CHAPTER 5

STUDIES ON THE MORPHOLOGICAL AND ULTRASTRUCTURAL CHANGES OF *STREPTOCOCCUS FAECALIS* GROWN UNDER PANTOTHENATE-DEFICIENT CONDITIONS

Most morphological changes caused by environmental condition in growing or aging cultures result from unbalanced growth and developement (109). Among the various inducing agents starvation of growth factors required by cell wall, cytoplasmic or nuclear synthesis has been reported (109) to lead to an unbalanced growth and developement.

Cell walls of bacteria contain mucopeptide as the major component causing rigidity of bacterial shapes. Hexosamines (N-acetylglucosamine and N-acetylmuramic acid) make up the major part of mucopeptide. Deficiency of both of these hexosamines (or their precursors) has been reported to produce large, branched and haemoptysis forms in Lactobacillus bifidus var pennsylvanicus (132).

Observations made in chapter 2 (Fig. 2.1) indicated that during log phase growth in pantothenate-sufficient medium cell membrane to cell-wall ratio of normal cells remains constant unlike in similar cells grown in pantothenate-deficient conditions. In the latter cases, a gradual change was noted along with the exponential growth. This hinted that some possible anatomical/morphological changes of cells undergoing pantothenate deficiency. In the previous chapter (Ch. 4) we observed inhibition of cell divisions in pantothenate-deficient cells, specially in pantothenate-limited cells, with marked drop in survivor percentages.

All these above studies coupled with our observations made in chapter 4 led us to undertake the present investigations on the morph-

ological and ultrastructural changes following pantothenate deficiency in S. faecalis cells. This chapter records our (a) morphological observations with light microscopy and scanning electron microscopy; and (b) ultrastructural observations with transmission electron microscopy.

5.1 MATERIALS AND METHODS

5.1.1 Preparation of log phase cultures of deficient cell types

Normal, pantothenate-limited and pantothenate-starved cultures of S. faecalis R ATCC NO. 8043 and S. faecalis ATCC NO. 9790 were grown as stated previously in chapter 2. Appropriate amounts of different cultures were collected at different AOD levels of exponential growth and chilled. These samples were used for microscopic observations under light microscope, scanning electron microscope and transmission electron microscope after different treatments as described below.

5.1.2 Specimen preparation for light microscopic observations

Chilled cultures obtained at different AOD levels were uniformly spread on the glass slides. Fixation of the cells was done under an electric lamp (100 watt, 220 volt) held at a distance of about 15 cm from the slide for about 3 min., later the slides were cooled to room temperature. Freshly prepared crystal violet solution (123) was used for staining (30 sec.) the cells. Excess dye was washed out by drop wise addition of tap water and the slides were later air dried. Observation was made with a Karl-Zeiss phase-contrast microscope and photographs were taken at 800X instrumental magnification.

5.1.3 Specimen preparation for scanning electron microscopic observations

One loop of the culture (adjusted to 100 AOD approx. by appropriate dilution with normal saline) was spread uniformly on a glass slide (1 cm x 1 cm x 0.1 cm) and fixed slowly by holding it under a warm electric bulb as stated earlier. It was cooled and stained with freshly prepared crystal violet solution (3 gm crystal violet dissolved in 20 ml of 95 percent ethanol and volume made upto 100 ml with 1 percent ammonium oxalate solution and filtered). The excess dye was removed by drop wise addition of water from a pipette. The glass slide with stained culture when air dried were kept in a vacuum dessicator containing fused $CaCl_2$ for about 7 days. Metal coating of specimens was done by sputtering (40 sec.) in a Edward's Sputter coater Model NO. S150. Specimens were examined (at 40° - 45° C) in a stereo Phillips Scanning Electron Microscope (PSEM - 500) and using an accelerating voltage of 25 - 50 KV (spot size being 160 - 300 Å).

