INDUCTION OF SPHEROPLASTS BY D-SERINE

IN A SPECIES OF ERWINIA

By

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CHAPTER I

INTRODUCTION

Bacteria, in common with plants, possess a well defined cell wall which can be differentiated from surface slime, capsular components, and the protoplasmic membrane in a variety of ways (15,29,30,37,38).

The bacterial cell wall accounts for about 20% of the dry weight of the cell (7). The contribution by the wall to the weight of the cell will vary with the age of the culture and as shown by Shockman, Kolb and Toennies (40), using Streptococcus fecalis, it may vary from 27% during the exponential growth phase to 38% during the stationary growth phase.

Chemical study of cell walls from different bacteria has led to the discovery of striking features in composition in both Gram-negative and Gram-positive bacteria. The walls of Gram-positive bacteria contain as few as three or four major amino acid constituents, usually D- and L-alanine, D-glutamic acid, lysine or diaminopimelic acid and glycine. These amino acids plus the amino sugars, N-acetylglucosamine and muramic acid are the constituents of a polymer referred to as mucopeptide, mucopolymer, murien, glucosaminopeptide, glycopeptide or peptidoglycan (39). Gram-negative bacteria possess the same type of mucopeptide as in Gram-positive bacteria but in much smaller amounts and, in addition, contain lipoprotein and lipopolysaccharide components. Thus the cell wall of Gram-negative bacteria

contains many more and diversified components than Gram-positive bacteria (10,39).

The different components of the cell wall of Gram-positive and Gram-negative bacteria exist as macromolecular units (50 billion. MW) (46). Their participation in the construction of the cell wall of bacteria is indisputable. These polymers display unique chemical features and contain compounds such as muramic acid and diaminopimelic acid which are found only in bacterial cell walls, blue-green algae, and streptomycetes (8).

Mucopeptide is greatly decreased or absent in halobacteria, protoplasts, and spheroplasts. Normally, it comprises 2-15% of the Gram-negative cell wall (10,44,45,46). It has been demonstrated that mucopeptide confers rigidity; thus maintaining the shape of the bacterial cell as well as protecting the cell from osmotic lysis (plasmoptysis) in distilled water (5,13,37,44).

Teichoic acids may also be present in the cell wall of some bacterial species; these are usually restricted to the Gram-positive bacteria. Teichoic acids are polymers of ribitol or glycerol phosphate which always contain D-alanine in an O-ester linkage and which, particularly in the ribitol forms, may also contain various sugar components such as glucose or glucosamine bonded to carbon #4 of ribitol (1,3). Teichoic acids of varying chain lengths are intimately bound to the mucopeptide component, presumably via a direct P to C bonding (1,3).

In Gram-negative bacteria the mucopeptide is sandwiched between the cell membrane and the lipoprotein-lipopolysaccharide complex. Lipopolysaccharide possesses antigenic properties and is found

outermost in the cell wall of Gram-negative bacteria (10,44).

Since rigidity and shape are conferred by the mucopeptide, removal or destruction of this layer results in the formation of protoplasts in Gram-positive bacteria and spheroplasts in the case of Gram-negative bacteria. When hypertonic conditions are provided (usually 0.5M sucrose) protoplasts and spheroplasts do not undergo plasmoptysis and they remain viable. Spheroplasts, but not protoplasts, can revert to a bacillary form under appropriate conditions (37).

The viability of spheroplasts will vary depending on the method of induction as well as the organism studied and conditions of observation. Spheroplasts induced by glycine (20) and penicillin (24,27) are found to be viable and capable of wall synthesis. In contrast, spheroplasts formed by lysozyme (21,23) are usually nonviable which suggests that loss of viability accompanies complete removal of cell wall mucopeptide. The most extensively used method for induction of spheroplasts in Gram-negative bacteria employs treatment with lysozyme and EDTA (20,23,37). Nevertheless penicillin and D-isomers of some amino acids can serve the same purpose (14,25,34).

In addition to the macromolecular structural components making up the cell wall, recent studies have shown that microorganisms contain enzymes within their cell wall capable of hydrolyzing the wall mucopeptide component (37).

The inhibitory effect of D-amino acids, especially D-serine, has been studied vigorously in our laboratories using a species of Erwinia (12,15,16,17). Growth in the presence of D-serine causes damage to the cell membrane (induces "leakiness"), decreases the total cell wall mucopeptide content by 30-40%, is incorporated into the mucopeptide

while replacing glycine, causes accumulation of pyruvic acid in the growth medium, inhibits division of the nuclear body and inhibits the synthesis of pantothenic acid and co-enzyme A. It has also been reported that growth in the presence of D-serine results in formation of filamentous cells and occasionally spheroplasts (12). Although both responses are dose dependent, spheroplasting occurs only at high concentrations of D-serine (0.0168M and above). Because of the decrease in mucopeptide content, Grula, Smith, and Grula (15) considered the filamentous cells to be partial spheroplasts even though they do not undergo appreciable lysis when suspended in distilled water.

When mucopeptide has been removed either from Gram-positive or Gram-negative bacteria, plasmoptysis occurs, as pointed out previously, unless hypertonic conditions are employed to aid in maintaining stability of the resultant protoplasts or spheroplasts. A study was made by Corner and Marquis (6) on the relative effectiveness of sugars, short peptides and amino acids as osmotic stabilizers for protoplasts of Bacillus megaterium. The study indicated that the protoplast membrane could act as a porous differential membrane and that its effective porosity increases when it is stretched during osmotic swelling. The protoplast membrane also behaves as a highly extensile structure in contrast to membranes such as those of erythrocytes. In effect, osmotic bursting of bacterial protoplasts could not be related to the ultimate tensile strength of maximally extended membranes. Rather, it appears that when protoplasts swell in hypotonic solutions, their surface membranes might become sufficiently stretched so that they admit stabilizing solutes. There will then be a rapid influx of solutes and water, resulting in rapid stretching of the membrane and

rupture due to the process of brittle fracture. Thus, bursting generally occurs without full extension of the membrane.

The stability of spheroplasts and protoplasts has been shown to be greatly increased by the presence of divalent cations (18,29). The solute must not be able to penetrate the osmotic barrier of the protoplast or spheroplast at an appreciable rate if it is to give protection from lysis.

All studies performed in our laboratory which relate to the effect of D-serine on cell division have employed growing cultures; therefore, D-serine has always been added to the various growth media prior to inoculation (time zero). Although such addition allows the necessary time for full expression of the effect of D-serine on cell division, growth is usually greatly retarded and many of the cells undergo lysis. Because several studies, primarily those involving analysis of enzymes and "leaked" compounds, were contemplated, Dr. E. Grula thought it necessary to obtain a growing system wherein yields of nondividing cells would be increased.

One of the parameters he studied involved addition of D-serine to cells in various growth media after some growth was evident (12-14 hr). He observed that an additional 4-6 hr incubation period allowed for increased yields of filamentous cells. When incubation was extended for 10-15 hr after addition of D-serine, optical density (OD) of some cultures (different medium formulations were utilized) continued to increase whereas others drastically decreased. In those cultures where great decreases in OD occurred, examination using optical microscopy revealed that nearly all of the cells had lysed. Cell remains were evident only as poorly staining

rod-shaped ghosts.

Examination of cells from cultures where increases in OD had occurred revealed that nearly 100% of the cells were now present as spheroplasts (confirmed by plasmoptysis of the rounded cell forms in distilled water). Since these spheroplasts were stable in the growth medium which was not hypertonic, two questions were immediately apparent. 1) Why did the cells form spheroplasts? 2) Why didn't the spheroplasts lyse in the growth medium after they were formed?

