CHAPTER 4

STUDIES ON THE VIABILITY OF AND MACROMOLECULAR SYNTHESIS IN *STREPTOCOCCUS FAECALIS* GROWN UNDER PANTOTHENATE-DEFICIENT CONDITIONS

Importance of cytoplasmic membrane in cellular macromolecular synthesis and nuclear division has been amply stressed (108,112). Reusch and Burger (117) and Ryter (76) discussed the important role of cytoplasmic membrane (inclusive mesosomes) in cell division, cell envelope synthesis, energy metabolism in bacteria. Henning et al (110) showed how macromolecular synthesis e.g., DNA, RNA, protein and phospholipid synthesis stopped leading to cellular death in an exponential way in an oleate-auxotroph of E. coli when oleate was removed from the growth medium. They attributed most effects to be due to defective de novo membrane synthesis with altered phospholipid composition.

Leakage and damage of cell membrane could naturally lead to loss of membrane bound enzymes (109 - 111) and other important nutrients. Pantothenate deficiency has been reported to cause leakiness of S. faecalis (49). Krystyna Myszkowskar (52) reported deficiency of pantothenate to cause lowering of cellular proteins in Lactobacillus arabinosus. Presumably, the effect is due to abnormal membrane formed in the vitamin-deficient cells. Neither CoA nor ACP is known to be involved in protein or nucleic acid synthesis. Puddu et al (56) reported that pantothenate deficiency in rats did not affect the DNA and RNA content of liver per unit weight.

Pantothenate deficiency has been reported to cause marked loss of cellular CoA in S. faecalis (49). This might lead to alteration and or change of membrane-lipids affecting membrane functions adversely (118).

As far as is known cell wall is not involved in any macromolecular synthesis like the cytoplasmic membrane as stated above. Its function is more of mechanical in nature i.e., giving protection to the cytoplasmic membrane which it encloses. It has been reported (109), however, removal of cell wall by lysozyme (in an osmotically protected medium), prevention or interference of cell wall formation by some antibiotics like penicillin, cycloserine or similar other agents and starvation of cell wall precursors like hexosamines (N-acetylhexosamine and N-acetylmuramic acid) in the growth medium lead to impairment of septation process and unbalanced growth and abnormal developement. (aberrant forms). Starvation of growth factors which are needed preferentially for cell wall and cytoplasmic membrane synthesis may lead to unbalanced growth and abnormal developement (109).

Results of studies in the chapter 3 showed that in pantothenate deficiency the cell membrane and cell wall were both effected, though the effects upon the former being more marked. These observations and the considerations of the importance of these two integumental structures led us to undertake the present investigations. Results of our studies on the macromolecular synthesis (DNA, RNA and protein) and viability of S. faecalis R 8043 under different pantothenate nutritions are reported in this chapter.

4.1 MATERIALS AND METHODS

4.1.1 Preparation of different cultures

Normal, pantothenate-limited and pantothenate-starved cultures of both S. faecalis R 8043 and S. faecalis 9790 were grown as reported earlier. Necessary amounts of cultures were collected at different AOD levels of exponential growth phase, washed twice with 0.85 percent saline at 0 - 4° C and the pellets obtained were used for the estimation of RNA, DNA and protein.

4.1.2 RNA Estimation by Orcinol method (119)

Reagents used:

i) Recrystallized orcinol (10 gm) was dissolved in ethanol (100 ml).

ii) $FeCl_3.6H_2O$ (0.1 gm) was dissolved in concentrated HCL (100 ml).

iii) Perchloric acid (PCA) (0.5 N)

iv) Standard RNA solution: 5 mg of yeast RNA (Sigma chemical Co., U.S.A.) dissolved in 0.5 N PCA and then 1 ml diluted to 5 ml to obtain 200 µg/ml RNA solution. This was then heated at 90° C for 20 min.

Appropriate volumes of growing cultures (equivalent to about 4 mg dry cells) of normal, pantothenate-starved and pantothenate-limited cells were chilled and centrifuged at 0 - 4° C at 6000 rpm for 15 min. These were washed twice with 0.85 percent saline. Cold extracts of cultures in 5 ml of 0.5 N of PCA showed no detectable amount of RNA and hence hot extraction method was applied. The pellet obtained after cold

centrifugation in each case was extracted with 5 ml of 0.5 N PCA at 90° C for 20 min. and the hydrolysate was used for determination of RNA.

PCA extracts (0.0 - 0.5 ml) of the samples of RNA solutions were taken and volume in each case was made upto 0.5 ml with 0.5 N PCA. 2.5 ml of water was added to each tube. Later 0.3 ml of orcinol reagent, 3.0 ml of $FeCl_3$ reagent were added one after another to each tube, mixed over vortex and then heated on a boiling water bath for 45 min. Tubes were cooled, deep green color developed. Color values were measured at 670 mμ in a Beckman Spectrophotometer Model DB1402. RNA values of the experimental samples were determined from the standard curve of RNA. Average of four identical samples were calculated for plotting in the graph (Figs. 4.1 and 4.2).

RNA values of samples obtained were checked by subtracting their respective DNA values from the total values obtained by U.V. absorption methods stated later.

4.1.3 DNA Estimation by Diphenylamine reaction method (120, 121)

Reagents used:

i) Diphenylamine reagent: This was prepared by dissolving 1.5 gm of steam distilled diphenylamine in 100 ml of redistilled (A.R.) acetic acid and adding 1.5 ml of conc. H_2SO_4. The reagent was stored in the dark. On the day of its use 0.1 ml of aqueous redistilled acetaldehyde (A.R. 10 mg/ml) was added to each 20 ml of the reagent required.

ii) 1 N PCA and 0.5 N PCA: This was prepared by appropriate dilution of PCA stock solution.

iii) Deoxyribose (DOR) standard: This was a 10 µg/ml of DOR solution in 0.5 N PCA.

Growing cells equivalent to 6 mg of dry weight were chilled and washed with 0.85 percent saline. The pellet obtained was suspended in 1.0 ml cold water and 1.0 ml of 1 N PCA was added and mixed over vortex and centrifuged. As no DNA could be detected in the cold extracts hot extraction with PCA was followed. To the pellet of cells 3.0 ml of 1 N PCA was added and digestion (70° C) followed for 30 min. