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A COMPARATIVE STUDY OF THE CELL WALLS OF RHIZOBIUM JAPONICUM
AND RHIZOBIUM LEGUMINOSARUM IN FREE-LIVING AND BACTEROID FORMS

by

Karen Lee Hooker

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Peter F. Wong
Major Professor

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I.

LITERATURE REVIEW

LITERATURE REVIEW

General Description of the Genus

The genus Rhizobium contains those bacteria able to form nodules on the roots of plants in the family Leguminosae. Rhizobia are gram negative rods, occurring singly or in pairs, and are motile when young, having peritrichous, polar, or subpolar flagella. Often prominent poly- β -hydroxybutyrate granules occur within the cell (Bergey's Manual, 1976).

Rhizobia are aerobic chemoheterotrophs, growing best at 25 to 30C on complex media, particularly with yeast extract. Many species are able to use nitrate, ammonia, or an amino acid as a sole nitrogen source. Atmospheric nitrogen is used primarily by bacteroids in symbiosis with a legume host (Vincent, 1977).

Variations Within the Genus

A major distinction between members of the genus is made between fast and slow growers. Rhizobium leguminosarum is an example of a fast grower. Relatively fast growth on mannitol-yeast extract agar occurs with a mean generation time of 2-4 hours, and the pH of the medium is lowered to a moderate degree. Colonies are large, gummy, and colorless. Slow growers, such as R. japonicum, have a mean generation time of 6-8 hours and produce an alkaline end point on the same medium. The colonies are small, colorless or white, and with a lesser amount of gum (Vincent, 1977). This extra-cellular gum is not known to have any morphological role.

Other differences between fast and slow growers have been found. Fast growers efficiently use almost all carbon sources, whereas the slow growers are more specific in these requirements (Fred, et al., 1932; Ishizawa, 1953; Neal and Walker, 1935). A summary of the data for fast growers (R. trifolii, R. leguminosarum, R. phaseoli, and R. meliloti) shows that 100% of the strains tested responded to glucose, fructose, arabinose, and sucrose as carbon sources (Graham, 1964). In contrast, the data for R. japonicum shows that 70-89, 47-53, 56-75, and 0% of the strains tested responded to those sources respectively (Elkan and Kwik, 1968).

The slow growers also have a less demanding vitamin requirement than the fast growers. In a test using 39 strains of R. japonicum, only biotin had any effect out of 10 vitamins tested (Elkan and Kwik, 1968). Graham and Parker (1964) have shown that the slow growers have a greater acid tolerance than the fast growers, although the slow growers were more sensitive to an alkaline pH.

Comparison Between Cultured Rhizobia and Bacteroids

All rhizobia have the ability to form nodules on specific leguminous hosts. They are found as greatly enlarged oval, club, or Y-shaped cells in Pisum and Vicia spp., and as slightly swollen rods in Phaseolus vulgaris and Glycine max (Schneider, 1892). How rhizobia can be so variable is not known.

In culture, cells usually lack nitrogenase, whereas it is present in the bacteroid. Another metabolic difference between the two forms of rhizobia is in the nature of their hemoproteins. Both forms of R. japonicum contain nonautoxidizable cytochromes c and b. Cytochromes a-a₃ and autoxidizable b are unique to the cultured cell. Bacteroids uniquely contain a

soluble CO-reactive cytochrome c and cytochrome p-450 (Bergersen, 1971). The cytochrome patterns of R. leguminosarum also differ between cultured and bacteroid forms (Kretovich, et al., 1973).

Metabolic Changes During Bacteroid Development

During bacteroid development, there is a progressive loss in ribosome number and the amount of DNA per Rhizobium, and bacteroids in the nodules do not divide (Dilworth and Williams, 1967). Young R. leguminosarum bacteroids, which are most active in synthesizing proteins, have a low nitrogenase activity. In more developed bacteroids, which have a high nitrogenase activity, protein synthesis decreases sharply. It is presumed that bacteroids during early development obtain fixed nitrogen from the plant, but at a later stage, become capable of producing ammonia, which is excreted to be used by the plant for amino acid synthesis (Scott, et al., 1976).

Pleomorphic bacteroid-like forms can be induced in culture by utilizing such substances as amino acids (Strijdom and Allen, 1966), and plant extracts, even in strains (e.g. R. phaseoli) that form few pleomorphic forms in the nodule (Buchanon, 1909). Nucleic acid synthesis was not impaired in R. leguminosarum "artificial bacteroids", but protein and cell wall synthesis was (Jordan and Coulter, 1965).

Cell Wall Structure

The detailed wall structure of Rhizobium is not known, though it is probably similar to that of other gram-negative bacteria. The innermost constituent of gram-negative cell walls is a rigid layer of mucopeptide

(murein) with covalently linked lipoprotein. On top of the murein is an outer membrane consisting of lipopolysaccharide (LPS), proteins, lipoprotein and extracellular and capsular polysaccharides. The cell surface constituents are noncovalently bound to the rigid layer (Osborn, 1969).

The walls of R. trifolii contain glucose, rhamnose, glucosamine, muramic acid, glutamic acid and diaminopimelic acid, as well as the usual range of amino acids found in gram-negative walls. In this case, lysine, aspartic acid, glycine, serine, valine, methionine, leucine, and tryptophane were present (Humphrey and Vincent, 1962).

Cell Shape Determinants

Weidel and Pelzer (1964) showed that the murein layer could be isolated intact from Escherichia coli cell envelopes by combined mechanical and detergent treatment. The isolated layer, or sacculus, retains the shape and dimensions of the cells from which it was made. Therefore, murein appears to have a relatively rigid structure and may play an important role in determining bacterial cell morphology. In rod shaped organisms, inhibition of murein synthesis or its degradation by lysozyme leads to spheroplast formation. However, it is not clear whether murein constitutes the only device for maintaining shape. It has been shown that plasmolyzed E. coli rods treated with lysozyme under certain conditions become osmotically sensitive, but remain rod shaped (Birdsell and Cota-Robles, 1967). Certain halophilic bacteria, totally lacking murein, can grow and maintain their rod shape (Stoekenius and Rown, 1967). While defects in the murein layer may lead to less LPS present in the wall (Hofshneider and Martin, 1968),

little evidence exists on the role of other cell wall constituents in determining cellular morphology.

Rhizobial LPS Studies

Recently, much work has been done to characterize rhizobial LPS and to determine its possible role in bacteroid formation and host specificity. The LPS in R. trifolii has been shown to be chemically similar to that studied in the Enterobacteriaceae (Humphrey and Vincent, 1969a; Luderitz, et al., 1966). A firmly bound lipid, 2-keto-3-deoxyoctonate, glucose, mannose, fucose, glucosamine and in one capsulated strain, a heptose were found. The LPS of R. trifolii was unusual in its low phosphorus content and the presence of glucuronic acid. Since the phosphorus content of R. trifolii is 0.14%, compared to 1-4% for enterobacteria, it is unlikely that it plays a major structural role in rhizobia, as a structural link between di-heptose units in the polysaccharide core (Humphrey and Vincent, 1969a). Similar results have been reported with R. leguminosarum except that heptose values have been greater than those found in R. trifolii (Planque and Kijne, 1977). The markedly anionic LPS of R. trifolii strain TAL reflected the surface characteristics of the cells (Humphrey and Vincent, 1969b; Marshall, 1967).

Composition analyses of rhizobial LPS showed that the differences in the LPS are as great among strains of a single species as they are among the different species (Graham and O'Brien, 1968; Carlson, et al., 1978). Phage lysis patterns among the rhizobia tested showed no correlation with nodulation groups (Carlson, et al., 1978).

This data does not rule out the possibility that LPS plays a role in determining Rhizobium-legume interactions. A successful symbiosis may require an interaction between the O-antigens of the rhizobia and the lectins of the legumes. Lectins, plant proteins with hemagglutinating activity, are known to associate strongly with cell surface polysaccharides (Lis and Sharon, 1973) and the O-antigens are exposed surface polysaccharides on gram-negative bacteria (Luderitz, et al., 1966). Bohlool and Schmidt (1974) have presented evidence that soybean lectin is capable of binding to 22 of 25 infective strains of R. japonicum (the symbiont of soybeans), while soybean lectin does not bind to any of 23 other strains from 5 species of rhizobia which do not nodulate soybeans.

Lectins have been reported to bind to either acid polysaccharide of capsular origin (Dazzo and Hubbel, 1975) or to LPS specifically of the Rhizobium species infective on the host from which the lectins were isolated (Wolpert and Albersheim, 1976). This discrepancy was possibly resolved when three different types of polysaccharide were found to be produced by R. leguminosarum (Planque and Kijne, 1977). One polysaccharide fraction, which precipitated pea seed lectins, was present in both the LPS and capsular polysaccharide preparations.

The heterogeneity of bacterial LPS was examined in E. coli and two fractions were found (Leive and Shovlin, 1968; Levy and Leive, 1969). Newly synthesized LPS was insensitive to brief exposure to ethylenediamine-tetraacetate (EDTA) and appears to maintain an equilibrium with the LPS fraction that is sensitive, which is released from the cells. The insensitive LPS is newly synthesized and may be located internally to the

fraction which is released by EDTA treatment (Leive and Shovlin, 1968). It has also been suggested that two fractions of LPS, differing in their polysaccharides, exist in rhizobial cell walls (Planque and Kijne, 1977). Pea lectins bind only to one fraction, which compared to the other, is larger in molecular size and lacking in heptose.

These data and the work of Van Brussel (1977) which indicates low LPS percentages in R. leguminosarum bacteroids, suggest that a portion of the polysaccharide may be removed from the rhizobial LPS during symbiosis. This loss or alteration of the cell's LPS may be necessary for an effective symbiosis to take place.

