CHAPTER 3

STUDIES ON THE AUTOLYTIC ACTIVITY AND SENSITIVITY OF PANTOTHENATE-DEFICIENT *STREPTOCOCCUS FAECALIS* TOWARDS PENICILLIN, CHLORAMPHENICOL AND LYSOZYME

It would appear from the results obtained in chapter 1 and 2 that pantothenate deficiency (starvation and limitation) causes severe curtailment of membrane synthesis and gradual but significant (upto about 16 percent) lowering of wall synthesis along with exponential growth (Fig. 2.1). Disorganisation or damage of membrane structure caused by curtailment of cell membrane synthesis during growth is likely to lead to leakiness of cell (leakiness resulting from the compositional changes of lipids) (110). Loss of some enzymes occurring within the periplasmic space and or bound to membrane and other factors and also derangement of some transport systems are quite likely under the situation (110, 111).

Importance of the integrity of the membrane structure in cellular protein synthesis has been amply stressed (112) and as such, it is very likely that disorganisation of cytoplasmic membrane might affect cellular protein contents. The functional significance of membrane bound ribosomes has yet to be clearly established, although role of plasma membrane in post-transcriptional processing of proteins destined for export is evident from the work of Lampen and his colleagues (112).

The most important enzymes namely D-alanine-carboxypeptidase and transpeptidase which are essentially needed for cross polymerisation of wall mucopeptide are located on the outside surface of the plasma membrane (58). These two enzymes are the prime targets of penicillin antibiotics. Dislocation of these two enzymes during curtailment of membrane synthesis (during exponential growth under pantothenate deficiency) might lead to change in the sensitivity of the cells towards peni-

cillins.

O-acetyl substituent has been shown to be important in governing the sensitivity of bacterial wall towards lysozyme in some mutant strains of Micrococcus lysodeikticus (114) but in others O-esters cannot account for the greater resistance of the walls to digestion with lysozyme. There are many other intriguing possibilities of chemical changes in the cell walls of bacteria leading to alteration of its sensitivity towards lysozyme.

Studies of Shockman and his colleague (102) with 1-C^{14}-acetate indicated that the acetyl groups (primarily N-acetyl groups) of hexosamine residues of peptidoglycan came from acetate of the growth medium, presumably through acetylation by cellular CoA. Naturally the question arose whether the change of acetyl content of hexosamines in pantothenate deficiency state also affected (a) the sensitivity of the cells towards lysozyme and (b) the 'built-in autolytic system' of the cells. Absence of N-acetyl groups on the glucosamine residues of the peptidoglycan has been reported (115) to produce lysozyme resistance in Bacillus cereus.

To answer the questions poised in the above discussion, comparative studies were undertaken with three types of cells i.e., normal, pantothenate-starved and pantothenate-limited cells, in order to determine their response towards (a) penicillin, (b) lysozyme and (c) the 'built-in autolytic enzyme system'. Studies on the response of these cultures towards chloramphenicol has also been included in the present investigation for interest. This chapter records the results obtained

with S. faecalis R ATCC NO. 8043 in the above directions.

3.1 MATERIALS AND METHODS

3.1.1 Bacterial strain and culture medium

Medium and other details followed were same as stated in chapter 1 and 2.

3.1.2 Preparation of pantothenate deficient cultures, growth conditions, and measurements of turbidity

All these were same as stated earlier (Ch. 1 and 2).

3.1.3 Reagents used

Sterile solutions (obtained by Millipore membrane filter, 0.45 μ pore size) of (1) lysozyme (Sigma Chemical Co., U.S.A., crystal), (2) penicillin (Crystaline Sodium penicillin - G, donated by Prof. S. Banerjee, Dey's Medical Stores, Calcutta,) and (3) chloramphenicol (donated by Prof. S. Banerjee, Dey's Medical Stores, Calcutta.) were used.

