eighty grams (wet weight) of bacteria were suspended in one liter of 0.5 M NaCl, mixed with a magnetic stirrer for one hour, and centrifuged at 11,000xg for twenty minutes. The supernatant fluid was dialyzed against distilled water which had been deionized to 18 mOhms by passage through a 4-bowl Milli-Q purifier (Millipore) and then lyophilized. This fraction will be referred to as the salt-extracted polysaccharide (SEP). The cell paste was extracted as above with 0.5 M NaCl three times to remove remaining SEP. The pelleted bacteria were suspended in 200 ml of water at 65°C and then mixed with 200 ml of 90% phenol at 65°C. This mixture was shaken vigorously at 65°C for 15 minutes, cooled on ice for 15 minutes, and centrifuged at 14,000xg for 20 minutes at 5°C.

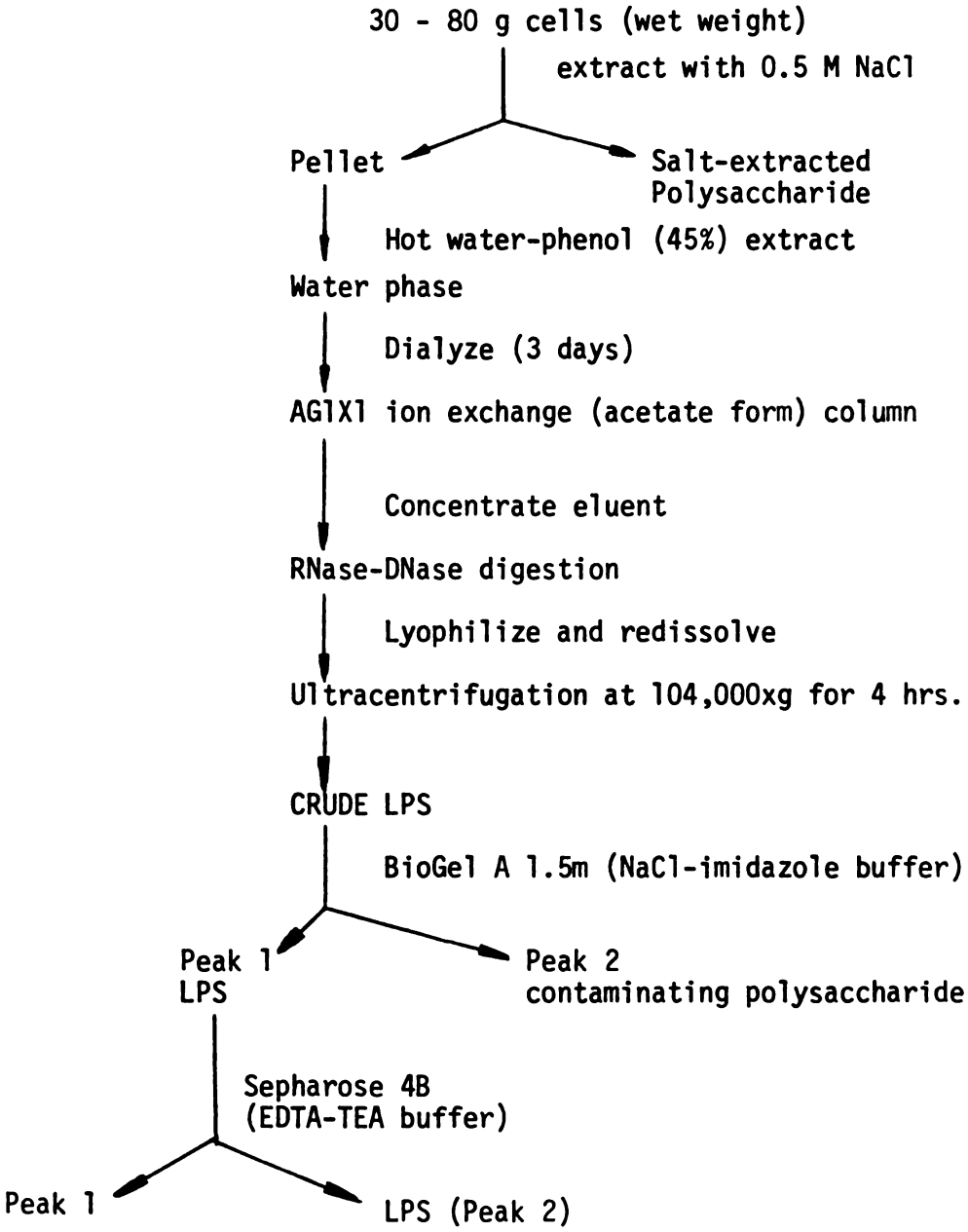


Figure 1. Purification of LPS.

The aqueous phase, which contained the LPS, was collected and the phenol phase was re-extracted for 15 minutes with an equal volume of water at 65°C. The two aqueous phases were pooled and dialyzed at 5°C against three 14-liter changes of deionized, distilled water. The dialyzed extract was passed through a 1cm x 15cm column containing Dowex AG1X1 (Sigma) in the acetate form using deionized, distilled water as solvent. The eluted polysaccharide was concentrated to 100 ml by flash evaporation at 40°C, made 10 mM in $MgSO_4$ and 50 mM in Trizma base (Sigma) and adjusted to pH 7.0 by dropwise addition of 0.3 N HCL with constant stirring. One mg of DNase I (Sigma) and one mg of RNase A (Sigma) were added. The solution was stirred overnight at 5°C and lyophilized.

This lyophilized material was dissolved in 4 ml of 20 mM imidazole-HCl (pH 7) and 100 mM NaCl. The LPS was pelleted by ultracentrifugation at 104,000xg at 5°C for four hours. The LPS pellet was redissolved in 1.5 ml of the imidazole-NaCl buffer and chromatographed on a BioGel A 1.5m column (40cm x 2.5cm) equilibrated in the same buffer. Fractions (2.5 ml) were assayed for carbohydrate by phenol-sulfuric acid (29). The LPS voided this column while a contaminating polysaccharide eluted in the included volume. The peak which contained LPS was dialyzed against deionized distilled water and lyophilized.

One ml of 10 mM EDTA (free acid form) titrated to pH 7 with triethylamine (TEA) was used to dissolve the lyophilized material, which was then applied to a Sepharose 4B column (30cm x 1.5cm) equilibrated with the EDTA-TEA buffer. The LPS eluted at approximately 142 ml of volume (v_e/v_o = 1.10) as the second carbohydrate peak.

Purity of the lipopolysaccharide was analyzed by immunoelectrophoresis on 7cm microscope slides at 10 mA/slide (56), instant thin layer chromatography using isobutyric acid:NH_4OH:water in the ratio 63:5:32, SDS-polyacrylamide gel electrophoresis (69) stained with Periodate-Schiff reagent and an ultraviolet absorption scan. Markers for rhizobial LPS (2-keto-3-deoxyoctanoate, heptose, and uronic acids) were assayed across the total carbohydrate peak (measured by the phenol-sulfuric acid assay) eluted from the Sepharose 4B column with the EDTA-TEA buffer as described above (7,59,72).

Preparation of Antisera

Antiserum to K = 90 LPS was prepared in rabbits according to the passive hemadsorption procedure of Dazzo and Brill (23). The immune response was assayed by a rising agglutination titer with homologous, steamed K = 90 rhizobial cells. Rabbits were exsanguinated by ear artery bleed when their antiserum contained more than 6000 rhizobial agglutinating units/ml. Some of this antiserum was exhaustively adsorbed with washed K = 50 cells to remove antibodies directed towards antigenic determinants conserved on LPS during the shift from exponential growth to early stationary phase. For adsorption, one ml of antiserum was added to 0.05 ml packed cell volume, mixed, incubated at 37°C for 15 minutes, and then centrifuged at 1000xg and transferred to a second tube containing 0.05 ml packed cell volume. This adsorption procedure was repeated twenty times. Anti-K = 90 LPS adsorbed with K = 50 cells will be referred to as adsorbed antiserum and anti-K = 90 LPS as unadsorbed antiserum. Rabbit anti-white clover root antiserum

and anti-R. trifolii 0403 sonicated early stationary phase cells were prepared as previously described (24,25).

Serological Studies

The bacterial agglutination assay (23) was performed on R. trifolii 0403 grown to a Klett density of 50 and 90 units, harvested by centrifugation at 10,000xg for 15 minutes, and resuspended in PBS. Some cells were steamed in PBS for thirty minutes to remove extracellular polysaccharides. The cell pellets were washed by centrifugation three times in phosphate buffered saline (PBS) (10 mM K_2HPO_4-KH_2PO_4, 140 mM NaCl, 1 mM $MgSO_4$, pH 7.2). The cell pellets were then resuspended in PBS, held for 15 minutes to remove settling flocs, passed through a small glass wool column to remove nondispersable flocs (25), and then adjusted with PBS to a density of 160 Klett units (1.7×10^9 cells/ml).

Canine erythrocytes were sensitized with LPS by the method of Nowotny (56) and then used in passive hemagglutination studies (23).

Indirect immunofluorescence was performed with adsorbed and unadsorbed antisera and cells of R. trifolii 0403 grown in BIII broth. Cells fixed on slides were examined for immunofluorescence as previously described (27). Immunofluorescence was scored by determining the percentage of immunofluorescent rhizobial cells in ten randomly selected microscope fields at 400 times magnification. Positive controls were included using unadsorbed antiserum as well as negative controls using totally adsorbed (anti-K = 90 LPS exhaustively adsorbed with K = 90 cells) antiserum and pre-immune serum.

The enzyme-linked immunosorbent assay (ELISA) was used to quantitate the binding of antibody to cells according to Voller et al. (71) with the following modifications. The tubes used in this assay were pre-treated with 100 ug bovine serum albumin (Sigma) in one ml of PBS to prevent nonspecific binding of antiserum and rinsed with PBS containing 0.05% (v/v) Tween 20 three times. At various times, one ml was removed from shaken broth culture. After volume adjustment, the cell density was determined in a Petroff-Hauser chamber and then adjusted with sterile BIII broth to 4×10^7 cells/ml. The cells in one ml were added to the pretreated tubes and washed with PBS by centrifugation at 10,000xg three times. The cell pellets were incubated with 0.2 ml adsorbed antiserum for 4 hours at 37°C, centrifuged again, resuspended in 0.2 ml of goat anti-rabbit IgG conjugated to horseradish peroxidase (Gibco, Calif.) (10 mg/ml), incubated for 4 hours at room temperature, and then washed as above. The substrate consisted of 0.4 mg orthophenylenediamine (Sigma) and 0.2 ml hydrogen peroxide per ml of phosphate-citrate buffer (0.1 M K_2HPO_4 adjusted to pH 5.2 with 0.1 M citric acid). The reaction was initiated by addition of 0.2 ml of freshly prepared substrate to each sample, then incubated for 50 minutes in the dark at room temperature, and finally stopped by addition of 50 ul of 4N H_2SO_4. Each sample was centrifuged as above and the absorbance of the orthodinitrobenzene in the supernatant fluid was measured at 492 nm with a Beckman DU-Gilford modernization spectrophotometer using a blank sample carried through the entire procedure with PBS. Controls included unadsorbed antiserum and anti-K = 90 LPS adsorbed with K = 90 cells substituted for adsorbed antiserum.

Inhibition of passive hemagglutination was used to identify probable haptenic determinants. Adsorbed antiserum (0.4ml) was incubated for thirty minutes at 37°C with 0.2 ml of PBS containing various sugars and then tested for passive hemagglutination (23) using canine erythrocytes sensitized with K = 90 LPS. Most sugars were purchased from Sigma and used at 30 mM concentration; quinovosamine was a gift from Dr. Laurens Andersen, University of Wisconsin, and from Dr. Malcolm Perry, National Biological Council, Canada, and was used at 7 mM concentration. The α-methyl and β-propyl derivatives of quinovosamine were also tested. LPS (500 ug) from cells in exponential and early stationary phase were preincubated with the adsorbed antiserum and examined as inhibitors of passive hemagglutination.

R. trifolii bacteriophages produce endoglycanohydrolases which digest rhizobial capsular polysaccharide into oligsaccharide fragments. These fragments may carry the haptenic determinant and therefore possess the ability to act as inhibitors in this passive hemagglutination system. K = 90 SEP was partially digested by R. trifolii bacteriophage BYØ1 and BYØ11. Ethanol was added to 80% (v/v). The resulting mixture was ultracentrifuged at 104,000xg for 4 hours at 5°C. The ethanol soluble fragments were concentrated by flash evaporation and assayed as probable inhibitors of passive hemagglutination.