II

INTRODUCTION

INTRODUCTION

In the transition from bacteria to bacteroids, members of the genus Rhizobium must undergo biochemical and morphological changes within the root nodule. The most visible of these transitions is morphological. All free living rhizobia exist as rod shaped cells, but in the bacteroid state much variability occurs between the species. Rhizobium leguminosarum bacteroids occur as swollen pleomorphic, Y-shaped cells, while the bacteroids of R. japonicum exist as slightly swollen, but still rod shaped cells. The cause of this variation among genus members is not known, but in light of the importance of the cell wall to bacterial morphology, alterations in the cell wall may be responsible.

The goals of this study were to compare some aspects of the cell walls of R. japonicum and R. leguminosarum in free living and bacteroid forms. The comparative study was undertaken utilizing two different approaches. First, the flexibility of the walls was tested by incubating whole cells in different test solutions and observing their susceptibility to osmotic shock. Secondly, the lipopolysaccharide (LPS) was extracted and the amount present in the cell envelopes was measured. Because both R. japonicum and R. leguminosarum bacteroids have a smaller amount of LPS in their cell envelopes than do the culture grown cells, experiments were carried out to determine the fate of the LPS during the transition from bacteria to bacteroids.

MATERIALS AND METHODS

MATERIALS AND METHODS

Bacterial Strains

R. leguminosarum strain 128 C76 and R. japonicum strain 61 A118 were used for these experiments. The cultures were originally obtained from Dr. J. C. Burton of The Nitragin Company, Milwaukee, Wisconsin. These strains form effective nodules on broad beans and soybeans respectively.

Media and Rhizobium Cultivation

A chemically defined medium and a mannitol-yeast extract medium were used for these experiments. The compositions of the media are indicated in Tables 1 and 2. The media were prepared using deionized water and autoclaved at 121C for 20 minutes.

After inoculation of media with stock cultures grown on mannitol-yeast extract agar, 250 ml Erlenmeyer flasks containing 100 ml of medium were incubated on an Ederbach rotary shaker at 25C and 200 rpm. Late exponential phase (3-4 day old) cultures were used to inoculate larger broth cultures in 3l Fernbach flasks containing 1 l of medium. The flasks were placed on a rotary shaker at 200 rpm. At the late exponential phase of growth, as determined by O.D. at 617 nm, the rhizobia were harvested by centrifugation at 10,000g for 10 minutes.

Plant Cultivation

The plants used for bacteroid cultivation were broad beans (Vicia faba cv. Windsor) and soybeans (Glycine max cv. Prize). The broad beans were purchased from the Charles M. Lilly Company, Portland, Oregon. Soybeans were purchased from the Burpee Seed Company, Clinton, Iowa.

The seeds were surface sterilized in 75% ethanol for approximately 10 minutes, rinsed with water and soaked in 20% Clorox bleach for 5 minutes and rinsed with deionized water. The seeds were then sown in 6 inch plastic pots in a sterilized mixture consisting of 50% vermiculite and 50% perlite. The pots were flushed with a nitrogen free nutrient solution and were maintained in a Percival growth chamber. The photoperiod was 16 hours with 8 hours of darkness, and the temperature was 26C during the day and 22C at night. One week after planting, the seedlings were inoculated with Rhizobium grown in mannitol-yeast extract medium. All plants produced pink colored nodules which were mainly on the primary root.

Harvest of Cells

Culture grown rhizobia were harvested in late exponential growth by centrifugation at 10,000g for 10 minutes and washed a total of three times with cold 0.3M sucrose. The final pellet was used for the osmotic shock experiments.

The nodules were collected from the plants 4 weeks after inoculation with rhizobia and washed in cold water to remove external debris. They were homogenized in cold 0.3M sucrose (1g nodule/4 ml solution) in a Sorval Omni mixer for 2 minutes. The homogenate was squeezed through four layers of cheesecloth to remove the plant material and centrifuged for 3 minutes at 500g to remove the remaining plant debris. The supernatant was centrifuged at 4500g for 10 minutes, to obtain the bacteroids. The cells were then washed three times with cold 0.3M sucrose and the final pellet was used for the osmotic shock experiments. Microscopic examination of the

isolated bacteroids revealed relatively pure preparations of bacteroids with no contamination by plant material and small amounts of contamination by bacterial cells.

Osmotic Shock Experiments with Intact Cells

Pellets of culture grown cells and bacteroids were resuspended in various test solutions to give an absorbance reading of 0.4 to 0.5 at 617 nm. This wavelength was used to measure the turbidity of the suspensions during incubation in the test solutions and the effect of osmotic shock. The compositions of the test solutions used for these experiments are given in the legend to Figure 1. The suspensions were incubated at room temperature in 1 cm spectrophotometer tubes and optical density at 617 nm was measured at 10 minute intervals for a total of 110 minutes with a Bausch and Lomb Spectronic 70 (van Brussel, 1973). After 80 minutes of incubation, the bacteroid suspensions were centrifuged for 10 minutes at 2000g and the bacterial suspensions at 4200g. To apply osmotic shock, the supernatant was carefully pipeted off, the inside of the tubes wiped, and a volume of cold deionized water equal to the initial volume was added to the pellet. Readings at 617 nm were continued for 20 minutes to observe the effect of osmotic shock on the optical density of the suspensions.

The supernatant obtained before and after osmotic shock were observed in a Beckman DB-GT spectrophotometer at 260 and 280 nm to determine the amount of cytoplasmic material released from the cells. These wavelengths were used as rough measures of nucleic acid and protein losses respectively. The amount of cytoplasmic material released was determined as a percentage of the total 260 and 280 nm material released by sonication of an equivalent amount of the cell type.

Enzymes

The enzymes used for the osmotic shock experiments were lysozyme (Grade 1) and trypsin (Type 111). These enzymes were purchased from the Sigma Chemical Company, St. Louis, Missouri.

Isolation of the Cell Envelope

The method of Anea and Nikaido (1976) was used to isolate the cell envelope. Cells grown in mannitol-yeast extract medium and bacteroids were harvested as previously described but washed 3 times in cold deionized water instead of 0.3M sucrose to avoid RNA contamination in the LPS fraction. Each g of cells or bacteroids was resuspended in 10 ml of 1 mM Tris-Cl pH 7.6 buffer containing 1mM $MgCl_2$. The cells were disrupted at 4C with a French pressure cell at 10,000psi. The crude extract was centrifuged at 6,000g for 20 minutes and the supernatant centrifuged for 60 minutes. The pellet was washed 2 to 3 times by resuspending in the same buffer and centrifuging. The final washed pellet (cell envelope) was resuspended in the same buffer, quickly frozen in iced methanol and lyophilized.

Lipopolysaccharide Extractions

The cell envelopes, isolated and described above, of culture grown Rhizobium and bacteroids were extracted with 15 ml of 90% phenol and 15 ml di H_2O at 68C for 10 minutes (Westphal and Jann, 1965). The minimum amount of cell envelopes extracted was 25 mg. After centrifugation at 1000g for 30 minutes, the cell envelopes of bacteria and bacteroids separated into an aqueous phase, a phenolic phase, an interphase and a hard pellet. The aqueous and phenolic phases were carefully separated, and the extraction

was repeated with both the combined interphase and insoluble residue by the addition of 15 ml deionized water. The combined aqueous phase material was dialyzed against several changes of 4l of deionized water at 4C to remove traces of phenol. The percentage of LPS present in the cell envelope was determined by the dry weight of the nondialyzable material. The amount of heptose present in the LPS was estimated with the cysteine- H_2SO_4 reaction (Dische, 1955) modified by Osborn (1963), using D-glucoheptose as a standard. Two hours after the start of the reaction, the absorbance at 505 and 545 nm was determined for the samples and the standard. Subtraction of these values and comparison to the standard curve was used to estimate the amount of heptose present.

Bacteroid cell envelopes were also extracted with a 90% phenol, chloroform, and petroleum ether mixture (2:5:8) at 1g cells per 15 ml of the mixture to determine the presence of R-lipopolysaccharides (Galanos, et al., 1969). After centrifugation at 3000g for 15 minutes, the supernatant was set aside and the pellet was extracted two more times. The pooled supernatants were put in a Buchi rotary evaporator at 40C to remove the chloroform and ether. The concentrated phenol suspension was transferred to a Sorval centrifuge tube and deionized water was added dropwise until the R-LPS precipitated. This R-LPS was further purified by washing three times with 5 ml of 80% phenol followed by three washings with 5 ml of petroleum ether. The R-LPS was resuspended in deionized water and ultracentrifuged at 100,000g for 4 hours. The sediment was resuspended in 2 to 3 ml of deionized water and lyophilized.

EDTA Extractions of Lipopolysaccharide

EDTA treatment of culture-grown rhizobia and bacteroids was performed as described by Leive and Shovlin (1968). The cells and bacteroids were washed 2-3 times with a 0.12M Tris-Cl pH 8 buffer and brought to 37C in a water bath. The final volume contained 0.2g wet weight of cells per ml, and 0.01M EDTA. After 5 minutes of gentle agitation, $MgCl_2$ was added to give a 0.05M final concentration, and the cells were centrifuged at 9500g for 20 minutes. The supernatant was dialyzed at 4C, first against 4l of 0.1M sodium phosphate, pH 7, for 24 hours and then against several changes of 4l of deionized water for two days. The nondialyzable material was lyophilized.

The lyophilized LPS was used to determine if an interaction occurs with Concanavalin A (Grade IV, Sigma Chemical Company). The LPS and Con A were suspended in a 0.05 sodium phosphate buffer, pH 6.8, and the reaction was measured as the time needed for precipitation of the rhizobial LPS.