While staining slides with crystal violet for light microscopic observations, we ran, as a trial, a small piece of stained glass slide containing S. faecalis cells (after drying for 7 days in dessicator over fused $CaCl_2$) for preliminary examination in a scanning electron microscope. When we found the procedure to work satisfactorily for our purpose we standardized the method using 3 percent crystal violet solution for examination of our various experimental slides for scanning electron microscopic observations. A later examination with glutaraldehyde (2.5 percent in phosphate buffer 0.3 M, pH 6.5) fixation for 16

- 24 hr. of the cells (133) gave identical scanning electron microscopic pictures. Glutaraldehyde was very expensive and not available locally, and as such our procedure (134) with crystal violet was followed satisfactorily.

5.1.4 Specimen preparation for transmission electron microscopic observations

For this we used a modified procedure based on the method followed by Kellenberger et al (135) and Higgins et al (136). Cells were fixed by addition of glutaraldehyde, washed, counterstained with Kellenberger fixative, dehydrated in ethanol, infiltrated and embedded in Araldite, thin sectioned and counterstained in uranyl acetate and lead citrate (137). Details followed are given below.

Fixation and embedding:

i) 28.0 ml of the culture was mixed with 4.0 ml of Calcium carbonate neutralised (pH 6.5) glutaraldehyde (25 percent).

ii) Fixed cells were centrifuged at 1800g for 5 min.

iii) Supernatant was decanted and the pellet resuspended in 0.01 M phosphate buffer (pH 6.3) containing 0.08 M Potassium chloride and 0.01 M Magnesium acetate. This was left overnight at room temperature (27° C).

iv) Suspension was centrifuged for 5 min. at 1800g. The supernatant was decanted. The pellet was resuspended in the above buffer and centrifuged for 5 min. at 1800g.

v) The pellet was next resuspended in veronal acetate buffer, allowed to stand for 30 min. and centrifuged (1800g for 5 min.).

vi) The pellet was resuspended in a mixture of 30 ml of veronal acetate buffer and 3 ml of Kellenberger's fixative (135) and centrifuged at 1800g for 5 min.

vii) The supernatant was decanted and the pellet was resuspended in 2 ml of Kellenberger fixative to which 0.2 ml of Tryptone medium (containing 1 percent of Difco Tryptone and 0.5 percent NaCl in distilled water) was added.

viii) Above suspension was allowed to stand overnight at room temperature (16 hr.).

ix) To the suspension obtained 8.0 ml of Kellenberger buffer was added and the whole was centrifuged for 10 min. at 5000 rpm.

x) The supernatant was decanted and the pellet was resuspended in washing solution (stated in step (iii) above). It was kept there for 15 hr. and centrifuged for 15 min at 5000 rpm.

xi) The pellet was again resuspended in washing solution. After waiting for 2 hr. the suspension was centrifuged for 15 min. at 5000 rpm.

xii) To the pellet 3 drops of 2 percent of molten agar was added at 45° C and mixed carefully.

xiii) This was centrifuged at 5000 rpm for 2 min. at 50° C (hot water jacket).

xiv) The tube was cooled to solidify the agar.

xv) The agar containing bacteria was poured on a glass slide previously cleaned thoroughly. The solid agar was cut with a razor blade into 1 mm cubes and these cubes were put into 30 percent alcohol for 10 min.

xvi) The cubes were then transferred into 50 percent alcohol and kept for 10 min. This process was repeated once again.

xvii) These were then transferred to 70 percent alcohol and kept for 20 min. This step was also repeated once.

xviii) These were then put into 90 percent alcohol and kept for 15 min.

xix) The cubes were then put into 100 percent alcohol and kept there for 30 min. This was repeated once again.

xx) These were then put into propylene oxide for 30 min. This process was repeated once again.

xxi) These were put into Araldite (502) and propylene oxide mixture in the ratio of 1:1 for 30 min. with lid

xxii) These were then put into fresh Araldite propylene oxide mixture with lid intact for 30 min. and left at room temperature for overnight without lid.

xxiii) These were then put into Araldite mixture for 1 hr.

xxiv) Again these were put into fresh Araldite mixture and kept for 2 hr. (without DMP-30). Capsule was made with Araldite mixture containing DMP-30. The capsule was kept overnight at 40° C, kept 24 hr. at 45° C and then at 60° C for next 24 hr.

