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Studies on Muropeptide Amino Acids of Rhizobium japonicum

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STUDIES ON MUROPEPTIDE AMINO ACIDS OF RHIZOBIUM JAPONICUM

(TITLE)

BY

Mohammed Alauddin

THESIS

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ABSTRACT

Alauddin, Mohammed. M.S., Eastern Illinois University.
Studies on muropeptide amino acids of Rhizobium japonicum.
Major Professor: Dr. William A. Weiler.

The isolation of Rhizobium japonicum cell walls was performed by ultrasonication and the enzymatic digestion with DNase, RNase and pronase separately. Crude cell walls were washed with M NaCl and 5% SDS and were isolated by 20,000 g centrifugation. Muropeptide amino acids were freed by hydrolysis at 121C for 10 minutes in 6N HCl.

Detection of Rhizobium japonicum murein layer amino acids was done by 2-dimensional instant thin layer chromatography on silica gel plates. The solvents found most favorable, were n-butanol: acetic acid: water and n-propanol: 34% ammonium hydroxide. Amino acid spots were developed by spraying ninhydrin on the chromatograms, followed by heating at 100C for 10 minutes. Spots were identified by their Rf values compared to those of known amino acids run in the same solvent system.

The eight amino acids and sugars identified in the murein layer of Rhizobium japonicum cell walls were DAPA, lysine, alanine, glutamic acid, glucosamine, serine, (valine, methionine or isoleucine) and leucine.

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INTRODUCTION

Bacteria are the friends of man. Indeed, they are essential to all life. Many of them live close to man, other animals or plants and do more good than harm.

A distinguishing characteristic of bacteria is their complex surface structure, a rigid cell wall outside the cell membrane. The constituents of bacterial cell walls are proteins, lipids and polysaccharides whereas cell walls of plants, many ascomycetes, oomycetes and the yeasts (ascomycetes) are largely composed of cellulose. In zygomycetes and higher fungi the cellulose is replaced by a polymerized acetylated glucosamine known as chitin and by other substances of obscure nature.

Only a few years ago, it seemed that the ultimate in microscopy had been attained with the bright field, dark field, and ultraviolet microscopes. After the invention of phase and electron microscopy, the use of fluorescent dyes and other histochemical methods has offered exciting new opportunities for the study of microbial structure.

Salton (1964) studied bacterial cell walls by electron microscopy. He observed the cell wall of Gram positive bacteria as one fairly homogeneous dense layer. Woutera (1965) investigated Bacillus

subtilis as an example of the Gram positive cell type in which the thick wall (ED) and plasma membrane (CM) are united along their entire length (Fig. 1a). On the other hand, cell walls of Gram negative bacteria showed two electron dense layers (ED) separated by an electron transparent layer (ET) with two membranes (CM); Salton (1964), Forsberg et al. (1970), (Fig. 1b).

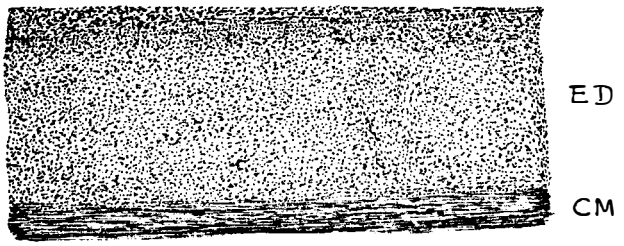
Cell wall studies begin with autolysis, a process by which enzymes of a cell which has ceased to metabolize, digests its own protoplasm. This is followed by heterolysis, the lysis of the cell by external materials.

Baumrucker (1971) investigated the lysis of the family Rhizobiaceae giving a good deal of information about the family and some insight into its cell wall composition. In view of this work, the author began to study the cell wall of Rhizobium japonicum by applying some recent techniques and apparatus.

The purpose of this paper is to describe the isolation of the cell wall of Rhizobium japonicum. It is a Gram negative rod, motile by means of monotrichous flagella, whose growth on mannitol agar is slow and scant. Rhizobium japonicum is an aerobic, nitrogen-fixing bacterium, which may grow symbiotically in root nodules of Soja max (soy bean) plants.

Gram positive

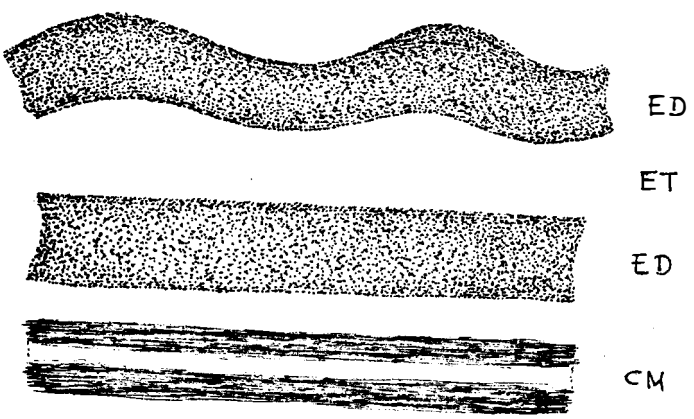
(a)



electron micrograph of Bacillus subtilis cell wall

Gram negative

(b)



electron micrograph of Gram negative cell wall

Fig. 1

HISTORICAL

The Bacterial Cell Wall

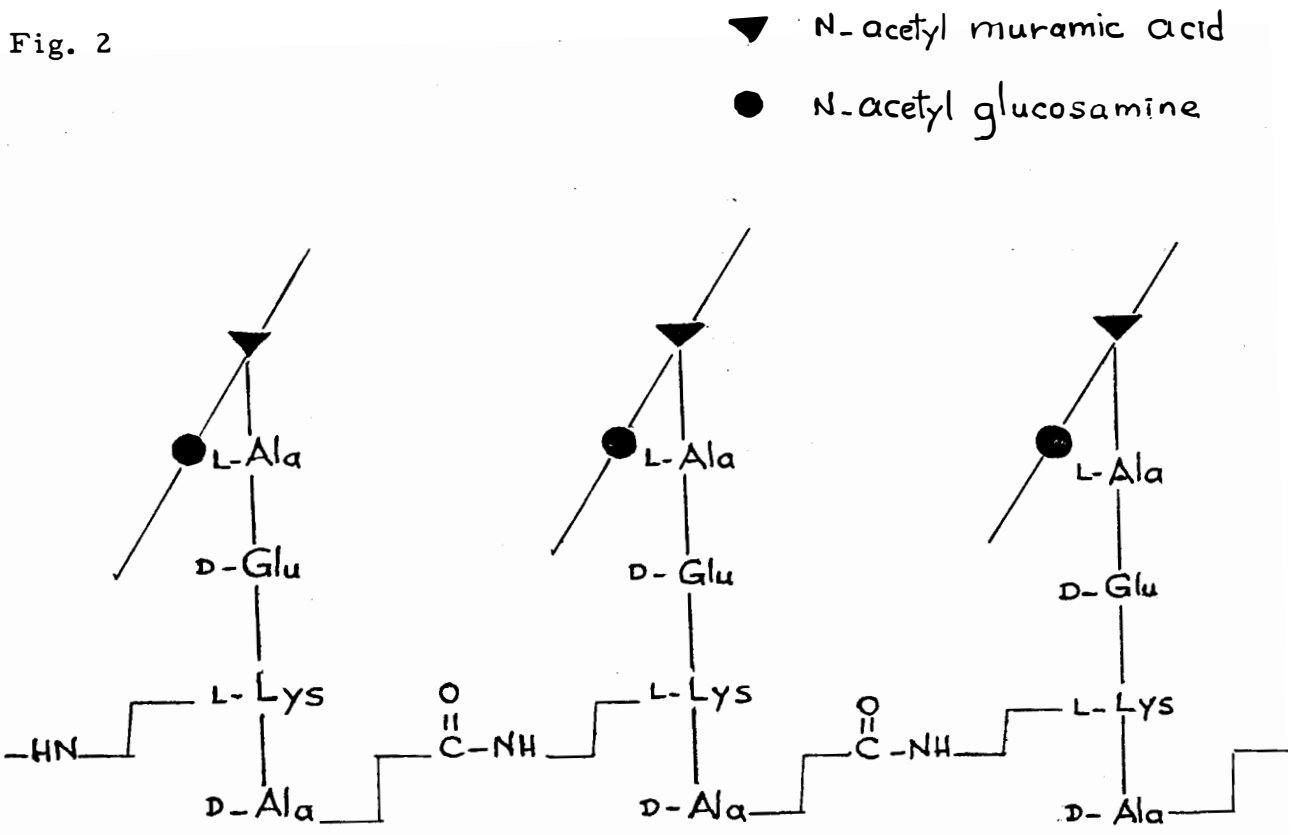
The direct demonstration of the bacterial cell wall has been achieved by a number of means. These include micro-dissection, differential staining, and electron microscopy. Cell rupture by ultrasonic vibration reveals differences in bacterial species. On rupture, jagged lines of fraction occur in the cell wall because of its rigidity. Because of the low refractive index of the cell wall, it was difficult or impossible to see it by either bright or dark field microscopy; hence, electron microscopy was a necessary tool in such studies. In addition, the bacterial cell wall has only slight affinity for dyes and thus was not demonstrable by early workers with the staining techniques available to them.

The bacterial cell wall is a strong and rigid structure that protects and supports the weaker and biochemically more active parts of the cell. Its thickness varies from 10 to 25 nm, according to the species of the organism. In general, Gram negative bacteria possess thinner walls than Gram positive bacteria. In both groups of bacteria the cell wall protects the cell from mechanical damage and can maintain a high concentration of salt within the cell, without osmotic rupture.