IV

RESULTS

RESULTS

Morphology of Culture-Grown Bacteria and Bacterioids

The morphology of log phase culture-grown cells and bacterioids is shown in Plates 1 to 4. The plates were taken using phase contrast microscopy at a magnification of 1,250. The morphological differences between R. japonicum cells and bacterioids (Plates 1 and 2) were subtle. The bacterioids retained the rod shape of the free-living cells, but were slightly swollen. In contrast, the morphological changes undergone in the nodule were highly visible for R. leguminosarum (Plates 3 and 4). The bacterioids showed no resemblance to the rod shaped culture-grown cells. They were present in the nodule mainly as enlarged, pleomorphic, Y-shaped cells (Plate 4).

Osmotic Shock Experiments Using Intact Cells

The effects on absorbance at 617 nm of incubation in the various test solutions and subsequent osmotic shock, for both culture-grown and bacterioid cells are given in Figures 1 to 4. The amount of cytoplasmic material released as measured by absorbance at 260 and 280 nm before and after osmotic shock is given in Figures 5 to 12. Light microscopy was used to observe morphological changes during incubation and after osmotic shock.

Incubation in Sucrose

Shortly after the start of the experiment, the absorbance at 617 nm of the R. leguminosarum bacterioid suspension (Fig. 4) started to increase. This increase was accompanied by observed cell plasmolysis. After osmotic

shock, the absorbance fell to nearly that at the start of the experiment. The plasmolyzed cells appeared to return to normal size and shape with some cell breakage. Similar results were seen with R. leguminosarum (Fig. 3) except that cell plasmolysis was to a lesser degree and the effect of osmotic shock was not as drastic.

Incubation in 0.3M sucrose had little effect on R. japonicum cells and bacteroids. The culture-grown cells remained normal during incubation and after osmotic shock. There was an increase in absorbance after osmotic shock in the bacteroids (Fig. 2) which corresponded to an increase in the optical density of the cells. A large amount of cytoplasmic material was lost from R. japonicum bacteroids after osmotic shock, corresponding to 50% of the cell's 260 and 280 nm absorbing materials (Figs. 7 and 8).

However, at sucrose concentrations greater than 0.3M, R. japonicum exhibited a high degree of plasmolysis and did not form a good pellet upon centrifugation. This phenomena was also seen to a lesser extent in R. leguminosarum bacteroids. This was not a buoyancy effect of the high sucrose concentration, but a change in the density of the cells. Centrifugation at 15,000g for 30 minutes still did not produce a hard pellet from R. japonicum cells and R. leguminosarum bacteroids. The other cells easily formed hard pellets using these conditions and also using centrifugation at 10,000g for 10 minutes.

Incubation in Sucrose and Phosphate Buffer at pH 6

In all of the suspensions, few observed changes occurred in either the absorbance or the morphology of the cells. R. leguminosarum bacteroids exhibited a slight rise in the absorbance at 617 nm and some plasmolysis

during incubation (Fig. 4). This increase corresponded to the release of 30% of the cell's 260 and 280 nm absorbing materials (Figs. 11 and 12). There was also a large amount of material lost after osmotic shock, corresponding to the decrease in absorbance at 617 nm. R. japonicum also exhibited a decrease in absorbance after osmotic shock (Fig. 1) and the release of over 30% of its 260 nm material and almost 50% of the 280 nm absorbing material (Figs. 5 and 6). R. japonicum bacteroids showed a slight increase in absorbance after osmotic shock and appeared morphologically unchanged (Fig. 2).

Incubation in Sucrose, Phosphate Buffer at pH 6 and Lysozyme

During incubation, all suspensions remained fairly constant and little or no cell breakage occurred. After osmotic shock, R. leguminosarum remained the most stable. The absorbance at 617 nm fell slightly after osmotic shock, but after 10 minutes returned to nearly that at the start of the experiment (Fig. 3). R. leguminosarum bacteroids and R. japonicum both showed decreases in absorbance after osmotic shock (Figs. 1 and 4) and the release of a large amount of cytoplasmic materials (Figs. 5 and 6, 11 and 12). R. japonicum lost the majority of its cellular contents after osmotic shock (Figs. 5 and 6) and the cells were almost completely disrupted. R. leguminosarum bacteroids released over 50% of their 260 and 280 nm materials and showed a lot of cell breakage, but not complete disruption (Figs. 11 and 12). R. japonicum bacteroids showed an immediate rise in absorbance after osmotic shock (Fig. 2), followed by a decrease to the level prior to osmotic shock. The loss of cytoplasmic material in the bacteroids was much less than that of R. japonicum culture-grown cells (Figs. 5-8).

Incubation in Sucrose and Phosphate Buffer at pH 8

A gradual decrease in absorbance at 617 nm was observed during incubation of R. japonicum (Fig. 1). The cells exhibited a tendency to become spherical and release a small amount of 260 and 280 nm absorbing material (Figs. 5 and 6). R. leguminosarum bacteroids released almost 30% of their cytoplasmic material during incubation (Figs. 11 and 12) and showed fluctuations in the absorbance at 617 nm. The other suspensions showed no morphological or absorbance changes during incubation (Figs. 2 and 3). After osmotic shock, a decrease in absorbance occurred in all suspensions except R. japonicum bacteroids (Fig. 2). The greatest decrease in absorbance after osmotic shock occurred in R. japonicum, where the reading at 617 nm fell to almost half of the initial reading (Fig. 1). Considerable cell breakage occurred whereas the other suspensions exhibited little morphological changes. The greatest losses of 260 and 280 nm absorbing material were in R. japonicum (Figs. 5 and 6) and R. leguminosarum bacteroids (Figs. 11 and 12). The least amount of cytoplasmic loss due to osmotic shock was in R. japonicum bacteroids (Figs. 7 and 8).

Incubation in Sucrose, Buffer at pH 8 and Trypsin

In all suspensions, a slight decrease in absorbance at 617 nm occurred ten minutes after the start of the experiment (Figs. 1-4). No morphological changes could be seen. During the remainder of the incubation period, the absorbance of all suspensions except R. leguminosarum bacteroids remained fairly constant. There was much fluctuation in the absorbance at 617 nm of R. leguminosarum bacteroids (Fig. 4) and the release of over 20% of 280 nm

material and almost 40% of the cell's 260 nm material (Figs. 11 and 12). After osmotic shock, there was a decrease in absorbance of all suspensions except R. japonicum bacteroids (Fig. 2). The largest loss of cytoplasmic material after osmotic shock occurred in R. japonicum (Figs. 5 and 6).

Lipopolysaccharide Extractions of the Cell Envelopes of Culture-Grown Cells and Bacteroids

The cell envelopes of bacteria and bacteroids separated into an aqueous phase, a phenol phase, an interphase, and a hard pellet after extraction with aqueous phenol. The material from the aqueous phase contains the LPS, which after dialysis and lyophilization, was white and fluffy in all extractions. The amount of LPS obtained from the culture-grown and bacteroid cell envelopes is given in Table 3. The recovery of the total cell envelope after phenol extraction, dialysis, and lyophilization was between 86.4 and 97.6% for all cell envelopes. The bacteroid cell envelopes had the least amount of aqueous phase material compared to the bacterial cell envelopes.

To determine if part of the LPS of the bacteroid cell envelopes was lost during bacteroid isolation, phenol extractions were carried out on the combined bacteroids and plant material obtained by centrifugation. The amount of LPS in the combined extract of R. leguminosarum bacteroids and plant debris was $2.8 \pm 1.1\%$ more than in the bacteroid cell envelope alone. No LPS was detected in the plant debris from R. japonicum.

Heptose determinations were carried out using the modified Dische-cysteine- H_2SO_4 reaction and measured 2 hours after the start of the experiment. The standard used is D-glucoheptose which has an absorption maximum of around 505 nm. The graphs obtained from these reactions are

given in Figures 13 and 14. An absorption maximum at 405 nm was present in all cell envelope aqueous phase material. This absorbance was caused by deoxyhexoses (rhamnose and fucose) and glucose (Humphrey and Vincent, 1969a). All graphs appeared to be similar indicating little or no difference in the sugar makeup of the various cell types (Figs. 13 and 14). The amounts of heptose indicated in the culture-grown cell envelopes were approximately the same (Table 4). In R. japonicum bacteroids, there was slightly less than half the amount of heptose indicated in the culture-grown cells. No heptose was indicated in R. leguminosarum bacteroids.

The bacteroids were extracted to determine the presence of rough form lipopolysaccharide (R-LPS). These extractions were repeated three times per organism and did not indicate the presence of any R-LPS as described by Galanos and coworkers (1968). However, in R. japonicum bacteroids, a white fluffy material was consistently obtained which correlated to 12-14% of the cell envelope. This material did not form a pellet upon centrifugation, and did not have the translucent appearance that was expected. A Dische reaction was run on this material, and a spectrum similar to that for R. leguminosarum bacteroid phenol extracted LPS was obtained. No heptose is present in the sample and the sugars appeared to be present in lesser amounts than in LPS extracted by the phenol method.

The culture-grown cells were briefly extracted with EDTA to see if the cells would release any of their LPS. In both cases, the amount of LPS released by the rhizobial cells comprised between 35 and 48% of the LPS found in the phenol extracted cells. The Dische reaction run on the released

LPS was identical to the spectra obtained in Figures 13 and 14. The bacteroids were also extracted with EDTA and no released LPS was detected. The released LPS from the culture-grown cells was used to determine if binding occurs with Con A. An interaction was seen between the LPS and Con A for both species (Table 5).

DISCUSSION

Several factors must be taken into account when interpreting the results of the osmotic shock experiments. It is difficult to compare culture-grown cells and bacteroids directly by osmotic shock. It is possible that larger and more irregular cells (e.g. R. leguminosarum bacteroids) may be more sensitive to osmotic shock even when their cell walls are the same. Mitchell and Moyle, (1956) showed that under certain conditions, Mycoplasma laidlawii in cylindrical form is more sensitive to osmotic shock than when spherical, even though the cell wall has not been altered.

