CHAPTER 2

STUDIES ON THE PANTOTHENATE DEFICIENCY AND SYNTHESIS OF INTEGUMENTAL STRUCTURES (CELL WALL AND MEMBRANE) OF *STREPTOCOCCUS FAECALIS*.

Most bacteria, yeasts, fungi and algae are surrounded by a rigid wall was apparent to the early cytologists starting from Leeuwenhoek. Vincenzi, in 1887, was the first to investigate what he believed to be the wall of B. subtilis. The isolation of microbial surface structures was made possible by the application of the methods of biochemistry, biophysics and electron microscopy. Weibull (94) was one of the first to use combination of such methods. Mudd, Polevitsky, Anderson and Chambers (95) showed by electron microscopy that sonic disintegration of bacteria left a resistant wall. Weidel was the first to use autolytic methods for the isolation of cell walls of E. coli.

The walls and its immediate vicinity are particularly important part of the organism. The wall itself is interesting because it must possess the structural rigidity for its function in protecting cytoplasm and its membrane from the considerable osmotic forces that bacteria encounter, yet it is fairly porous and it must be permeable to nutrients and metabolites. As it represents normally about 30 percent of the dry weight of the cell, an appropriate proportion of the metabolic activity of bacteria is concerned with the synthesis of wall and its precursors.

Cell wall of S. faecalis like other gram-positive bacteria contains the peptidoglycan chains and also some teichoic acids. The structure of peptidoglycan polymer present in S. faecalis has been shown in the Fig. 0.7. The peptidoglycan may account for about 50 - 95 percent of the cell wall structure in gram-positive bacteria, depending on the

particular species, growth conditions, etc.

Functionally the bacterial cytoplasmic membrane occuring next to wall is another important surface structure and in addition to being involved in active transport and selective permeability it has to fulfil the role of anchoring DNA of the chromosome and ensure nuclear separation during cell division. Its presence was very clearly demonstrated by electron microscopy of plasmolysed cells and also by actual isolation and observation of protoplast membrane obtained by lysozyme digestion of the cell wall of gram-positive organism (96,97). Membrane ghosts derived from protoplasts of S. faecalis contain a number of important enzymes. The total membrane fraction represents 10 - 20 percent of the dry weight of gram-positive cells (98) and the membrane contains 15 - 30 percent (99) of lipid. About 90 - 95 percent of the total lipid is present as phospholipids (100, 101). Cell wall is essentially free of lipid and the cytoplasm does not contain more than 4 percent of the cellular lipid phosphorus. Therefore, lipid phosphorus may serve as a true index of membrane substance.

Studies of incorporation of 1-C^{14} acetate in S. faecalis cell wall fraction by Dezelee and Shockman (102) showed that 1-C^{14} acetate was rather efficiently incorporated into peptidoglycan. Their observations were suggestive of 1-C^{14} acetate incorporation into N-acetyl groups of glucosamine and muramic acid residues and some possibly also in N-acetyl amino sugars of teichoic acid and O-acetyl of amino sugars.

Synthesis of N-acetyl hexosamines which are cell wall building blocks and the synthesis of lipids, which are membrane building blocks,

both involve acetyl transport; acetyl transport is dependent on pantothenate in the form of Coenzyme A (49). Observations from chapter 1 showed that pantothenate is the essential growth factor for S. faecalis and presumably the metabolic conversion of pantothenate to CoA is prerequisite for acetyl transport which is needed both for the formation of cell wall and membrane. These considerations along with our observations in chapter 1 in regard to dependence of peak-growth, lysis and secondary growth under pantothenate starvation (i.e. in absence of exogenous pantothenate) phenomena led us to investigate the role of pantothenate in the biosynthesis of N-acetyl-hexosamine (as presumptive measure of cell wall) and membrane substance (measured as lipid phosphorus).

Results of our investigations with S. faecalis R ATCC NO. 8043 in regard to the above are recorded in this chapter.

2.1 MATERIALS AND METHODS

2.1.1 Preparation of culture and growth measurement

These were same as described earlier (Ch. 1). Culture used here was S. faecalis R ATCC NO. 8043.

2.1.2 Total hexosamines (as a presumptive measure of cell wall substance and its precursors)

Determination of hexosamines was made following Good and Bessman's modification of the Morgan - Elson method (103).

The reagents used and details of the procedure followed are given below:

Reagents:

Acetic anhydride Reagent: Acetic anhydride (Ac_2O) 0.15 ml was added to 10 ml of acetone. This reagent was good for use only for the day.