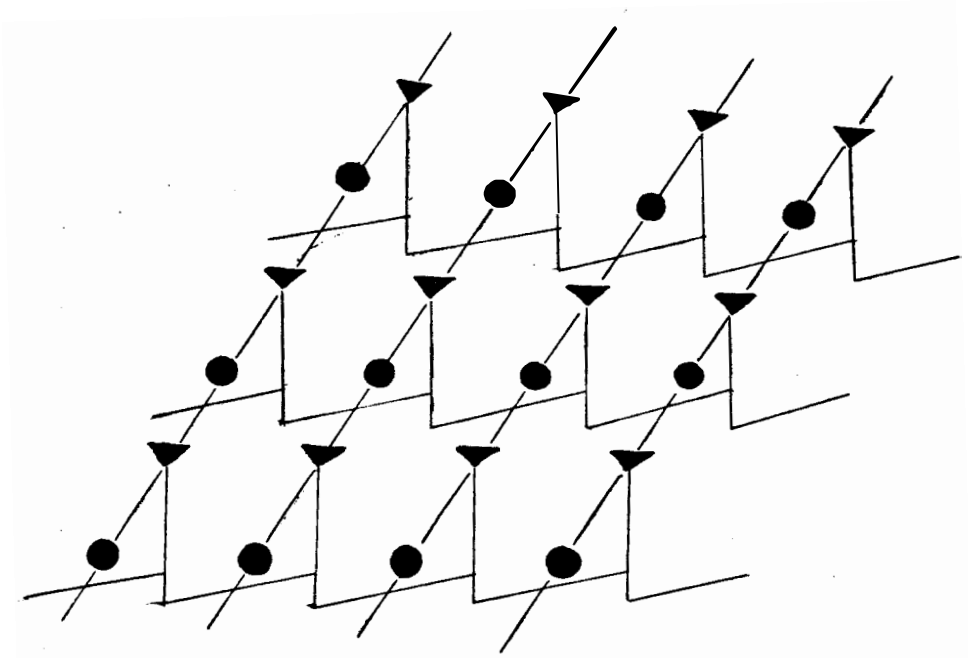
At the molecular level the cell walls of Gram positive bacteria differ from those of Gram negative species (Figs. 2 and 3), particularly in their amino acid composition. The common constituent of all bacterial cell walls is the mucopeptide layer. The mucopeptide layer (also called muropeptide, murein, murein sacculus, glycopeptide, glycosaminopeptide, peptidoglycan and rigid layer) is the inner-most layer of the cell wall. Mucopeptide is the supporting layer for the bacterial cell wall. Loss of this layer causes cells to burst or to form osmotically unstable protoplasts, spheroplasts, or L-forms. Self-lysis (autolysis) of the mucopeptide occurs generally, as the cell ages. Rogers and Perkins (1968) reported 80 to 90% mucopeptide in Gram positive bacterial cell walls, whereas Salton (1964) reported only 5 to 10% mucopeptide present in Gram positive bacterial cell walls.

The major components of the mucopeptide from both groups are two amino sugars (N-acetyl glucosamine and N-acetyl muramic acid) and four amino acids (D-alanine, L-alanine, D-glutamic acid, and L-lysine or meso-diaminopimelic acid) (Taylor et al., 1969, Hoshino et al., 1972). Additional amino acids are present in the Gram positive mucopeptide. They are in peptide form (several amino acid units) attached in cross-linkages, called bridges. L-lysine or meso-diaminopimelic acid (DAPA) of one peptide subunit attaches them to the carboxyl group of D-alanine of another subunit. Davis et al. (1968) described the Staphylococcus aureus mucopeptide as homopolymers

Fig. 2



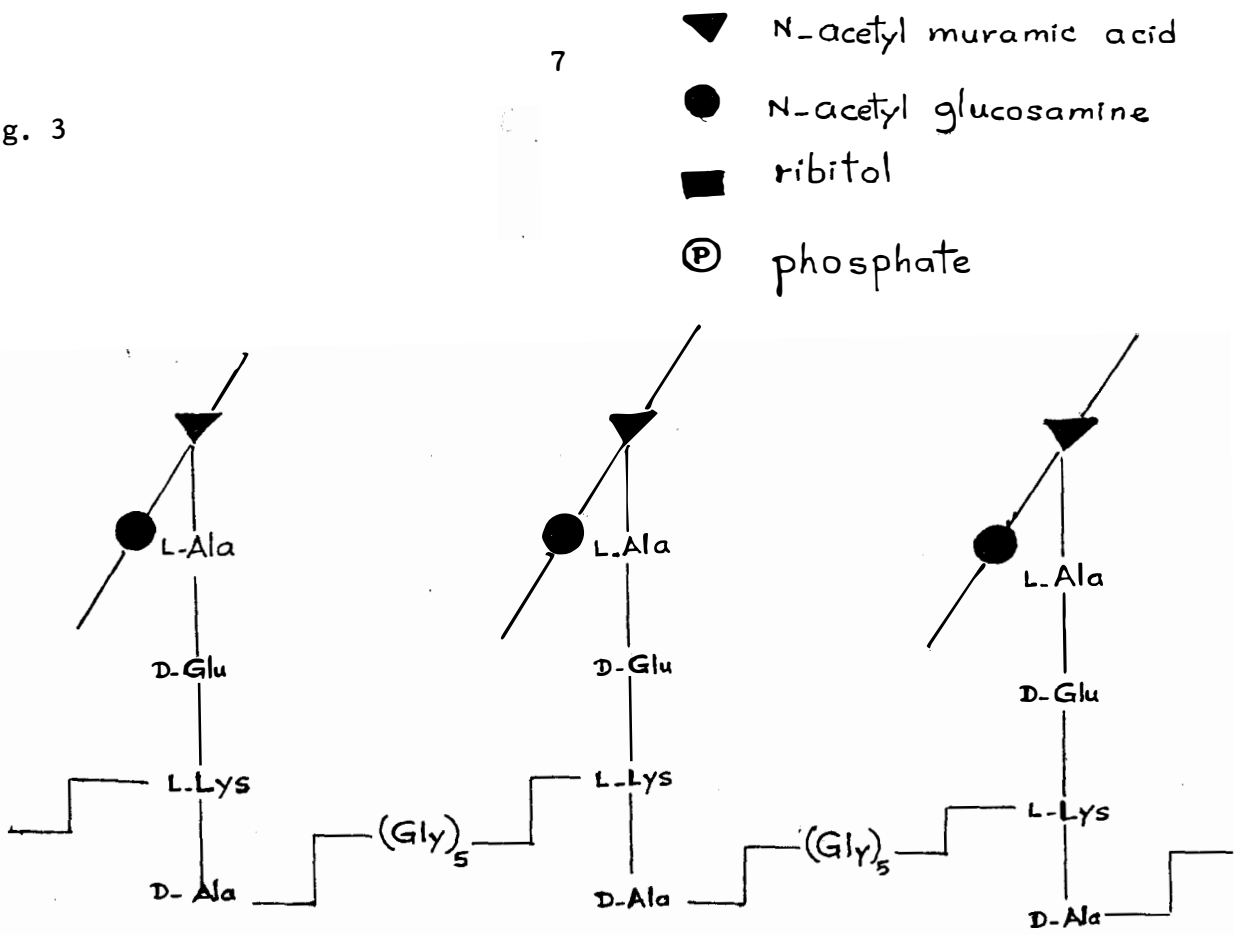
Cross-linking of mucopeptide in Escherichia coli



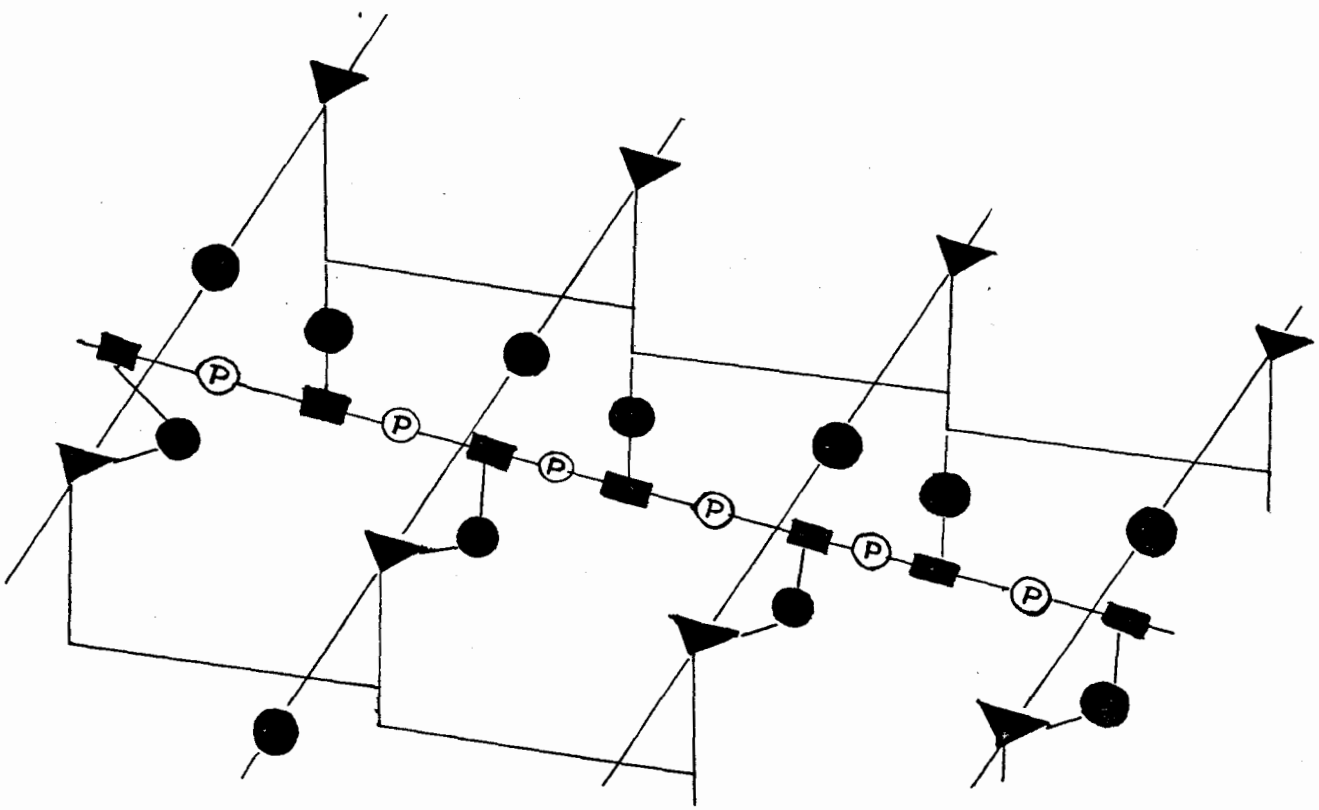
Arrangement of several mucopeptide chains

Vertical lines = tetrapeptide side-chains

Fig. 3



Cross linking of mucopeptide in Staphylococcus aureus



Arrangement of several mucopeptide chains

of N-acetylglucosamine linked B-1,4 to N-acetyl muramic acid. These chains are usually considered as repeating polymers (Fig. 3). The tetrapeptide side chains appear regularly on alternate N-acetyl muramic acid residues, but it is not known whether the cross-linking pentaglycine chains form a regular, parallel array of bridges between successive pairs of tetrapeptides along two parallel polysaccharides, or whether they form a less regular belt. Mandelstam and Strominger (1961) proposed the structure of the mucopeptide of Staphylococcus aureus (Copenhagen) with the possible mode of attachment of the teichoic acid polymer.