Purification of Monovalent Fab Fragments

IgG from adsorbed antiserum and preimmune rabbit IgG were purified as described elsewhere (Dazzo and Brill, 1979). The purity of IgG was examined by immunoelectrophoresis (56) using goat anti-rabbit IgG

(Difco, Detroit, Michigan). Purified IgG of normal rabbit serum was a gift of Dr. Lee Velicer, Michigan State University, East Lansing, Michigan. IgG was digested with mercuripapain (Sigma) and the Fab fragments purified by cation exchange chromatography (56). Both peaks of Fab eluting from the CM52 column were pooled, concentrated by high pressure ultrafiltration with a PM-10 membrane (Amicon Corp., Lexington, Mass.), and dialyzed at 4°C against PBS. Protein content was determined by the method of Lowry et al. (49).

Trifoliin Inhibition Assay

Trifoliin was purified from seeds of white clover (Trifolium repens var. Louisiana Nolin) (28). Competition between the Fab fragments from IgG of adsorbed antiserum and trifoliin for binding to R. trifolii 0403 was performed as described elsewhere (Dazzo and Brill, 1979), using cells at a culture density of 90 Klett units (early stationary phase). Both exponential and early stationary LPS and early stationary SEP at a concentration of 250 ug dry weight LPS/ml were tested as inhibitors of trifoliin-meditated agglutination of R. trifolii 0403 grown on plates for five days. The polysaccharides were incubated with trifoliin (80 ug protein/ml) at 30°C for one hour. The polysaccharide-treated trifoliin (0.2 ml) was then incubated with rhizobial cells (harvested, washed with PBS, and adjusted to K = 160) for four hours. The degree of inhibition of trifoliin agglutination was recorded.

Inhibition of Root Adherence

Cells of early stationary phase (grown in broth, washed as above, and adjusted to K = 60) were pretreated with purified Fab fragments (200 ug protein) and then suspended in 1.5 ml of filter-sterilized Fahraeus nitrogen-free solution (33). The treated cells were incubated with five 24 hour old sterile white clover seedlings with rotary shaking at 30°C for 15 minutes. The seedling roots were rinsed gently with Fahraeus solution and examined by phase-contrast microscopy to determine the degree of rhizobial adherence to the clover root hairs as previously described (23).

Biochemical Analysis of LPS

The content of lipid A was determined by the gravimetric method of Carlson et al. (12). Carbohydrate content was determined by the phenol sulfuric acid assay (29) using D-glucose as standard. O-pyruvyl group substitution was determined according to Sloneker and Orentas (65) using pyruvic acid as standard. O-acetyl group substitutions were quantitated according to Hestrin (39) using acetylcholine as standard. Uronic acid was quantitated by the metahydroxydiphenyl assay of Blumenkrantz et al. (7) using glucuronic acid as standard. Two mg of K = 50 and K = 90 LPS were sized on a Sepharose 4B gel filtration column (2cm x 100cm) equilibrated in 10 mM EDTA-TEA buffer, pH 7.0, and calibrated with blue dextran, glucose, and dextrans at 200,000 and 500,000 daltons (Sigma).

The sugar composition of the LPS was analyzed quantitatively by gas chromatography of the alditol acetate derivatives. One to three

mg lyophilized samples of LPS were mixed with 0.5 ml of 2N trifluoroacetic acid, sealed, and hydrolyzed for 60 minutes at 121°C. The resulting monosaccharides were converted into their alditol acetates by the procedure of Hakomori and Siddiqui (36). Inositol (1.0 ug) dissolved in deionized, distilled water was added as an internal standard after hydrolysis. The solution was then reduced with 5 mg of $NaBH_4$ in 0.3 ml of water for 2.5 hours at room temperature. The solution was neutralized with three drops of glacial acetic acid and then extracted with three -0.3 ml portions of chloroform to remove fatty acids. The chloroform-extracted aqueous solution was evaporated to dryness under a stream of nitrogen at 80-100°C and further evaporated after three consecutive additions of one ml of methanol containing one drop of glacial acetic acid to remove Na borate. The residue was acetylated in a sealed vial containing 0.5 ml of acetic anhydride:pyridine (1:1) by heating at 100°C for 15-30 minutes. The solution was evaporated to dryness under a stream of nitrogen. One ml of chloroform and one ml of water were added. Excess reactants (acetic anhydride, pyridine) entered the water layer. The chloroform layer containing the alditol acetates was evaporated to dryness under a stream of nitrogen at room temperature, redissolved in 50 ul of chloroform, and analyzed by gas-liquid chromatography.

Chromatography was performed with a Varian 2700 dual column gas chromatograph equipped with six foot glass columns containing 3% ECNSS-M on GasChrom Q, 100-120 mesh support (Alltech) and flame ionization detectors. The flow of nitrogen carrier, hydrogen, and compressed air was 30, 30, and 300 ml/min. respectively. The temperature program was

13 minutes at 165°C, 4°C/minute increase to 185°C, hold for 5 minutes at 185°C, 4°C/minute increase to 205°C, and then hold for 15 minutes at 205°C. The area of each peak was determined by triangulation. The ratio of the peak area of the internal standard, inositol (which had the same area in K = 50 and K = 90 LPS chromatograms) to each sugar component in its respective chromatogram was calculated. Direct comparisons could then be made between the peak areas of the chromatograms of the LPS from cells at the two different culture ages. Tentative identification of peaks was made by comparison to retention times of standard derivatives. Identification was confirmed whenever possible by combined gas chromatography-mass spectrometry. For some of the components, comparisons to published chromatograms have been used for tentative identification of alditol acetates which were consistent with predicted mass spectra. Many of the peaks did not match the alditol acetates of the sugar standards available.

Combined gas chromatography-mass spectrometry was performed by staff operators at the Mass Spectrometry facility of the Department of Biochemistry, Michigan State University. A Hewlett Packard model 5840A gas chromatograph and a Hewlett Packard model 5985 quadropole mass spectrometer were used. Alditol acetate derivatives of the hydrolysate of K = 90 LPS and of sugar standards were chromatographed on a six foot column containing 3% OV225 on 80-100 mesh Supelcoport (Supelco). The temperature program was a one minute hold at 190°C and then 2°C/minute increase to 240°C. The flow rate of the carrier gas, helium, was 30 ml/min. The ionization temperature of the mass spectrometer was 200°C.

RESULTS

Assessment of LPS Purity

LPS from cells at exponential and early stationary phases of growth were shown to be homogeneous by a variety of criteria. Single bands were produced when the LPS was assayed by immunoelectrophoresis, thin layer chromatography (R_f = 0.148 for K = 50 and K = 90 LPS), and SDS-polyacrylamide gel electrophoresis. The ultraviolet absorption spectrum of the LPS dissolved in EDTA-TEA buffer showed no absorption maxima characteristic of nucleic acid or protein. Also, no protein was detected by the method of Lowry et al. (49). The LPS caused gelation of the Limulus lysate reagent which reacts with endotoxic lipid A. Two mg of each LPS was applied to a Sepharose 4B column (in separate experiments) and the eluted fractions were assayed for KDO, heptose, uronic acid, and total carbohydrate. All markers co-chromatographed in a constant component ratio across the single peak of polysaccharide (Figures 2a and 2b) providing further evidence for carbohydrate homogeneity of the LPS preparation.

Comparison of Cell Surface Determinants in Exponential and Early Stationary Phases of Growth by Immunochemical Methods

A comparison of bacterial agglutination titers with adsorbed antiserum showed important differences in antigenic determinants on cells in exponential and early stationary phase (Table 1).

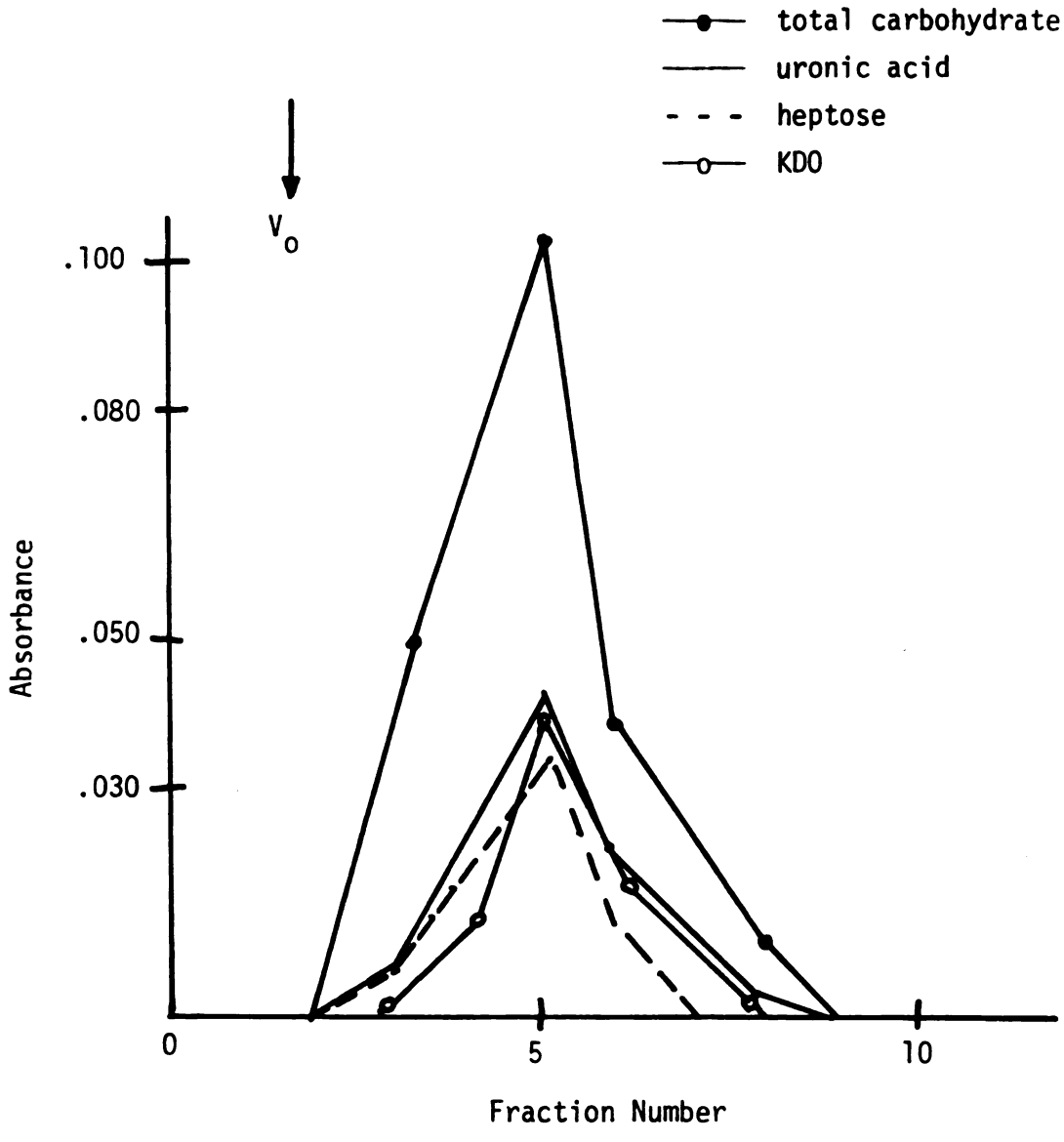


Figure 2a. Purity column: K = 50 LPS.