Borate Buffer (1.12 M KH_2BO_3): To 6.92 gm (= 112 mM) H_3BO_3, dissolved particularly in water, 56 mE KOH was added and the volume was made upto 100 ml, pH was checked and adjusted to 9.2 $\pm$ 0.1.

DAB Reagent: To 2.00 gm dimethylaminobenzaldehyde (recrystallized), 200 ml acetic acid was added, and after dissolution 2.50 ml conc. HCl was added. The reagent was used on the same day of preparation.

Glucosamine standard: Glucosamine hydrochloride (GCN.HCl) (53.9 mg) was dissolved in 25 ml; 10 ml of this solution was made upto 50 ml with 1.0 M NaCl to have 36 percent glucosamine per ml of 1 M NaCl.

Procedure:

Collection of samples: Fresh log cells equivalent to about 80 µg glucosamine (i.e., about 3 mg dry cells) were used for each determination. Culture aliquots were centrifuged and drained. Since ordinary culture medium contained no hexosamine, no washing was necessary.

Hydrolysis: 1.00 ml 2.00 N HCl was added to each tube which was capped tightly and subjected to 100° C for 12 hrs. for hydrolysis. The tubes were then cooled and to each 1.00 ml 2.00 N NaOH was added and volume in each was made upto 2.05 ml.

Developement of Color: To each tube (taken in a rack) 0.20 ml Ac_2O reagent, 1.00 ml Borate buffer reagent were added and the whole was mixed well over a vortex mixer. The tubes were capped tightly and put into a boiling water bath at 100° C for 3 min. Right after, the rack with tubes was put into an ice bath, with some shaking for 5 min. Next 7.00 ml DAB reagent was added. The tubes were capped tightly and the contents were mixed and later incubated for 20 min. at 38° C.

Reading and Calculations: Immediately the outside wall of the tubes were dried and the color was read in a Coleman spectrophotometer at 750 mμ against acetic acid (HAc) blank and compared with the standard series run between 0.0 - 36.0 μg glucosamine per tube contained in 1.0 ml of 1 M NaCl solution. GCN contents of the experimental tubes were calculated from the standard curve. Average of three values was used for plotting the results. Results of separate hydrolysates did not differ by more than 2 percent (Fig. 2.1).

2.1.3 Lipid phosphorus

Lipid phosphorus as a presumptive measure of cell membrane substance (and precursors), was determined following Toennies et al (49, 104). Details of the procedure followed is given below:

Procedure:

Preparation of dry cells: Adequate volume of growing culture, equivalent to about 2 mg dry cells, was collected directly in a screw capped tube and spun for 30 min. at 3000 rpm (at 0 - 2° C). Supernatant was carefully removed from the test tube by completely inverting it.

Any supernatant remaining at the shoulder was removed as much as practicable with the help of a bent pasteur pipette. The tubes with the wet bacterial cells were taken in a beaker and placed inside a vacuum desicator containing conc. H_2SO_4. The desicator is connected to a vacuum pump which was run for 30 min. and the whole was left as such for additional 30 min. It was found that this treatment was sufficient to give superficially dry cells suitable for lipid phosphorus assay.

Treatment of dry cells with water: To the dry cells in the test tube 0.10 ml of distilled water (ordinary) was added and the whole was allowed to blend for 2.5 min. after mixing over a vortex mixer.

Extraction of lipids: To each tube containing the cell suspension 1.0 ml methanol (absolute spectro quality reagent) was added quantitatively, mixed well, capped tightly and was placed in a water bath at 95° C and the extraction was continued for 1 hr. Then the tubes were carefully removed from the bath and allowed to cool to room temperature. Then 2.00 ml of $CHCl_3$ was added quantitatively to each, mixed well, capped tightly and allowed to stand overnight. After overnight standing 4.00 ml of neohexane (NH, spectro grade) and 7.00 ml of 2 M KCl solution were added to the tube. The whole was shaken for 1 min. (145 times) by hand and later centrifuged for 25 min. at room temperature (25° C). After spinning, the organic phase i.e., 4.00 ml of NH plus 2.00 ml of $CHCl_3$ containing the whole bacterial lipids was collected above the aqueous KCl phase (containing whole of the methanol). 5.0 ml of this phase was very carefully pipetted out (with accropet) to a digestion vessel (10 ml Kjeldahl flask) for determination of phosphorus

by digestion method.