Gram positive bacterial cell walls have some non-murein components, principally the teichoic acids and polysaccharides, which possess antigenic properties. Although Salton and Horne (1951) reported that cell walls of Gram positive bacteria contain no protein, cell wall analysis generally involves the use of proteolytic enzymes, suggesting that during isolation, the cell wall proteins may have been digested. Salton (1964) reported little or no lipid in the cell walls of Gram positive bacteria.

Teichoic Acid

Teichoic acid, an organic phosphate polymer found only in Gram positive bacteria, has been isolated by treating the cell walls of Gram positive bacteria with trichloroacetic acid. Ghuysen and Strominger (1963) used 10% trichloroacetic acid at 60C for 12 hours

and obtained 95% of the cell wall teichoic acid (as cell wall organic phosphate). The composition of teichoic acid is glycerol or ribitol phosphate, D-alanine and N-acetyl glucosamine.

Lipopolysaccharide

The space between the two electron-dense layers of Gram negative bacterial cell walls is lipopolysaccharide. These complex macromolecules are responsible for many biological properties like 0 antigen determinants, bacteriophage receptor sites, and endotoxins.

Lipopolysaccharide consists of lipid A (composed of fatty acids, glucosamine and phosphate) linked to a polysaccharide "core". The "core" is composed of KDO (ketodeoxyoctulosonic acid), heptose and phosphate. The end of the lipopolysaccharide core consists of antigenic side chains, composed of mannose, rhamnose, abequose and galactose.

Lipoprotein

The outside layer of the Gram negative cell wall is the lipoprotein layer. It appears to be a double membrane and is a permeability barrier. Little is known about this lipoprotein.

Methods of Cell Wall Isolation

Cell Disruption

Disruption of bacterial cells is the first step for the qualitative and quantitative analysis of the cell wall. A number of cell disruption methods have been used in isolating cell walls. Among them are auto-

lysis, osmotic lysis, heat treatment, and mechanical disintegration.

The autolysis of living cells was applied by Weidel (1951) for the isolation of Escherichia coli cell walls. According to him, the preparation behaved homogeneously in an ultracentrifuge, but was heterogeneous due to the presence of those small enzymatically digested molecules. Autolysis was applied by Vaituzis and Doetsch (1969) for the isolation of cell walls of Vibrio metchnikovii, Bacillus licheniformis, and Pseudomonas aeruginosa. Autolysis was achieved by washing 5 ml amounts of 18 hour broth cultures and suspending them in 5 ml of sterile distilled water at room temperature or at 4C for periods up to 4 weeks. After that, most of the cells showed varying amounts of membrane separation and cytoplasmic clearing.

Salton (1964) ruptured Escherichia coli cells at 70C. As a result, a naked coagulated protoplast was obtained. Some of the chemical components were lost and he found this method unsatisfactory.

Gerhardt and Judge (1964) ruptured the cell walls of Saccharomyces cerevisiae and Bacillus megaterium by passing a heavy suspension (50 mg of cells per ml) through a refrigerated pressure unit (Ribi et al., 1959) at 17,000 psi for the yeast and 30,000 psi for Bacillus megaterium. Breakage of the cell wall was judged by phase and electron microscopy.

Kagramanova (1968) stated that the more plastic outer layer of the cell wall of Proteus gelatica could be destroyed by acetone, EDTA

(ethylenediamine tetraacetic acid), or freezing and thawing of the cells. Wallinder and Neujahr (1971) ruptured cell walls of Lactobacillus fermenti by freezing and storing at -20C. The length of time of frozen storage was not mentioned. Hoshino et al. (1972) used the procedure of Sharon and Jeanloz et al. (1963) for Micrococcus lysodeikticus cell wall rupture and the yield according to them was 4.6 to 4.8 g cell walls.

The use of glass beads to disrupt the cell wall is one of the more successful methods. Ghuysen and Strominger (1963) used glass beads in a Nossal disintegrator for the rupture of Staphylococcus aureus cell walls. Ralston and McIvor (1964) also used glass beads for rupturing Staphylococcus aureus cell walls. Gaffar et al. (1966) used glass beads for breaking of the cell walls of Clostridium acidiurici. Burge and Draper (1967) isolated Proteus vulgaris cell walls by shaking at 4C in 100 ml portions of grade 12 Ballotine glass beads at 500 vibrations per minute for 10 hours. According to them, this technique broke the cells adequately and detached the flagella. Hofschneider and Martin (1968) used glass beads for the rupture of Proteus mirabilis cell walls by prolonged shaking of the bacteria in an aqueous suspension of 0.8% (w/v) sodium dodecyl sulphate. Braun and Rehn (1969) ruptured Escherichia coli cell walls by shaking with glass beads. Braun and Sieglin (1970) isolated cell walls of Escherichia coli by glass bead disruption. Johnson and Cummins (1972) disrupted cell

walls of anaerobic coryneforms, classical propionibacteria, and strains of Arachnia propionica by suspending in 0.15 M NaCl, 0.01 M EDTA with glass beads. In general, using this method, 80-90% of the cells were broken, but it was time consuming.

Ultrasonic cell disruption is the most recent successful technique. More than 99% of the cells can be disrupted in a few minutes. Murray and Elson (1964) studied the location of mucopeptide in Escherichia coli. The intact or spheroplast cells were broken by ultrasound (without fixation) using a 20 kc ultrasonic apparatus. They ran this apparatus 1 minute for the intact cells and 30 seconds for the spheroplasts and obtained more than 90% cell disruption. Edwards and Noller (1964) also sonically disrupted the Escherichia coli cell walls in almost the same way. Yamaguchi (1965) used sonic oscillation (10kc) for breaking cell walls of actinomycetes. The time of sonication was 10-40 minutes. The degree of breakage was checked by phase microscopy. Araki et al. (1966), working with Escherichia coli, broke the cells with 10 kc sonic oscillation for 7 minutes in EDTA. According to them, 40% of the cells were disrupted. The cell walls of Rhizobium japonicum (Fottrell and Mooney, 1969) were broken by an ultrasonic disintegrator for 1.5 minutes at less than 5C. Vaituzis and Doetsch (1969) used ultrasonic treatment of Vibrio metchnikovii, Bacillus licheniformis and Pseudomonas aeroginosa for tearing flagella with membrane fragments still attached to their terminal portions from the cells. Accord-

ing to them 10 seconds treatment at 20 kc in a Mullard ultrasonic disintegrator was adequate.

Isolation of the Mucopeptide

After the rupture of bacterial cell wall, the inner protoplasm disintegrates readily and the more resistant walls can be purified for chemical and physical study. The most important constituent of the cell wall is the mucopeptide, a very stable complex, capable of surviving a variety of chemical treatments. This capability facilitates its separation from all other cell wall components.

Murray et al. (1965) investigated the location of mucopeptide in sections of the cell wall of Escherichia coli and other Gram negative bacteria. They demonstrated its location by treating the sections with uranium, lanthanum, thalium, or lead salts. The inner-most layer alone was lost in spheroplasts produced by penicillin poisoning or by treatment with lysozyme-EDTA and was removed from isolated cell walls by lysozyme alone. The inner most layer was considered, therefore, to contain mucopeptide characteristic of bacteria.

The removal of other cell-wall components of Gram negative bacteria (lipopolysaccharide, lipoprotein, and protein) depends on mucopeptide stability.

Mucopeptide is phenol insoluble whereas the lipopolysaccharide-protein complex is phenol soluble (Weidel, 1951). Hofschneider and Martin (1968) isolated the mucopeptide from the cell wall of Proteus

mirabilis with phenol and water extraction. The insoluble material was composed of lipopolysaccharide and mucopeptide. After dialysis and differential centrifugation, the sediment obtained contained the mucopeptide. Protease digestion yielded a soluble and insoluble fraction. The insoluble fraction was the mucopeptide, and the purity of which was not ascertained.

Trichloroacetic acid has been used by various researchers for the isolation of the mucopeptide with favorable results. Wallinder and Neujahr (1971) obtained purified cell walls of Lactobacillus fermenti by incubation with 10% trichloroacetic acid at 2C for 24 hours. Smit et al. (1971) isolated the lipoprotein, lipopolysaccharide, and mucopeptide fraction of Escherichia coli and Proteus mirabilis cell walls with phenol (Westphal and Jaun, 1965) and trichloroacetic acid (Staub, 1965) extraction.

Hatton (1969) isolated the cell wall mucopeptide of Micrococcus lysodeikticus by a fractional procedure. The mucopeptide of the cell wall was isolated by mechanical disintegration. Its purity was monitered in regard to its quantitative chemical composition and susceptibility to lysozyme. Drying of the cell wall or mucopeptide at elevated temperature rendered it more susceptible to solubilization by hot trichloroacetic acid.