3.1.4 Measurement of lysis of cells

Techniques followed were almost the same as reported by Shockman et al (93). Details of culture collection, washings, and optical density measurements were as follows:

Appropriate volumes of cultures (approximately equivalent to 3 mg of dry cells) were harvested from log phase growth at different AOD levels and chilled in crushed ice. In all the cases, chilled cultures were rapidly filtered on a sterile membrane filter (0.45 μ pore size, Millipore membrane filter) and washed twice with sterile cold water (0 - 4° C). The membrane filter containing the cells in each case was removed to sterile culture tube containing 6 ml of sterile cold buffer (0.3 M phosphate) and the cells were detached from the filter by subjecting the whole tube with the filter over vortex mixer and continuing shaking for 30 sec. Membrane filter was removed aseptically. Turbidities of the suspensions (after incubation in a water bath at 38° C) were read in the colorimeter (Photochem Colorimeter C-110, red filter) at appropriate intervals. Samplings were done in such a way that the initial turbidities in all the cases were around 600 AOD per ml.

3.2 RESULTS AND DISCUSSION

From the Fig. 3.1. it is apparent that the rate of autolysis of exponentially growing normal and pantothenate-starved cells did not significantly alter at different harvesting AOD levels unlike the pantothenate-limited cells. In pantothenate-limited cells the rate of autolysis was found to be unusually fast (time for 50 percent reduction of AOD being 7.5 min.) at low harvesting level (i.e., 150 AOD) but at higher harvesting levels this rate was very much slowed down (times for 50 percent reduction of AOD at 300 AOD level being 54 min. and for 25 percent reduction of AOD at 600 AOD level of harvest being 101 min.).

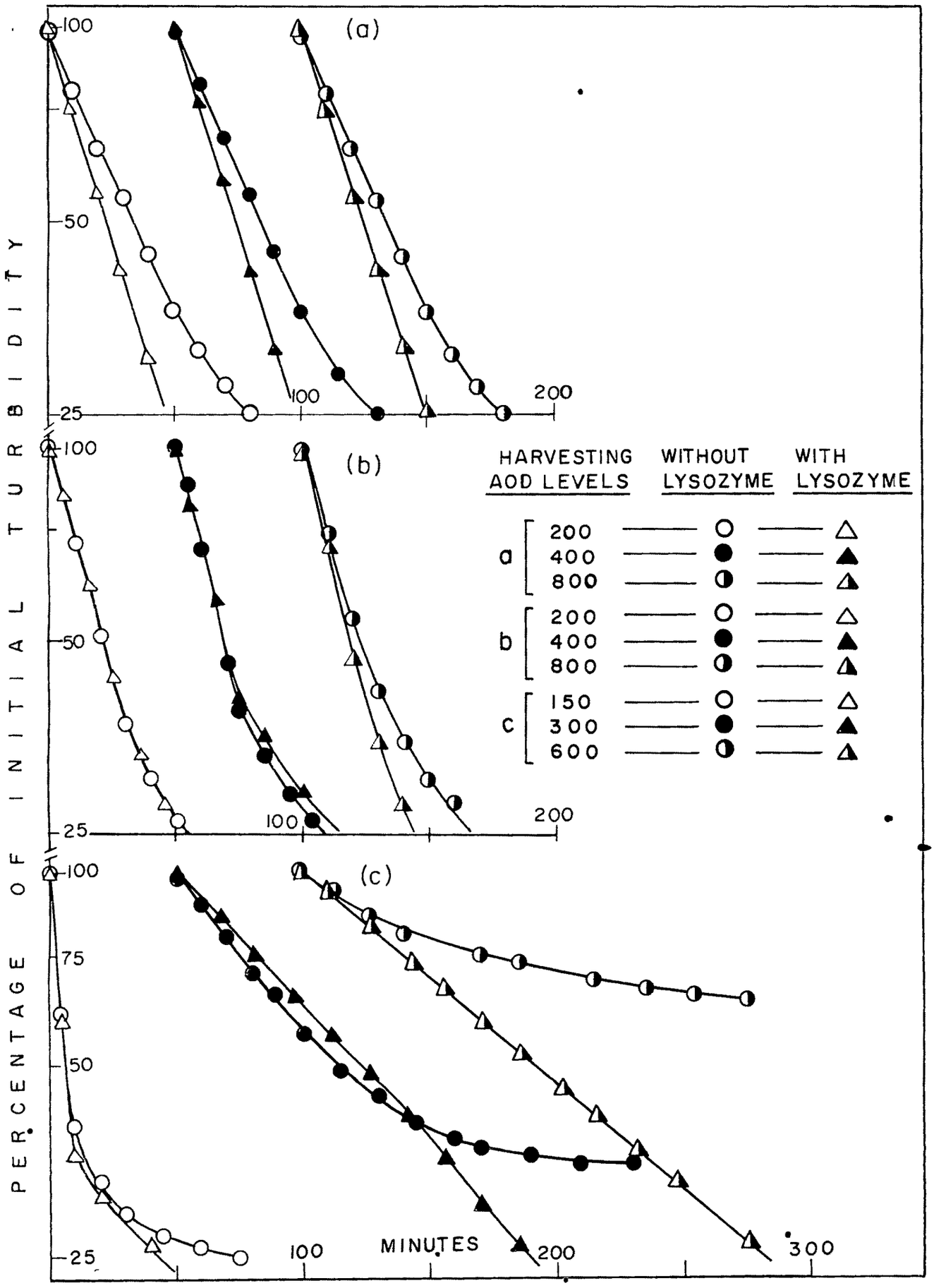
PERCENTAGE OF INITIAL TURBIDITY
100
75
50
25
(a)
(b)
(c)
100
200
300
MINUTES
HARVESTING AOD LEVELS
WITHOUT LYSOZYME
WITH LYSOZYME
a
200
400
800
b
200
400
800
c
150
300
600