Evaporation of organic solvents from the digestion vessel: The digestion vessel (with its loose cap) was next placed in water bath (70° C) for 10 - 15 min. and temperature of the bath was later increased to 100° C and digestion vessel was kept for further 10 - 15 min. for complete removal of the solvents. Remaining traces of solvents (odour) were removed by suction.

Digestion with H_2SO_4: To each digestion tube 0.2 ml of 18 N H_2SO_4 was added. Digestion was followed for 5 min. since the fuming of H_2SO_4. Later cooled $2\frac{1}{2}$ min. and added 0.1 ml of H_2O_2 (3 percent). Heating was done further for 5 min. and again cooled for $2\frac{1}{2}$ min. Next 0.1 ml H_2O_2 added and redigested for 5 min.

Volume adjustment after digestion: After digestion 0.94 ml of NaOH (4 N) was added to the digestion vessel and mixed. Next with an accropet pipette the total volume of the digest, thus obtained (inclusive of alkali) was measured and the final volume adjusted to 2.5 ml with distilled water. Uniform concentration of the digest was assured by sucking in and out the solution and washing the sides of the digestion vessel (upto the neck) with the same.

Analysis of aliquots of lipid digest: Analysis was done colorimetrically (104) in screw capped test tube.

The phosphate standards (in duplicates) containing 0.50, 1.00 and 2.00 µg of phosphorus were diluted to 2.0 ml with distilled water and 1.0 ml of freshly prepared molybdate-hydrazine reagent (105) was

added. Tubes were capped and, together with 1.00 ml of digest (in duplicate) treated in the same way, heated for 10 min. in boiling water bath. Optical density was noted at 830 mμ in Beckman Model DB 1402 spectrophotometer, using quartz absorption cells. Average of three separate determinations was used for plotting the results (Fig. 2.1).

2.1.4 Cultures used for cell wall and membrane determination

The organism used was Streptococcus faecalis R ATCC No. 8043. Cultures obtained under three different nutritional conditions (with respect to pantothenate) were used for all the experiments done in present chapter. Same nomenclatures of these three nutritional types have been used throughout this dissertation wherever needed. The nomenclatures used for different cultures are stated below:

(a) Normal culture: These were normal cells grown in the complete synthetic medium (Ch. 1) containing high pantothenate (i.e., 400 mμg/ml).

(b) Pantothenate-limited culture: These were cells grown in the same complete synthetic medium (Ch. 1) containing 20 mμg/ml pantothenate.

(c) Pantothenate-starved culture: The cells were first grown in the same complete synthetic medium as above containing 400 mμg/ml pantothenate and grown upto about 500 - 1000 AOD. Later these cells were washed two times with the same pantothenate-free but otherwise complete synthetic medium and re-incubated in the same medium at an initial bacterial concentration of about 100 AOD.

2.2 RESULTS AND DISCUSSION

It is evident from the Fig. 2.1. that with the increase of AOD, cell wall content also increased linearly in case of both normal and pantothenate-starved cultures though the rate of increment in the latter case was slightly lower. In case of pantothenate-limited culture cell wall synthesis was like that of the normal culture upto about 300 AOD and thereafter this gradually lagged behind and reduced to about 20 - 25 percent near the peak AOD. At 600 AOD, cell wall content of pantothenate-starved culture dropped down about 16 percent compared to that of the normal culture.

Membrane synthesis continued (almost in parallel with wall synthesis; this was found to be the case when lipid phosphorus values were plotted against time in a semi-log paper) throughout the exponential growth phase in normal culture (Fig. 2.1). In pantothenate-starved culture membrane synthesis lagged from the beginning and tended to attend a plateau at around 600 AOD. With pantothenate-limited culture membrane synthesis showed lagging from about 150 AOD and thereafter the rate of its synthesis gradually went down. After 300 AOD the synthesis of membrane of this type of culture was much diminished.