Sodium dodecyl sulfate, a detergent, is also used for the isolation of mucopeptide. Braun and Rehn (1969) isolated the murein-

lipoprotein complex of the cell wall of Escherichia coli by treatment with 4% hot sodium dodecyl sulfate.

Only in recent years have microbiologists been able to determine the chemical composition of cell wall mucopeptide. Taylor et al. (1969) isolated the murein layer of Salmonella typhi. It was shown to be composed of muramic acid, glucosamine, alanine, glutamic acid and diaminopimelic acid (DAPA) in the molar ratio of 0.9:1.1:1.6:1.0:0.8 respectively. The murein of Escherichia coli B prepared by the same method gave the following ratios: 0.8:1.0:1.9:1.0:0.9. Twelve amino acids typical for Gram positive organisms and 2 amino sugars were identified in cell walls of Pseudomonas gelatica by Kagramanova and Ermoleva (1969). Recently, Shmeleva and Birger (1972) found the same amino sugars and amino acids in cell walls of Corynebacterium diphtheriae.

Isolation of Lipopolysaccharide

This is the middle layer of the Gram negative cell wall. The most successful method for the purification of lipopolysaccharide is the treatment of defatted cell walls with 45% phenol (Key et al., 1970). There is little modification of this method by other investigators. For example, Volk (1968) extracted the lipopolysaccharide from the cell wall of a Xanthomonas sp. with a hot 45% phenol treatment. Hofschneider and Martin (1968) isolated the lipopolysaccharide from the cell wall of Proteus mirabilis by a phenol-water (9:1, v/v) extraction. Weckesser

et al. (1972) isolated the lipopolysaccharide from the cell wall of Rhodopseudomonas capsulata by treatment with hot phenol. According to them, hydrolysis of the isolated lipopolysaccharide in weak acid yielded a polysaccharide fraction which was degraded from a lipid fraction (lipid A). Lipid A was shown to be a fatty acid esterified glucosamine polymer. The chemical analysis of the lipopolysaccharide demonstrated the presence of D-glucose, D-galactose, D-rhamnose, 3-0-methyl-L-rhamnose, D-glucosamine, 2-keto-3-deoxyoctonate and neuraminic acid.

Variations in methodology for the isolation of lipopolysaccharide are few. Burge and Draper (1967) treated the cell walls of Proteus vulgaris with ether-ethanol 1:1 (v/v) until the solvent became colorless. The sediment was subjected to cold (4C) phenol water treatment. The lipopolysaccharide component was contained in the aqueous phase and was separated as an opalescent supernatant.

Additional methods for lipopolysaccharide isolation were compared by Heath and Ghalombor (1963) in the preparation of lipopolysaccharide from Escherichia coli cell walls. Phenol extraction (Westphal et al., 1952), trichloroacetic acid extraction (Boivin et al., 1933), ether extraction (Ribi et al., 1959), digestion of acetone dried cells with pronase followed by precipitation with Mg^{++} (Osborn et al., 1962) were used. Among them, phenol and trichloroacetic acid gave better results.

Isolation of Lipoprotein

The outer electron dense layer of the Gram negative bacterial cell wall is the cell wall membrane, generally called lipoprotein. Little work has been done on the lipoprotein layer. Murray et al. (1965) found that the lipoprotein layer was outside of the limits of the wall profile usually visualized in sections. The outer layers generally loosened during embedding, but in Escherichia coli and some other Gram negative bacteria, the layers all stayed tightly adherent to each other. Hofschneider and Martin (1968) treated cell walls of Proteus mirabilis with phenol-water (9:1, v/v). The soluble portion was lipoprotein, which when dialyzed against distilled water yielded a water insoluble lipoprotein, a tough, gummy precipitate.

Purification of Cell Wall Components

In the process of cell wall purification, digestion with different enzymes is applied. Lysozyme, deoxyribonuclease, ribonuclease, and trypsin, are most commonly used in the purification of bacterial cell walls. Two of them are proteolytic enzymes, trypsin and pronase. They are not capable of splitting all peptide linkages in a protein molecule; each cleaves only the linkages adjacent to certain specific amino acids. The enzymes effect a splitting of proteins into fragments of varying lengths, but do not produce many free amino acids.

Lysozyme has been used to break the B-1, 4 linkage present between N-acetyl glucosamine and N-acetyl muramic acid (Jeanloz

et al., 1963 and Sharon et al., 1966) in the bacterial cell wall. Hoshino et al. (1972) digested cell walls of Micrococcus lysodeikticus with lysozyme. Braun and Wolff (1970) used lysozyme, lysozyme-pronase and 4% SDS (sodium dodecyl sulfate) for Escherichia coli cell wall isolation. According to them, with pronase digestion, lipoprotein could be degraded to the dipeptide lysyl-arginine which remained bound to the mucopeptide. The resulting mucopeptide-lysyl-arginine was separated from the rest of the undissolved degradation products of lipoprotein by 4% SDS and was further degraded by lysozyme. Edwards and Noller (1964) digested Escherichia coli cell walls with lysozyme or trypsin. They observed a marked increase in the amount of protein released by trypsin. Release of reducing sugars by lysozyme increased when trypsin was also present.

Digestion with deoxyribonuclease (DNase) has been applied frequently for the isolation of Gram positive and Gram negative cell walls. Braun and Sieglin (1970) isolated cell walls of Escherichia coli and digested them with DNase before the cells were ruptured. The disrupted cells were treated with EDTA and after several washings, 4% boiling sodium dodecyl sulfate was added dropwise. The supernatant upon centrifugation, yielded the mucopeptide-lipoprotein complex. Digestion of mucopeptide-lipoprotein was done with pronase for 5 hours at 37C. The pellet after centrifugation was treated with 4% (boiled) SDS. The mucopeptide-lysyl-arginine yield was 75 mg per

200 mg of frozen cell paste thawed. The insoluble mucopeptide lipoprotein complex was treated with ultrasound for 30 seconds and was treated with trypsin for 35 minutes at 24C. Wallinder and Neujahr (1971) treated cell walls of Lactobacillus fermenti with trypsin and DNase followed by heating in boiling water for 10 minutes. This treatment was followed by SDS solubilization at 37C for 24 hours and by washing in M NaCl. At each step the "cell walls" were observed. After trypsin treatment, they found cell debris and larger particles. After DNase digestion, smaller particles were observed. SDS treated cell walls appeared to be purified. Gaffar et al. (1966) digested cell walls of Clostridium acidiurici with trypsin, RNase for 4-5 hours at 37C and about 18 hours with pepsin to obtain purified cell walls. Kolenbrander and Ensign (1968) isolated the mucopeptide of Spirillum serpens by digesting the cell wall with SDS, DNase - pronase, and EDTA. During the process, they washed the cell walls with boiling water several times. Ralston and McIvor (1964) digested cell walls of Staphylococcus aureus with RNase, DNase, and trypsin until material absorbing light at 260 and 280 nm had been removed.

Identification of Cell Wall Components

Hydrolysis

After the purified cell walls or their components are isolated, they are hydrolyzed to their constituent monomers. By this, water is introduced at the points of cleavage of larger molecules, liberating

the constituent monomers, and yielding peptides and amino acids.

Four normal HCl (hydrochloric acid) was used for cell wall hydrolysis at 100C for 4, 8, and 14 hours by Shuster and Betts (1969) for Pseudomonas saccharophila, Anderson et al. (1967) for Micrococcus lysodeikticus and Staphylococcus aureus and by Martin and Kemper (1970) for Clostridium perfringeus, respectively (Table 1). Braun and Wolff (1970) also used 4N HCl for the hydrolysis of Escherichia coli cell walls. Hydrolysis for 15 hours seemed to reduce the amounts of lysine and DAPA. According to them, hydrolysis in 6N HCl increased the lysine-DAPA value to 1 but muramic acid and glucosamine were more destroyed. They did not mention the temperature at which hydrolysis was done. Six normal HCl hydrolysis was also done at 100-110C temperatures for 16-22 hours by Gray and Wilkinson (1965), Braun and Rehn (1969), Wallinder and Neujahr (1971), and Beaman et al. (1971) for the cell walls of some Gram negative bacteria, Escherichia coli, Lactobacillus fermenti, and Nocardia rubra, respectively.

Identification of Cell Wall Components

After hydrolysis, the hydrolysate is dried and washed to remove HCl and is either spotted on a chromatogram paper or used in different types of detection methods for the analysis of amino acids, amino sugars, and sugars.

Paper chromatography is the most common method for the detection of amino acids but thin layer chromatography, a very recent

BACTERIAL CELL WALL HYDROLYSIS

Name of Organism	Normality HCl	Temp.	Hours	Author
M. lysodeikticus and *S. aureus*	4N	100C	8	Anderson *et al.* (1967)
P. saccharophila	4N	100C	4	Shuster and Betts, (1969)
C. perfringeus	4N	100C	12	Martin and Kemper, (1970)
E. coli	4N	100C	15	Braun and Wolff, (1970)
E. coli and other Gram negative bacteria	6N	105C	16	Gray and Wilkinson, (1965)
S. pyogenes	6N	105-110C	16	Gunetileke and Anwar, (1966)
E. coli	6N	105-110C	16	Hatton, (1969)
M. lysodeikticus	6N	105-110C	16	Braun and Rehn, (1969)
E. coli	6N	--	15	Braun and Wolff, (1970)
L. fermenti	6N	105-110C	16	Wallinder and Neujahr, (1971)
Nocardia rubra	6N	100C	22	Beaman *et al.* (1971)

Table 1

method, seems superior as far as the sharpness of separation, high sensitivity, and great speed is concerned.