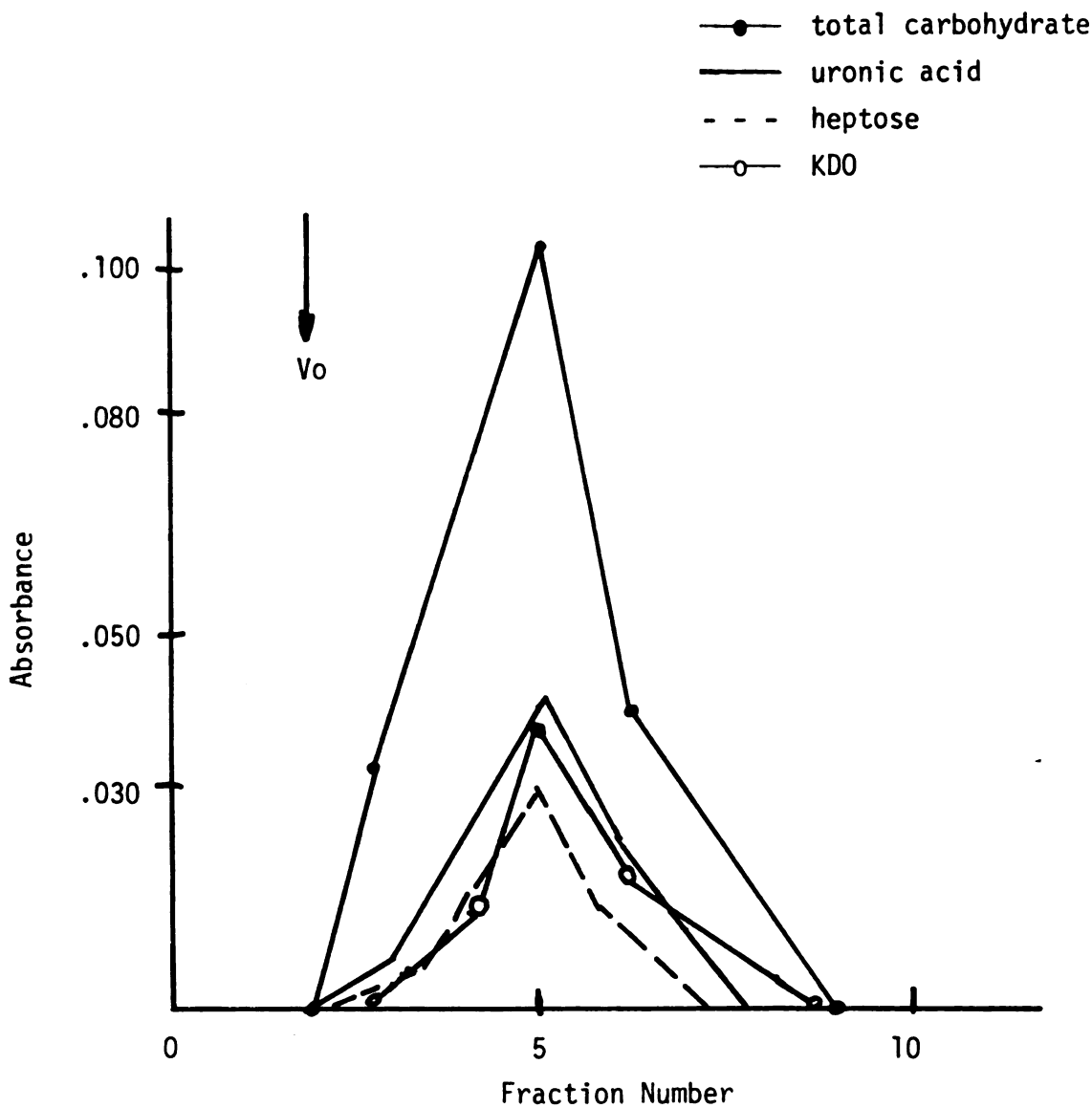


Figure 2b. Purity column: K = 90 LPS.

Table 1. Differential agglutination of R. trifolii 0403 by antiserum against cell surface antigens.

Antiserum	Exponential Phase Cells (agg. units/ml)	Early Stationary Phase Cells (agg. units/ml)
Unadsorbed anti-K = 90 LPS	6400	6400
Anti-K = 90 LPS adsorbed with K = 50 cells	5	640
Anti-R. trifolii 0403 sonicated cells (early stat) adsorbed with K = 50 cells	-[a]	1280

[a]No agglutination detected

Unadsorbed antiserum had the same agglutination titer with cells in either exponential or early stationary phase. Exhaustive adsorption of the antiserum removed 96% of the agglutinating titer using homologous cells. The residual agglutinating titer of anti-K = 90 LPS after adsorption was 128-fold higher with cells in early stationary phase than with cells in exponential phase. There was even a greater difference (>1280-fold) in residual agglutinating titer with adsorbed antiserum to cells in early stationary phase that had been disrupted by sonic oscillation. Steamed bacteria did not autoagglutinate in this assay.

Passive hemagglutination confirmed the results of the bacterial agglutination assay (Table 2). The residual hemagglutinating titer of adsorbed antiserum was eighty-fold higher using erythrocytes sensitized with K = 90 LPS than with K = 50 LPS. There was even a greater difference in residual agglutinating titer (>320-fold) with adsorbed antiserum to cells in early stationary phase which had been disrupted by sonic oscillation. The passive hemagglutinating titer with anti-clover root antiserum was 8-fold higher using erythrocytes sensitized with K = 90 LPS than with K = 50 LPS. Suspensions of sensitized canine erythrocytes did not autoagglutinate. Unsensitized erythrocytes did not agglutinate by any of the antisera used in the above experiments.

Indirect immunofluorescence was used to examine cells throughout the growth curve for antigenic determinants which appear on LPS during the shift from exponential growth to early stationary phase of growth. Four phases were apparent (Figure 3). A small percentage of cells exposed the unique determinants detected by adsorbed antiserum for a

Table 2. Passive hemagglutination of canine erythrocytes sensitized with K = 50 LPS or K = 90 LPS.

Antiserum	Sensitizing Antigen: K = 50 LPS (agg. units/ml)	Sensitizing Antigen: K = 90 LPS (agg. units/ml)
Anti-K = 90 LPS	2560+	2560+
Anti-K = 90 LPS adsorbed with K = 50 cells	---[a]	80
Anti-*R. trifolii* 0403 sonicated cells (early stat) adsorbed with K = 50 cells	---[a]	320
Anti-clover seedling roots (*Trifolium repens*)	20	160

[a]No hemagglutination detected.

+Titer was stopped at this point.

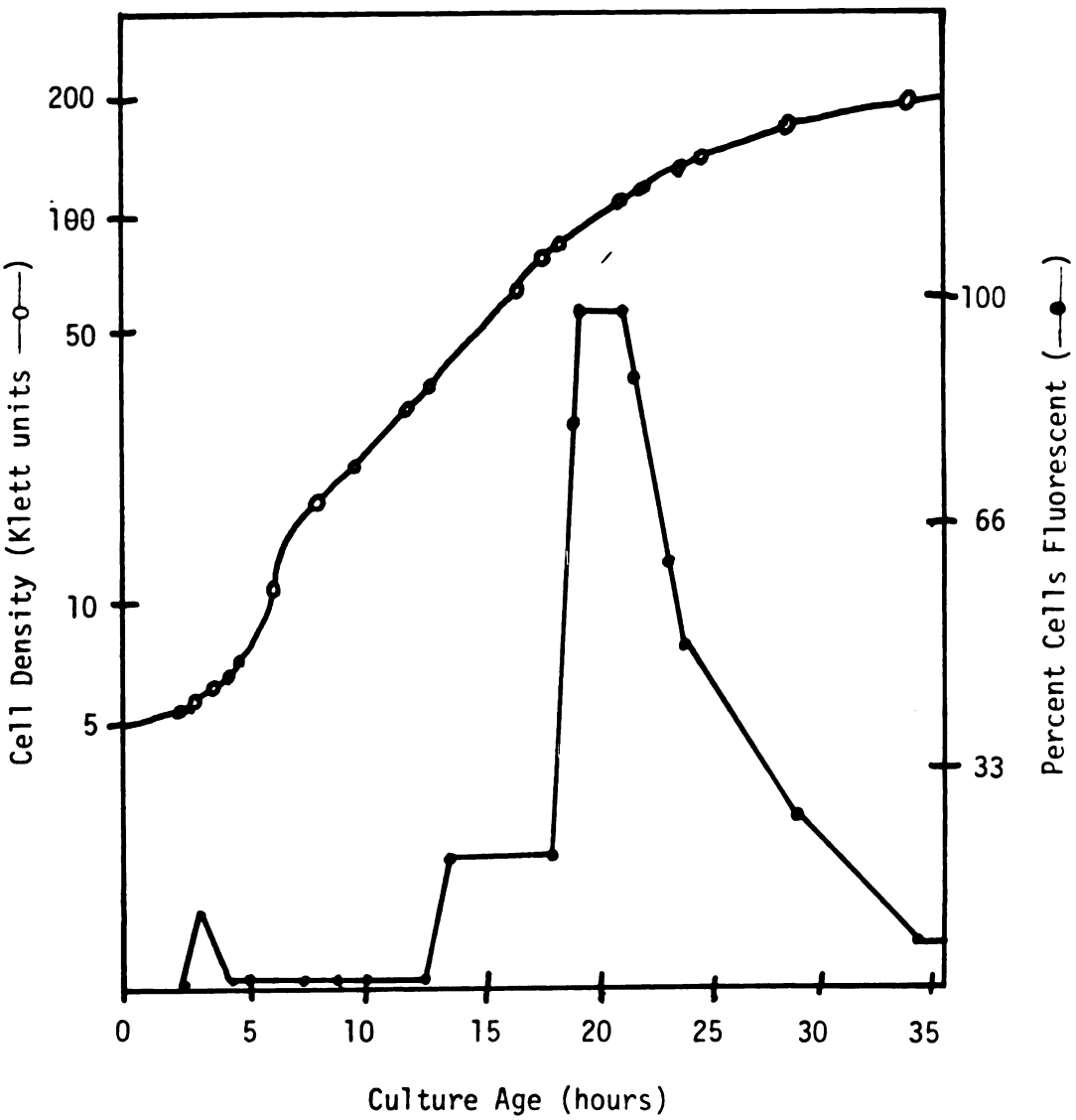


Figure 3. Effect of culture age on the percentage of cells which are immunofluorescent with anti-K = 90 LPS adsorbed with K = 50 cells.

brief period when they left lag phase (K = 5 to K = 15). The adsorbed antiserum did not bind to cells in exponential phase (K = 15 to K = 75). Most of the cells (98%) entering stationary phase (K = 80 to K = 115) were immunofluorescent. Cells in late stationary phase (K = 115 to K = 150) no longer bound adsorbed antiserum, i.e., the antigen(s) was no longer detected on the cell surface by the adsorbed antiserum. Cells representative of each of the above growth phases were incubated with unadsorbed antiserum and a high percentage (98%) were immunofluorescent. This result demonstrated that the immunodominant determinants were conserved on the cell surface from lag phase through late stationary phase. Rhizobium trifolii 0403 cells did not autofluoresce or react with the FITC-conjugated rabbit anti-globulin in the absence of anti-rhizobial antiserum.

The ELISA using adsorbed antiserum confirmed the results of the quantitative immunofluorescence studies (Figure 4). The same four phases of antigenic reactivity were found. Cells just leaving lag phase (K = 5 to K = 15) bound more antibodies than cells in exponential phase (K = 15 to K = 75). In early stationary phase (K = 80 to K = 115), cells bound the maximal amount of antibody. Later in stationary phase (K>135), few antibody binding sites remained exposed on the cell surface. Only background levels of antibody were bound to cells if pre-immune serum or anti-K = 90 LPS adsorbed with K = 90 cells were used (absorbance at 492 nm was 0.020-0.025). At K = 9, 42, 96, and 137 which represent the four different phases of growth, ELISA was repeated with unadsorbed antiserum. In each case, the absorbance remained at the high level of 0.686 ± 0.006. The plot of optical density vs.