Feeling that an answer to both questions might lie in the chemical composition of the cell walls, I set out first to repeat the critical growth experiments of Dr. Grula. Since formation of spheroplasts is dependent on both the concentration and time of addition of D-serine I initially experimented with that part of his system which involved the concentration and time of addition of D-serine. The final procedure which I evolved utilized 2.205g of D-serine in 14 ml water which is added at 14-15 hr to actively growing cells (OD 0.60-0.65) in 86 ml of culture medium. Such addition causes 90-99% spheroplast formation after an additional 14 hr of incubation. Higher concentrations of D-serine cause lysis and and growth beyond an OD of 0.60-0.65 decreases the percent of spheroplasting obtained while increasing lysis. Lower concentrations of D-serine or addition of D-serine before an OD of 0.60-0.65 is reached results in formation only of filamentous cells.

After the critical nutritional experiments had been done I then advanced to the second portion of my study and chemically analyzed the

contents of the peripheral layers (envelopes) of the spheroplasts and normal cells of *Erwinia* species.

CHAPTER II

METHODS AND MATERIALS

Organism and Spheroplast Induction

A species of Erwinia most closely related to Erwinia carotovora was used throughout this study (11). Stock cultures were maintained by alternately transferring the cells (24 hr intervals) on nutrient agar or nutrient agar containing 1.0% of glucose and incubating at room temperature. All inoculations into defined media were made using only cells grown for 24 hr on nutrient agar slants. Prior to inoculation, cells were twice washed in sterile 0.85% NaCl, resuspended to an OD of about 0.1 (540 mμ using a Coleman Jr. Spectrophotometer) and 1.0 ml of the suspension added to 86 ml of defined growth medium contained in 250 ml Erlenmyer flasks.

The defined growth medium contained the following per 100 ml: $MgSO_4 \cdot 7H_2O$, 2.0 mg; KH_2PO_4, 136 mg (0.01 M); K_2HPO_4, 174 mg (0.01 M); aspartic acid, 420 mg (3.15×10^{-2}M); glucose, 150 mg (8.3×10^{-3}M); NH_4Cl, 480 mg (8.9×10^{-2}M); DL-serine, 617.4 mg (5.88×10^{-2}M) and the following trace minerals: $CaCO_3$, 10.0 μg; $CuSO_4 \cdot 5H_2O$, 1.0 μg; $FeSO_4$ $(NH_4)_2SO_4 \cdot 6H_2O$, 50.0 μg; KI, 1.0 μg; $MnSO_4 \cdot H_2O$, 20 μg; MoO_3, 1.0 μg; $ZnSO_4 \cdot 7H_2O$, 5.0 μg; and H_3BO_3, 0.5 μg. Final pH of the medium was 6.8 before autoclaving.

Sterilization was accomplished by autoclaving for 15 min at 15

lbs pressure. Glucose and serine were autoclaved separately (stock solutions, 2.5 g/100ml and 4.41 g/100 ml respectively) for 10 min at 12 lbs pressure and added to the medium aseptically.

After inoculation, cultures were incubated at 25C on a rotary shaker. After 14-15 hr incubation, 14.0 ml of serine was aseptically added to the medium. To obtain maximum spheroplast formation, cells were allowed to grow until an OD of 0.60-0.65 was reached at which time serine was added. Incubation for an additional 14 hr results in almost total conversion (90-99%) of the cells to the spheroplast state.

For control cultures the same procedure was followed but instead of serine, 14.0 ml of sterile deionized water was added to the growth medium.

Isolation of Cell Envelopes

After incubation (total of 28-29 hr) cells (spheroplasts plus rod cells present in the culture) were harvested by centrifugation (5000 rpm for 20 min). The pellet was then suspended in cold (5C) deionized water to cause plasmoptysis of the spheroplasts. After 10 min the suspension was spun at slow speed (2000 rpm for 10 min using the RC-2 model centrifuge) to separate the unlysed and intact rod cells from the spheroplast cell envelopes. The supernatant was then centrifuged at 30,000 rpm (Spinco model L) for 30 min to sediment the spheroplast cell envelopes. These cell envelopes were then washed three times in cold phosphate buffer (0.1M pH 7.0) followed by two washes in chilled deionized water prior to lyophilization.

To obtain an envelope fraction from control cells, washed cells were suspended in very small amounts of water and frozen in the X-press. These cells were then broken by raising the pressure to 20,000 lb/sq inch in the X-press using a Carver laboratory hydraulic unit. Envelopes were obtained by first centrifugating the broken preparation in phosphate buffer (0.1M pH 7.0) at 2000 rpm for 10 min to remove unbroken cells. The supernatant, which contains the cell envelopes, was then processed in the same way as given above.

Determination of Total Carbohydrate in Cell Envelopes

Lyophilized samples (5.0-10.0 mg) of both spheroplast and control cell envelopes were weighed and hydrolyzed in 1.0 ml of 2N HCl for 2 hr at 100-105C *in vacuo*. Anthrone reagent was used for quantitation (0.2% solution of anthrone in concentrated H_2SO_4). Tubes containing samples (1 ml plus 2 ml anthrone reagent) were incubated in a boiling water bath for 10 min then allowed to cool. Color was read at 620 mμ using a Coleman Jr. Spectrophotometer. A standard curve was constructed using glucose as a standard.

Determination of DNP-Phosphatidylethanolamine

The method used for the determination of phosphatidylethanolamine was essentially that of Axelrod, Reichenthal, and Brodie (2). Cells were sedimented from control and spheroplast media, resuspended in 30 ml methanol in screw cap bottles, flushed with nitrogen and then placed in a 55C water bath for 30 minutes. Chloroform (60 ml) was then added and extraction was continued overnight under nitrogen at room temperature. The cells were removed using millipore filtration.

An equal volume of 2M KCl was then added to the filtrate, the bottles inverted about 20 times and then permitted to stand for a few min to allow separation of the immiscible layers. The top methanol layer was removed by aspiration and the chloroform layer washed twice more with 2M KCl and finally with deionized water.

Samples of phospholipid from the chloroform layer were transferred into 15 ml graduated centrifuge tubes, placed in a beaker of warm water and evaporated to dryness using a stream of air. One ml of 2N NaOH was added to each tube; the tube was then covered with a glass marble and immersed in a boiling water bath. After hydrolysis for 2 hr the tubes were cooled and 1 ml of 2N HCl added. The samples were then diluted with water to 3.0 ml volumes and centrifuged. Samples (2ml) were transferred to 15.0 ml glass stoppered centrifuge tubes and 0.1 ml of DNFB (2,4-dinitroflourobenzene) reagent added followed by 1.0 ml of 2.5% $NaHCO_3$. The tubes were then stoppered and placed in a water bath at 75-80C for one hr after which they were cooled prior to the addition of 8.0 ml of chloroform. After vigorous shaking the tubes were centrifuged and the top layer removed by aspiration. Six mls of the chloroform layer were transferred to small beakers, immersed in a warm water bath, and the chloroform removed under a stream of air. Ten ml of petroleum ether and 4.0 ml of 4N HCl were added and the contents again shaken for 5 minutes. After standing and separation of the immiscible layers, 3.0 ml of the acid phase (top layer) were transferred to a cuvette and the OD read at 420 mμ. Quantitation was accomplished by comparison of OD readings with a standard curve prepared using ethanolamine.

Isolation of Lipopolysaccharide

The method used for extraction of lipopolysaccharide from whole cells was essentially that of Westphal and Jann (45). Lyophilized cells were suspended in water (1 g/16.0 ml water) and heated to 65-68C with stirring. An equal volume of aqueous 90% (w/v) phenol, preheated to the same temperature, was added and the mixture stirred (65-68C) for 15 minutes. The mixture was cooled in ice and centrifuged at 2,000 x G for 30 min below 10C to allow separation of the immiscible layers. The phenolic layer and gummy white precipitate were extracted three times the same way the first extraction was carried out. The aqueous layers were then combined and dialyzed against 4 liters of deionized water for 24 hr to remove phenol. The resulting solution was centrifuged at 105,400 x G for 4 hr in a Spinco model L centrifuge using the #40 head to sediment the lipopolysaccharide. The jelly-like lipopolysaccharide was then suspended in deionized water, frozen and lyophilized prior to further analysis.