Detection of amino acids using different solvent systems was done by Gray and Wilkinson (1965). They used 2-dimensional chromatography with 2-butanol-88% formic acid-water (75:15:10, by vol.), followed by phenol-water-5N ammonia (80:20:0.3, by wt.) for Gram negative bacterial cell wall amino acids. Spot detection was done with ninhydrin spray. The presence of cysteine and methionine was detected by treating the cell wall hydrolysates with H_2O_2 followed by chromatography. The presence of DAPA was confirmed by descending chromatography using a mixture of methanol-water-10N HCl-pyridine (32:7:1:4, by vol.). Hatton (1969) used descending chromatography for Micrococcus lysodeikticus cell walls with n-butanol-pyridine-water (6:4:3, v/v/v); 0.6% ninhydrin in butanol was sprayed and heated for 1 hour for color development. Amino sugars were separated by 2-dimensional chromatography. Pyridine-water (4:1, v/v) in the first direction and N-butanol-pyridine-water (3:1:1, v/v/v) was used in the second direction for 18 hours. Shuster and Betts (1969) used paper chromatography techniques for the separation of amino acids in the cell wall of Pseudomonas saccharophila. The solvents used were ethylacetate-pyridine-water (12:5:4, v/v/v) and 2-butanol-acetic acid-water (40:10:22, v/v/v). Ninhydrin and alkaline $AgNO_3$ spray were used for the detection of amino acids and amino sugars, respectively. Volk (1968) worked on

Xanthomonas sp. cell walls and used descending chromatography with pyridine-ethylacetate-water (20:72:23) and used a $AgNO_3$ dip for sugars.

Braun and Rehn (1969) used thin layer chromatography for the detection of Escherichia coli cell wall lipids. Lipid A components were detected in the solvent system heptane-ethylacetate (4:1) by exposure to iodine vapor and dipping in a 0.25% solution of rhodamine B in 96% ethanol followed by viewing under 366nm light.

Gas liquid chromatography, amino acid analysis, and other methods are also used for the detection of amino acids and amino sugars of the bacterial cell wall. Anderson et al. (1967) used gas-liquid chromatography on 10% diethyl glycol-succinate and 3% SE-30 columns for the separation of fatty acid methyl esters from the cell walls of Micrococcus lysodeikticus and Staphylococcus aureus.

Amino acid analysis was used for the detection of cell walls of Clostridium sp. by Martin and Kemper (1970). Glucosamine and basic amino acids were separated on a 0.9 x 24 cm column (A). Resolution of muramic acid, neutral and acidic amino acids was performed on a 0.9 x 54 cm column (B) and was eluted with 0.2 N sodium-citrate-HCl buffer.

In reviewing the literature cited for the isolation techniques for Gram positive and Gram negative cell walls, the author began an approach to a standard method for the isolation of Rhizobium japonicum cell wall muropeptides.

MATERIALS AND METHODS

Bacterial Cultures

The strain of Rhizobium japonicum used was obtained from Dr. Forrester Davisson of Urbana Laboratories, Urbana, Illinois.

Escherichia coli B (Purdue University Culture Collection) was used as a control.

General

Glassware

All glassware used was washed thoroughly in detergent and rinsed twice in distilled deionized water.

Sterilization

All glassware was sterilized in a Castle autoclave at 15 psi., 121C for 15 minutes. All media were similarly sterilized.

Reagents

Reagents (Appendix B) were prepared in distilled water. Fresh reagents were prepared for each experiment. All but heat-labile reagents were sterilized by autoclaving.

Centrifugation

A Sorvall Superspeed RC2-B automatic refrigerated centrifuge was used for all centrifugation.

Incubation

A Precision Thelco, model 6 incubator was used at 30C for the growth of Rhizobium japonicum on slants. Enzymatic digestion took place at 37C.

Incubation of spectrophotometric flasks for cell growth was done in a gyrotory water bath shaker, Brunswick, Model G-76.

Sonication

Cell wall rupture was done by ultrasonication, using a Biosonik III, Bronwill Model BPIII.

Spectrophotometry

A Bausch and Lomb Spectronic 20 was used to monitor cell growth. Absorption was measured at 440 nm.

Resuspension

A Vortex Jr. mixer, Model K-500-J was used.

Thin Layer Chromatography

Detection of amino acids present in the cell walls of Rhizobium japonicum was done by thin layer chromatography. Gelman (silicic acid) and Eastman Kodak (silica gel) instant thin layer plates were used. A

Gelman chromatogram tank was used to run the solvent. Ninhydrin (Appendix B) was sprayed on the plates for the detection of amino acids.

Chromatography Oven

Heating of the chromatograms for drying and amino acid detection was done in a chromatography oven at 100C.

Standard Cell Growth Procedure

A stock culture of Rhizobium japonicum was kept on DMYE slants (Baumrucker, 1971) at 7C. Working stocks were transferred to other DMYE slants and incubated at 30C for about 3 days. To begin the experiment, a loop-full of freshly grown cells was transferred to 100 ml of DMYE broth in a 750 ml spectrophotometric flask which was incubated in the 30C gyrotory water bath shaker at 140 RPM. Growth of cells was observed spectrophotometrically and a growth curve was made for Rhizobium japonicum and Escherichia coli B.

Standard Procedure for Cell Wall Isolation

The standard procedure for Rhizobium japonicum cell wall isolation is diagrammed in Fig. 4. Variations in digestion time, sonication time, hydrolysis time and centrifugation speed and time were tested.

Early stationary phase cells of Rhizobium japonicum and Escherichia coli (Figs. 5a and 5b) in DMYE and TGYE, respectively, were