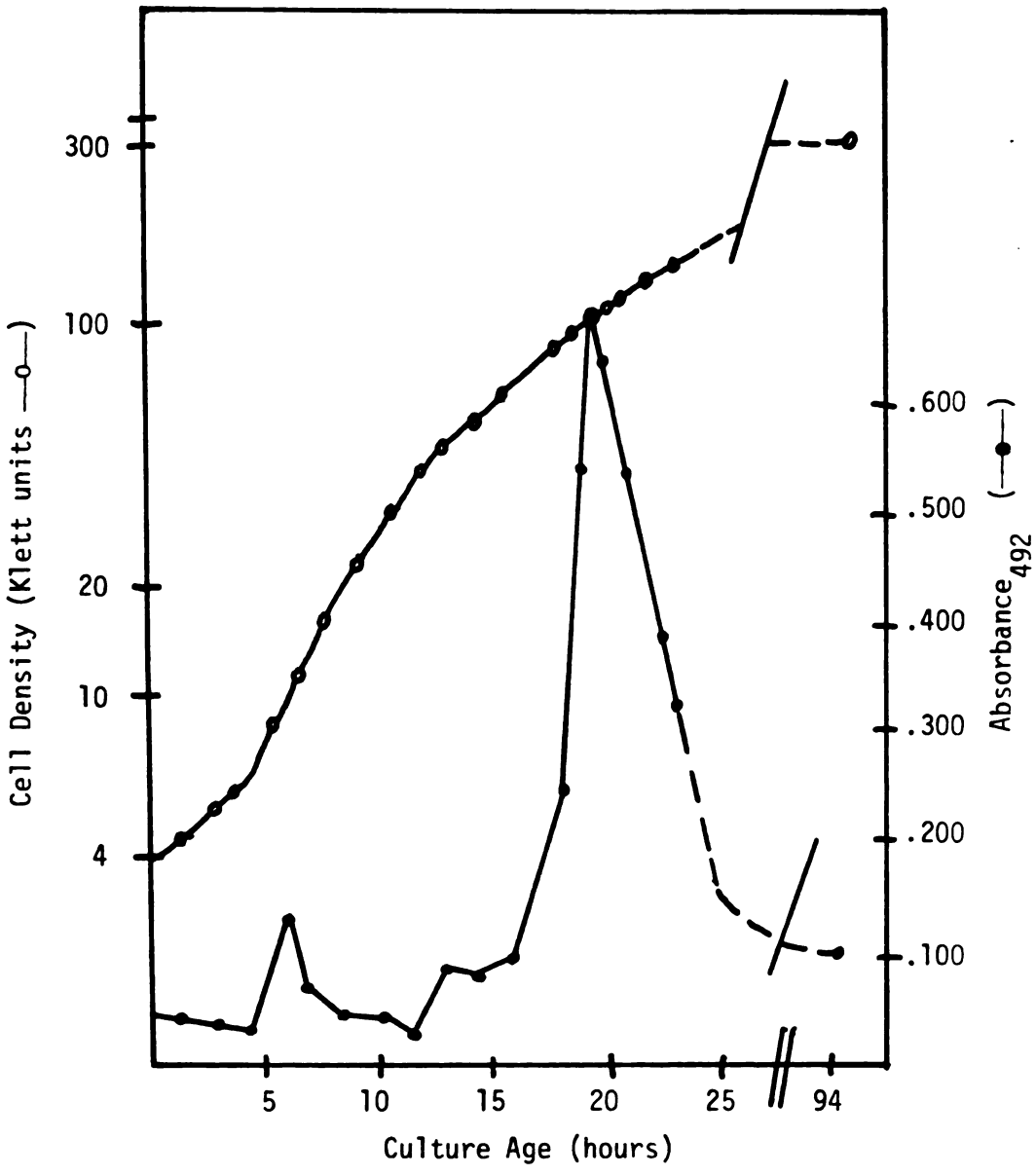


Figure 4. Effect of culture age on the binding of anti-K = 90 LPS adsorbed with K = 50 cells to 10^7 cells of R. trifolii 0403 as quantitated by ELISA.

concentration of orthodinitrobenzene (the product of this immunoperoxidase reaction) remained linear through the range of absorbance of 0-0.850.

Rhizobium trifolii 0403 grown on BIII plates also demonstrated this transient response in the ability to bind antibodies in the adsorbed antiserum. The ELISA indicated that approximately 6.2 times more antibody bound to 10^7 cells grown on plates for 5 days than when grown for three days.

Hapten Inhibition Studies

Monosaccharides and LPS were incubated with adsorbed antiserum to see which inhibited the specific hemagglutination of K = 90 LPS sensitized erythrocytes by adsorbed antiserum (Table 3). Uninhibited adsorbed antiserum had 80 agglutinating units/ml. Quinovosamine was the most potent hapten identified, decreasing the titer four-fold (at 7 mM concentration). Two-deoxyglucose at 30 mM concentration also decreased the titer four-fold. Glucosamine, N-acetyl glucosamine, and mannosamine decreased the titer by two-fold. None of the other monosaccharides tested were inhibitory at 30 mM. K = 90 LPS completely adsorbed the hemagglutination activity while K = 50 LPS showed no inhibition (at least a 970-fold difference in inhibitory activity). Ethanol-soluble products of phages BYØ1 and BYØ11 digestion of K = 90 SEP carried antigenic determinants that bound to the adsorbed antiserum.

Blocking of Trifoliin Agglutination

Trifoliin agglutinated R. trifolii 0403 grown in BIII broth harvested at early stationary phase with a specific agglutinating activity of 12,800 units/mg protein. Rhizobium trifolii cells at

Table 3. Hapten inhibitors of passive hemagglutination using erythrocytes sensitized with K = 90 LPS and adsorbed antiserum.

	Inhibitory Activity
Positive sugar haptens	Inhibitory units/ 30 umoles of sugar
2-Deoxy-D-glucose	60
Quinovosamine	240
α-Methyl quinovosamine	240
β-Propyl quinovosamine	240
D-glucosamine	40
D-mannosamine	40
N-acetyl-D-glucosamine	40
Negative sugar haptens	
D-glucose	
2-Deoxy-D-galactose	
D-fucose	
L-rhamnose	
D-glucuronic acid	
α-Methyl mannoside	
D-allose	
D-tagatose	
LPS inhibitors	Inhibitory units/ mg glucose eq
K = 50 LPS	0
K = 90 LPS	970
Phage digest inhibitors	Inhibitory units/ mg glucose eq
BYØ1	200
BYØ11	120

K = 90 were pre-incubated with Fab fragments of IgG isolated from either pre-immune serum or adsorbed antiserum (200 ug protein). To determine if they blocked the trifoliin binding site, these cells were then incubated with trifoliin (70 ug protein). Pre-immune Fab showed no inhibition of agglutination. The immune Fab inhibited bacterial agglutination by 97%, i.e., the immune Fab fragments had 4000 specific inhibitory units of activity/mg protein. Rhizobial cells did not agglutinate when mixed with immune or pre-immune Fab fragments in PBS.

K = 50 LPS did not block the agglutination of cells grown on BIII plates for five days by trifoliin. K = 90 LPS did interact with trifoliin, i.e., 6,400 inhibitory units of activity/mg glucose equivalent were detected. K = 90 SEP an even more potent inhibitor of trifoliin agglutination, i.e., 10,160 specific inhibitory units/mg glucose equivalent was found.

Inhibition of Root Adherence

Rhizobium trifolii 0403 cells in early stationary phase were treated with either immune or pre-immune Fab and then assayed qualitatively for their ability to bind to sterile clover seedling roots. The results of this experiment were similar to results described in the report of Dazzo and Brill (23) and are illustrated by a comparison of Figures 3a and 3b of that paper. Pre-immune Fab did not inhibit rhizobial attachment to the root hair region of sterile clover seedlings while immune Fab significantly inhibited bacterial attachment. Untreated rhizobial cells attached as well to root hairs as did cells

treated with pre-immune Fab fragments. Uninoculated, sterile clover seedlings showed no attached bacteria.

Biochemical Analyses of the LPS

Mild acetic acid hydrolysis cleaves LPS at the KDO linked between the R core and lipid A, thus effectively separating the polysaccharide and lipid A moieties of the LPS. The relative amount of lipid A was lower when isolated from cells in early stationary phase than in exponential phase. Conversely, the relative amount of polysaccharide released by mild acid hydrolysis (remaining R-core-O antigen after lipid extraction) was higher in LPS isolated from early stationary phase than exponential phase (Table 4). Carbohydrate assayed by the anthrone and phenol-sulfuric acid assays gave similar results. Both assays were used because not all monosaccharides produce chromophores with the same extinction coefficients. The extent of O-acetyl and O-pyruvyl group substitutions in the LPS was different depending on the age of the culture. The amount of O-acetyl substitutions in the LPS was lower when this polysaccharide was isolated from cells in early stationary phase than in exponential phase (Table 5). O-pyruvyl substitutions were detected in K = 90 LPS but not in K = 50 LPS.

K = 50 LPS contained 200±15 nanomoles uronic acid/mg glucose equivalent as compared to K = 90 LPS which contained 180±22 nanomoles/mg glucose equivalent. Uronic acids were not considered an important component of the unique immunodeterminant of K = 90 LPS since their relative content remained the same with culture growth and because they were not effective haptens in the passive hemagglutination assay.

Table 4. Relative amounts of lipid A and polysaccharide present in K = 50 LPS and K = 90 LPS.

	Component Composition[a]		
	Lipid A[b]	Carbohydrate[c]	
LPS isolated at:		phenol-H_2SO_4	anthrone
K = 50	380±11.4	300±7.8	310±13.3
K = 90	300±10.0	330±4.5	352±5.5

[a]Average ± standard deviation of duplicate samples.

[b]μg lipid A/mg dry weight LPS

[c]μg glucose equivalent/my dry weight LPS

Table 5. O-acetyl and O-pyruvyl substitutions in K = 50 LPS and K = 90 LPS.[a]

	Group Substitution			
	O-acetyl		O-pyruvyl	
LPS Type	μmoles/mg glucose equivalent	μmoles/mg dry weight LPS	μmoles/mg glucose equivalent	μmoles/mg dry weight LPS
K = 50	12.00	3.24	---[b]	---[b]
K = 90	7.75	2.60	3.49	1.22

[a]Values represent the average of duplicate experiments.

[b]None detected.

The LPS was sized by gel filtration. The peak of K = 90 LPS eluted before K = 50 LPS (Figure 5). The V_e/V_o for K = 50 LPS and K = 90 LPS were 1.13 and 1.06, respectively. Using four average molecular weight markers, a plot was made of the log of the molecular weight of the sample as a function of the eluted volume (Figure 5). The estimated molecular weights under these chromatographic conditions were 3.9×10^6 daltons for K = 50 LPS and 4.4×10^6 daltons for K = 90 LPS.

Gas Chromatography and Mass Spectrometry

Gas chromatograms of the alditol acetate derivatives of sugars in K = 50 LPS and K = 90 LPS hydrolysates are shown in Figures 6a and 6b, respectively. Fifteen peaks were resolved. An attempt was made to identify each peak and calculate its content relative to the internal standard, inositol hexaacetate (Table 6). These analyses would indicate how the LPS changed in composition with culture age. Some major differences in peak areas were found for the following saccharide constituents: quinovosamine (peak K); peak G1 (tentatively identified as N-methyl-3-amino-3,6-dideoxyhexose); peak F (tentatively identified as a deoxyhexose); and peak A (unidentified).

Figure 7 is a chromatogram of the alditol acetate derivatives of K = 90 LPS prepared at the mass spectrometry facility in the Department of Biochemistry at Michigan State University. Identification of the following sugars in the LPS has been confirmed by comparisons to the retention time and mass spectra of alditol acetates of: D-fucose, D-mannose, D-galactose, D-glucose, quinovosamine, heptose, and D-glucosamine. The unusual sugars, 2-O-methyl-6-deoxyhexose (peak at