FIG. 3·1

Again at low AOD (150 AOD) level in pantothenate-limited culture the rate of autolysis was found to be much faster than those of the normal and pantothenate-starved cells. Results also indicated that the rate of autolysis of pantothenate-starved cells was significantly higher than that of normal cells.

Lysozyme enhanced the rate of lysis in normal culture as expected (Fig. 3.1-a). But lysozyme addition had almost no effect on the lysis of pantothenate-starved culture (Fig. 3.1-b). In case of pantothenate-limited culture (Fig. 3.1-c) the effect of lysozyme addition varied at different AOD levels of exponential growth (upto peak-growth). At AOD 150, lysozyme addition had no significant change upon the rate of autolysis. At AOD 300, addition of lysozyme to pantothenate-limited cells did not seem to produce any significant effect upto 60 percent reduction of initial turbidity. Towards the end of exponential growth phase of pantothenate-limited cells lysozyme enhanced significantly the rate of lysis.

Holden et al (115) studied the lysozyme sensitivity of Lactobacillus plantarum, normal and pantothenate-deficient cells. They found that the lysozyme sensitivity reduced at different AOD levels of normally growing cells unlike our observations with S. faecalis R 8043. They also reported the lysozyme sensitivity to be markedly increased in pantothenate-limited culture agreeing with our observations reported here with pantothenate-limited S. faecalis cells at low AOD (150 AOD) level and to some extent in pantothenate-starved cells. They did not, however, report the rate of autolysis of the above cells and observed the

pantothenate-deficient cells at only one harvesting level and the level of pantothenate was very low (5 mμg/ml compared to 20 mμg/ml in our experiment). The disagreements between our observations and those of Holden could be due to the use of different organisms and somewhat different conditions (different pantothenate levels and harvesting AOD levels). Holden et al (115) correlated the increase of lysozyme sensitivity of L. plantarum with decline of O-acyl content of the cells. Findings of Dezelee and Shockman (102) with S. faecalis with radio active C^{14} acetate suggested that the C^{14} acetate contributed primarily to N-acetyl at position 2 of the cell wall hexosamines. Some incorporation also was found at O-acetyl group. Acetyl transfer is known to be dependent on pantothenate in the form of CoA or ACP. In pantothenate-deficient culture both these acetyl contents are expected to be low.