Fig. 4

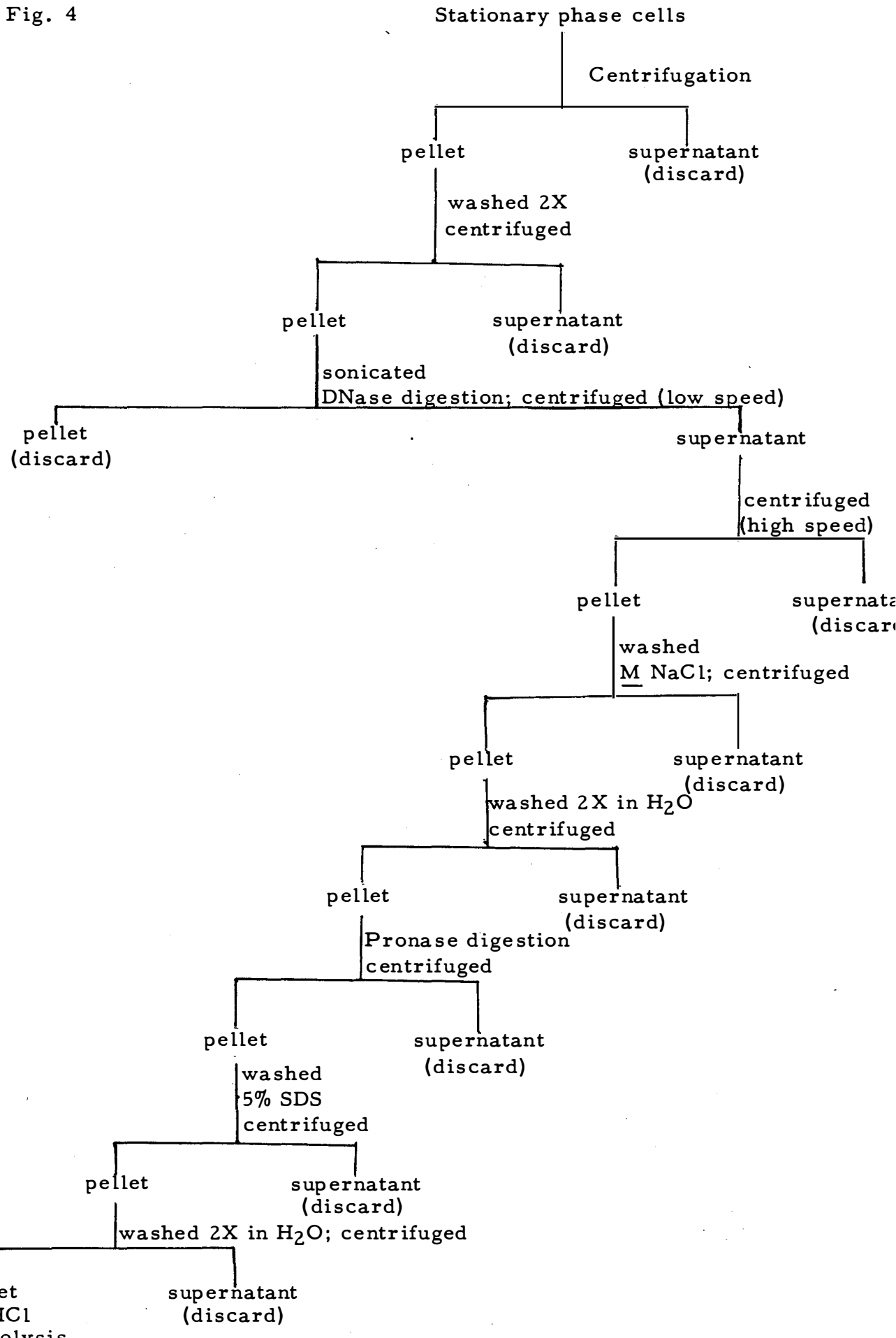
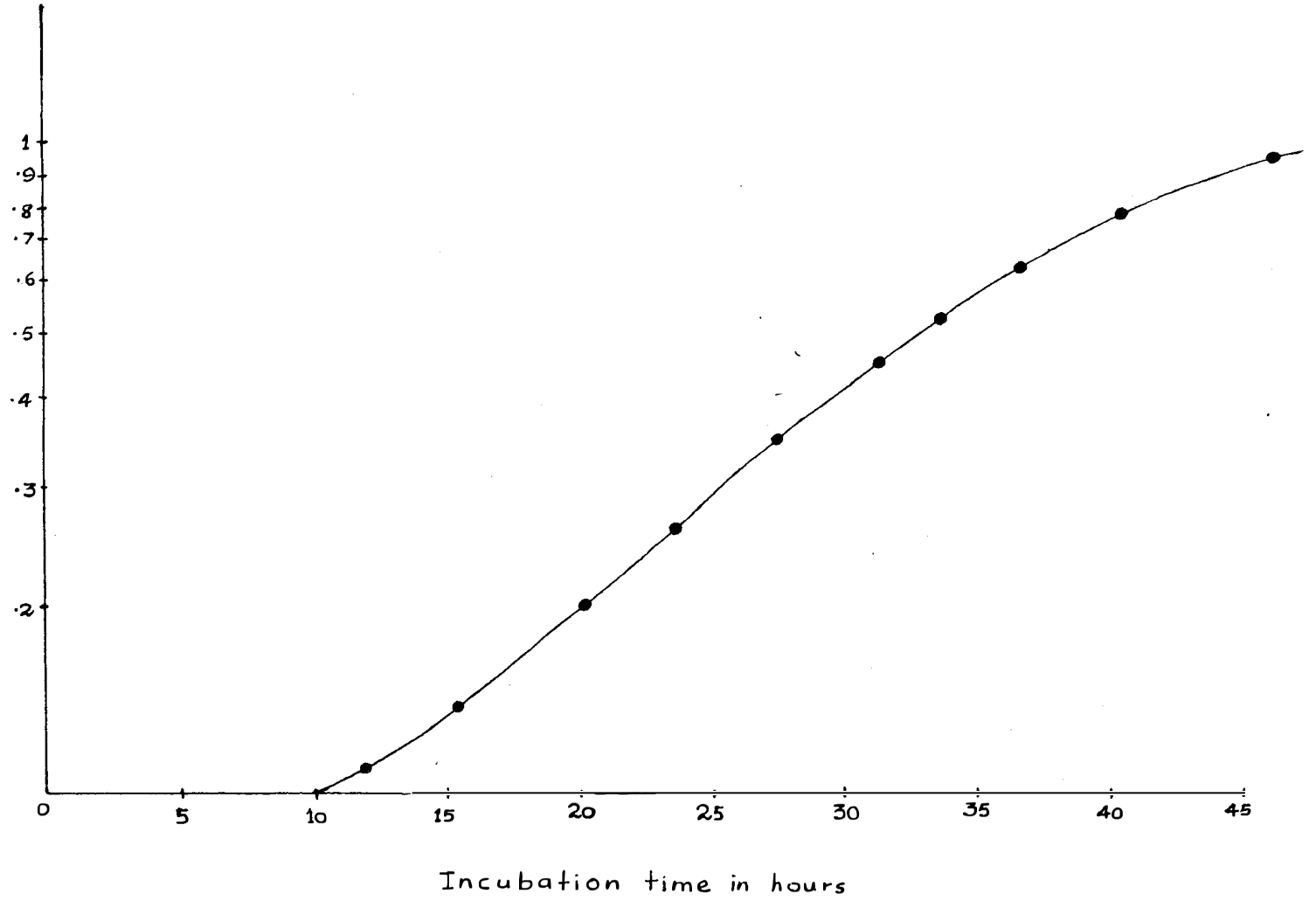
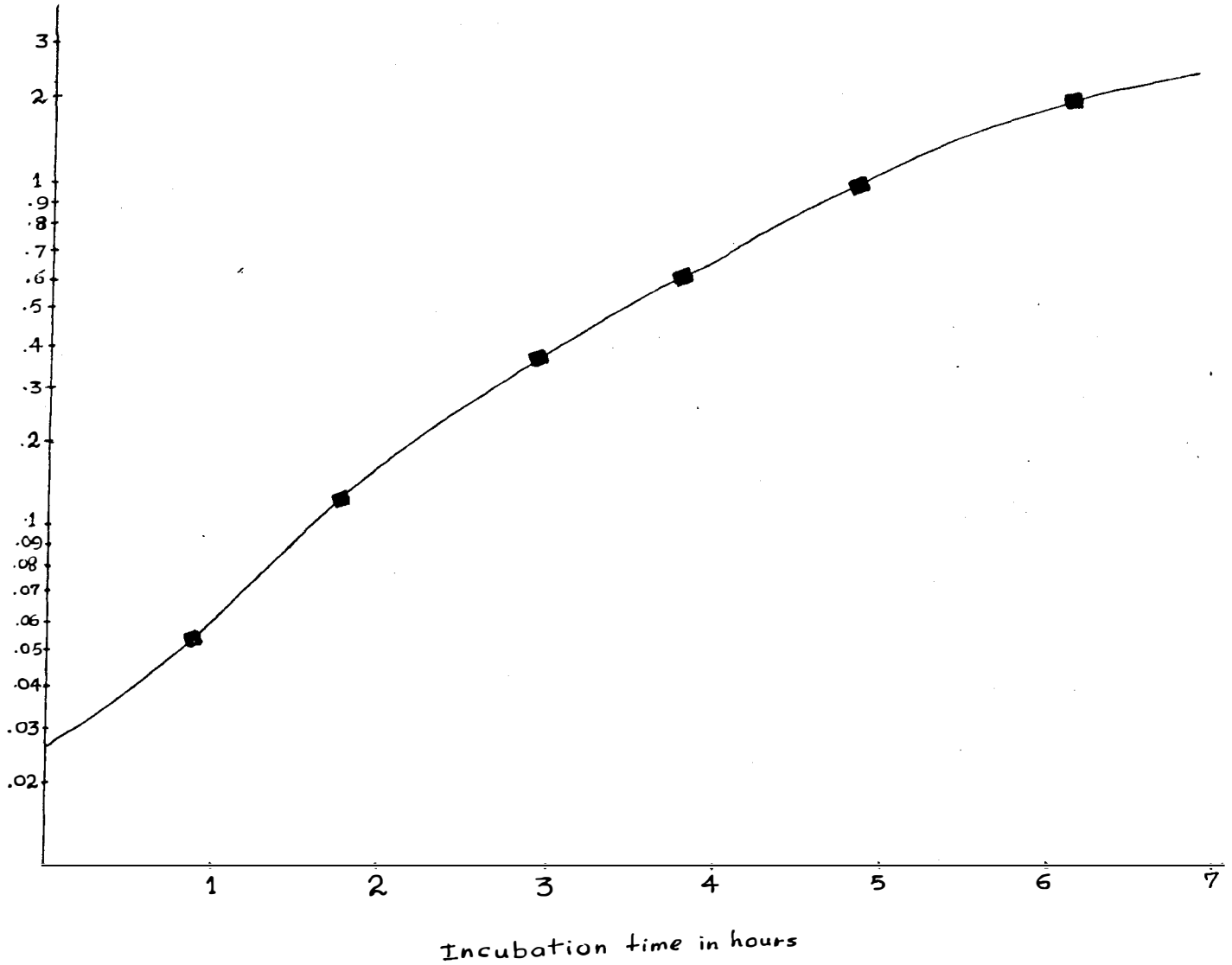

Stationary phase cells
Centrifugation
pellet
supernatant (discard)
washed 2X centrifuged
pellet
supernatant (discard)
sonicated DNase digestion; centrifuged (low speed)
pellet (discard)
supernatant
centrifuged (high speed)
pellet
washed M NaCl; centrifuged
pellet
supernatant (discard)
washed 2X in H_2O centrifuged
pellet
supernatant (discard)
Pronase digestion centrifuged
pellet
supernatant (discard)
washed 5% SDS centrifuged
pellet
supernatant (discard)
washed 2X in H_2O; centrifuged
pellet 6N HCl hydrolysis
supernatant (discard)

Fig. 5a.



Growth of the Rhizobium japonicum strain.

Fig. 5b.



Growth of the Escherichia coli Strain.

centrifuged at 15,000 g for 15 minutes. The cells were washed twice with sterile water and centrifuged each time at 15,000 g for 15 minutes. The supernatant was discarded. The pellet was resuspended in 10 ml distilled water and sonicated at setting #70 on the Biosonik III for 2 minutes. The sonicated cells were digested with DNase (1 ml) and incubated at 37C for 30 minutes.

The DNase digested cell walls were centrifuged at 3,000 g for 10 minutes. The pellet (presumed to be whole cells) was discarded; and the supernatant was centrifuged again at 20,000 g for 30 minutes. The supernatant was discarded. The pellet was mixed in 5 ml of M NaCl and centrifuged at 20,000 g for 30 minutes. The supernatant was discarded. The pellet was washed and centrifuged twice in 5 ml of distilled water and centrifuged at 20,000 g for 30 minutes. The supernatant was discarded.

The pellet was mixed in 0.5 ml pronase and 4.5 ml distilled water and incubated at 37C for 30 minutes. The pronase digested cell walls were centrifuged at 20,000 g for 30 minutes. The supernatant was discarded.

The pellet was mixed with 5 ml of 5% SDS and centrifuged at 20,000 g for 30 minutes. The supernatant was discarded. The pellet was washed twice with 5 ml of distilled water and centrifuged at 20,000 g for 30 minutes. The pellet was resuspended in 2 ml of 6N HCl and hydrolyzed at 121C for 10 minutes. The hydrolysate was dried and

resuspended in 0.25 ml of distilled water for spotting on chromatograms.

(Most of the centrifugations done at 10,000 g and 15,000 g at the beginning of the experiments did not give good yields. Therefore, 15,000 g and 20,000 g centrifugations were subsequently used. Long (12 hours) (Gaffar et al., 1966; Kolenbrander and Ensign, 1968) and short (30 minutes) enzymatic digestions did not show any changes in the amino acid spots detected on the chromatograms. Five per cent (boiling) SDS was also used (Braun and Sieglin, 1970; 4% boiling SDS) but did not show any difference in the detection of amino acids. Long (12 hours) (Braun and Wolff, 1970) and short (10 minutes) acid hydrolysis also did not change the qualitative analysis of amino acids.)