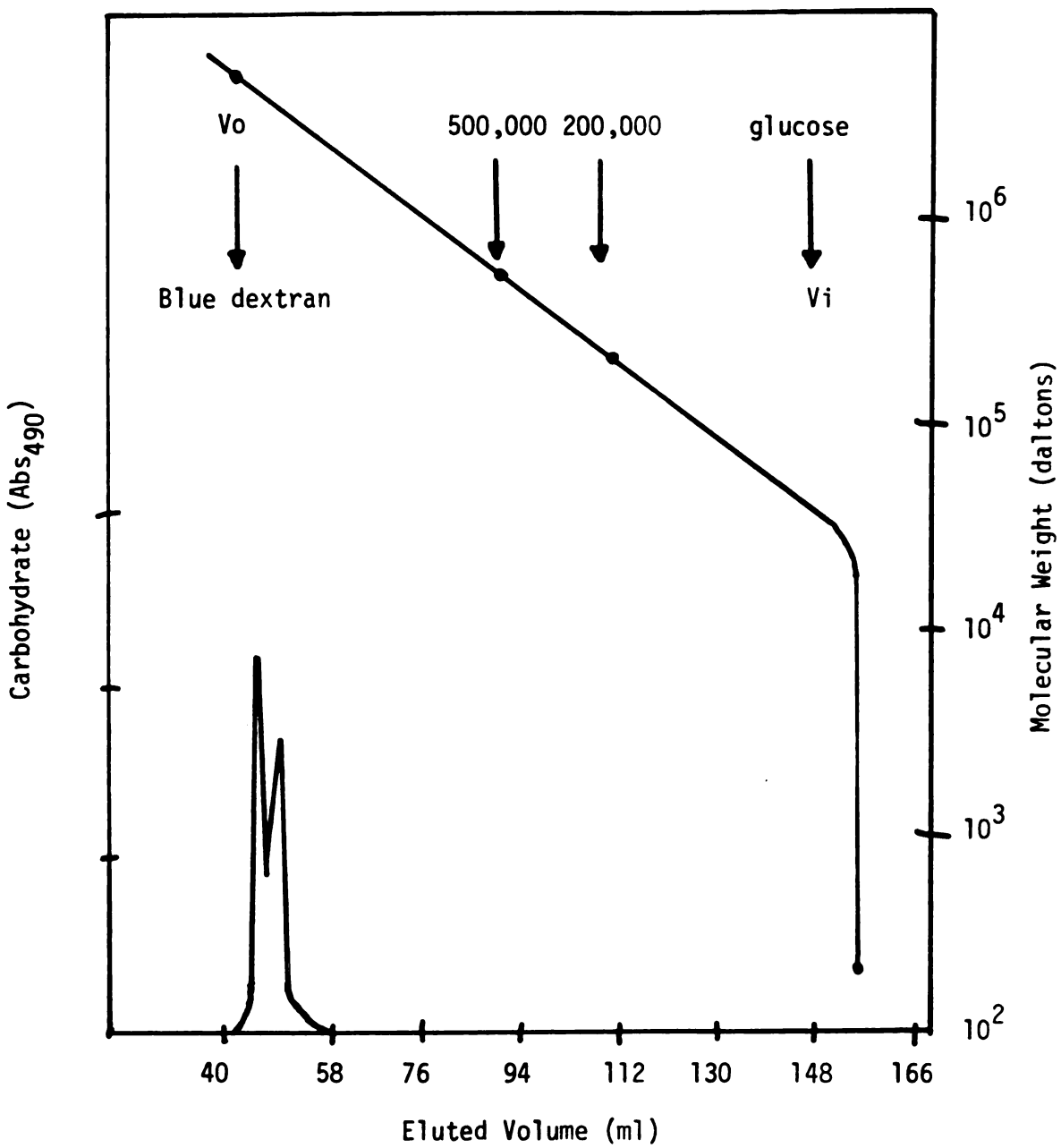


Figure 5. Sizing of K = 50 LPS and K = 90 LPS on a Sepharose 4B gel filtration column. Arrows indicate elution volume of markers used for calibration.

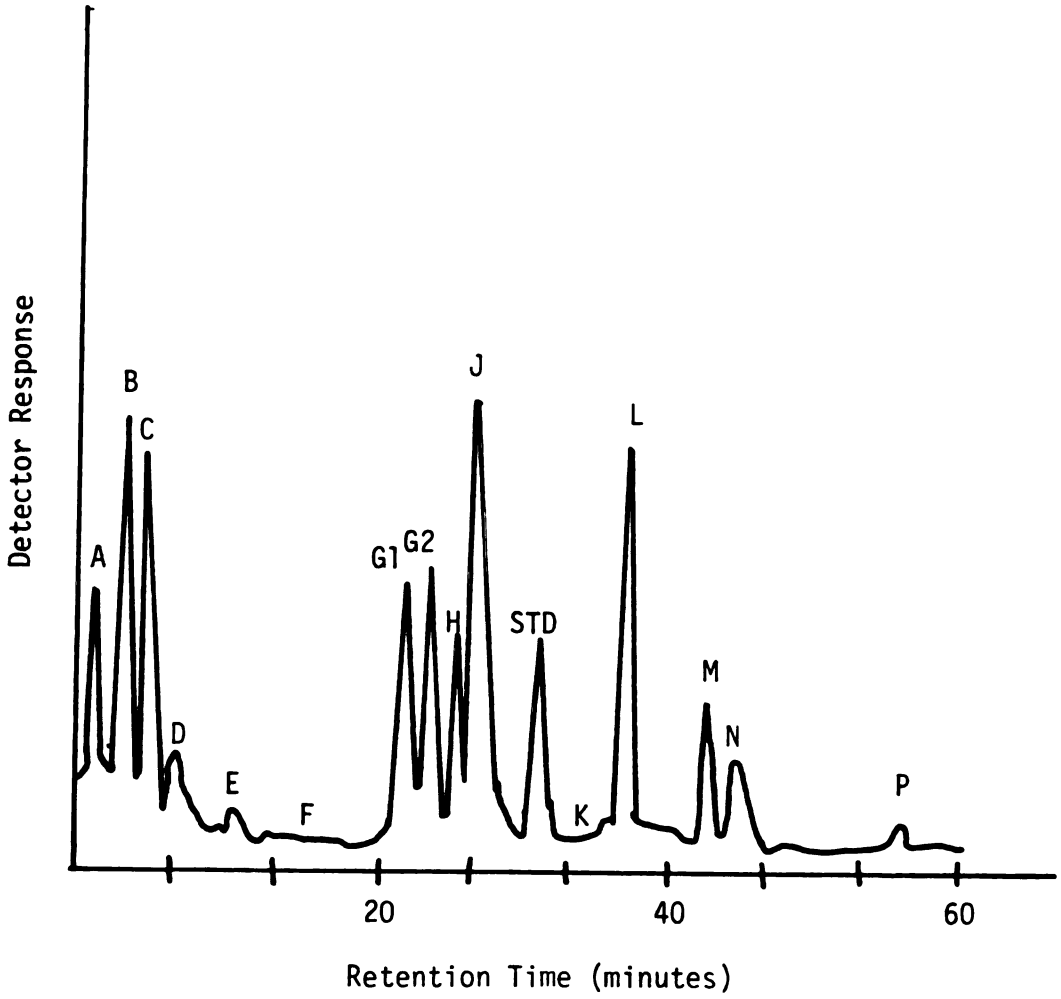


Figure 6a. Gas chromatogram of the alditol acetates of the hydrolysate of K = 50 LPS.

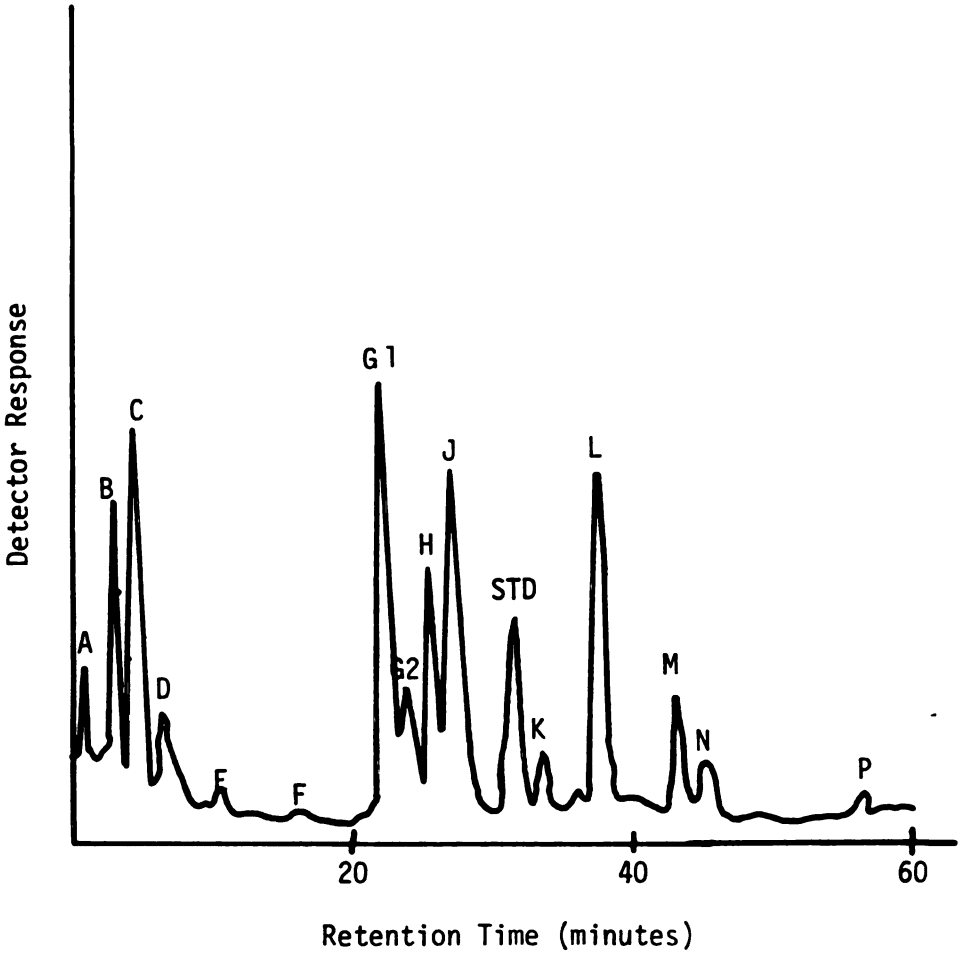


Figure 6b. Gas chromatogram of the alditol acetates of the hydrolysate of K = 90 LPS.

Table 6. Comparison of peak areas of monosaccharide constituents of K = 50 LPS and K = 90 LPS.[a]

Peak Label	LPS Constituent[b]	K = 50 LPS	K = 90 LPS
A	unidentified	0.184	0.070
B	unidentified	0.490	0.32
C	2-O-methyl-6-deoxyhexose*	0.403	0.557
D	fucose	0.197	0.285
E	unidentified	0.087	0.105
F	deoxyhexose*	0.017	0.096
G1	N-methyl-3-amino-3,6-di-deoxyhexose*	1.12	2.28
G2	mannose	1.25	0.65
H	galactose	1.00	1.19
J	glucose	2.75	2.2
Standard	inositol	1.00	1.00
K	quinovosamine	--	0.68
L	heptose	1.24	0.93
M	glucosamine	0.246	0.175
N	unidentified	0.166	0.316
P	unidentified	0.101	0.088

[a]Values are peak area of inositol (mm^2)/peak area of each sugar (mm^2) as determined on the 3% ECNSS-M column.

[b]*Indicates tentatively identified monosaccharides.

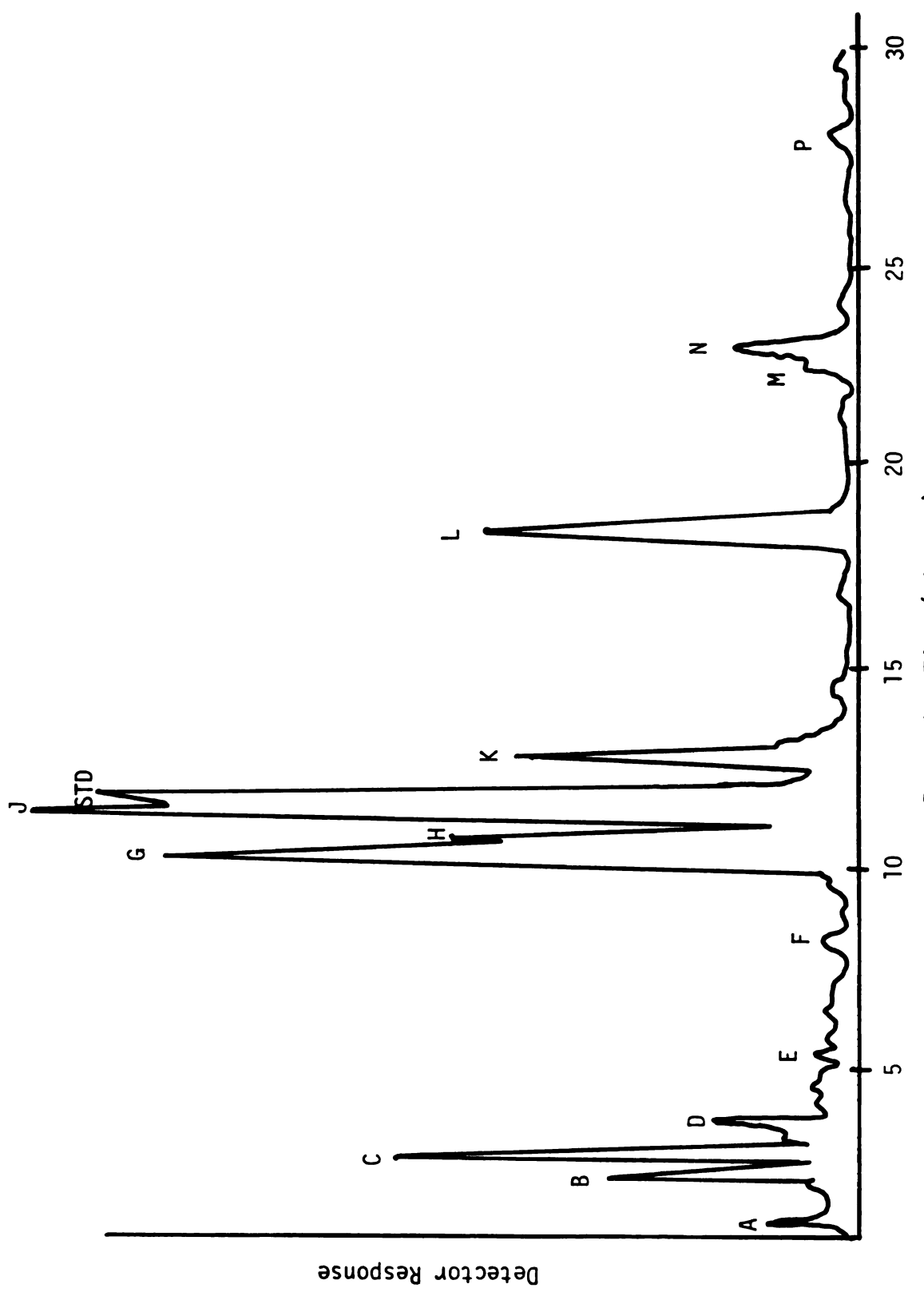


Figure 7. Gas chromatogram of the alditol acetates of the hydrolysate of K = 90 LPS on 3% OV-225.