Observations made in the present experiment in regard to marked decrease of membrane biosynthesis and gradual fall in wall hexosamines under pantothenate deficiency are in consonance with those reported by Toennies *et al* (49). Reduction of wall hexosamines in pantothenate-deficient state is perhaps to be related to the defective membrane formation and its dysfunction in regard to permeability of glucose or

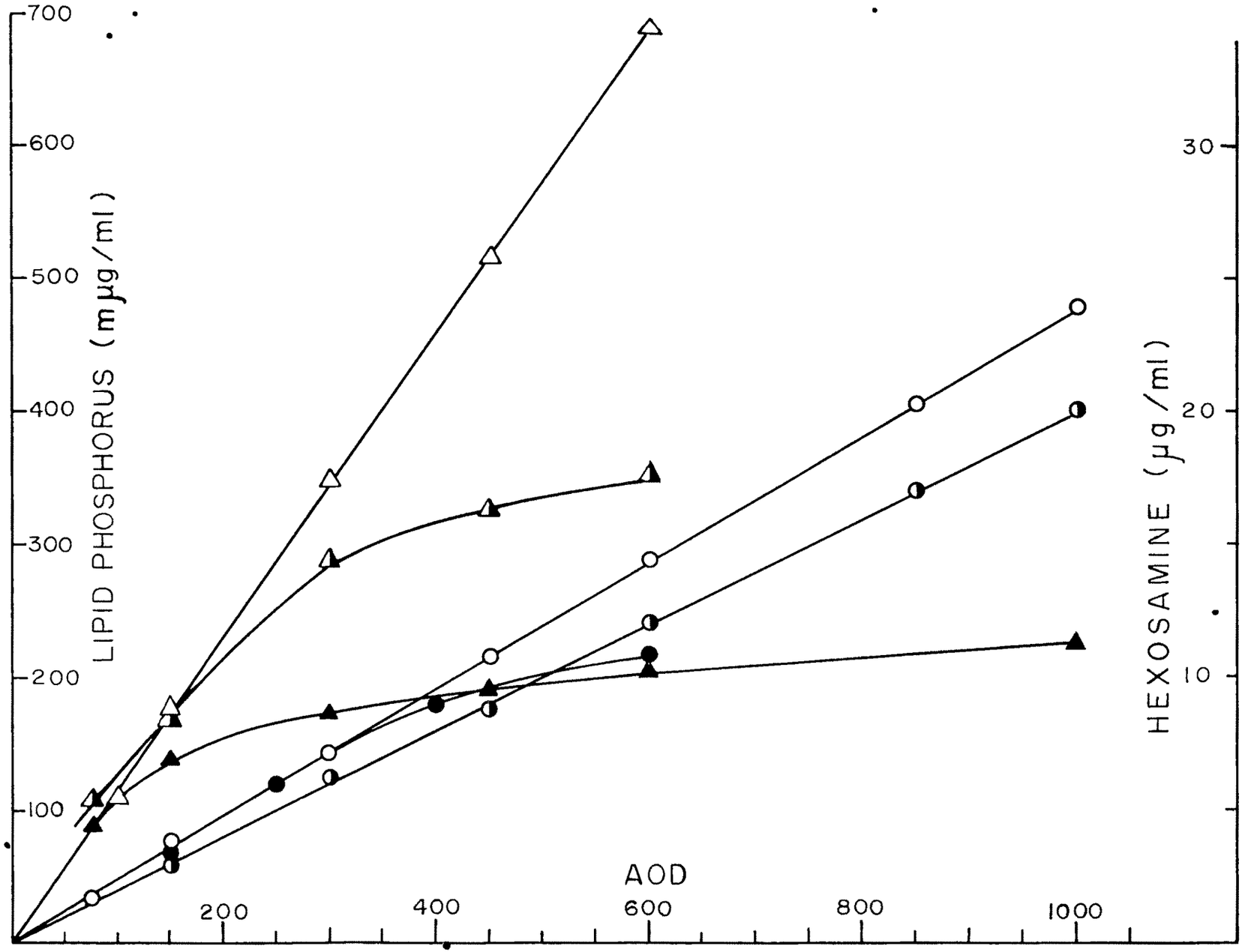
700
600
500
400
300
200
100
LIPID PHOSPHORUS (mμg/ml)
30
20
10
HEXOSAMINE (μg/ml)
AOD
200
400
600
800
1000

FIG. 2·1

glucosamine and its derivatives. The severe curtailment of membrane biosynthesis, as observed in case of pantothenate-deficient conditions, seems to be related to marked reduction in lipid constituents of the membrane.

In the present method employed for estimation of wall synthesis, one can not evaluate the extent of acetylation of NH_2 group of the hexosamine residues of the peptidoglycan. The degree of acetylation of the NH_2 group of hexosamine residues is very likely to vary in proportion to the degree of pantothenate deficiency (106).