Sugar Identification

Samples of lipopolysaccharide were weighed and then hydrolyzed 2N HCl for 2 hr at 100-105 C <u>in vacuo</u>. Chromatography was accomplished using Whatman #1 paper (22 x 22 cm and 10 x 40 cm) by the ascending and descending methods respectively. The following solvents were used: a) pyridine - n-butanol-water (4:6:3), b) isopropanol-formic acid-water (40:2:10), c) isopropanol - acetic acid-water (4:1:1), and d) tertiary butanol-methyl ethyl ketone- 88% NH_4OH-

water (50:30:10:10). Neutral sugars were detected using aniline acid-oxalate reagent (0.8 g oxalic acid dissolved in 200 ml water and mixed with 1.8 ml aniline). Amino sugars were detected using either ninhydrin (0.5 g/100 ml acetone or the Morgan-Elson reagents (31,36). Glucose was detected using glucose-oxidase (Worthington Co.).

Disc Gel Electrophoresis

The system used was essentially that of Weber and Osborn (41).

Lyophilized cell envelopes were incubated at 37C for 2 hr in a solubilizing agent containing the following per 100 ml volume: 0.078 g NaH_2PO_4, 0.386 g $Na_2HPO_4.7H_2O$ and 1.0 g sodium lauryl sulphate (SLS). The final pH of the solubilizing agent is 7.0-7.15.

The gel buffer contained 7.8 g $NaH_2PO_4.H_2O$, 38.6 g $Na_2HPO_4.7H_2O$ and 2.0 g SLS per liter. For the 10% acrylamide solution, 22.2 g of acrylamide and 0.6 g of methylene bisacrylamide were dissolved in 100 ml water. Insoluble material (if present) was removed by filtration through Whatman #1 filter paper.

For a run consisting of 12 gels, 7.5 ml of gel buffer was mixed with 6.75 ml of acrylamide solution. After standing for 3-5 min. 0.75 ml of ammonium persulphate (14 mg/ml) and 0.02 ml N,N,N'N'-tetra methylethylenediamine were added. After mixing, each tube was filled with 1.0 ml of the solution. Before the gels were allowed to polymerize a few drops of water were carefully layered over the surface of the gels.

An equal volume of glycerine was mixed into the cell envelope sample just before it was put on the gels. Equal volumes of samples were always applied to the top of the gels.

Gel buffer diluted 1:1 with water was then carefully layered on top of the samples and both the upper and lower reservoirs of the electrophoretic tanks were filled with the diluted gel buffer. Electrophoresis was carried out at 2.5 mA/tube with the positive electrode in the lower chamber. A marker dye (methyl green B) was always employed to aid in determining the length of time required to complete a gel run. After electrophoresis was complete (usually about 8 hr) the gels were removed by squirting water with a syringe between the gels and the tubes.

Staining of proteins present in the samples was accomplished by adding a solution containing 1.25 g of Coomassie Brilliant Blue in a mixture of 454 ml of 50% methanol and 46 ml glacial acetic acid to the gels and allowing them to sit for 4 hr at 37C. The gels were then removed from the staining solution, transferred to capped tubes and rinsed once with distilled water after which they were placed in destaining solution (75.0 ml glacial acetic acid, 50.0 ml methanol and 875.0 ml water). Destaining was aided by shaking the tubes at 37C for 14-20 hr and 6-10 changes of fresh destaining solution were required to resolve the blue protein zones. After destaining, the gels were stored in small tubes containing 8.0% acetic acid.

Preparation of Cells for Thin Sectioning

Pellets of spheroplasts and normal cells were fixed with 0.1 M glutaraldehyde in 6.25% sodium cacodylate buffer (35) for one hr at room temperature, spun down, washed with acetate-Veronal (A-V) buffer (22) twice and then fixed in 1.0% OsO_4 (prepared by adding 1.0 ml of 0.2% tryptone and 0.2% $CaCl_2$ in A-V buffer to one ml of 2.0% OsO_4 in

A-V buffer) overnight at room temperature in tightly capped tubes. The cells were then sedimented again and washed three times in A-V buffer. The pellets were washed, suspended in 3.0% agar, and allowed to set for hardening after which the agar was sliced into blocks about 1 mm thick using a razor blade.

The blocks were then suspended in 0.5% uranyl acetate in A-V buffer for 4 hr at room temperature in tightly capped vials.

The blocks were then dehydrated using the following ethanol series: 25%, 50%, 75%, 90%, 100% and 100% for 15 min each step. Dehydration was completed using 2 washes with 100% propylene oxide each for 15 minutes.

The blocks were set in 50% resin and 50% propylene oxide for 2 hr, 75% resin and 25% propylene oxide overnight and finally 100% resin for 24 hours.

Embedding was accomplished by placing a few blocks in capsules (size "00") containing 100% resin. At the end of 24 hr the capsules were placed in an oven (62-65) for 72 hr for polymerization.

Sections were cut using a Sorvall MT-2 ultramicrotome. Sections were picked up on 400 mesh copper grids, dried and observed using a RCA EMU-3G electron microscope.