m/e 117) and N-methyl-3-amino-3,6-dideoxyhexose (peaks at m/e 128, 142, 230, 244) were tentatively identified by their fragmentation patterns and comparisons to published retention times (12). One peak, F, has a mass spectrum consistent with a 2- or 3-deoxyhexose derivative. Five peaks remain unidentified as of this writing. Comparison of the mass spectra and gas chromatograms of peak K and the acetylated derivative of quinovosamine are shown in Figures 8 and 9. A blank sample containing chloroform was derivitized according to the alditol acetate procedure used. No peaks were produced during GC-mass spectral analysis.

Peak F was of particular interest because of its tentative identification as a deoxyhexose. The retention time of the acetylated derivative of 2-deoxyglucose was one minute less than the retention time of peak F. Treatment of 2-deoxyglucose with trifluoroacetic acid before acetylation did not decompose this deoxysugar, change its retention time, or its mass spectrum. Therefore, peak F cannot be considered a decomposition product of 2-deoxyglucose as a result of the trifluoroacetic acid hydrolysis.

Figure 8. Gas chromatogram of the alditol acetates of the hydrolysate of K = 90 LPS on 3% OV-225.

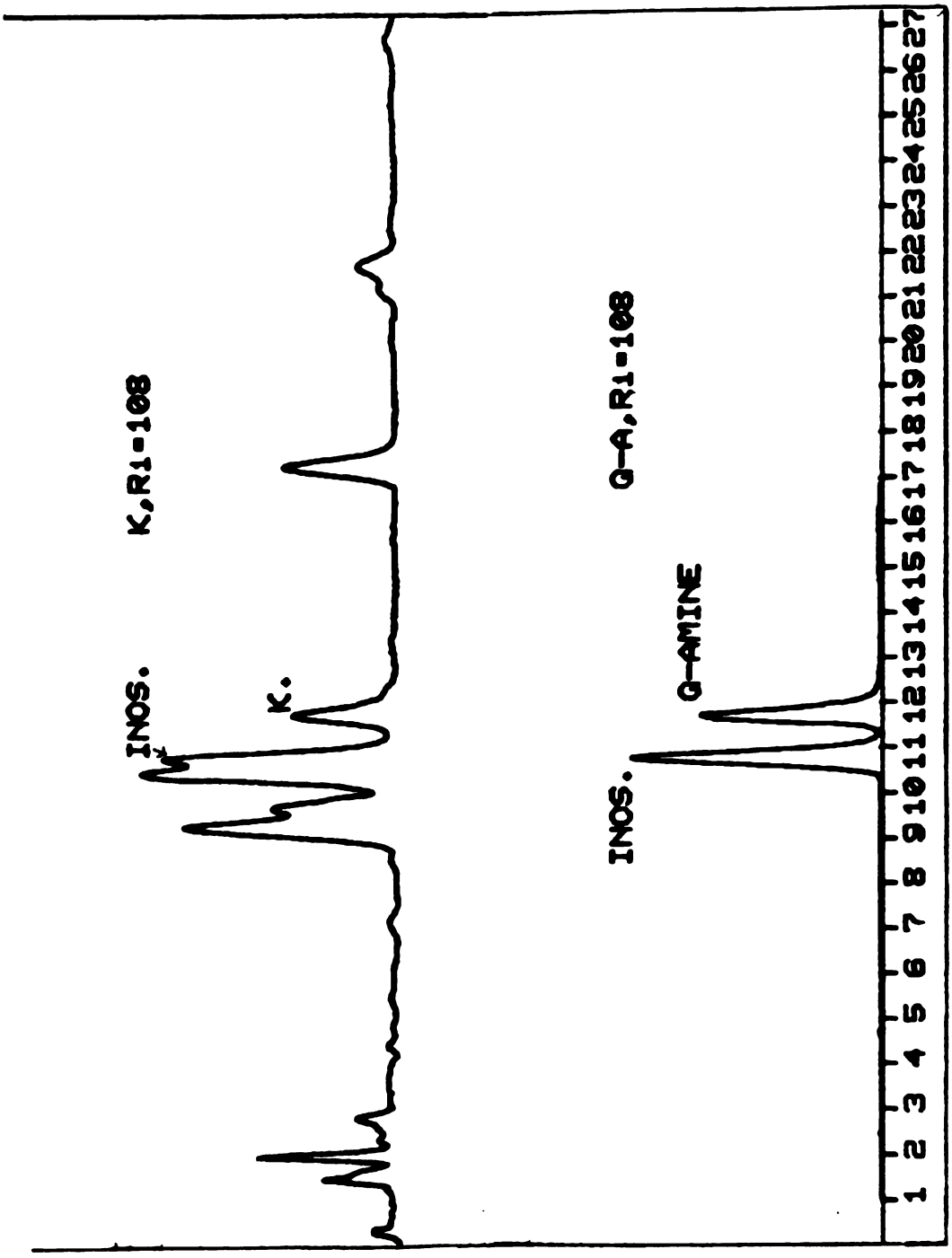
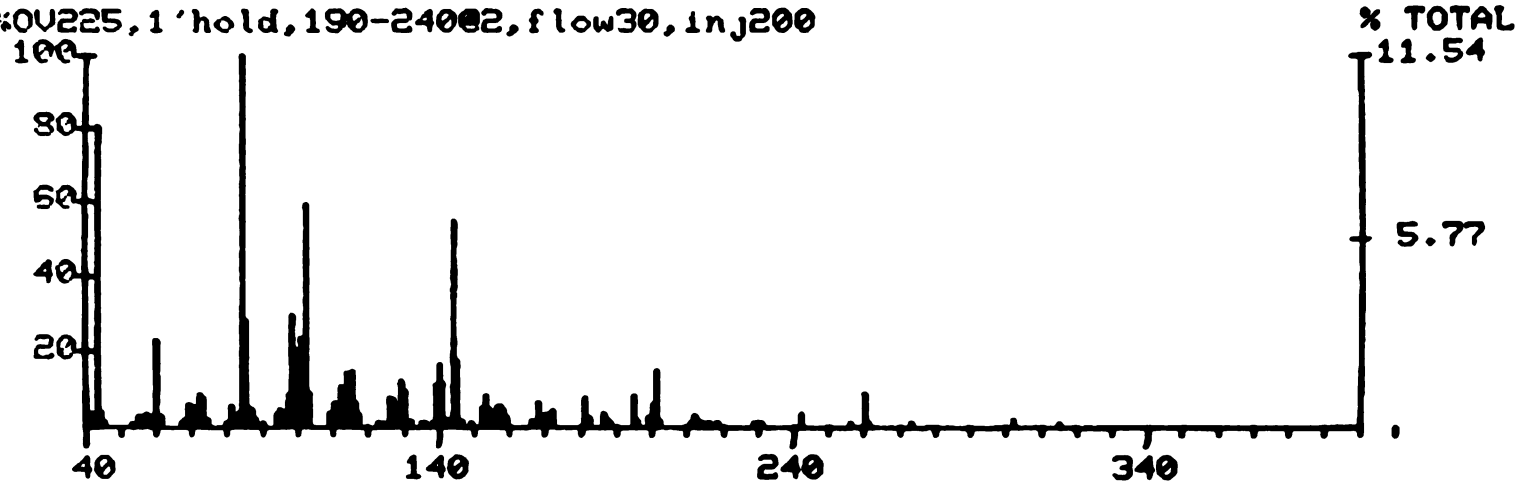
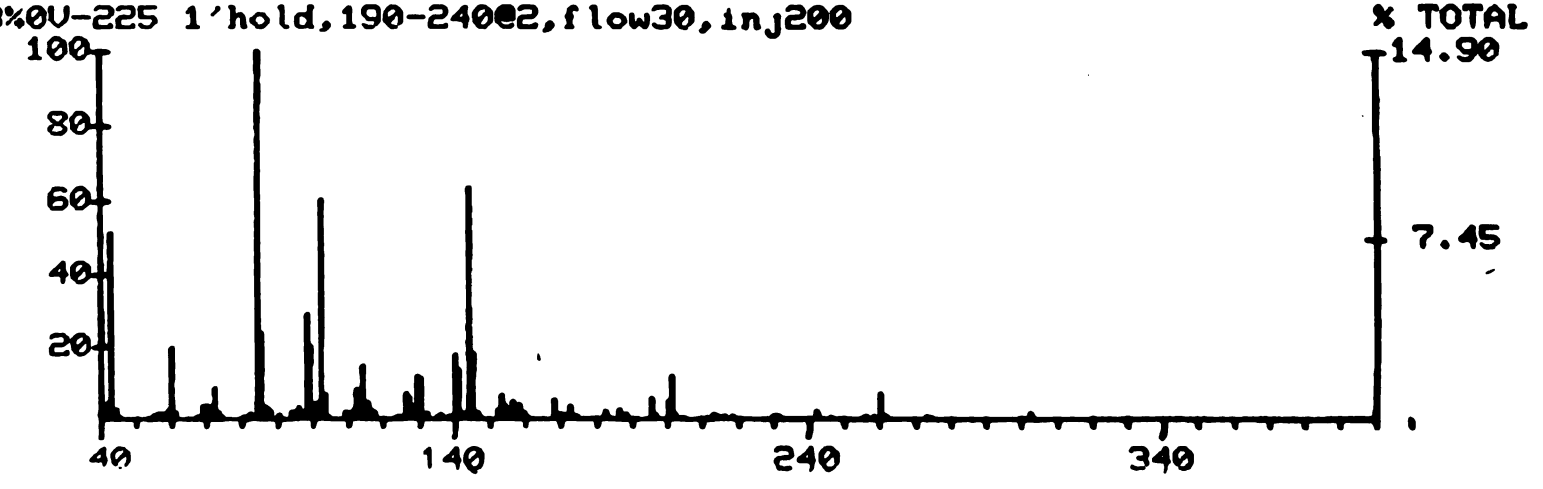

K,R1-108
INOS.
K.
Q-A,R1-108
Q-AMINE
INOS.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27

Figure 9. Comparison of the mass spectra of the alditol acetate of quinovosamine (with internal standard) to that of peak K from the hydrolysate of K = 90 LPS on 3% OV-225.