Dezelee and Shockman (102) demonstrated that the growth of S. faecalis 9790 in the present synthetic medium containing sufficient amount of pantothenate (i.e., 400 mμg/ml) was dependent on the acetate concentration used. Limitation of acetate like limitation of pantothenate caused growth peak to be attained and prompt lysis to be followed thereafter. Peak of the growth depended on the concentrations of acetate. Their further studies with 1-C^{14} acetate under pantothenate-sufficient condition indicated acetate to be efficiently incorporated into the peptidoglycan, presumably, in the NH_2 group at position 2 of the hexosamine residues (no significant incorporation was detected at the lactyl moiety at position 3 of the muramic acid residues) of the peptidoglycan. Both these findings are strongly suggestive of the fact that the lysis observed in acetate limitation is perhaps due to lessening in the degree of acetylation of the NH_2 group of the hexosamine residues.

The degree of acetylation of the NH_2 group is presumably, dependent both on the availability of the acetate and the acetylation factors

e.g., CoA and ACP. In the studies of Dezelee and Shockman (102) acetylation factor(s) was in abundance and the lysis naturally followed owing to limitation of acetate. In our observations reported here (Figs. 1.1, 1.3 and 1.4; Ch. 1) the lysis was presumably, due to limitation of acetylation factor(s) caused by limitation of pantothenate. This could cause differences in the degree of acetylation of the hexosamine residues of the peptidoglycan. This means that NH_2 group of hexosamines will be less and less acetylated in pantothenate deficiency state.

Recent studies of Kelemen and Rogers(107) shows that acetamido group of hexosamine residues of the peptidoglycan is strongly hydrogen bonded. Infrared studies indicate hydrogen bonds between -CO- of acetamido group of one glycan chain with the -NH- group of the acetamido group of the other glycan chain running parallel to it. Though individually hydrogen bonds are weaker (3 - 7 Kcal/mole) in nature but presence of billions of these bonds will naturally strengthen the binding of the glycan chains leading to the rigidity of the cell wall structure. Presence of free NH_2 group (not acetylated as a result of limitation of acetyl factors), at position 2 of the hexosamine residues of peptidoglycan chains will much diminish the chances of hydrogen bond formation and thus would lead to weakening of cell wall structure.

Importance of cytoplasmic membrane in cellular macromolecular synthesis inclusive of cell envelopes synthesis, nuclear division and cell division has been reported before (108). Removal of cell wall with lysozyme in an osmotically protected medium, prevention or interference of cell wall formation by some antibiotics like penicillins, cycloserine

or similar other agents and starvation of cell wall precursors like hexosamines (N-acetylglucosamine and N-acetylmuramic acid) in the growth medium cause inhibition of cell division (109). Deficiency of growth factors needed preferentially for cell wall and cytoplasmic membrane synthesis could lead to unbalanced growth and developement (109). Accordingly, the severe curtailment of membrane biosynthesis and gradual decrease of wall synthesis as observed in the present studies with pantothenate-deficient S. faecalis R 8043 could lead to inhibition of cell division and nuclear division. It would be further evident from Fig. 2.1 that in case of normal cells the ratio of cell wall contents to cell membrane contents was maintained constant throughout the exponential growth unlike in the pantothenate-deficient cells. This is indicative of gradual deformation of anatomical and morphological features of S. faecalis under the vitamin-deficient conditions.

Analysis of the present biochemical data does not reveal anatomical changes, if any, occurring as well, as a result of restriction of pantothenate and needs to be studied by microscopic observations. Microscopic studies relating to the anatomical changes have been included in the chapter 5.

2.3 SUMMARY

Syntheses of cell wall (in terms of total hexosamines) and membrane (in terms of lipid phosphorus) in pantothenate-sufficient and pantothenate-deficient exponential cells of S. faecalis R ATCC NO. 8043 have been observed.

(i) In case of pantothenate-limited cells (20 mμg pantothenate per ml) membrane synthesis continued initially upto 150 AOD, like that of the normal cells. Membrane synthesis thereafter gradually lagged. Under this condition wall synthesis was similar to that of normal cells upto about 300 AOD. Later the wall synthesis began to slow down.

(ii) In case of pantothenate-starved culture membrane synthesis showed severe lagging almost from the beginning of growth. Wall synthesis under this condition though continued along with the growth but this too showed signs of lagging (16 percent approx.) from that of normal culture.

(iii) Possible effect of partial acetylation of NH_2 group in hexosamine residues of peptidoglycan of pantothenate-deficient cells, upon the rigidity of cell wall has been discussed.