The chromatogram tank was saturated by placing a filter paper pad of the same solvent into it. The spotted chromatogram was placed into the tank which was closed with the glass cover. Within 15 minutes the chamber was saturated. The solvent was added slowly until the chromatogram sheet was immersed 5 mm. The solvent was allowed to run 4-5 hours. The developed chromatogram was removed and the solvent line was marked for Rf values. The chromatogram was then dried in the air-hood at 100C for 10 minutes. Ninhydrin (Appendix B) was sprayed uniformly on the chromatogram. It was dried again in the air-hood for about 10 minutes. Amino acid spots were detected by keeping the chromatogram in chromatography oven for an additional 10 minutes.

RESULTS

The experimental aspects of this thesis consist of two parts.

I. Isolation of the cell wall.

II. Identification of the amino acids in the cell wall.

I. Isolation of the cell wall

Cell wall isolation of Rhizobium japonicum consists mainly of the following steps:

a. Sonication to break open the cells.

b. Centrifugation to separate whole cells from broken cell walls.

c. Enzymatic digestion to remove non-murein components.

d. Lipophilic solubilization to remove lipids.

e. Acid hydrolysis to "free" the amino acids present.

a. Sonication

After the cells were grown to early stationary phase, they were harvested and washed by centrifugation before sonication.

Low intensity sonication was done in some cases, before washing the cells, because some cells were not sedimenting and a mucoid layer settled over the "button" of cells. This mucoid material was due to the presence of heavy capsule production in Rhizobium japonicum. The control, Escherichia coli, never showed this mucoid material in

the isolation of its cell walls.

It was also observed that when the cells of Rhizobium japonicum were stored in refrigerator at 7C for about 12 hours and then centrifuged, no mucoid material was observed.

After the cells were washed, high speed sonication (setting #70) was carried out to break the cell walls. Since the author had no idea of the efficiency of cell wall rupture in Rhizobium japonicum, 90 seconds sonication was chosen (Fottrell and Mooney, 1969) which gave favorable results. About 98% of the cells were broken. In order to get better cell rupture, 2 and 5 minutes sonication was also tried. More than 99.99% of the cells were broken in 2 minutes sonication. Prolonged sonication (5 minutes) further fragmented the cell walls making them more difficult to centrifuge and digest in the subsequent stages. Hence, 2 minutes of sonication was used for the rupture of Rhizobium japonicum cell walls.

b. Centrifugation

All centrifugations before sonication were done at 15,000 g. The ruptured cells, after sonication, were centrifuged at 20,000 g for 30 minutes. This speed of centrifugation was found very efficient for good sedimentation. Prior to this, in the early stages of experimentation, 15,000 g centrifugations were used instead of 20,000 g but the pellet was not very hard. This resulted in a very low yield. Therefore, high speed centrifugation (20,000 g) was applied for the remainder of the experimentation.

c. Enzymatic Digestion

The enzymes used for Rhizobium japonicum cell wall isolation were DNase, RNase, trypsin and pronase. The ruptured cell walls were digested with DNase and trypsin early in the experimentation. RNase and pronase were also tried. Salton (1964) mentioned an unpublished observation that, in the case of Gram negative bacteria only, trypsin digestion did not separate the mucopeptide components from other proteinaceous material. Hence, pronase digestion was applied in the later experiments and separation of the murein amino acids was facilitated.

d. Lipophilic Solubilization

Lipophilic solubilization was carried out by treating the cell walls with sodium dodecyl sulfate (SDS), at 1% and 5% concentrations. It had been observed (Braun and Sieglin, 1970) that 5% SDS washes the cell walls better. Five per cent hot (boiling) SDS was also tried; but no gross difference was observed. Toluene was also tried for washing the crude cell walls in some experiments. The use of toluene was not found appealing because there was a problem in washing the cell walls and in removing traces of toluene after treatment.

M NaCl washing tends to avoid prolonged washings of the cell walls (Salton, 1964). Hence, M NaCl was used to advantage.

e. Acid Hydrolysis

Six normal HCl was found very suitable for the hydrolysis of

Rhizobium japonicum murein layers. Braun and Wolff (1970) stated that four normal HCl hydrolysis decreases the amount of lysine and DAPA isolated, whereas six normal HCl increases both amino acids.

Long (12 hours) and short (10 minutes) hydrolysis at 121C were tried for Rhizobium japonicum mureins, but no difference in the identification of amino acids on the chromatograms was observed.

II. Identification of Amino Acids

The identification of amino acids for Rhizobium japonicum murein layers was done by using thin layer chromatography. One dimensional and two dimensional chromatography with Gelman silicic acid and Eastman Kodak silica gel instant thin layer sheets were run. Since the silicic acid sheets contained fiber glass fibers, they were found suitable to run only as one dimensional chromatograms. Two dimensional solvent systems did not give favorable results and no amino acid spots were observed. On the other hand silica gel plates were run in 2-dimensions.

The solvents used in these experiments (Randerath, 1963) were:

1. 96% ethanol/water (63:37 w/w)
2. n-propanol/water (64:36 w/w)
3. 96% ethanol/34% NH_4OH (77:23 w/w)
4. Chloroform/methanol/17% NH_4OH (2:2:1 v/v/v)
5. n-butanol/acetic acid/water (60:20:20 w/w/w)
6. n-propanol/34% NH_4OH (67:33 w/w)

Among these solvents, n-butanol/acetic acid/water and n-propanol/ 34% NH_4OH gave the best results. The Rf values of known amino acids and those of the Rhizobium japonicum murein layer were determined with these solvents. Two dimensional chromatograms, using the same solvents were also used.

The experimentally determined Rf values for the known amino acids and glucosamine are given in Table 2. The known amino acids (Appendix B) were spotted on silica gel instant thin layer sheets and were run using both solvents (BuOH:HoAC:H_2O and n-propanol:34% NH_4OH) separately. The amino acid spots were detected by spraying ninhydrin reagent (Appendix B). The Rf values of the known amino acids were compared to the Rf values of murein layer amino acids of Rhizobium japonicum in one dimension (see Figs. 6 and 7). Two dimensional chromatography, using the two solvents, yielded satisfactory results (Figs. 8 and 9).

The Rf values of known amino acids (Table 2) were compared to the Rf values of the murein amino acids of Rhizobium japonicum (Table 3). The following amino acids could be tentatively assumed to be in the murein layer of Rhizobium japonicum:

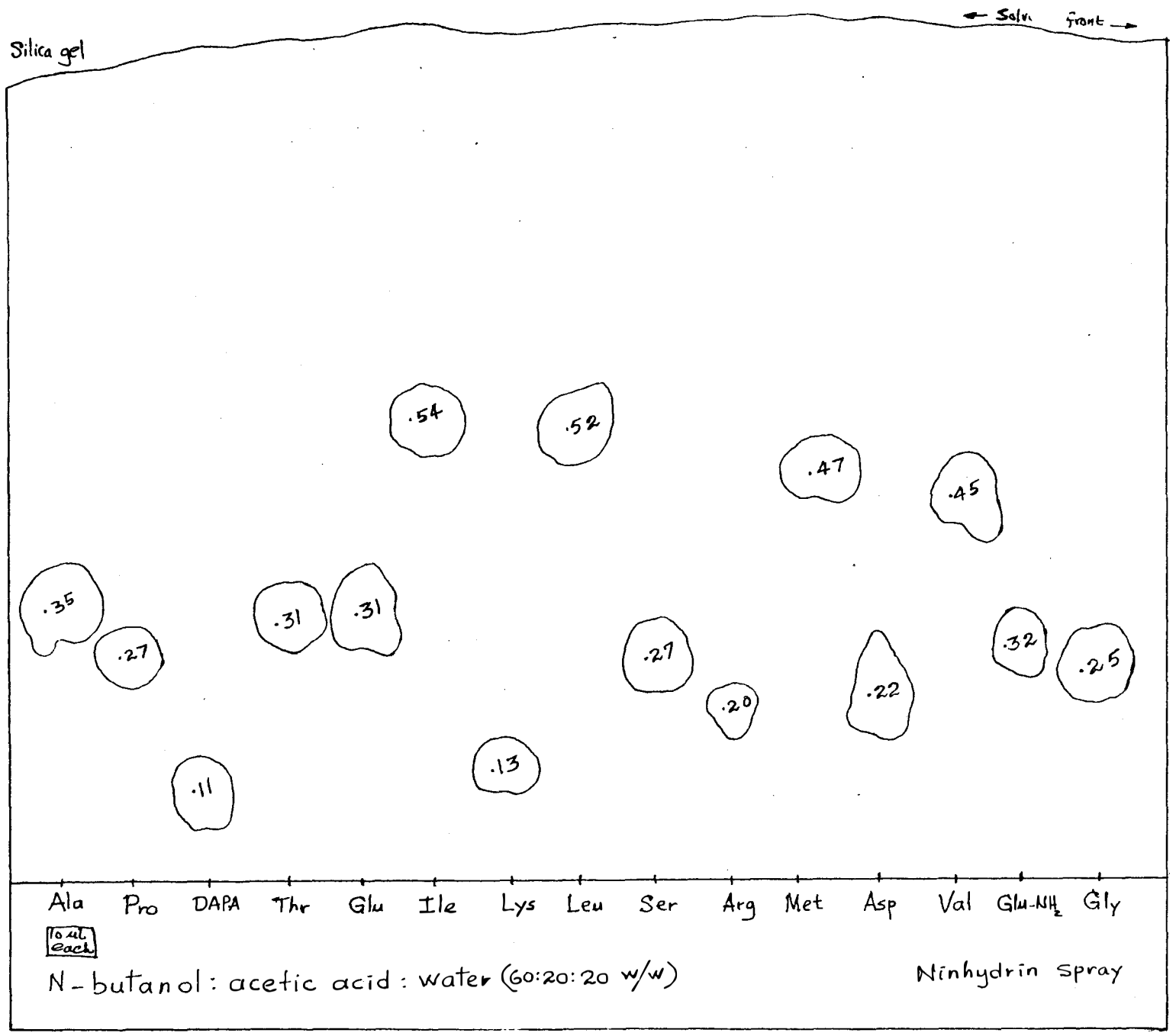
1. Diaminopimelic Acid (DAPA)
2. Lysine
3. Alanine
4. Glutamic Acid