FRN 5021 SPECTRUM 375 RET. TIME: 12.8 R_I = 108
BASE PK: 84.1 BASE PK ABUND: 1080. TOTAL IONS: 9357.
DAZZO/URBANO 1073 791213-4 +INOSITOL MGJ2-5-80
3%OV225,1'hold,190-240@2,flow30,inj200



FRN 5010 SPECTRUM 355 RET. TIME: 11.7 R_I = 108
BASE PK: 84.1 BASE PK ABUND: 3124. TOTAL IONS: 20971.
ALDITOL ACETATES-STD.QUINOVOSAMINE+INOSITOL MGJ 2-4-80
3%OV-225 1'hold,190-240@2,flow30,inj200



DISCUSSION

The goal of this research is to determine how culture age affects the selective adherence of Rhizobium trifolii to the root hairs of its host, clover. This is a portion of long-range goal to determine the biochemical basis of host specificity in this nitrogen-fixing symbiosis. Our approach was to examine the structure of one of the trifoliin-binding polysaccharides of R. trifolii 0403, namely, the LPS from these cells in early stationary phase, and determine how it differs from the LPS isolated from cells at exponential phase. Identification of differences in structure of the corresponding polysaccharides should yield important clues to the molecular nature of the trifoliin-binding site important to host-symbiont recognition.

Bacterial agglutination and passive hemagglutination assays show that, as expected, cells in exponential and early stationary phase share antigenic immunodominant determinants in common, giving rise to high titer agglutinating antibody with K = 90 LPS as the immunogen. In addition, antigenic determinants unique to the surface of cells in early stationary phase have been detected with antiserum made specific to these determinants by exhaustive adsorption with cells in exponential phase. This procedure removes the antibodies to immunodominant determinants conserved on cells as they advance from exponential to early stationary phase and leaves in solution antibodies against determinants

which appear on cells when they enter stationary phase. Passive hemagglutination using anti-clover root antiserum demonstrates that LPS from cells in early stationary phase is more antigenically cross-reactive with clover root antigens than is LPS from cells in exponential phase. Antigenic cross-reactivity of at least two surface polysaccharides has been demonstrated between R. trifolii 0403 and the root epidermal surface of its clover hosts, T. repens and T. fragiferum (25).

The appearance of these unique antigenic determinants can be detected with adsorbed antiserum by immunofluorescence and ELISA. These determinants are exposed for brief periods as the culture leaves lag phase and again as it enters early stationary phase. These experiments demonstrate that unique antigenic determinants accumulate on cells in discrete stages of growth, and it is during this transient period of time that the rhizobia can attach in greatest numbers to the root hairs of their symbiotic host, clover (27). The results correspond to the transient appearance of the lectin receptor on R. trifolii which is antigenically cross-reactive with the host cell (27).

There are differences in the LPS isolated from R. trifolii 0403 in exponential and early stationary phase. The quantitative analysis of lipid A and total carbohydrate contents suggest that the carbohydrate chains increase in size as the culture advances from exponential to early stationary phase. Similar results have been reported for the O antigen of several Salmonella species grown at different rates in continuous culture (15). Gel filtration studies showed that K = 90 LPS is larger than K = 50 LPS. It may be possible that the addition of a

new polysaccharide linked to K = 90 LPS may be responsible for the difference in molecular weight.

The differences in specific inhibitory activity (inhibitory units/mg glucose equivalent) of K = 50 and K = 90 LPS suggest that structural differences important to this recognition process cannot be explained simply by an increase in polymerization of the same oligosaccharide repeating sequence on this polysaccharide.

This conclusion is further supported by comparison of the component structures of K = 50 and K = 90 LPS. Pyruvyl and O-acetyl groups have been shown to be constituents of rhizobial polysaccharides (31,45). These groups may be important components of the unique antigenic determinants because their relative quantity changes with culture age. In particular the O-pyruvyl substitutions may be important since they are not found on the LPS of cells at exponential phase.

Most monosaccharides, as demonstrated by gas chromatography and mass spectrometry, are present in similar amounts in LPS from R. trifolii 0403 cells in exponential and early stationary phase. However, the quantity of four monosaccharides changes at least two-fold in the LPS of cells as they advance from exponential to early stationary phase. Sugar inhibition experiments were done to identify probable haptenic determinants of the unique antigenic structures. Quinovosamine and 2-deoxyglucose were the most effective haptens suggesting that these sugars carry moieties important to the recognition of unique antigenic determinants on K = 90 LPS. Glucosamine, N-acetyl glucosamine, and mannosamine (which, like quinovosamine, have an amino group on carbon position 2), also are effective haptens. Quinovosamine

and peak F (mass spectrum consistent with a deoxyhexose) show the greatest increase in the LPS consistent with an increase in trifoliin binding ability. These results suggest that these components may also be of importance to the unique antigenic determinant on K = 90 LPS which bind trifoliin.

Partial characterization of LPS from R. trifolii 0403 in late exponential phase did not reveal the presence of quinovosamine (12). In our study, however, quinovosamine was found in the LPS of this rhizobial strain when the cells were in early stationary phase. These results emphasize the complexities of the biology of bacterial polysaccharides and offer an important explanation for the anomalous inconsistencies of rhizobial polysaccharide composition reported from different laboratories. Other studies (63) on LPS from R. leguminosarum, both wild type and mutant EXO-1 (which expresses reduced polysaccharide content on the cell surface) were reported to contain quinovosamine. Rhizobium trifolii and R. leguminosarum are closely related species and have been known to cross-infect their respective hosts on occasion (38). These studies further support the importance of quinovosamine in the structure and function of LPS from related rhizobial strains.

Related studies (3) have shown that the galactose residues in the capsular polysaccharide responsible for binding R. japonicum, the soybean symbiont, to the soybean lectin become methylated as the culture shifts from exponential growth to stationary phase and can no longer bind to the soybean lectin. This work supports the concept that non-carbohydrate substituents may be important in the functional role

of binding rhizobial polysaccharides to lectins from their corresponding legume.

Digestion of K = 90 SEP by rhizobiophage produced small fragments which carried the antigenic determinants unique to K = 90 LPS. Presumably, the endoglycanohydrolases associated with these bacteriophages (Hrabak, Sherwood, and Dazzo, unpublished observation) cleave glycosidic linkages in the polysaccharide sufficiently distal from the trifoliin-binding sequence so as to leave it intact. This approach may be useful to elucidate which of the candidates identified by this study are part of the structure of the minimal saccharide sequence in the bacterial polysaccharide which is important to the recognition process.

Two-deoxyglucose specifically inhibits the adsorption of R. trifolii 0403 cells to clover root hairs (26). The sugar, 2-deoxyglucose, specifically prevents the binding of R. trifolii cells and their capsular polysaccharide to roots (21,26). The clover lectin, trifoliin, is specifically eluted from the surface of clover roots when 2-deoxyglucose is present (21,28). Two-deoxyglucose inhibits the binding of trifoliin to cells (21,25) and purified capsular polysaccharide (25), as well as the binding of clover root antibody to capsular polysaccharide and LPS (23). In such hapten inhibition studies, it is believed that the hapten occupies the site on the lectin or antibody normally occupied by the polysaccharide. However, the effective inhibitors may only be analogs of the true haptenic site on the polysaccharide. This has been shown by the accumulation of studies designed to determine if 2-deoxyglucose is indeed present

in the polysaccharides which bind trifoliin. In the first study (27), gas-liquid chromatography of tri-methylsilylated derivatives of sugars in sulfuric acid hydrolysates of the capsular antigen contained a peak with retention time suspected of being the TMS-derivative of 2-deoxyglucose. Subsequently, descending paper chromatography showed that 2-deoxyglucose had the same R_f as a weakly staining component in HCl hydrolysates of trifoliin-binding capsular material and LPS-containing polysaccharide from two distinct strains of R. trifolii (23). However, Janssen et al. (45), argued that the hydrolysis procedures used in previous experiments by Dazzo and Hubbell would have destroyed any 2-deoxyglucose present. Only recently has literature been published on this subject by Hirsch and Feather (40) demonstrating that 2-deoxyglucose, when heated in aqueous mineral acids, decomposes to a substituted furan. Therefore, gas chromatographic analysis was repeated in the present study according to the trifluoroacetic acid hydrolysis procedure of Carlson et al. (12) and alditol acetate derivitization which does not destroy deoxysugars. Peak F from the K = 90 LPS hydrolysate had a retention time near (plus one minute) the alditol acetate derivative of 2-deoxyglucose. Mass spectrometry suggests that this sugar is not 2-deoxyglucose but another 2,3, or 4 deoxyhexose based on the diagnostic fragmentation pattern showing peaks at m/e of 145 and m/e of 231. Its retention time does not match the alditol acetate derivatives of the deoxyhexoses, rhamnose, fucose, or 2-deoxygalactose.

Monovalent Fab fragments of anti-K = 90 LPS adsorbed with K = 50 cells were potent inhibitors (97% inhibition) of the

agglutination of cells in early stationary phase by trifoliin. This result indicates that trifoliin and the Fab fragments recognize the same or similar determinants on the surface of cells in early stationary phase. These results also suggest that antibodies in the adsorbed antiserum recognize the antigenic determinants unique to K = 90 LPS which are important to the binding of rhizobial cells to the clover root hair lectin, trifoliin. Monovalent Fab fragments of adsorbed antiserum blocked the attachment of R. trifolii cells in early stationary phase to clover root hairs demonstrating that the exposure of this unique antigenic determinant is critical to the adherence of cells at this age to clover root hairs.

These studies support the hypothesis that the architecture of the rhizobial cell surface is not constant in composition but changes with culture age. The influence of the microbial growth phase on the adsorption of bacteria to epithelial surfaces occurs in both animal and plant hosts (16,35). This effect of culture age can be explained as a transient appearance of lectin receptors on the rhizobial cell surface (27). An acidic heteropolysaccharide and a LPS-containing preparation of R. trifolii 0403 have already been shown to share antigenic determinants cross-reactive with clover roots (25). Studies with monovalent antigen binding fragments of anti-clover root antibody have suggested that these two polysaccharides bind R. trifolii 0403. Inhibition of agglutination of this strain with trifoliin by various polysaccharide fractions has shown that K = 90 SEP (equivalent to the acidic heteropolysaccharide of earlier studies) (23) has considerably more trifoliin binding specific activity than K = 90 LPS. This last

experiment indicates that, as predicted (23), at least two polysaccharides from the surface of R. trifolii 0403 cells in early stationary phase are important to the attachment of rhizobia to the root hairs of its symbiotic host, clover. This study leaves open for further inquiry the relation of LPS from early stationary phase to the major acidic heteropolysaccharide in the capsule of R. trifolii 0403 which is specifically recognized by trifoliin at this culture age.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Bal, A. K., S. Shantharam, and S. Ratnam. 1978. Ultrastructure of Rhizobium japonicum in relation to its attachment to root hairs. J. Bacteriol. 133: 1393-1400.

2. Bauer, W. D. 1977. Lectins as determinants of host specificity. Basic Life Sci. 9: 283-297.

3. Bhuvaneswari, T. V., S. G. Pueppke, and W. D. Bauer. 1977. Role of lectins in plant-microorganism interactions. I. Binding of soybean lectin to rhizobia. Plant Physiol. 60: 486-491.

4. Bishop, P. E., F. B. Dazzo, E. R. Appelbaum, R. J. Maier and F. B. Dazzo. 1977. Intergeneric transfer of genes involved in the Rhizobium - legume symbiosis. Science 198: 938-940.

5. Bjorndal, H., C. Erbing, B. Lindberg, G. Fahraeus, and H. Ljunggren. 1971. Extracellular polysaccharide from Rhizobium meliloti. Acta Chem. Scand. 25: 1281-1286.

6. Bleiweis, A. S., M. C. Taylor, J. Deepak, T. A. Brown, and J. Wetherell. 1976. Comparative chemical compositions of cell walls of Streptococcus mutans. J. Dental Res. 55: A103-A108.

7. Blumenkrantz, N. and G. Asboe-Hansen. 1973. New method for quantitative determination of uronic acids. Anal. Biochem. 54: 484-489.

8. Bohlool, B. B., and E. L. Schmidt. 1974. Lectins: a possible basis for specificity in the Rhizobium-legume root nodule symbiosis. Science 185: 269-271.

9. Buttke, T. M., and L. O. Ingram. 1975. Comparison of lipopolysaccharides from Agmenellum quadruplicatum to Escherichia coli and Salmonella typhimurium by using thin layer chromatography. J. Bacteriol. 124: 1566-1573.

10. Calvert, H. E., M. Lalonde, T. V. Bhuvaneswari and W. D. Bauer. 1978. Ultrastructural studies of the binding sites for soybean lectin on the cell surfaces of the nitrogen-fixing bacterial symbiont Rhizobium japonicum. Plant Physiol. Abstr. 4: 59.