Sectioning and staining: Ultrathin silver grey sections were made with the help of LKB ultramicrotome. Thin sections were counterstained with uranyl acetate and lead citrate solution (137).

Electron microscopic observations: Cells were observed under Siemens Elmiskop 101B electron microscope using 80 KV. Photographs were taken at an instrumental magnification of 20,000X.

5.2 RESULTS AND DISCUSSION

It appears from the light microscopic photographs (Fig. 5.1) that pantothenate deficiency caused remarkable changes, namely, swelling and spherical to longitudinal extension of *S. faecalis* R 8043 cells and the effect was more marked in the case with the pantothenate-limited cells. These changes were also associated with declumping and dechaining of the cells. This effect was found to be less prominent in case of pantothenate-starved cells (photograph of *S. faecalis* 9790 only is presented, Fig. 5.2-c). Similar morphological changes also occured in case of pantothenate-deficient *S. faecalis* 9790 cells (Fig. 5.2). Shapes of pantothenate-limited cells (both *S. faecalis* R 8043 and *S. faecalis* 9790) varied from ellipsoidal to dumb-bell types. Further observations by D. N. Das *et al* (134) have shown that with further lowering of pantothenate concentrations (below 20 mμg/ml) division-inhibition and further lengthwise elongation of the *S. faecalis* cells took place.

Fig. 5.1 Light microscope photographs of normal and pantothenate-limited S. faecalis R ATCC NO. 8043. X1600. 'a' represents normal cells and 'b' represents pantothenate-limited cells, both harvested at about 200 AOD.