It is to be further noted that in the case of pantothenate-starved culture the 'built-in autolytic system' was so altered as to increase its rate of autolysis. Obtention of increase in resistance of the pantothenate-limited cells (beyond 150 AOD) towards both lysozyme and its own 'built-in autolytic system' is of interest to note. Araki et al (114) also reported from experiments with both whole cells and isolated cell-wall of B. cereus that removal or absence of N-acetyl groups on glucosamine residues of the glycan increased resistance to lysozyme. Marked lagging in membrane synthesis and gradual decrease in wall synthesis as noted in chapter 2, in case of pantothenate-limited culture, are very likely to produce membrane damage and leakiness. Leakiness under this condition has already been reported by Toennies et al (49). Membrane damage accompanied with leakage of some essential

factors in pantothenate-limited cultures could affect the 'built-in autolytic system' of the organism as reported here (Fig. 3.1). The comparative insensitiveness of pantothenate-limited cells to lysozyme and its own 'built-in autolytic system' might also be due to orientation (accompanying membrane and cell wall damage) of some specific sites needed for lysozyme or autolytic actions. The differences in regard to autolysis rates of pantothenate-starved and pantothenate-limited cells are to be noted (Fig. 3.1). These can not be explained satisfactorily until and unless further work be done with purified cell wall and membrane of these two types of deficient cells. It seems that the 'built-in autolytic system' like lysozyme may also be dependent on various probable factors like ratio of amino sugars, number of disaccharide units, amount of O-acetyl and N-acetyl contents, branching points, frequency of cross-linkage between the glycopeptide residues and amino acid substituents attached to muramic acid. Holden et al (115) reported that lytic and binding reactions between L. plantarum and muramidase were markedly dependent on the physiological age of the cell and were enhanced by pantothenic acid deficiency.

Results in the Fig. 3.2 shows that the addition of penicillin caused lysis at various AOD levels and addition of chloramphenicol caused protracted growth in both types of the pantothenate-deficient cells as in the normal cells. These observations indicated that cell wall and protein synthesis were both continuing in the vitamin-deficient cells. These data, however, did not reveal any difference in regard to rates of synthesis of the macromolecules which could be affected under

LEGEND OF FIG. 3.2

Effects of addition of penicillin and chloramphenicol on *S. faecalis* R ATCC NO. 8043 growing exponentially. 'a', indicates normal cells; 'b', indicates pantothenate-starved cells; and 'c', indicates pantothenate-limited cells. The arrows indicate the addition of antibiotics. Symbols: O, represent growth curve of the control; ●, represents growth curve after addition of penicillin; Δ, represents growth curve after addition of chloramphenicol. Minutes shown on the descending parts of the curves are half-times of lysis after addition of penicillin. Concentrations of penicillin and chloramphenicol were 20 and 10 μg/ml respectively in the final culture medium.

membrane deficient conditions. Half-times of lysis as observed both for normal and deficient cells here (Fig. 3.2) did not seem to indicate any significant correlation between the different cell types. In pantothenate-deficient cells (both types) addition of chloramphenicol near the peak AOD showed to have no significant influence upon the rate of lysis of the cultures. This indicated that the lysis observed was not an active process and did not require the synthesis of proteins (enzymes) as reported by U. Henning (110) who worked with an oleate-starved fatty acid auxotroph of E. coli K-12.

3.3 SUMMARY

Studies have been made in regard to autolysis and responses towards lysozyme, penicillin and chloramphenicol with S. faecalis R ATCC NO. 8043 and the results obtained were as follows:

(i) Rate of autolysis of normal and pantothenate-starved cells did not alter significantly at different AOD levels of exponential growth. Rate of autolysis of pantothenate-starved cells was found to be significantly higher than that of the normal cells. At low AOD level (150 AOD) autolysis rate of pantothenate-limited cells was much higher compared to those of other two types of cultures. With increase of AOD the rate of autolysis of this type of cells decreased remarkably.

(ii) Addition of lysozyme had no significant effect on the lysis rate of pantothenate-starved culture. In case of pantothenate-limited

culture addition of lysozyme significantly enhanced the rate of autolysis of these cells only towards the end of exponential growth phase. Lysozyme addition did not seem to produce any significant effect on the autolysis of pantothenate-limited cells upto its mid-log phase of growth.

(iii) Addition of penicillin caused lysis at various AOD levels of pantothenate-deficient cells as in normal cells. Addition of chloramphenicol caused protracted (non-exponential) growth. These observations indicated that cell wall and protein synthetic systems were both active in pantothenate-deficient conditions. Lysis observed in pantothenate-deficient cells was not affected by the addition of chloramphenicol. This indicated that lysis observed in the pantothenate-deficient cells was not an active process and did not require the synthesis of enzymes.