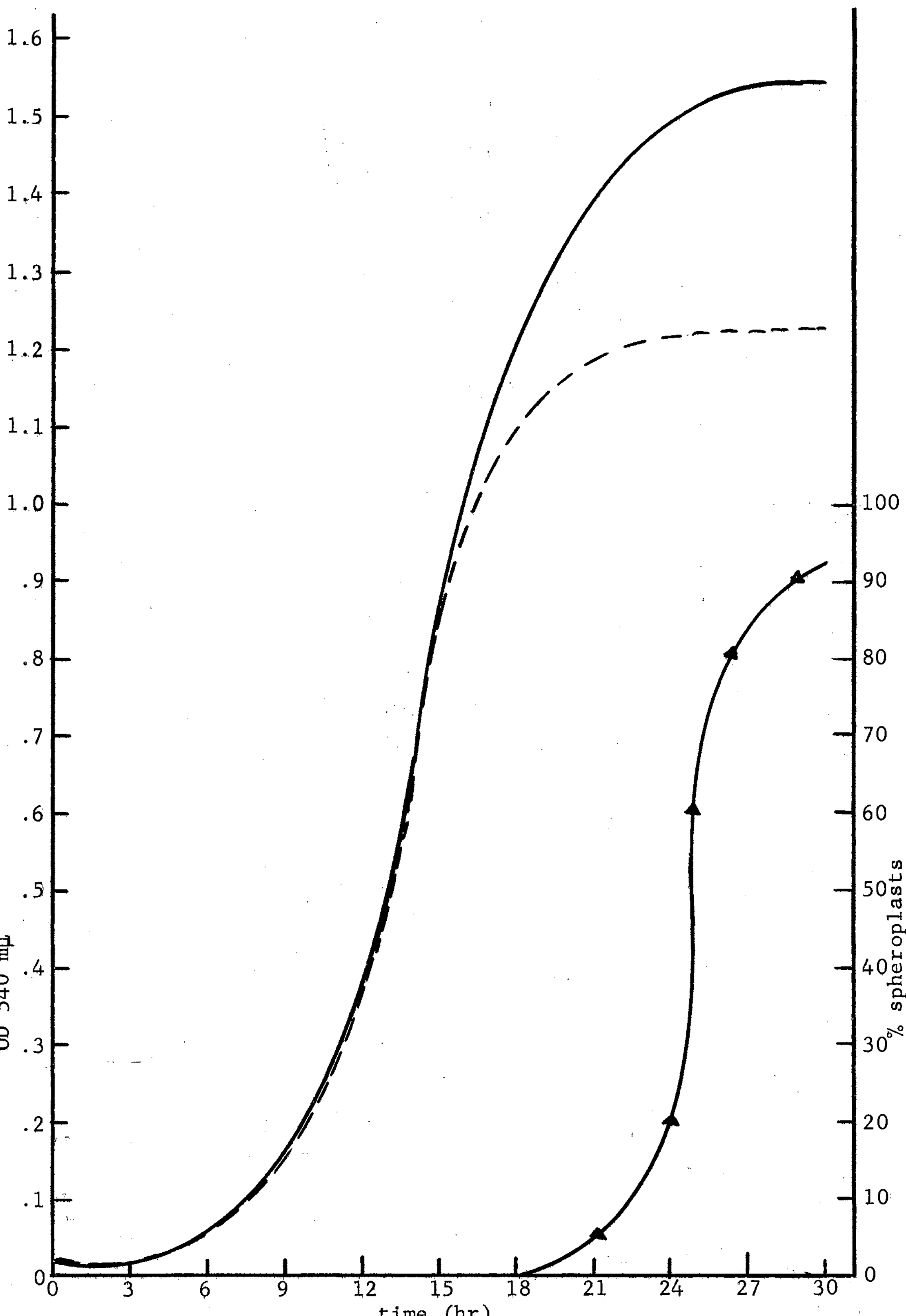
CHAPTER III

RESULTS

Before envelope components from spheroplasts could be isolated in the relatively pure form needed for chemical analysis, nutritional control had to be adjusted so that most (as near as 100% as possible) of the cells would undergo morphological transformation into the spheroplast form within a predictable period of time. Since the two major parameters for control appeared to involve time of addition and amount of serine, varying concentrations of DL-serine ($1.0x10^{-1}$ to $3.0x10^{-3}$ M) were added to growing cultures (OD 0.04-1.0) and incubation continued an additional 7-18 hr after addition of serine. These types of studies revealed that maximum (90-99%) spheroplast formation resulted when: a) the final concentration of DL-serine in the medium was $5.88x10^{-2}$M, b) addition of serine was made 14-15 hr after time of inoculation when OD of the culture was 0.60-0.65, and c) cells were allowed to incubate for an additional 14 hour (total incubation time approximately 28 hr). Variation in any of these conditions results in a decreased amount of spheroplasting.

As shown in Fig. 1 addition of serine at 14 hr does not bring about a decrease in growth rate for about 2 hours. Spheroplast formation is initiated 4-5 hr after addition of serine, but does not sharply increase until about 10 hr have passed. Within the

Figure 1. Growth and spheroplast induction. (-) growth of cells in the control medium (no serine); (---) growth of the cells in the spheroplast medium (serine added at 14 hr); (▲) percent spheroplasts induced in the spheroplast medium.


1.6
1.5
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
OD 540 mμ
0
3
6
9
12
15
18
21
24
27
30
time (hr)
100
90
80
70
60
50
40
30
20
10
0
% spheroplasts

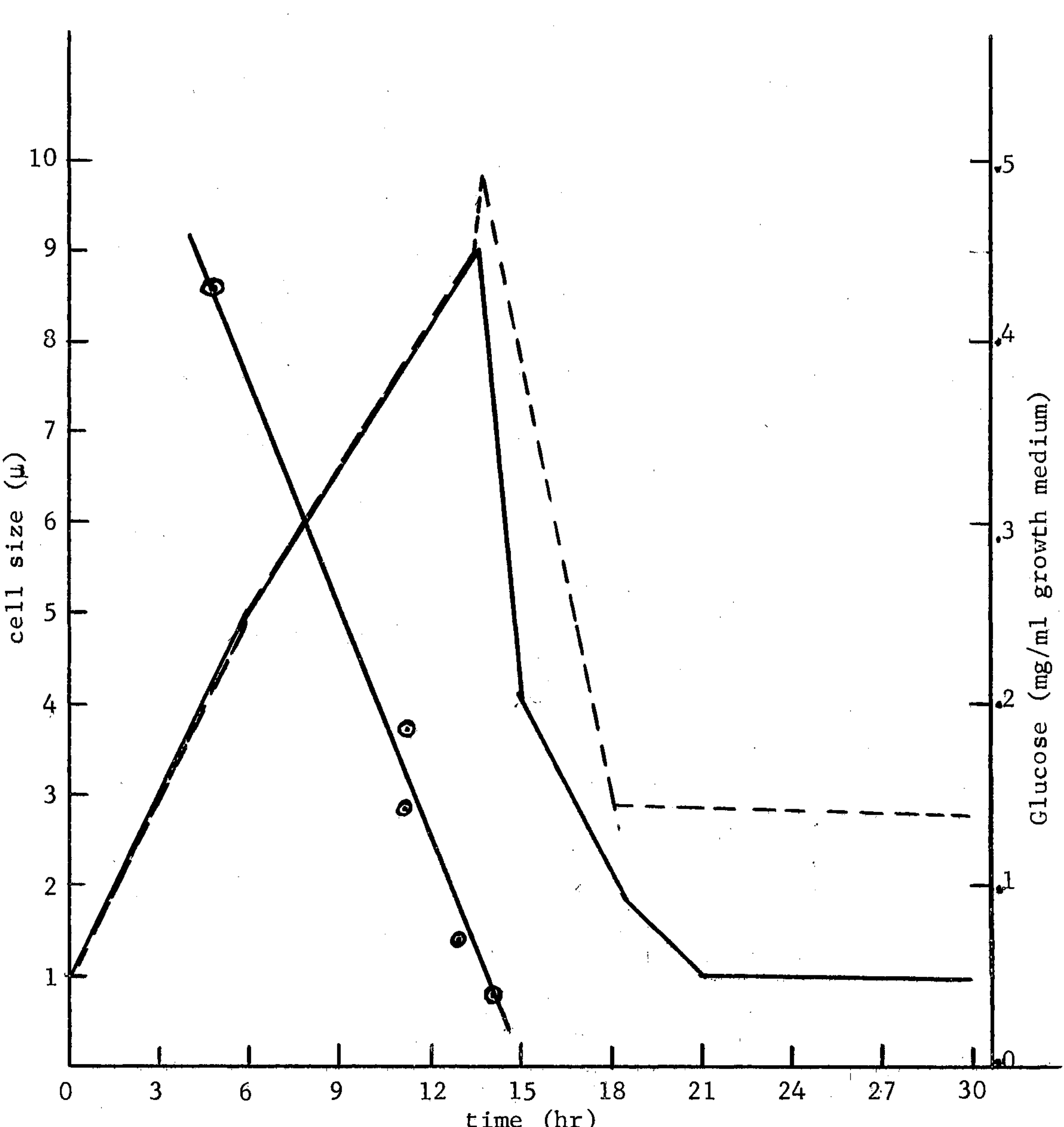
following 3 hr (10-13 hr) after addition of serine) spheroplast formation is completed (about 90-99% of the cells are converted). As also shown in Fig. 1, formation of spheroplasts does not result in lysis of the culture. Both the induction of spheroplasts by D-serine and their stability in the absence of osmotic protection were unexpected events. It should further be noted (Fig. 1) that spheroplast formation occurs during the stationary phase of growth.

Since we were also interested in cell size as well as the effect of glucose on formation of spheroplasts, size of cells was recorded and samples (0.5ml) removed each hr to measure disappearance of glucose. Data relative to these two parameters are given in Fig. 2.

Cells in the spheroplast medium elongated somewhat more than cells in the control medium; however, soon after 14 hr, cells in both media started to decrease in size (divide) before maximum formation of spheroplasts was initiated. This initiation of division was far more rapid in the control medium and might be related to the low level of glucose remaining in the medium at that time. It has been shown that glucose can inhibit cell division in this organism (16). Although the data shown in Fig. 2 do not prove the point, it appears that spheroplast formation can also be correlated to disappearance and/or very low levels of glucose in the growth medium.