The bacteroids, in vivo, may differ from cultured rhizobia in their internal osmotic pressure. This may cause a different degree of observed plasmolysis in the bacteroids and culture-grown cells in the incubation mixtures and different reactions to osmotic shock. Differences in the osmotic values of the incubation mixtures and temperature changes may influence the lysozyme sensitivity of the cells (Birdsell and Cota-Robles, 1967). These conditions were the same for culture-grown cells and bacteroids, and therefore should not have influenced the results of these experiments.

R. japonicum cells and R. leguminosarum bacteroids were shown to be more sensitive to osmotic shock after incubation in lysozyme than R. japonicum bacteroids and R. leguminosarum cells. But only R. leguminosarum appeared resistant to lysozyme induced lysis in the test conditions utilized.

The isolated sacculus of gram-negative bacteria is readily attacked by lysozyme, whereas the "in situ" murein is not (Weidel, et al., 1960). These authors concluded that the cell wall layers outside the sacculus function as a permeability barrier which prevents lysozyme from reaching its substrate. The relative insensitivity of R. leguminosarum to lysozyme is expected, but the sensitivity of R. japonicum and the bacteroids is not. It is particularly surprising that R. japonicum bacteroids are less sensitive to lysis than the culture-grown form, especially since they are morphologically similar.

Sutton and coworkers (1977) suggested that the osmotic sensitivity of R. leguminosarum bacteroids is not due to an adaptation to the high osmolarity of the nodule cytoplasm, but due to a change in the cell wall structure, perhaps by digestion by plant enzymes. The initial change in the cell wall may result in a greater permeability of the wall as well as greater osmotic sensitivity.

The cells probably differ in a component in the outer membrane which prevents lysozyme from reaching the [N-acetyl muramic acid]-[N-acetyl glucosamine] bonds. Van Brussel (1973) suggests a defective murein layer may be present in the bacteroids, due to an interference in cell wall synthesis. Increased permeability to substances, such as lysozyme, has been observed in bacteria with a fragmentary cell wall (Hofshneider and Martin, 1968). These authors observed L-forms of Proteus mirabilis with a lower LPS percentage than the wild type strains. Decreased amounts of LPS in these cells may be caused by a fragmentary murein layer, on which other cell wall constituents can not attach.

The stable shape of the bacteroid cell walls, particularly R. japonicum, and their stability in most of the incubation mixtures, suggests a strong murein layer rather than a defective one. However, the fact that R. leguminosarum exhibits a drastic shape change in the nodule, compared to R. japonicum seems to indicate a greater change in the cell wall of these cells. It is possible that the murein layer could be less complete in R. leguminosarum bacteroids, perhaps with differences in the nature of the crossbridges.

Similar observations have been made with Arthrobacter crystallopoietes which can grow as a rod or as a sphere depending on nutritional conditions (Ensign and Wolfe, 1964). It was found that the murein of the spheres had shorter polypeptide chains and possibly longer peptide crosslinks than that of the rod form. The spherical murein peptide crosslinks contain glycine and alanine residues while the rod murein crosslinks have only alanine. (Krulwich, et al., 1967). The spheres were shown to possess more N-acetyl muramidase activity than the rods and that this activity decreases during the sphere to rod transformation. A difference in the polypeptide crosslinks could account for the differences in the permeability of the rhizobial cell walls.

Some cell disruption occurred when R. leguminosarum bacteroids and R. japonicum were treated with trypsin, but the effect of trypsin is not nearly as drastic as that of lysozyme. The reactions of the cells during incubation in sucrose and buffer at pH 8 are basically the same as those during incubation in the same suspension plus trypsin. But in the culture grown cells, those incubated with trypsin are less sensitive to osmotic

shock than those in sucrose and buffer at pH 8. How trypsin is involved in decreasing the cell's susceptibility to osmotic shock is not known.

R. leguminosarum cells and R. japonicum bacteroids are sensitive to incubation in sucrose and release a larger amount of cytoplasmic material after osmotic shock than the other cells. It is not known why these cells are unstable in sucrose, but differences in the cell wall or membrane structures could account for the increased leakage of cellular contents.

From these observations, it appears that the cell wall structures of R. leguminosarum and R. japonicum are probably different. The differences may lie in the nature of their muurein crossbridges or in permeability barriers provided by LPS. Differences between the two species still exist when the cells are in bacteroid form. It is interesting to note that the fast grower, R. leguminosarum, changes to a less stable bacteroid form and the slow growing R. japonicum changes to a more stable form in the nodule.

The significance of the results of the phenol extraction and the estimation of heptose depends on the quantity of contamination in the cell envelope fraction. The material in the aqueous phase represents the LPS fraction of the cell envelope (Westphal and Jann, 1962). Any contamination by RNA or polysaccharides would be found in this fraction. Most of the RNA contamination was avoided by successively osmotically shocking the cells prior to the cell envelope isolation, and by phenol extracting the cells envelopes rather than the intact cells. Differences in the amount of polysaccharide contamination would lead to different heptose values of the LPS. The amounts of heptose were constant for all cell envelopes and except for R. leguminosarum bacteroids, correspond to the amount of LPS found in the cell envelopes, arguing against differences due to contamination.

The quantity of LPS in the bacteroids is less than half that found in free-living cells. In R. leguminosarum bacteroids 3% of the cell envelope LPS was lost during the bacteroid isolation procedure. No LPS was lost during the isolation of R. japonicum bacteroids. The heptose values for both species of Rhizobium were higher for the cultured cells than for the bacteroids. There is no heptose indicated in R. leguminosarum bacteroids and a small amount in R. japonicum bacteroids. The low values of heptose in R. japonicum were expected and correlated to the small percentage of LPS present in the cells. The fact that no heptose was indicated in R. leguminosarum bacteroids may indicate structural differences in the LPS of the bacteroids.

The smaller amount of LPS in the bacteroid cell envelopes indicates two possibilities. They may be cells with little LPS or they may have their LPS present in another form. The bacteroid LPS may occur partly in a rough form. The structure of the R-antigens, thought to be also the core of the O-polysaccharides, has not yet been fully elucidated. The R-LPS (glycolipids) would contain a low amount of monosaccharide and less hydrophilic properties than the smooth form LPS (Galanos, et al., 1969).

The bacteroid cell envelopes were extracted for the presence of R-LPS, and while none was indicated in R. leguminosarum bacteroids, a precipitate was obtained from R. japonicum bacteroids. This material corresponded to 13% of the cell envelope, but did not pellet upon ultracentrifugation. While this material was obtained in all extractions, it ran a spectrum similar to that obtained using the phenol-water method, except indicating lesser amounts of sugars. This material was consistently obtained in all R-LPS extractions and may indicate a type of LPS differing structurally from

that described by Galanos and coworkers (1969). The presence of an additional type of LPS, which has been structurally altered may explain why the bacteroids of R. japonicum are less sensitive to lysozyme and osmotic shock than R. leguminosarum bacteroids. Since no R-LPS was found in R. leguminosarum bacteroid cell envelopes, it is assumed from these data that these are cells existing with little LPS.

Intact R. leguminosarum and R. japonicum cells and bacteroids were treated with EDTA to determine if any LPS is released from the cells. In E. coli, EDTA causes an increase in permeability to substrates that normally can not enter and the loss of a large fraction of the surface LPS (Lieve, et al., 1968). Approximately 45% of the LPS of the culture grown Rhizobium cells is released, which agrees with previous research using E. coli (Lieve, et al., 1968). The released LPS had a Dische reaction similar to those of the aqueous phase material of these cells. No LPS was released from the bacteroids. The released LPS of the culture grown cells reacted with the plant lectin, Con A, utilizing a precipitation reaction. The specific interaction of the LPS of four species of Rhizobium with their host lectins has been demonstrated (Wolpert and Albersheim, 1976). It is possible that the EDTA released fraction of LPS may play a role in specific host recognition.

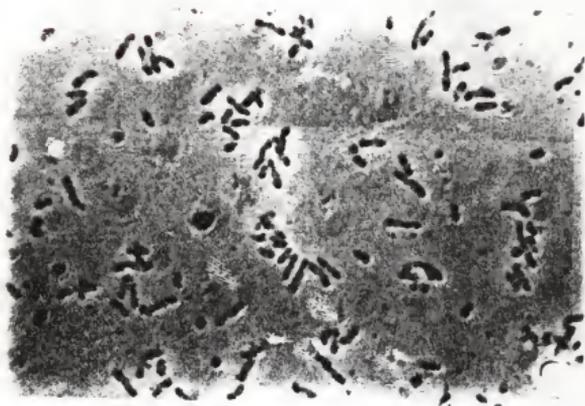
During symbiosis, the EDTA released LPS may be lost to the plant or sloughed off in the nodule cytoplasm. This would account for the low LPS percentages and increased permeability of the bacteroids. In the case of R. leguminosarum this LPS could be acted on by plant enzymes and structurally altered. The reactions of the cells to the plant environment and the degree of cell wall alteration is probably different between the fast and slow growing species of Rhizobium.

Plate 1: Rhizobium japonicum. Phase contrast microscopy of culture grown cells in the logarithmic phase of growth.



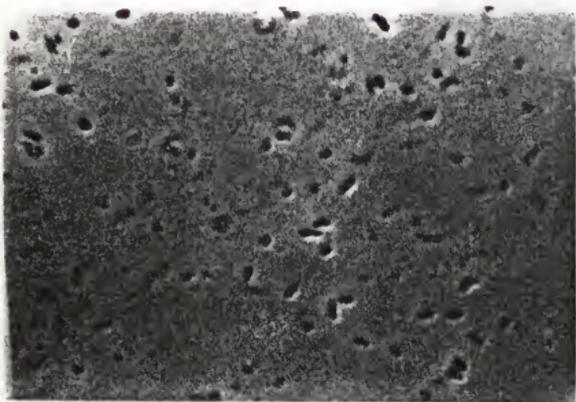
Magnification = 1250.