5. Glucosamine

6. Serine

7. Valine, Methionine or Isoleucine

8. Leucine



Known amino acids

ig. 6

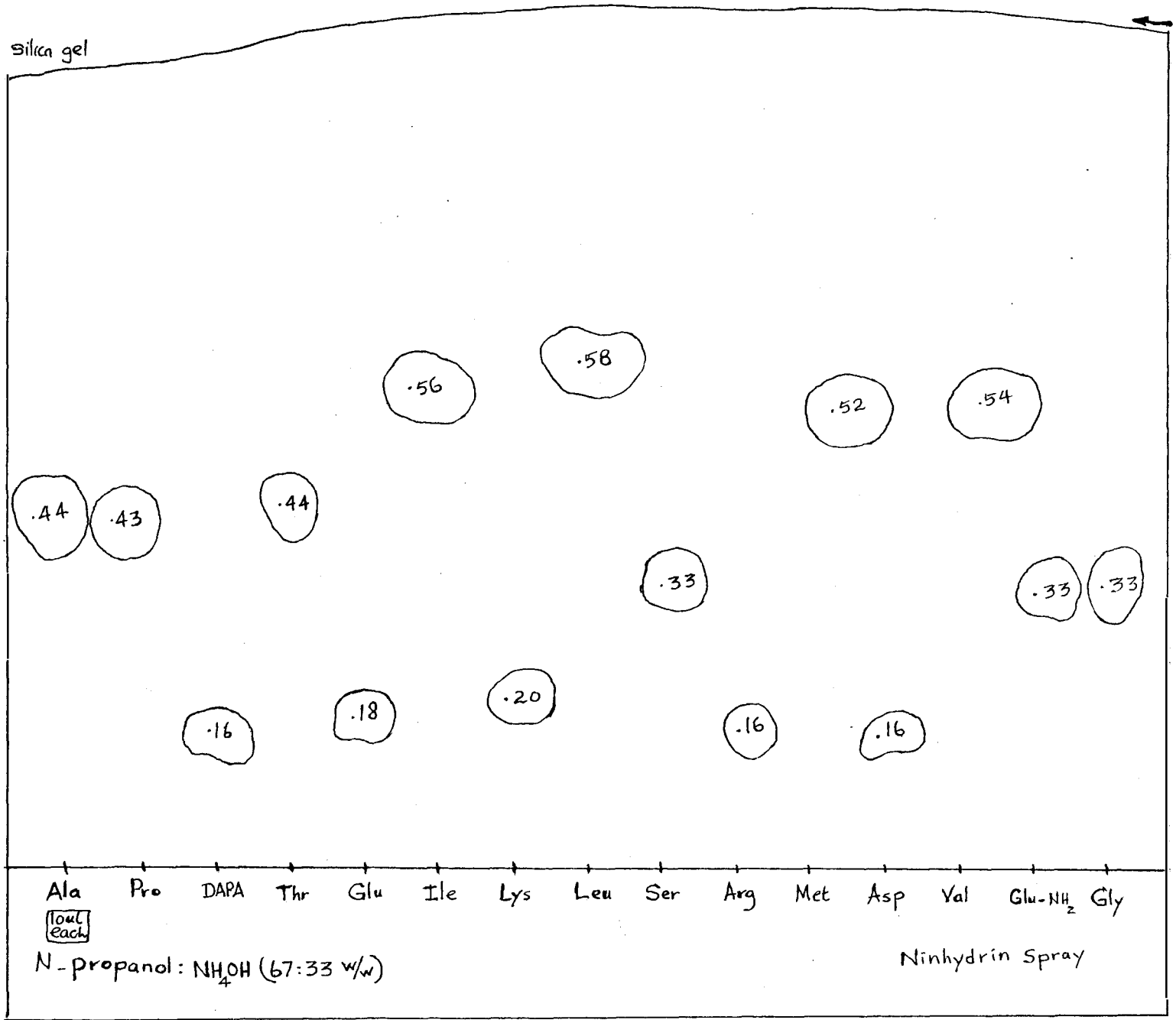

Silica gel
Solvent front
.44
.43
.44
.56
.58
.52
.54
.33
.33
.33
.16
.18
.20
.16
.16
Ala
Pro
DAPA
Thr
Glu
Ile
Lys
Leu
Ser
Arg
Met
Asp
Val
Glu-NH2
Gly
1 out each
N-Propanol : NH4OH (67:33 w/w)
Ninhydrin Spray

Fig. 7

Fig. 8

Solvent front

Solvent front

.55, .64

.46, .58

.48, .46

.35, .36

.28, .33

.20, .29

.12, .16

.10, .10

n-propanol : 34% NH_4OH
(67:33 w/w)

2nd direction

R_f 1 to 1.1

BuOH : HOAc : H_2O
(60:20:20 v/v)

1st direction

Ninhydrin spray

R_f 1 = *Rhizobium japonicum* to all spots

Silica gel
Solvent front
Solvent front
.64, .55
.56, .47
.50, .48
.35, .32
.31, .18
.27, .12
.17, .08
.09, .12
n-propanol : 34% NH4OH (67:33 w/w)
2nd direction
Rj 10 µL
BuOH : HOAc : H2O (60:20:20 w/w/w)
1st direction
Ninhydrin spray
Rj = Rhizobium japonicum 10 µL spotted

Fig. 9

Amino Acids	BuOH:HoAC:H_2O	n-propanol:34% NH_4OH
Ala	.35	.44
Pro	.27	.43
DAPA	.11	.16
Thr	.31	.44
Glu	.31	.18
Ile	.54	.56
Lys	.13	.20
Leu	.52	.58
Ser	.27	.33
Arg	.20	.16
Met	.47	.52
Asp	.22	.16
Val	.45	.54
Glu-NH_2	.32	.33
Gly	.25	.33

Rf values of commonly

occurring amino acids

Table 2

Solvent		Solvent	
n-BuOH:HoAC:H_2O (60:20:20 w/w/w)		n-propanol/34% NH_4OH (6 7:37 w/w)	
Rf values		Rf values	
(1)[a]	(2)	(1)	(2)
.10	.09	.10	.11
.12	.10	.16	.18
.20	.16	.29	.28
.28	.20	.33	.32
.35	.34	.36	.36
.48	.49	.46	.46
.46	.47	.58	.55
.55	.52	.64	.60

Rf values of amino acids from acid-hydrolyzed

Rhizobium japonicum murein layers

Table 3

[a]Rf values calculated from two separate 2-dimensional chromatograms.

DISCUSSION AND CONCLUSION

The isolation of cell wall murein layer of Rhizobium japonicum brought out the following points.

1. Ultrasonication for 2 minutes ruptured more than 99.99% of the bacterial cells (Fottrell and Mooney, 1969).

2. Enzymatic digestions, whether done for 30 minutes or 12 hours, did not alter the identification of the murein amino acids by instant thin layer chromatography. Since there was poor amino acid separation after trypsin digestion, Pronase was used.

3. Toluene did not wash the cell walls well and was very hard to remove. Subsequently SDS was used. One per cent SDS was not strong enough to wash the cell walls well and resulted a poor separation of amino acids on instant thin layer. On the other hand, 5% SDS gave better results and a pure murein layer was obtained. Five per cent hot (boiled) SDS was also used but it did not cause any difference in the detection of the murein amino acids.

4. Centrifugation at 15,000 g before sonication and 20,000 g after sonication gave the best results. 15,000 g centrifugation after sonication caused some "floating" cell walls which were lost with the supernatant, resulting in a very low yield at the end of the cell wall isolation.

5. Acid hydrolysis with 6N HCl (Braun and Wolff, 1970) was found very suitable for the complete digestion of Rhizobium japonicum cell wall amino acids. Ten minutes at 121C was sufficient for the hydrolysis of the murein layer of Rhizobium japonicum. Twelve hours at 121C did not yield any qualitative difference in the amino acids on chromatograms.

6. Thin layer chromatography was found to be a good technique for the detection of these amino acids. Six tenths per cent ninhydrin in water-saturated n-butanol gave good color development after it was sprayed onto the chromatogram and heated at 100C for 10 minutes.

In conclusion, the isolation of the Rhizobium japonicum cell wall murein layer was made by the use of some recent techniques. Silica gel (Eastman Kodak) chromatograms were used and the amino acids (eight in number) were detected by 2-dimensional chromatography.

The solvents, n-butanol/acetic acid/water (60:20:20 w/w/w) and n-propanol/34% NH_4OH (67:33 w/w) gave the best separation of amino acid spots on these silica gel chromatograms.

Detection of these amino acids confirms the isolation of purified Rhizobium japonicum cell wall murein layers.

APPENDICES

APPENDIX A

Media

1. Dextrose Mannitol Yeast Extract (DMYE broth) (Baumrucker, 1971)

Mannitol	5.0g
Dextrose	5.0g
Yeast Extract	1.0g
Sodium Chloride	0.2g
Distilled Water	1000.0g

pH adjusted to 7.2 by adding 5.5 ml of 0.1 N NaOH per liter.

For a solid medium, 1.5% agar (Difco) was added.

2. Tryptone Glucose Yeast Extract (TGYE broth) (Baumrucker, 1971)

Tryptone	5.0g
Glucose	5.0g
Yeast Extract	3.0g
Distilled Water	1000.0g

For a solid medium, add 1.5% agar (Difco).

APPENDIX B

Reagents

1. Known amino acids (Calbiochem and Sigma Chemical Company) were dissolved at 1mg/ml in distilled water.
2. DNase (Calbiochem) and RNase (Calbiochem) were dissolved at 1mg/ml in distilled water.
3. Pronase (Calbiochem) and trypsin (Nutritional Biochemical Corporation) were dissolved to 2.5mg/ml in distilled water.
4. Ninhydrin spray reagent: 0.6% Ninhydrin (Calbiochem) in 100ml of water saturated n-butanol.

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