I observed that spheroplast formation starts with a swelling at the middle or one end of the rod only. This swelling increases as the size of the rod part decreases, finally resulting in a spherical body. Cells in various stages of spheroplast formation can readily be observed 4 to 13 hr after addition of D-serine.

Figure 2. Size of cells in (-) control medium and (--) spheroplast medium and (⊙) disappearance of glucose during spheroplast induction. Cell size was determined by estimating size of all cells in at least five microscopic fields (average). Standardization was accomplished using an ocular micrometer. Glucose was depleted in both media at the time (14 hr) serine was added.


10
9
8
7
6
5
4
3
2
1
cell size (μ)
.5
.4
.3
.2
.1
0
Glucose (mg/ml growth medium)
0
3
6
9
12
15
18
21
24
27
30
time (hr)

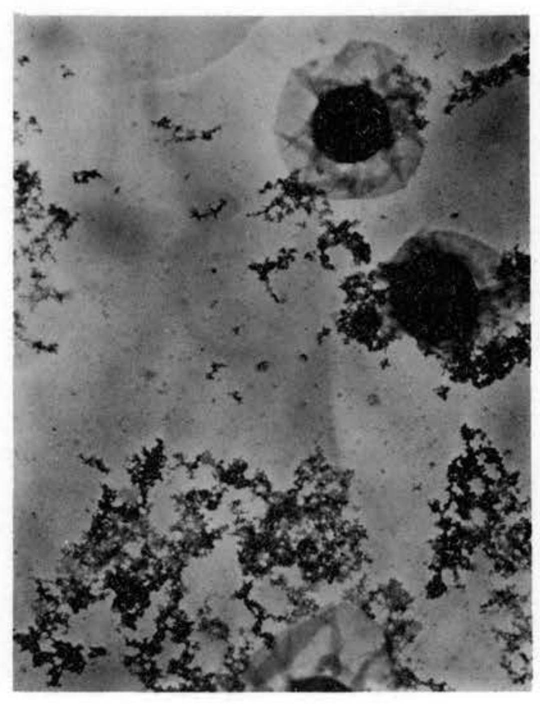
The spheroplasts as seen using the electron microscope (Fig. 3a) are completely circular and possess an opaque center surrounded by a transparent halo region. We suggest that the opaque area represents the plasmolyzed protoplast (cytoplasm plus cell membrane) whereas the halo material represents those portions of the cell wall which remain during and after transformation of the cell into the spheroplast state. Lark (25) claimed that a halo similar to the one we observed is part of the mucopeptide cell wall that oozes out of the cell when damage to the mucopeptide is effected by growing cells of *Alcaligenes fecalis* in the presence of D-methionine. He reached this conclusion after removing the halo by exposure of the spheroplasts to lysozyme. In our laboratory the halo we observed could be removed by excessive washing (4-5 times using a Vortex mixer for dispersion of cells) with ordinary stabilizing agents. Since most spheroplasts can synthesize lipoprotein and lipopolysaccharide (28) we think the halo represents these two macromolecular components of the cell wall. (Data relative to this point will be presented later.) As can be seen in Fig. 3b spheroplasts, when viewed in thin-section, do retain some portions of cell wall. Due to the fragility of the spheroplasts in distilled water we could not wash our preparations sufficiently to remove salts; thus all of our electron micrographs of spheroplasts show relatively heavy deposits of salts at the cell periphery. Because of this technical difficulty it is not possible for us to precisely depict the amount of cell wall remaining with the spheroplasts. For this reason, as well as to obtain more precise information at the molecular level, we set about to devise a technique for isolation of intact envelopes from spheroplasts.

Figure 3. a. Intact spheroplasts (17,000x).

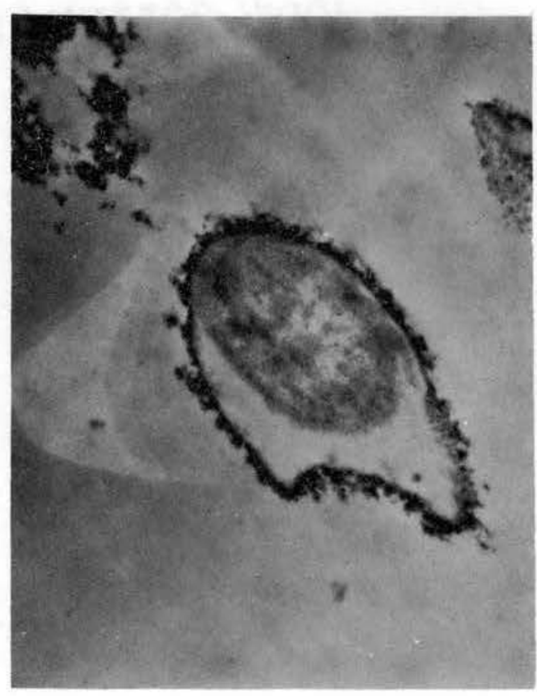
b. Thin section of a spheroplast cell (50,000x).

Both preparations were negatively stained using 2.0% uranyl acetate.

a



b



Envelope Isolation

When these studies were initiated, we knew that envelopes could readily be prepared from normal rod cells of Erwinia species. Briefly, either crushing with an X-press (20,000 lbs/sq in) or sonic oscillation causes complete breakage of the cells. Insoluble envelopes can then be washed in any type of menstruum prior to chemical analysis. Because we were not sure that the residual envelope surrounding the spheroplast form of Erwinia species would not completely dissociate into extremely small and nonsedimentable units of some type during preparation, we carefully checked ways in which to treat the spheroplast envelopes which are readily obtained by lysis in distilled water.

Initially we observed that plasmoptysis of spheroplasts using deionized water gave very small envelope fragments. Since plasmoptysis was carried out at room temperature, we thought that autolytic enzymes might have been responsible for what appeared to be excessive fragmentation of the isolated envelopes. Therefore, 5 min. after plasmoptysis had been accomplished, the lysed preparations were placed in a boiling water bath for 15 minutes. This was done to inactivate (denature) autolytic enzymes. Although envelope preparations were found to be somewhat larger in size, heating in this manner did not result in isolation of very large envelope units. We also tried plasmoptysis using cold deionized water (5C). This type of treatment, although not as good as hoped for, gave better results than heating. Therefore, all envelope isolation processes for chemical analysis were carried out using this

method.

Both sodium phosphate buffer (0.1M/pH 7.0) and 1.0% NaCl were utilized to wash cell envelopes. It was observed that NaCl has the disadvantage of making the envelopes clump together; therefore, cytoplasmic materials within the clump could not be completely removed. Phosphate buffer did not cause such clumping, hence; this buffer was was used throughout the experimentation (Fig. 4b). When it was necessary to remove salts prior to hydrolysis, envelopes were washed 2-3 times in cold deionized water.

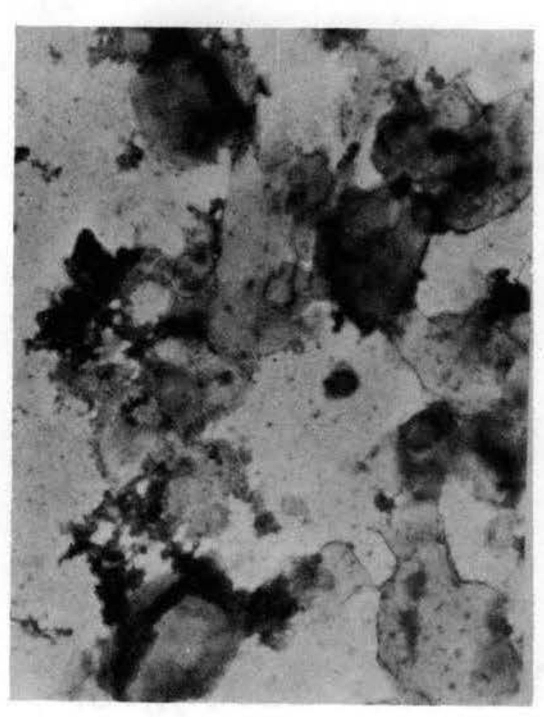
Mucopeptide Isolation and Quantitation

Formation of spheroplasts represents a transformation from rod to spherical body. Because it is generally acknowledged that the mucopeptide portion of the cell wall is responsible for shape of a cell and also protection from osmotic lysis, we thought it necessary to obtain quantitative data relative to the actual amount of mucopeptide present in the envelopes of spheroplasts.