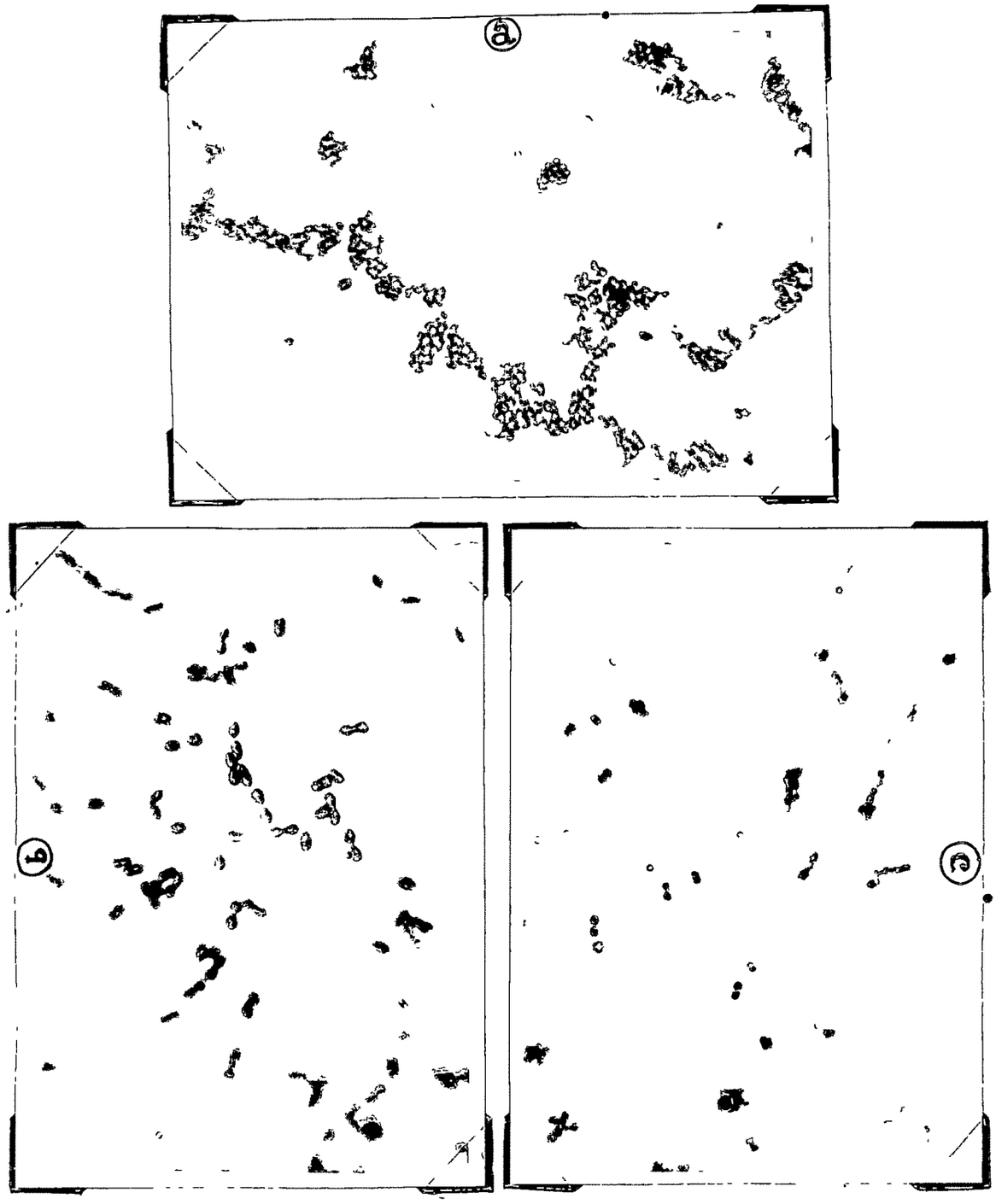


Fig. 5.2 Light microscope photographs of normal, pantothenate-limited and pantothenate-starved S. faecalis ATCC NO. 9790. 'a' represents normal, 'b' represents pantothenate-limited and 'c' represents pantothenate-starved cells. All the cells were harvested at 400 AOD.

To get the better perspective of the phenomena pantothenate-limited cells of S. faecalis were observed by scanning electron microscope (Figs. 5.3 and 5.4). The observations confirmed the light microscopic findings as reported above. Fig. 5.4 shows how gradual increase in the cell volume and change of shape of the cell occured along with growth (at 20 mμg pantothenate per ml) at different AOD levels. Similar morphological changes along with lowering of pantothenate concentrations in the medium, would also be very likely. Calculation of the volume changes using the formula $\frac{4}{3}\pi r^3$ for spherical shaped normal cells; and $\frac{4}{3}\pi ab^2$ (where a = $\frac{1}{2}$ of major axis, and b = $\frac{1}{2}$ of minor axis) for ellipsoidal shaped pantothenate-limited cell was made and it was found that the volume expansion of the pantothenate-limited cells (near about the peak-growth) was about 25 percent.

Counting experiments (Ch. 4, Fig. 4.3) indicated that pantothenate limitation caused significant lowering of viability and inhibition of cell division. Observations of chapter 2 indicated that pantothenate deficiency caused diminution of cell wall and membrane biosynthesis, the effect upon the latter being more prominent. The scanning pictures also indicated incompleteness of cell divisions (Fig. 5.4) in pantothenate-deficient conditions. All these morphological findings led us to undertake ultrastructural studies to evaluate the anatomical changes following pantothenate limitation.