Plate 2: Rhizobium japonicum bacteroids. Phase contrast microscopy of bacteroid cells.



Magnification = 1250

Plate 3: Rhizobium leguminosarum. Phase contrast microscopy of culture grown cells in the logarithmic stage of growth.



Magnification = 1250.

Plate 4: Rhizobium leguminosarum bacteroids. Phase contrast microscopy of bacteroid cells.



Magnification = 1250

Table 1: Ingredients of the defined medium used for Rhizobium cultivation.

<u>Ingredients</u>	<u>g/l</u>	<u>Ingredients</u>	<u>mg/l</u>
Mannitol	3.00	$\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$	0.05
Arabinose	3.00	H_3BO_3	1.45
KH_2HPO_4	1.00	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.043
K_2HPO_4	1.00	$\text{ZnCl}_2 \cdot 7\text{H}_2\text{O}$	1.08
KNO_3	0.80	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	2.50
MgSO_4	0.18	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.10
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	0.13	$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	4.00
		$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	5.50
		Riboflavin	0.10
		Nicotinic acid	0.10
		Thiamine HCl	0.40
		Ca Pantothenate	0.50
		Vitamin B ₁₂	0.10
		p-Amino benzoic acid	0.10
		Biotin	0.12
		Pyridoxine HCl	0.10
		Inositol	0.50

Table 2: Ingredients of the mannitol-yeast extract medium used for Rhizobium cultivation.

<u>Ingredients</u>	<u>g/l</u>
Mannitol	3.00
Yeast extract	1.00
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$	0.13
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.36
$\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$	1.00
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$: 16.00 g/l H_2O , 0.25 ml/l.	
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$: 0.1g/l H_2O , 1.0 ml/l.	

Table 3: The percentage of LPS in the cell envelopes of rhizobial cells and their bacteroids, extracted by the phenol-water method.

<u>Organism</u>	<u>% LPS in the Cell Envelopes</u> ¹
R. <u>leguminosarum</u> 128 C76	19.12 <u>±</u> 3.92
R. <u>leguminosarum</u> 128 C76 bacteroids	4.98 <u>±</u> 2.74
R. <u>japonicum</u> 61A113	20.88 <u>±</u> 4.64
R. <u>japonicum</u> 61 A118 bacteroids	7.97 <u>±</u> 3.72

¹Values are an average of at least 3 trials, using standard deviation from the mean.

Table 4: Percentage of heptose present in the rhizobial cell envelopes measured using D-glucoheptose as a standard.

<u>Organism</u>	% heptose in the cell envelope ¹
R. <u>japonicum</u> 61A118	2.87 \pm .53
R. <u>japonicum</u> 61A118 bacteroids	.93 \pm .09
R. <u>leguminosarum</u> 128 C76	3.69 \pm .37
R. <u>leguminosarum</u> 128 C76 bacteroids	- 0

¹Values are an average of at least 3 trials, using standard deviation from the mean.

Table 5: Interaction of EDTA-released LPS from rhizobial cells with Concanavalin A. The LPS and Con A were suspended in a 0.5 M sodium phosphate buffer, pH 6.8 at room temperature. Interaction was measured as the time required for complete precipitation.

<u>Organism</u>	<u>Ratio of Con A to LPS</u>	<u>Precipitation Time</u>
R. <u>japonicum</u> 61 A118	2:1	11.5 min.
R. <u>japonicum</u> 61 A118	1:1	9.5 min.
R. <u>japonicum</u> 61 A118	1:2	7.0 min.
R. <u>leguminosarum</u> 128 C76	2:1	*
R. <u>leguminosarum</u> 128 C76	1:1	15.0 min.
R. <u>leguminosarum</u> 128 C76	1:2	12.5 min.

* No observed precipitation occurred after 30 minutes.

Figure 1: Rhizobium japonicum culture-grown cells. Effect of incubation in various test solutions and osmotic shock on absorbance of the suspensions at 617 nm. Suspensions of cells are in:

- X 0.3M sucrose
- O 0.3M sucrose, 0.1M potassium phosphate, pH 6
- 0.3M sucrose, 0.1M potassium phosphate, pH 6
0.2 mg/ml lysozyme
- Δ 0.3M sucrose, 0.1M potassium phosphate, pH 8
- ▲ 0.3M sucrose, 0.1M potassium phosphate, pH 8
0.2 mg/ml trypsin

The arrow indicates the time of osmotic shock.

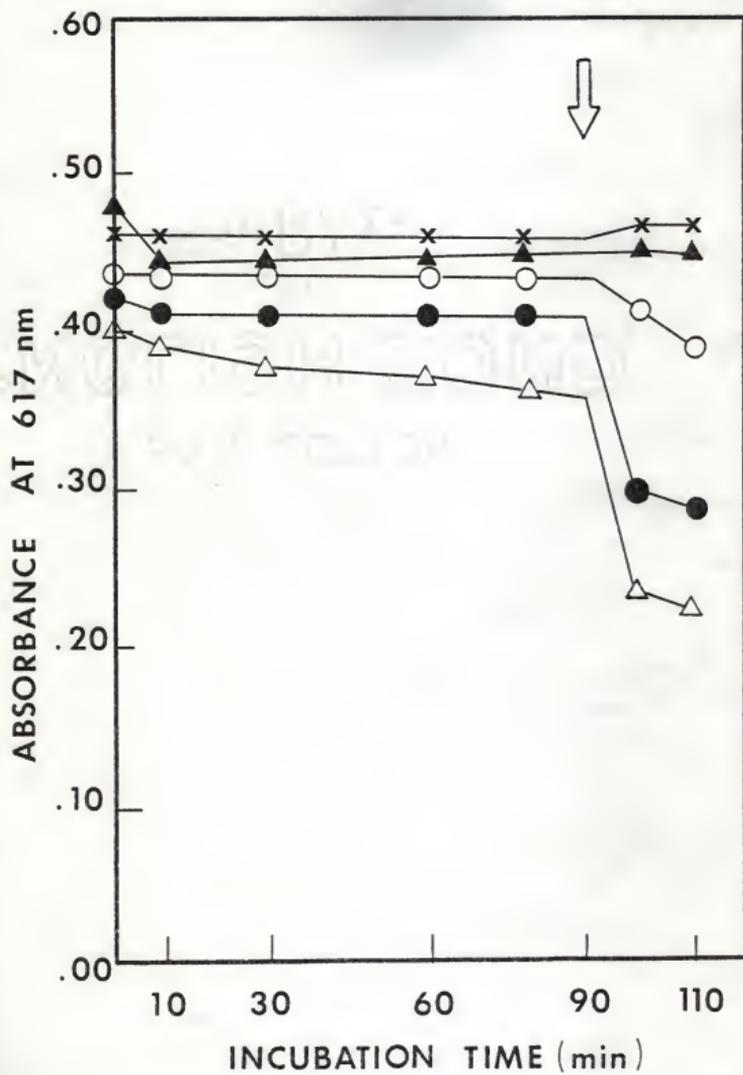


Figure 2: Rhizobium japonicum bacteroids. Effect of incubation in various test solutions and osmotic shock on absorbance of the suspensions at 617 nm. Symbols as in figure 1.

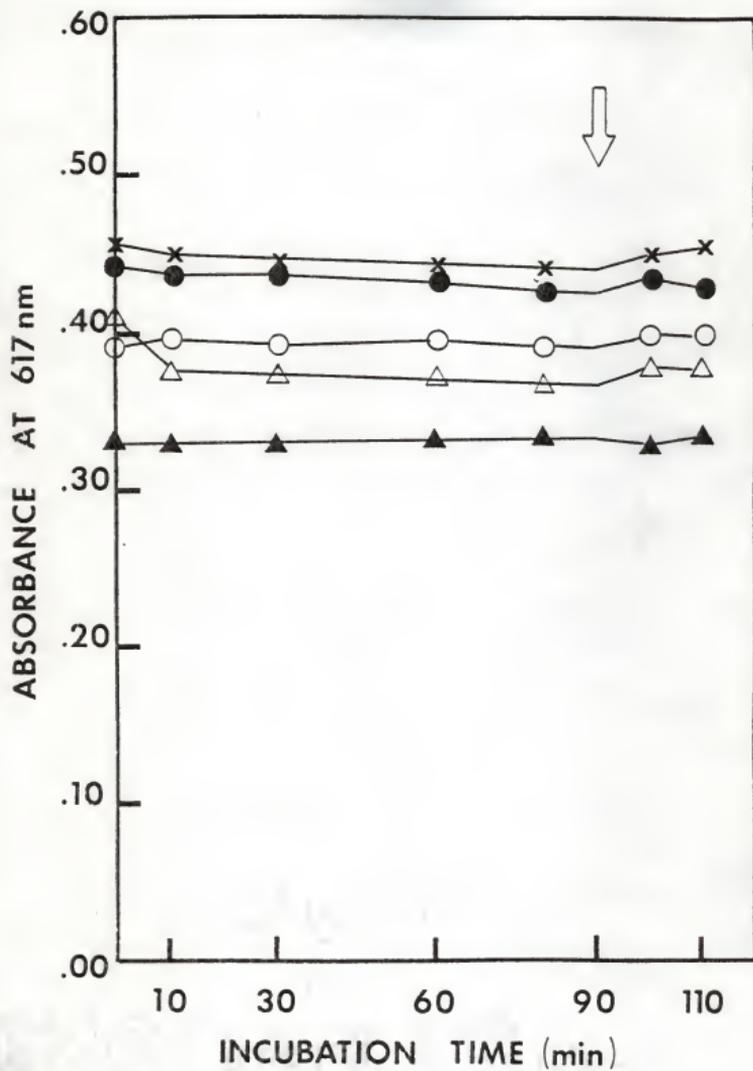


Figure 3: Rhizobium leguminosarum culture-grown cells. Effect of incubation in various test solutions and osmotic shock on absorbance of the suspensions at 617 nm. Symbols as in figure 1.