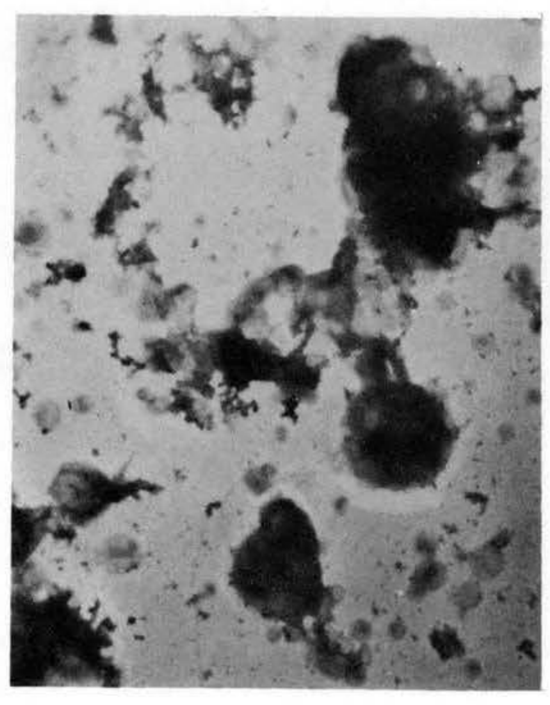
We first tried to isolate mucopeptide using the procedure of Grula, Smith and Grula(15). Our assay for mucopeptide was accomplished by observing for the presence of diaminopimelic acid (DAP) on paper chromatograms (19). Although no difficulty was experienced in obtaining mucopeptide from control cells, at no time could we demonstrate the presence of DAP in hydrolyzed samples of material made from spheroplasts. It appears that mucopeptide was completely absent from spheroplasts; however, it was also possible that it was present in very small pieces which were lost during the preparative procedures. To avoid such possible loss of mucopeptide, we simply

Figure 4. a. Isolated envelopes from control cells obtained by X-pressing. (25,000x).

b. Isolated envelopes from spheroplasts obtained by lysis with distilled water (25,000x).



b



isolated envelopes from spheroplasts and whole cells, stripped them using acetone-ammonia reagent, subject them to hydrolysis (6N HCl for 18 hr at 100-105C for amino acids or 4N HCl for 4 hr at 100-105C for amino sugars) and had the material analyzed for amino acids and amino sugars on the Beckman Amino Acid Analyzer in the Department of Biochemistry.

Results given in Table I clearly reveal that spheroplast envelopes are completely lacking in mucopeptide. This conclusion is justified because two compounds found only in mucopeptide (muramic acid and DAP) could not be detected. Thus it is easy to see why these cells exist in a rounded form instead of a rod shape.

It should be noted (Table I) that even though mucopeptide is not present in the envelope of spheroplasts, glucosamine is present; indeed it is present in amounts greater than those found in the envelopes of control cells. Although it has been shown that glucosamine is present in the mucopeptide of this organism in an amount exactly equivalent to muramic acid (13); more glucosamine (1-2 times more) is present in the envelope than can be accounted for by that amount present only in the mucopeptide (13). Because of the excess amount of glucosamine it was postulated that the additional glucosamine must be present in some envelope structure other than mucopeptide. If it is assumed that the most likely place for nonmucopeptide glucosamine is in the lipopolysaccharide (LPS) complex of the envelope, then it must also be concluded (Table I) that the amount of LPS in the envelope of spheroplasts is actually increased while the amount of mucopeptide is decreased to zero. In order to study this completely new and fascinating possibility, several studies

related to LPS content of the envelope were initiated.

TABLE I

AMINO ACID AND AMINO SUGAR CONTENT OF ENVELOPES FROM WHOLE CELLS AND SPHEROPLASTS.*

Compound	Spheroplasts	Control Cells
DAP	-	0.06
Glucosamine	0.12	0.09
Muramic Acid	-	0.04

*Expressed as μM/mg dry weight of envelopes.

Carbohydrate Content of Isolated Envelopes

To determine if an increase in total carbohydrates existed in envelopes isolated from the spheroplasts, different types of tests were performed.

Preliminary tests for reducing sugars using control and spheroplast envelopes were carried out using Nelson's alkaline reagent for reducing sugars. We obtained results showing that 3 times more reducing sugar is present in spheroplast than in control envelopes per mg of sample. Data obtained using a more elaborate procedure such as the anthrone test revealed that there is about 5 times more

carbohydrate material in spheroplast than in the control envelopes. (Table II.)

Because we knew that glucose is present in LPS from this organism and also to obtain the most precise data possible, enzymatic analysis utilizing glucose oxidase was employed to quantitate the amount of glucose present in envelopes of spheroplasts and control cells. Representative data from this type of determination are also given in Table II and it can be seen that spheroplast envelopes contain about 3 times more glucose than control envelopes. Therefore regardless of the type of determination done, (glucosamine, total carbohydrate, reducing sugar or glucose) an increase in the carbohydrate portion of LPS can be measured in spheroplast cell envelopes.

TABLE II

TOTAL CARBOHYDRATE AND GLUCOSE DETERMINATIONS ON ENVELOPES FROM CONTROL CELLS AND SPHEROPLASTS*

Test	Control Cells	Spheroplasts
Anthrone	0.8	4.4
Glucose-oxidase	1.9	4.0

*Expressed as mg/100 mg dry weight of washed envelopes

Isolation of Lipopolysaccharide (LPS)

Since mucopeptide makes up roughly 10-20% of the dry weight of cell envelopes in Gram-negative bacteria (10,44), the observed increase (3-5 times) in carbohydrate content could not be due to a decrease in total weight of the envelope brought about by absence of mucopeptide. The data obtained on carbohydrates instead seemed to indicate that LPS is synthesized in larger amounts. To confirm directly that the LPS content of spheroplast envelopes is increased, we decided to isolate LPS from control and spheroplast cells. The technique of Westphal and Jann (45) was used as given in the Methods and Materials Section. Weighing of the isolated material showed that at least 2 times more LPS is present in spheroplasts (32 mg/g dry weight of cells) than in control cells (16 mg/g dry weight of cells.

Carbohydrate determinations on the extracted and purified LPS were carried out using the Anthrone reagent. We observed that about 20% of the isolated LPS is present as carbohydrate both in control and spheroplast cells (Table III). Further testing of LPS using the glucose-oxidase test for glucose and the Morgan-Elson test (31) for glucosamine revealed the following information (also given in Table III). Both of these sugars are present in LPS and glucose is the major sugar in this complex. Because there appears to be only a relatively small increase both in glucosamine (7.0%) and glucose (12%) in LPS from spheroplasts, the data suggest that the increased amount of LPS in the spheroplasts can be viewed as an overall increase in all sugar components rather than an increase in just one monomeric component.