Ultrastructural observations with transmission electron microscope are given in Figs. 5.5 and 5.6. It would be evident from these studies that pantothenate limitation caused the following prominent eff-

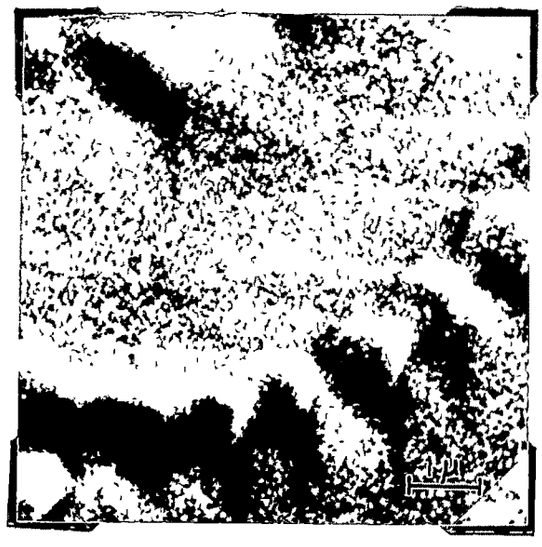


Fig. 5.3 Scanning electron micrograph of exponentially growing normal cells of S. faecalis R ATCC NO. 8043. The bar represents 1 μM X10,000.