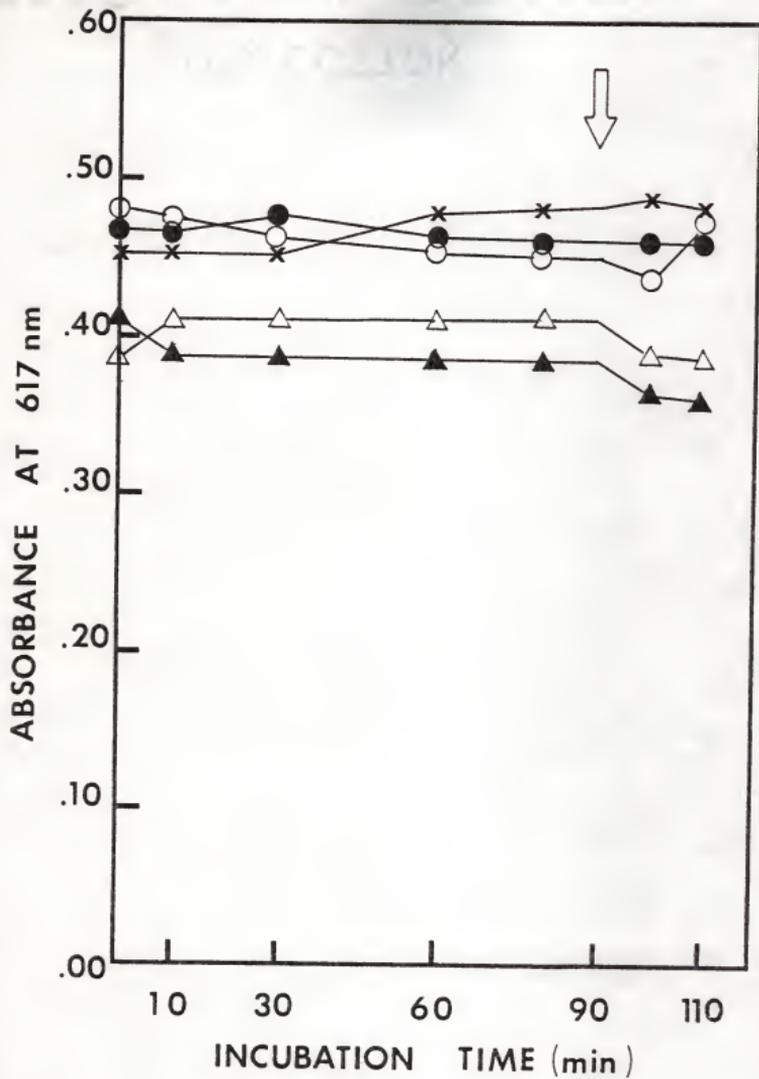


Figure 4: Rhizobium leguminosarum bacteroids. Effect of incubation in various test solutions and osmotic shock on absorbance of the suspensions at 617 nm. Symbols as in figure 1.

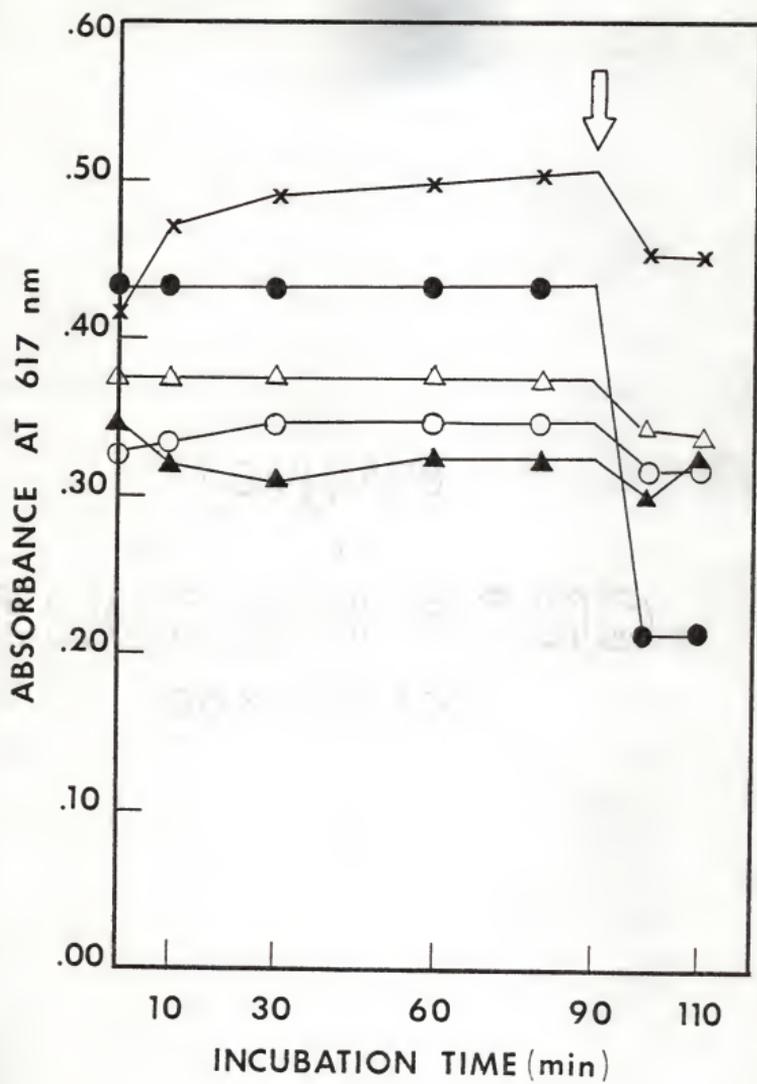
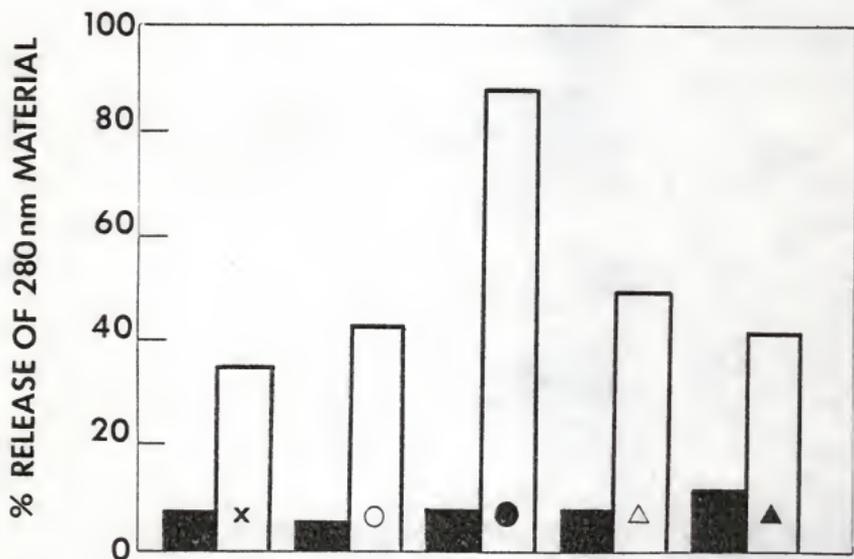
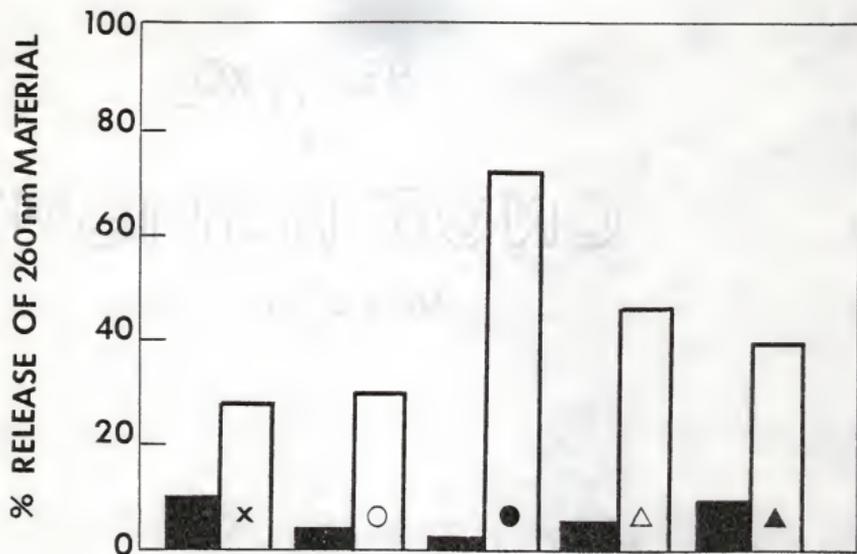


Figure 5: Rhizobium japonicum culture-grown cells. The amount of 260 nm material obtained from the supernatant after incubation in various test solutions and osmotic shock. Measured as a percentage of the total 260 nm material released from the cells by sonication. The shaded areas indicate the material obtained after osmotic shock. Symbols as in figure 1.

Figure 6: Rhizobium japonicum culture-grown cells. The amount of 280 nm material obtained from the supernatant after incubation in various test solutions and osmotic shock. Measured as a percentage of the total 280 nm material released from the cells by sonication. The shaded areas indicate the material obtained after osmotic shock. Symbols as in figure 1.