TABLE III

CARBOHYDRATE CONTENT OF ISOLATED LPS*

Test	LPS from Control Cells	LPS from Spheroplasts
Anthrone	20.0	19.7
Glucose-oxidase	15.3	17.4
Morgan-Elson	0.27	0.29

*mg/100 mg dry weight of LPS

Sugar Identification

Because what appeared to be relatively pure LPS had been isolated from _Erwinia_ sp., I attempted to identify the sugars present in the structure. Hydrolyzed samples of LPS (2N HCl for 2 hr at 100-105C) were run in the paper chromatographic systems given in the Methods and Materials Section. The Rf values of sugars we were able to identify are given in Table IV along with the Rf values and color reactions of the known compounds. As can be seen, four sugars match up very well (glucosamine, glucose, galactose and ribose). Although the Rf value of N-acetylglucosamine is identical to that of the sugars present in the LPS, color reactions are not the same; therefore, the identification of this compound is still tentative. No qualitative difference in sugar composition of LPS from spheroplasts and control cells was evident nor were there any large proportional differences between sugars as judged by direct observation of the stained chromatograms.

TABLE IV

R_f VALUES AND COLOR REACTIONS OF KNOWN SUGARS FROM LPS OF CONTROL OR SPHEROPLAST CELLS*

Sugar	R_f Value and Color	R_f Value and Color of Sugars from Control or Spheroplast Cells
Glucosamine	0.31 (brown)	0.31 (brown)
Galactose	0.37 (brown)	0.37 (brown)
Glucose	0.41 (reddish brown)	0.41 (reddish brown)
N-acetyl glucosamine	0.51 (yellowish green)	0.50 (brownish yellow)
Ribose	0.54 (red)	0.54 (red)

*Solvent: n-butanol-pyridine-water (6:4:3)

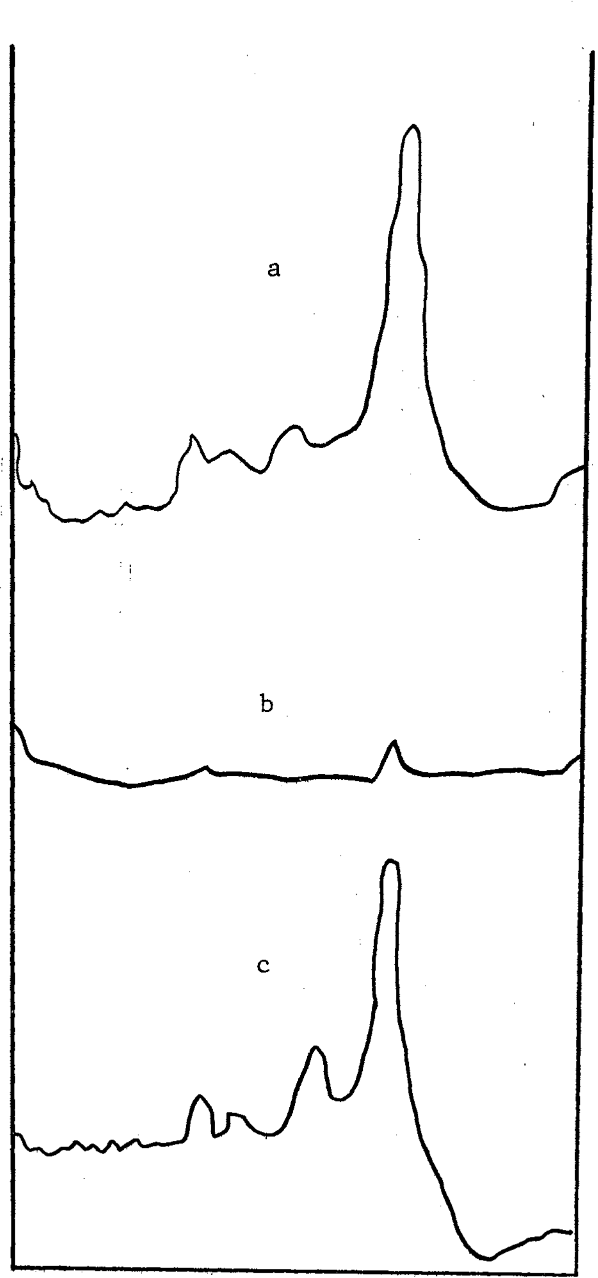
Spray: aniline-acid-oxalate

It should be mentioned that we did not try to determine presence or absence of 2-keto-3-deoxy-octonic acid (KDO): however, we did check for the presence of heptoses using the procedure of Dische (9), and these types of compounds are not present in LPS from Erwinia species.

Protein Studies Using Disc Gel Electrophoresis

Because both qualitative and quantitative data had been obtained relative to two of the three major macromolecular components of the cell wall of Erwinia sp., it appeared proper to obtain information relative to the lipoprotein layer in order to make this study more complete. To obtain both qualitative and quantitative data, detergent-solubilized envelope proteins were fractionated using polyacrylamide gel electrophoresis and the resulting gels analyzed using a Canalco Model K Gel Scanner. Scans are shown in Figure 5. About 10 protein bands could be resolved. The most striking feature of these scans relates to the presence of one large, sharp and distinct protein band in both types of envelopes. Electrophoresis runs using varying concentrations of solubilized envelopes (0.01-0.5 mg/0.04 ml solubilizing agent) from the spheroplast and control envelopes revealed in every case that there is less protein in the spheroplasts than in the control cells. To see if proteins were missing from the spheroplast envelopes, higher concentrations of solubilized envelopes were run. Since, under these conditions, patterns of proteins identical to those found in envelopes of control cells were observed, no qualitative difference in proteins appear to exist.

Figure 5. Scans of gels. (a) spheroplast envelope proteins (0.1 mg/0.04 ml solubilizing agent; (b) spheroplast envelope protein (0.02 mg/0.04 ml solubilizing agent); (c) control envelope proteins (0.1 mg/0.04 ml solubilizing agent).


a
b
c

Determination of Phosphatidylethanolamine (PTE)

Since changes could readily be demonstrated in all of the three major macromolecular constituents of the cell wall, we also wished to determine if any changes could be demonstrated in the cell wall membrane. The decreased levels of proteins present in envelopes from spheroplasts could indicate a decrease in the amount of different proteins in the cell wall lipoprotein complex. On the other hand, the data could also be used to indicate a decrease in the amount of proteins present in the "cell membrane complex." Because no method exists at present to separate and distinguish cell membrane lipoprotein from cell wall lipoprotein, we decided to assay envelopes for content of phosphatidylethanolamine (PTE) since this compound represents about 90.0% of phospholipid content of Erwinia species. Assay based on the procedure given in the Methods and Materials Section revealed that there is about 30.0% decrease in the amount of PTE present in envelopes of spheroplasts (44 μg/100 mg dry weight of control cells as opposed to 28 μg/100 mg dry weight of speroplasts). If it is assumed that the amounts of lipid and protein in these envelopes are related to each other in a stoichiometric manner and, also, that PTE is present only in the cell membrane of Erwinia sp., it follows then that both wall and membrane proteins are decreased in spheroplasts.

Stability of Spheroplasts

Weibull (43) first established that in cells of Bacillus megaterium an osmotic barrier is present at the surface of the protoplast and that the cell wall in all probability is permeable to small molecular solutes. The osmotic barrier corresponds to the

cytoplasmic membrane and is differentially permeable to various compounds depending on the organism. Protoplasts prepared by digesting away the cell wall with lysozyme have permeability properties similar to whole cells when protected from lysing in hypertonic media. Further, whereas intact bacilli resist changes in size or shape in media of varying concentration of sucrose or polyethylene glycol because of their rigid walls, protoplasts will shrink or swell as the solute concentration is raised or lowered over certain ranges.

Unlike spheroplasts formed from Gram-negative bacteria by different chemicals or antibiotics (26,42), spheroplasts induced by D-serine in Erwinia sp. can be maintained in the medium in which they are formed and undergo only minimum lysis for an extended period when kept on a shaker at 25C. Although we were able to easily establish that the serine-induced spheroplasts are not stable in distilled water, we decided to study how stable the spheroplasts actually are. Data given in Table V show that Mg^{++} ions alone are good stabilizers. Although NaCl even at 0.1M (equivalent to 0.58%) concentration allows for good stability, Carbowax 200 (organic) even at 20% concentration is a very poor stabilizer either in the presence or absence of Mg^{++} ions.