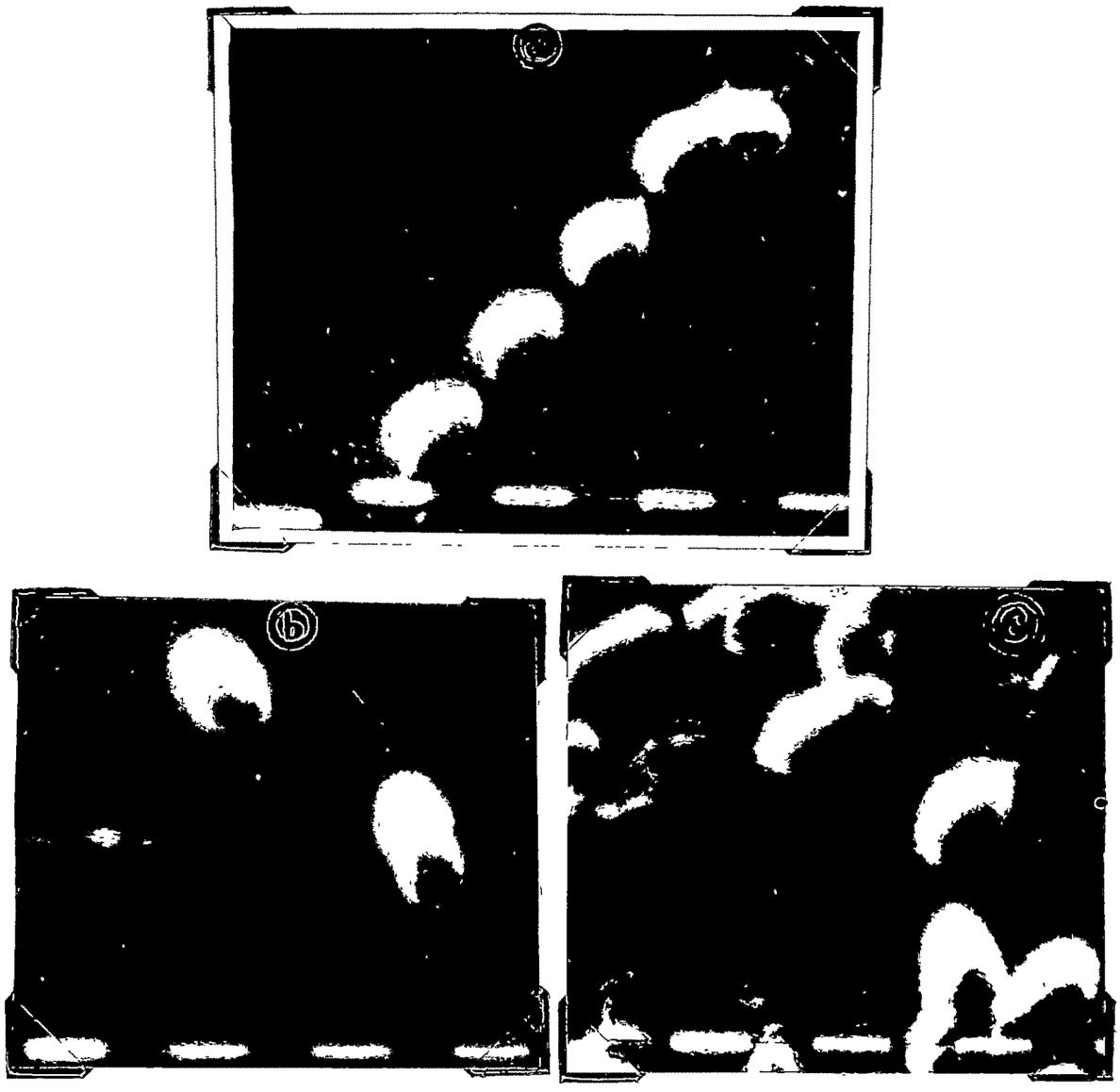
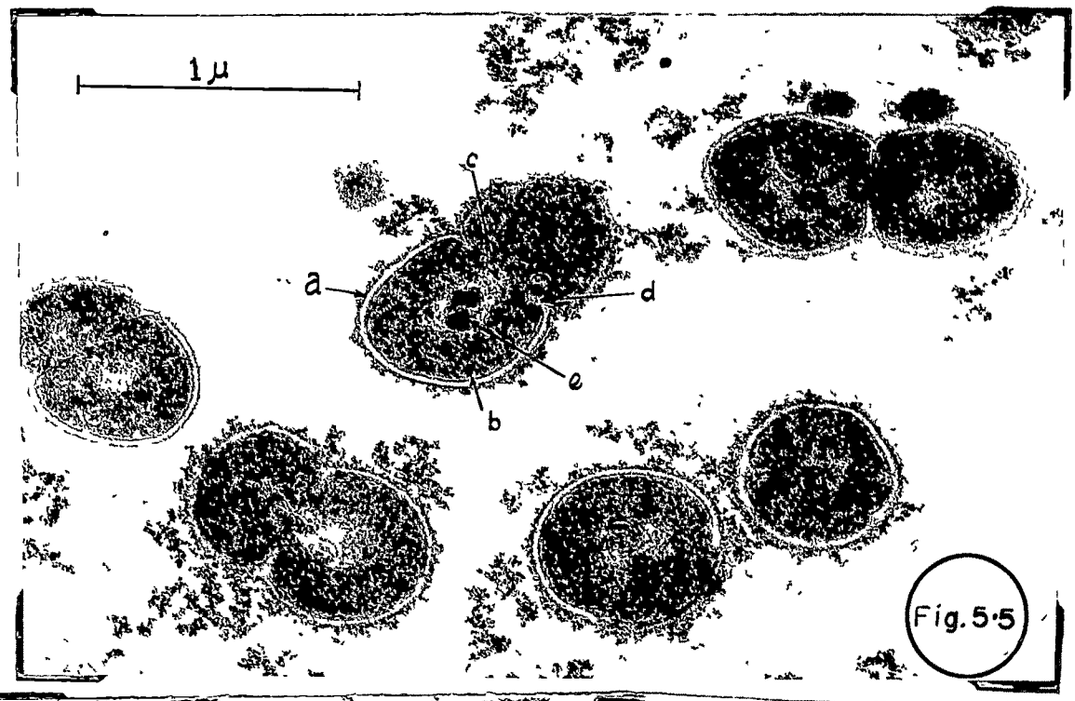