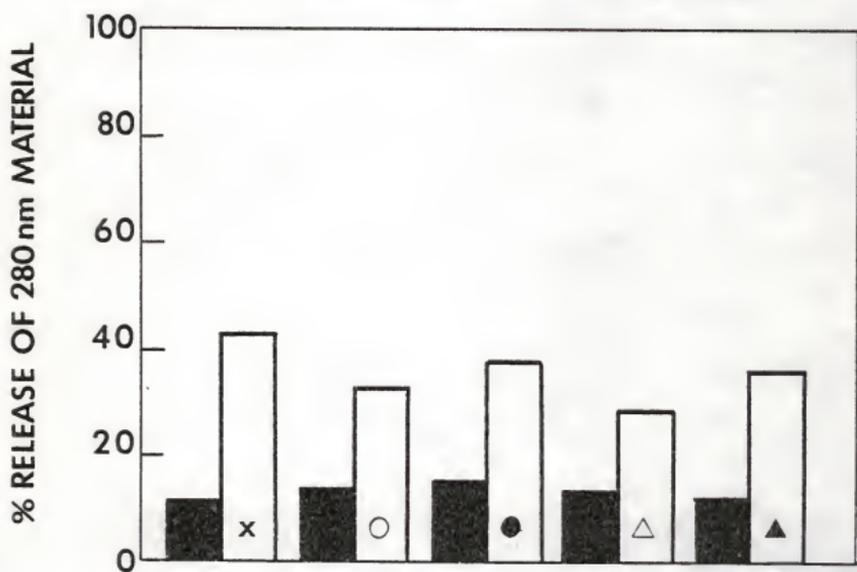
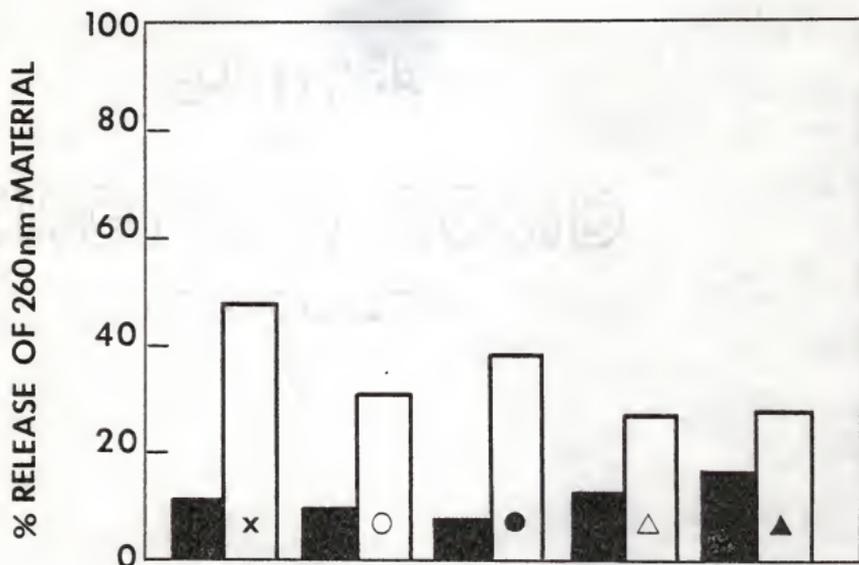


Figure 9: Rhizobium leguminosarum culture-grown cells. The amount of 260 nm material obtained from the supernatant after incubation in various test solutions and osmotic shock. Measured as a percentage of the total 260 nm material released from the cells by sonication. The shaded areas indicate the material obtained after osmotic shock. Symbols as in figure 1.

Figure 10. Rhizobium leguminosarum culture-grown cells. The amount of 280 nm material obtained from the supernatant after incubation in various test solutions and osmotic shock. Measured as a percentage of the total 280 nm material released from the cells by sonication. The shaded areas indicate the material obtained after osmotic shock. Symbols as in figure 1.

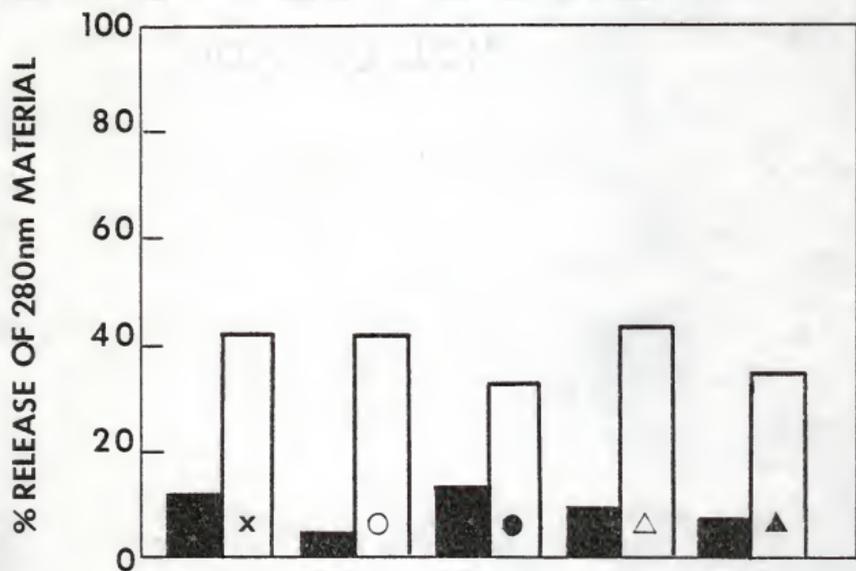
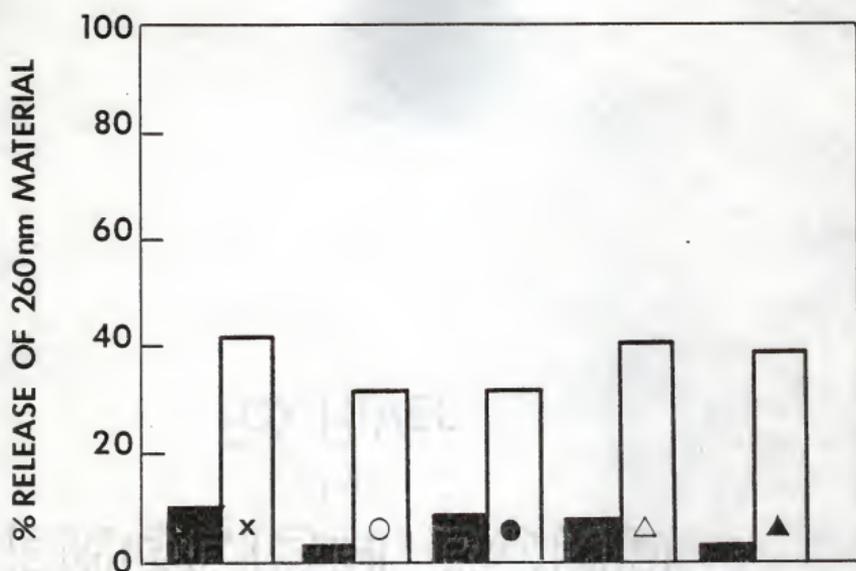


Figure 11: Rhizobium leguminosarum bacteroids. The amount of 260 nm material obtained from the supernatant after incubation in various test solutions and osmotic shock. Measured as a percentage of the total 260 nm material released from the cells by sonication. The shaded areas indicate the material obtained after osmotic shock. Symbols as in figure 1.

Figure 12: Rhizobium leguminosarum bacteroids. The amount of 280 nm material obtained from the supernatant after incubation in various test solutions and osmotic shock. Measured as a percentage of the total 280 nm material released from the cells by sonication. The shaded areas indicate the material obtained after osmotic shock. Symbols as in figure 1.

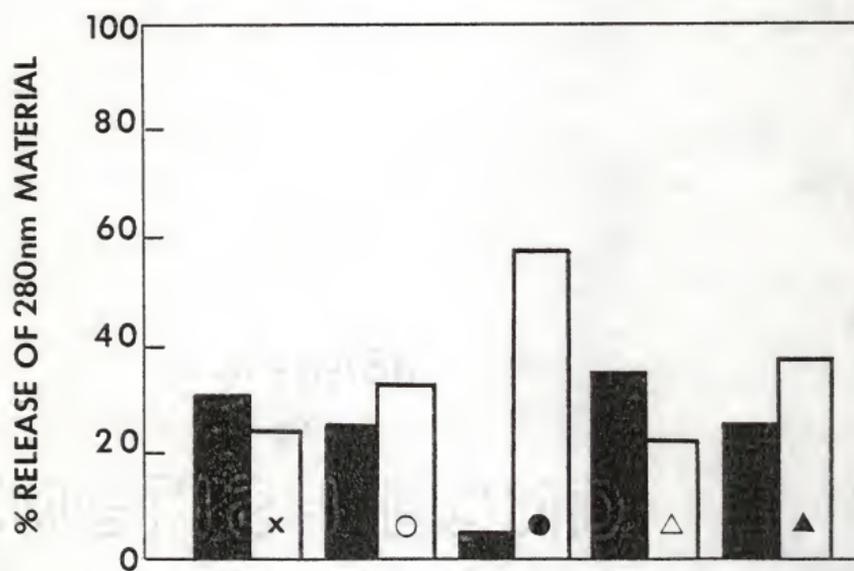
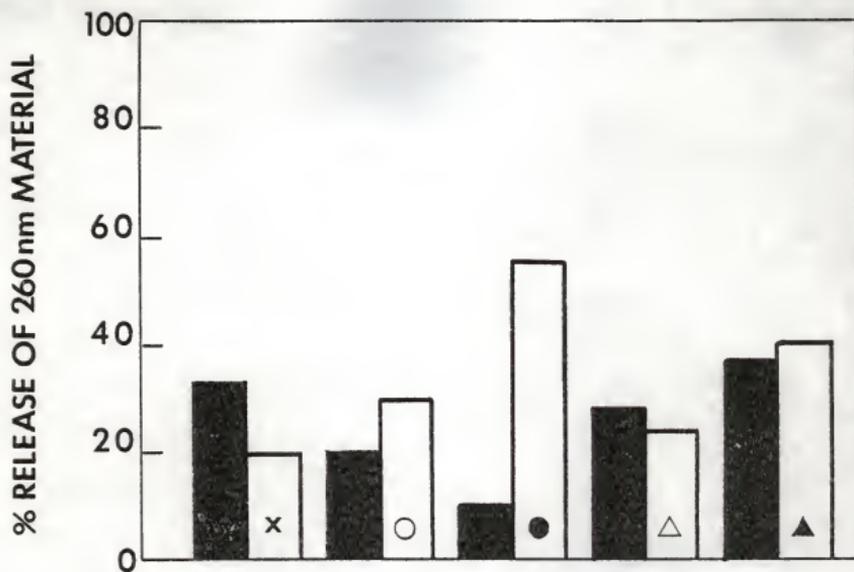


Figure 13: Rhizobium japonicum. Dische reaction of the aqueous phase material of culture grown and bacteroid cell envelopes. Measured 2 hours after the start of the reaction.