Effect of Pantoyl Lactone on Spheroplast Induction

Previous work done in our laboratory has demonstrated that pantoyl lactone overcomes division inhibition caused by D-serine (12). In order to observe the effects of pantoyl lactone on the process of spheroplast formation, varying concentrations of pantoyl lactone (1.5×10^{-2} M, 3.1×10^{-2} M and 4.6×10^{-2} M) were added to the medium at

TABLE V

SPHEROPLAST STABILITY*

Stabilizer	Concn. of Stabilizer	Time: 0 min	5 min	10 min	15 min	20 min
Growth medium	-	0.95	0.95	0.90	0.90	0.90
H_2O			0.30	0.29	0.28	0.28
$MgCl_2$	$1x10^{-3}$ M	"	0.75	0.73	0.73	0.73
NaCl	0.1 M	"	0.95	0.95	0.95	0.95
"	0.2 M	"	1.0	1.0	1.0	1.0
"	0.5 M	"	1.3	1.2	1.2	1.2
$MgCl_2$ +	$1x10^{-3}$ M	"				
NaCl	0.1 M	"	0.80	0.80	0.85	0.85
	0.2 M	"	1.0	0.95	0.95	1.0
	0.5 M	"	1.2	1.2	1.2	1.2
Carbowax 200	2.0%	"	0.34	0.33	0.33	0.32
	4.0%	"	0.34	0.33	0.33	0.32
	10.0%	"	0.33	0.32	0.32	0.31
$MgCl_2$ +	$1x10^{-3}$ M					
Carbowax 200	2.0%	"	0.43	0.42	0.42	0.41
	4.0%	"	0.43	0.42	0.42	0.40
	10.0%	"	0.43	0.44	0.44	0.44

*Lysis was followed by reading OD of the cell suspension at 540 mμ.

the same time serine was added. After 14 hr of incubation in serine-pantoyl lactone containing medium, samples were taken, stained with crystal violet and viewed under the phase contrast microscope. Only twenty to thirty percent of the cells in the 1.5×10^{-2} M pantoyl lactone-containing medium were transformed into spheroplasts. In media containing higher concentrations of pantoyl lactone, (3.1×10^{-2} M and 4.6×10^{-2} M) no more than 10.0% of the cells were present as spheroplasts. To determine if these "rods" were as resistant to distilled water as rods from control media, cells were sedimented and resuspended in cold distilled water and allowed to stand for 5 minutes. Although little or no decrease in OD occurs using rod-shaped cells from control media, there is 40-60% decrease in OD using "rods" from pantoyl lactone-containing media. Since the pantoyl lactone-produced "rods" were not nearly as resistant to plasmoptysis in distilled water as they should have been, it was decided to determine the content of mucopeptide within the cell wall.

Data given in Table VI show that pantoyl lactone-grown cells contain more mucopeptide than spheroplasts; however, they contain less than one-half the amount of mucopeptide present in rods grown in control media (compare to Table I). These data indicate that cells of Erwinia sp. can exist in the rod form without possessing the full amount of cell wall mucopeptide.

A further study of the pantoyl lactone effect showed that when incubation was continued for 24 hr after addition of serine and pantoyl lactone, 80%, 30-40% and 5-10% of the cells were present as spheroplasts in the 1.5×10^{-2} M, 3.1×10^{-2} M and 4.6×10^{-2} M pantoyl lactone treated cultures respectively. Such data show that the

TABLE VI

AMINO ACID AND AMINO SUGAR CONTENT OF SPHEROPLASTS AND "RODS" OBTAINED BY GROWTH IN THE PRESENCE OF PANTOYL LACTONE*

Mucopeptide Component	Spheroplast Envelopes **	Pantoyl Lactone "Rod" Envelopes **
DAP	0.007	0.015
Glucosamine	0.04	0.067
Muramic Acid	0.00	0.025

*expressed as µM/mg of dry weight of envelopes

**envelopes were obtained by X-pressing all cells present in the growth media (the spheroplast medium contained about 10% rod forms; the pantoyl lactone medium contained 10% or less spheroplast forms.)

"protective" effect of pantoyl lactone against D-serine is eventually overcome.

CHAPTER IV

DISCUSSION

Previous work from this laboratory has shown that growth of Erwinia sp. in the presence of D-serine results in a 30-40% decrease in mucopeptide content of the cell wall (15). Recently, Wise and Park have suggested that D-amino acids inhibit the transpeptidation reaction for mucopeptide cross linking in Staphylococcus aureus (46). It is likely therefore that D-serine could be inhibiting the transpeptidation reaction in Erwinia species. Although we are not sure of the precise way in which inhibition occurs, it has now been demonstrated that growth in the presence of serine can induce formation of spheroplast cell forms which are totally lacking mucopeptide.

If serine inhibits only the final cross-linking reaction, mucopeptide should still be present in the envelope of the spheroplasts. Further, if serine inhibits by yet another mechanism, which could involve direct incorporation or even the synthesis of mucopeptide precursor pieces, then the "old" cell wall mucopeptide, known to be present in the growing cells at 14 hr when serine was added and OD of the culture was 0.60-0.65, should still be present and attached to the "older" portion of the spheroplast cell. Because these spheroplasts are devoid of mucopeptide, at least two possibilities present themselves. 1) Formation of spheroplasts is an "explosive"

event which leaves behind a mucopeptide cell wall. We consider this unlikely because the mucopeptide layer completely surrounds a cell and, in addition, is the innermost layer of the wall. Therefore the entire layer or large parts of it could not be left behind unless cell wall lipoprotein and LPS, which surround it, were also left behind. This does not happen since spheroplasts contain 2-5 times more LPS than normal rod cell forms. 2) Growth in the presence of D-serine somehow activates an enzyme that hydrolyzes mucopeptide to soluble end products which diffuse away from the emerging spheroplast. Such an autolytic enzyme activity would explain our finding that spheroplasts are devoid of mucopeptide.

Regardless of how cells lose all of their mucopeptide, the absence of this rigid macromolecular layer accounts for the transformation of rods into the spheroplast state. Normally, it would be expected that spheroplasts which lack mucopeptide would lyse unless protected by hypertonic conditions. As reported in this thesis, spheroplasts produced as a result of growth in the presence of D-serine lyse in distilled water, but do not lyse in the growth medium. Since the only component which is increased in the envelope of the spheroplasts is LPS, it appears that it is this material which imparts sufficient strength to the envelope to allow the spheroplasts to be stable in the growth medium wherein several ions including Mg^{++} (8.0×10^{-5} M) are present. As shown in Table V both mono- and divalent ions aid stability of the serine-induced spheroplasts.

CHAPTER V

SUMMARY AND CONCLUSIONS

The time of addition and concentration of D-serine are important in spheroplast induction in our species of Erwinia. The cells must be in their logarithmic phase of growth when D-serine is added.

Amino acid and amino sugar analysis using isolated envelopes revealed that spheroplasts do not contain any mucopeptide; thus lack of this rigid layer of the cell wall results in formation of spheroplasts.

Quantitative data obtained using the anthrone and glucose-oxidase tests showed that 3-5 times more carbohydrate is present in spheroplast envelopes than in the envelopes of control cells. Direct isolation and quantitation of LPS revealed that 2 times more of this macromolecule is present in envelopes of spheroplasts. It is possible that stability of D-serine-produced spheroplasts in the growth medium could be due to this increase in LPS.

Analysis of envelopes from control and spheroplast cells using detergent polyacrylamide gel electrophoresis showed that spheroplast envelopes contained less protein than control envelopes. Because a decrease in PTE (about 30%) can also be demonstrated, it is suggested that proteins are decreased both in the lipoprotein layer of the cell wall and the cell membrane.

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