11. Carlson, R., and P. Albersheim. 1977. Host symbiont interaction: the structural diversity of Rhizobium lipopolysaccharides. Plant Physiol. 59: 51.

12. Carlson, R. W., R. E. Sanders, C. Napoli, and P. Albersheim. 1978. Host symbiont interactions: III. Purification and partial characterization of Rhizobium lipopolysaccharides. Plant Physiol. 62: 912-917.

13. Chaudhari, A. S., C. T. Bishop, and W. F. Dudman. 1973. Structural studies on the specific capsular polysaccharide from Rhizobium trifolii TA1. Carbohydr. Res. 28: 221-231.

14. Chen, A. T., and D. A. Phillips. 1976. Attachment of Rhizobium to legume roots as the basis for specific interactions. Plant Physiol. 38: 83-88.

15. Collins, F. M. 1964. The effect of the growth rate on the composition of Salmonella enteriditis cell walls. Aust. J. of Exp. Biol. and Med. Sci. 42: 255-262.

16. Costerton, J. W., G. G. Gessey, and K. J. Cheng. 1978. How bacteria stick. Scientific American. 238: 86-95.

17. Damirgi, S. M., L. R. Frederick, and I. C. Anderson. 1967. Serogroups of Rhizobium japonicum in soybean nodules as affected by soil type. Agron. J. 59: 10-12.

18. Dazzo, F. B. 1978. Adsorption of microorganisms to roots and other plant surfaces. In Adsorption of microorganisms to surfaces. G. Bitton and K. Marshall (eds.) J. Wiley and Sons. New York.

19. Dazzo, F. B. 1980. Determinants of host specificity in the Rhizobium-clover symbiosis. In Nitrogen Fixation, Vol. II. W. E. Newton and W. H. Orme-Johnson (eds.) University Park Press. Baltimore.

20. Dazzo, F. B. 1979. Lectins and their saccharide receptors as determinants of specificity in the Rhizobium-legume symbiosis. In Steve Subtelny (ed.) The Cell Surface: Mediator of Development. Academic Press, Inc. New York.

21. Dazzo, F. B., and W. J. Brill. 1977. Receptor site on clover and alfalfa roots for Rhizobium. Appl. Environ. Microbiol. 33: 132-136.

22. Dazzo, F. B., and W. J. Brill. 1978. Regulation by fixed nitrogen of host-symbiont recognition in the Rhizobium-clover symbiosis. Plant Physiol. 62: 18-21.

23. Dazzo, F. B., and W. J. Brill. 1979. Bacterial polysaccharide which binds Rhizobium trifolii to clover root hairs. J. Bacteriol. 137: 1362-1373.

24. Dazzo, F. B., and D. H. Hubbell. 1975. Antigenic differences between infective and non-infective strains of Rhizobium trifolii. Appl. Microbiol. 30: 172-177.

25. Dazzo, F. B., and D. H. Hubbell. 1975. Cross-reactive antigens and lectin as determinants of symbiotic specificity in the Rhizobium-clover association. Appl. Microbiol. 30: 1017-1033.

26. Dazzo, F. B., C. A. Napoli, and D. H. Hubbell. 1976. Adsorption of bacteria to roots as related to host specificity in the Rhizobium-clover symbiosis. App. Env. Microbiol. 32: 166-171.

27. Dazzo, F. B., M. R. Urbano, and W. J. Brill. 1979. Transient appearance of lectin receptors on Rhizobium trifolii. Current Microbiol. 2: 15-20.

28. Dazzo, F. B., W. E. Yanke, and W. J. Brill. 1978. Trifoliin: A Rhizobium recognition protein from white clover. Biochim. Biophys. Acta 539: 276-286.

29. DuBois, M. K., A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350-356.

30. Dudman, W. F. 1976. The extracellular polysaccharides of Rhizobium japonicum: compositional studies. Carbohydr. Res. 46: 97-110.

31. Dudman, W. F., and M. Heidelberger. 1969. Immunochemistry of newly found substituents of polysaccharides of Rhizobium. Science 164: 954-955.

32. Ellen, R. P., and R. J. Gibbons. 1974. Parameters affecting the adherence and tissue tropisms of Streptococcus pyogenes. Infect. Immun. 9: 85-91.

33. Fahraeus, G. 1957. The infection of clover root hairs by nodule bacteria studied by a simple glass slide technique. J. Gen. Microbiol. 16: 374-381.

34. Fred, E. B., I. L. Baldwin, and E. McCoy. 1932. Root nodule bacteria and leguminous plants. Univ. of Wisconsin Press, Madison, Wisconsin. 343 p.

35. Gibbons, R. J. 1977. Adherence of bacteria to host tissue. pp. 395-406, In Schlessinger, D. (ed.) Microbiology - 1977. Washington, D.C. A.S.M.

36. Hakomori, S. 1964. J. Biochemistry (Tokyo). 55: 205.

37. Hamblin, J., and S. P. Kent. 1973. Possible role of phytochem-agglutinin in Phaseolus vulgaris. I. Nature New Biol. 245: 28-30.

38. Hepper, C. M. 1978. Physiological studies on nodule formation. The characteristics and inheritance of abnormal nodulation of Trifolium pratense by Rhizobium leguminosarum. Ann. Botany 42: 109-115.

39. Hestrin, S. 1949. The reaction of acetylcholine and other carboxylic acid derivatives with hydroxylamine and its analytical application. J. Biol. Chem. 180: 249-261.

40. Hirsch, J., and M. S. Feather. 1979. The acid-catalyzed dehydration of 2-deoxy-D-glucose. J. Carbohydr., Nucleosides, Nucleotides. 6: 573-580.

41. Humphrey, B. and J. M. Vincent. 1969. The somatic antigens of two strains of Rhizobium trifolii. J. Gen. Microbiol. 59: 411-425.

42. Jann. B., K. Jann, G. Schmidt, I. Orskov, and F. Orskov. 1970. Immunochemical studies of polysaccharide surface antigens of Escherichia coli O100:K?(B):H2. Eur. J. Biochem. 15: 29-39.

43. Jann, K. and O. Westphal. 1975. In The Antigens. M. Sela (ed.), Vol. III. pp. 1-125. Academic Press, Inc., New York.

44. Janssen, P. E., L. Kenne, B. Lindberg, H. Lunggren, J. Longgren, U. Ruden, and S. Svensson. 1977. Demonstration of an octasaccharide repeating unit in the extracellular polysaccharide of Rhizobium meliloti by sequential degradation. J. Am. Chem. Soc. 99: 3812-3815.

45. Janssen, P. E., B. Lindberg, and H. Lunggren. 1979. Structural studies of the Rhizobium trifolii extracellular polysaccharide. Carbohydr. Res. 75: 207-220.

46. Kamberger, W. 1979. An Ouchterlony double diffusion study on the interaction between legume lectins and rhizobial cell surface antigens. Arch. Microbiol. 121: 83-90.

47. Law, I. J., and B. W. Strijdom. 1977. Some observations on plant lactins and Rhizobium specificity. Soil Biol. Biochem. 9: 79-84.

48. Li, D., and D. H. Hubbell. 1969. Infection thread formation as a basis of nodulation specificity in Rhizobium-strawberry clover associations. Can. J. Microbiol. 15: 1133-1136.

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

50. Luderitz, O. 1977. Endotoxins and other cell wall components of gram negative bacteria and their biological activities. In Microbiology - 1977. D. Schlessinger (ed.) pp. 239-246. Washington, D. C. A.S.M.

51. Maier, R. J., P. E. Bishop, and W. J. Brill. 1978. Transfer from Rhizobium japonicum to Azotobacter vinelandii of genes required for nodulation. J. Bacteriol. 134: 1199-1201.

52. McCoy, E. F. 1932. Infection by Bact. radicicola in relation to the microchemistry of the host cell walls. Proc. R. Soc. Lond. (Biol.) 110: 514-533.

53. Mort, A. and W. D. Bauer. 1978. The chemical basis of lectin binding to Rhizobium japonicum. Plant Physiol. Abstr. 4: 59.

54. Napoli, C. A. 1976. "Physiological and ultrastructural aspects of the infection of clover (Trifolium fragiferum) by Rhizobium trifolii NA30." PhD. Thesis, Univ. of Florida. 103 pp.

55. Napoli, C. A., F. B. Dazzo, and D. H. Hubbell. 1975. Production of cellulose microfibrils by Rhizobium. Appl. Microbiol. 30: 123-131.

56. Nowotny, A. 1969. Basic exercises in immunochemistry. Springer-Verlag. New York.

57. Nuti, M. P., F. C. Cannon, R. K. Prakash, A. A. Lepidi, and R. A. Schelperoort. 1979. Proc. Amer. Rhizobium Conference VII.

58. O'Gara, F., and K. T. Schanmugan. 1978. Mutant strains of clover rhizobium (Rhizobium trifolii) that form nodules on soybean (Glycine max.). Proc. Nat. Acad. Sci. U.S.A. 75: 2343-2347.

59. Osborn, M. J. 1963. Studies of the gram negative cell wall I. Evidence for the role of KDO in the lipopolysaccharide of Salmonella typhimurium. Proc. Nat. Acad. Sci. U.S.A. 50: 499-506.

60. Osborn, M. J., M. A. Cynkin, J. M. Gilbert, L. Muller, and M. Singh. 1972. Synthesis of bacterial O-antigens. Meth. Enzymol. 28: 583-601.

61. Planque, 'K, and J. W. Kijne. 1977. Binding of pea lectin to a glycan type polysaccharide in the cell walls of Rhizobium leguminosarum. FEBS Lett. 73: 64-66.

62. Porter, R. R. 1959. The hydrolysis of rabbit gamma-globulin and antibodies with crystalline papain. Biochem. J. 73: 119-126.

63. Saunders, R. E., R. W. Carlson, and P. Albersheim. 1978. A Rhizobium mutant incapable of nodulation and normal polysaccharide secretion. Nature. 271: 240-242.

64. Schlecht, S., and I. Fromme. 1975. Chemical and sarological characterization of Salmonella lipopolysaccharide from different phases of growth (In German with English summary). Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene. Abt. 1 Orig. 233: 199-222.

65. Sloneker, J. H. and D. G. Orantas. 1962. Pyruvic acid, a unique component of an exocellular bacterial polysaccharide. Nature 194: 478-479.

66. Solheim, B. 1975. Possible role of lectins in the infection of legumes by rhizobia. In Specificity in plant diseases, NATO Advance Study Inst., May 14-16, 1975.

67. Solheim, B. and J. Raa. 1973. Characterization of the substances causing deformation of root hairs of Trifolium repens when inoculated with Rhizobium trifolii. J. Gen. Microbiol. 77: 241-246.

68. Somme, R. 1974. Chemical analysis of exocellular, acid polysaccharides from seven Rhizobium strains. Carbohydr. Res. 33: 89-96.

69. Tsang, J. C., D. A. Brown, and D. M. Kranz. 1976. Detection of lipopolysaccharide components in isolated outer membranes of Serratia marcescens. Microbios Lett. 1: 209-217.

70. Umali-Garcia, M., D. H. Hubbell, M. H. Gaskins, and F. B. Dazzo. 1980. Association of Azospirillum with grass roots. App. Environ. Microbiol. 39: 219-226.

71. Voller, A., D. E. Bidwell, A. Bartlett. 1976. Microplate enzyme immunoassays for the immunodiagnosis of virus infections. Manual of Clinical Immunology. Rose N. Friedman W. (eds.) A.S.M. Washington, D.C. p. 506.

72. Waravdeker, V. S., and L. D. Saslaw. 1959. A sensitive colorometric method for the estimation of 2-deoxy sugars with the use of the malonaldehyde thiobarbituric acid reaction. J. Biol. Chem. 234: 1945-1950.

73. Wolpert, J. S., and P. Albersheim. 1976. Host-symbiont interactions. I. The lectins of legumes interact with the O-antigen containing lipopolysaccharides of their symbiont rhizobia. Biochem. Biophys. Res. Commun. 70: 729-737.

74. Zevenhuisen, L. P. T. M. 1972. Methylation analysis of acidic exopolysaccharides of Rhizobium and Agrobacterium. Carbohydr. Res. 26: 409-419.

75. Zevenhuisen, L. P. T. M., and H. J. Scholten-Koerselman. 1979. Surface carbohydrates of Rhizobium I. β-1,2-glucans. Antonie van Leeuwenhoek. 45: 165-175.