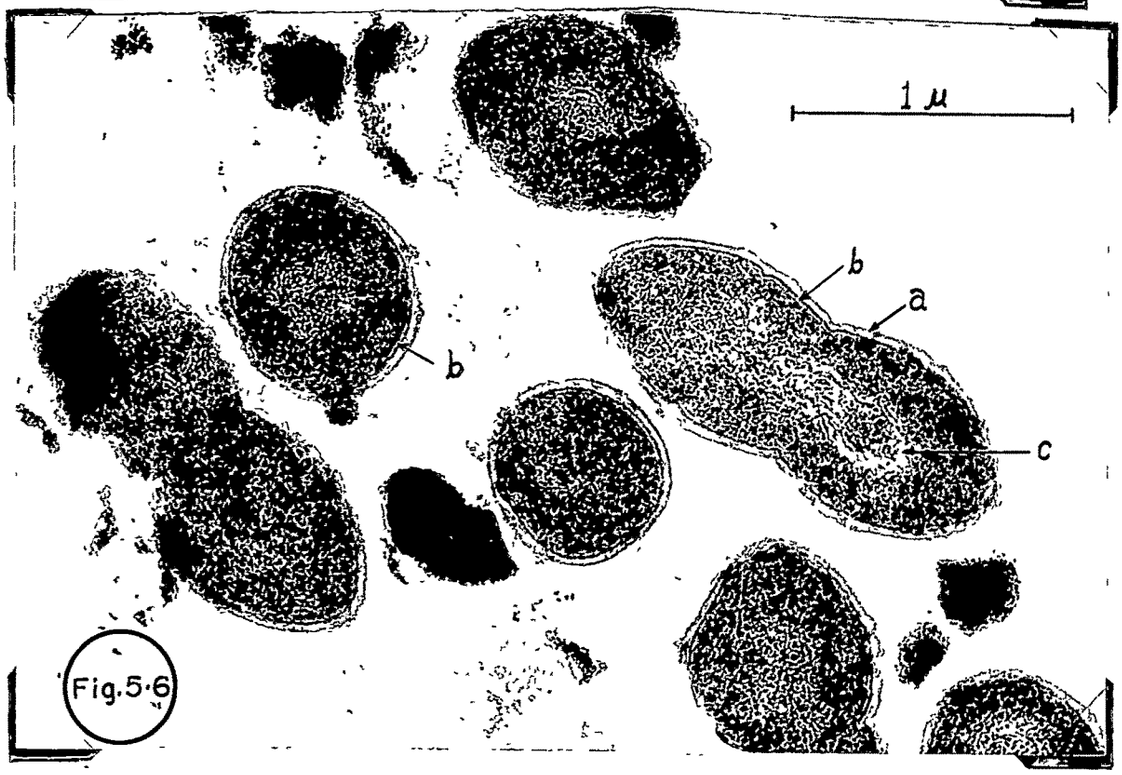