- o--o--o- R. japonicum (0.5 mg/ml)
- R. japonicum bacteroids (0.5 mg/ml)
- D- glucoheptose standard (0.05 mg/ml)

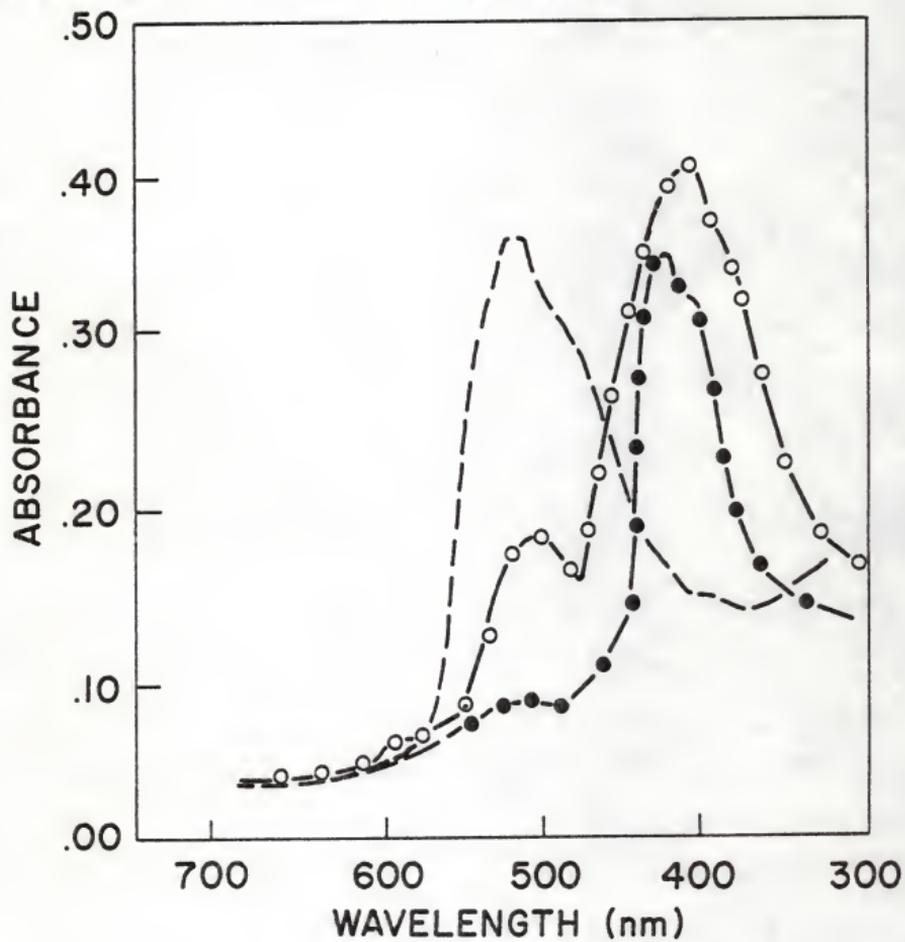
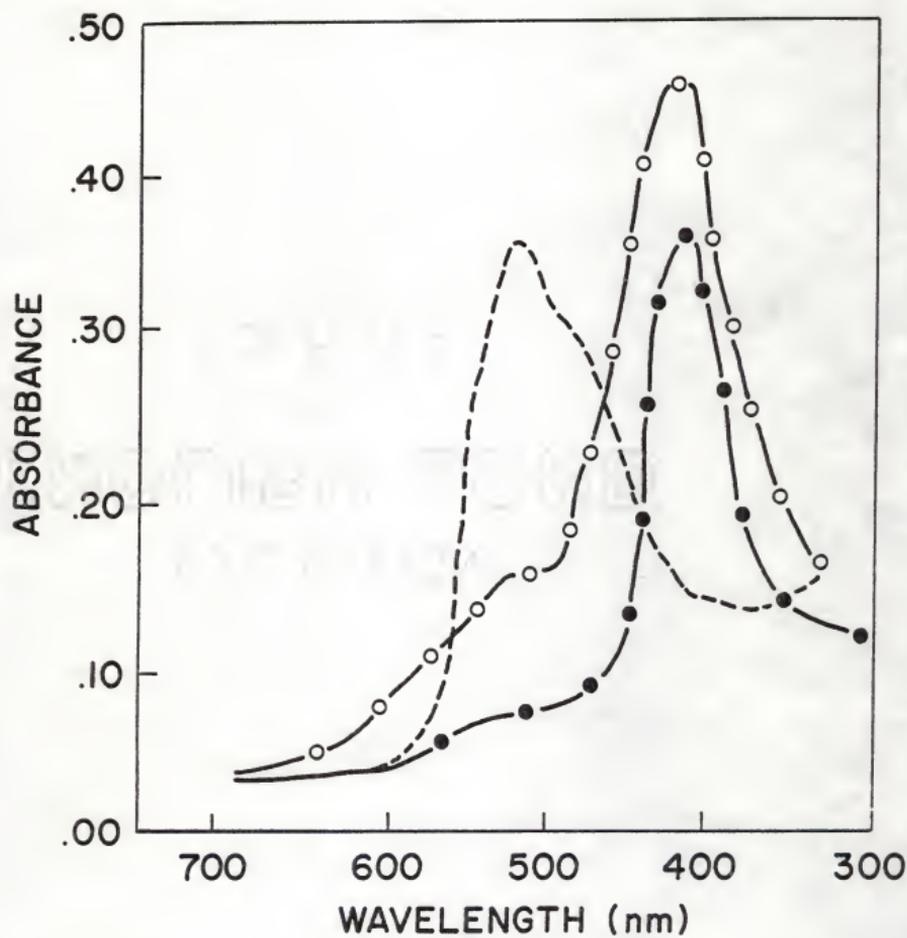


Figure 14: Rhizobium leguminosarum. Dische reaction of the aqueous phase material of culture-grown and bacteroid cell envelopes. Measured 2 hours after the start of the reaction.

- o--o--o- R. leguminosarum (0.52 mg/ml)
- R. leguminosarum bacteroids (0.5 mg/ml)
- D-glucoheptose standard (0.05 mg/ml)



VI

LITERATURE CITED

LITERATURE CITED

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A COMPARATIVE STUDY OF THE CELL WALLS OF RHIZOBIUM JAPONICUM
AND RHIZOBIUM LEGUMINOSARUM IN FREE-LIVING AND BACTEROID FORMS

by

Karen Lee Hooker

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The goals of this research were to study some aspects of the cell walls of a fast growing and a slow growing Rhizobium in free-living and bacteroid forms. R. leguminosarum strain 128 C76 was chosen as a representative fast grower, and exhibits a high degree of pleomorphism within the root nodules of broad beans (Vicia faba). The slow grower, R. japonicum strain 61 All8, maintains its rod shape in soybean (Glycine max) nodules.

Incubation in various test solutions and osmotic shock experiments were utilized to evaluate the flexibility of the cell walls. Only free-living R. leguminosarum appeared resistant to lysozyme in the test conditions utilized. R. leguminosarum bacteroids and R. japonicum were very sensitive to osmotic shock after lysozyme treatment. It was surprising that R. japonicum bacteroids were less sensitive to lysis than the culture-grown form, especially since they are morphologically similar. A high degree of plasmolysis occurred in R. leguminosarum cells and bacteroids when incubated in 0.3M sucrose. At higher concentrations of sucrose, R. leguminosarum bacteroids and R. japonicum were unable to form a pellet upon centrifugation due to changes in the density of the cells.

The cell envelopes of cultured rhizobia and bacteroids were isolated for the detection of lipopolysaccharide (LPS). The bacteroids contained less than half the LPS present in the free-living cells. In R. leguminosarum bacteroids, a small amount of LPS was lost during the extraction procedure. The amount of heptose present in the LPS was less in the bacteroids than in the cultured Rhizobium. No heptose was detected in R. leguminosarum bacteroids. The possibility of the LPS being present in a different form was discounted when no rough form LPS was detected in the ,

bacteroid cell envelopes. In R. japonicum bacteroids, an LPS-like material was consistently obtained during the R-LPS extractions. This material corresponded to approximately 13% of the cell envelope.

Treatment of free-living and bacteroid cells with ethylenediaminetetraacetate (EDTA) caused the release of approximately 45% of the LPS from free-living rhizobia. No LPS was released from the bacteroids due to EDTA treatment. The released LPS from the culture-grown cells interacted with Concanavalin A.