Fig. 5.4 Scanning electron micrographs of exponentially growing pantothenate-limited cells (at different AOD levels) of S. faecalis R ATCC NO. 8043. 'a', 'b' and 'c' represent pantothenate-limited cells at about 150, 300 and 600 AOD levels respectively. Each bar represents 1 μM. X10,000.





Figs. 5.5 & 5.6 Electron micrographs of thin sections of normal and pantothenate-limited cells of S. faecalis R ATCC NO. 8043. Fig. 5.5 represents normal and Fig. 5.6 represents pantothenate-limited cells. Arrows marked by 'a', 'b', 'c', 'd' and 'e' are cell wall, cytoplasmic membrane, nucleus, septum and mesosome respectively. The bar on each electron micrograph represents 1 μM. X40,000.

ects: (i) inhibition of septum formation; (ii) damage of peripheral walls and possibly also of cytoplasmic membrane; (iii) increase of cell size; (iv) inhibition of nuclear segregation; (v) thickening of cell wall (actual measurement by magnifying glass fitted with a scale was found to be about 15 percent thickening of wall).

Thickening of the cell wall compared to that of the normal as observed here is quite unexpected in view of our observations of gradual decrease of cell wall (hexosamines) biosynthesis in pantothenate-limited cells (Ch. 2, Fig. 2.1). This could be due to swelling of the cell wall itself because of loss of its compactness due to hydration. With the deficiency of pantothenate, hexosamines of the mucopeptide tends to be less acetylated causing gradual increase in the genesis of more of free amino groups in the position 2 of hexosamines (both glucosamine & muramic acid) residues of mucopolymers. It has been reported (107) that in the mucopeptide polymer, H atoms of the bulky imino groups in position 2 of a hexosamine residue are strongly hydrogen bonded with the carbonyl oxygen atoms in the position 2 of hexosamine residues of an adjacent chain of mucopeptide. Formation of this strong hydrogen bond was confirmed by I.R. studies (107). Due to pantothenate deficiency the production of unacetylated hexosamines is likely to lessen the chances of hydrogen bond formation between two lateral chains of the glycosaminopeptide. The bond length for hydrogen bond formation, if formed at all, between N-H and N-H (groups at position 2 of the hexosamine residues of the two peptidoglycan chains running parallel to each other) will be significantly larger and also much weaker in strength than that between N-H and C=O. Thus the lessening of the scope of hydro-

gen bond formation (i.e., decrease of compactness) at the said position as a result of lack of acetylation could weaken (reduction of tensile strength) the cell-wall structure very much. This will naturally cause loss of rigidity of the cell-wall structure of the pantothenate-deficient cells. This can satisfactorily explain the thickening and damage of cell-wall of the deficient cells (Fig. 5.6). However, further confirmation would need comparative gravimetric evaluation of isolated peptidoglycans of normal and pantothenate-limited cell walls.

The swelling of the pantothenate-deficient cells could be due to defective cytoplasmic membrane formation. Structural deformation of the 'unit membrane' following alteration (possibly both qualitatively and quantitatively) of lipid constituents is very likely to cause swelling (due to implosion of water from medium to the inside of the cell) and leakage of some nutrients and macromolecules (proteins and RNAs) as observed before (Ch. 4, Figs. 4.1 and 4.2). This has also been confirmed by Grula and Hopfer (111) working with a non-dividing Erwinia sp. The gradual swelling of the cytoplasm could produce excessive pressure upon the weak peripheral cell-wall and this could eventually lead to rupture and lysis of deficient cells as observed in the present investigations (Ch. 1, and this chapter).

The diminution of biosynthesis of cytoplasmic membrane (Ch. 2) and also possibly alteration in the lipid components might have caused the production of defective membrane in the pantothenate-deficient cells. The important functions of membrane inclusive of its role in cell division, cell wall and membrane synthesis have already been proposed.

Role of the membrane (mesosomal membrane) in nuclear division has been reported by Ryter (76). A large number of observations have documented the relationship between bacterial nucleus and plasma membrane. According to Ryter (76) mesosomal membrane is very importantly involved in bacterial nuclear division. Defective membrane as observed in pantothenate-deficient conditions reported here might have caused disfunctions and disappearance of the so-called mesosomal apparatus and caused thereby failure of nuclear segregation as reported here (Fig. 5.6). Grula et al (124-127) also reported secondary damage to plasma membrane in non-dividing filamentous cells. With lowered cell wall mucopeptide synthesis some filamentous cells can cause inhibition of cellular and nuclear division with no effects upon DNA synthesis. This supports our present observations reported here with S. faecalis.

5.3 SUMMARY

Studies have been made in regard to morphological and ultrastructural changes of pantothenate-deficient S. faecalis cells and following observations have been noted.

(i) Light microscopic studies with pantothenate-deficient cells showed that the deficiency of the vitamin caused the following effects; (a) cells were elongated and swelled, shape of the cells changed from spherical to ellipsoidal and dumb-bell forms with simultaneous de-chaining and declumping; (b) these changes were marked in the pantothenate-limited cells.

(ii) Studies with Scanning Electron Microscope also confirmed the light microscopic observations. It was found that in case of pantothenate-limited cells the cellular volume expanded considerably.

(iii) Ultrastructural examination of thin sections of pantothenate-limited and normal cells with Transmission Electron Microscope has been made. Electron micrograph indicated the following effects to be produced in pantothenate-limited cells: (a) inhibition of septum formation; (b) damage of peripheral cell-wall and also of cytoplasmic membrane; (c) increase of cell size (about three times longitudinally); (d) inhibition of nuclear division; and (e) thickening of cell wall.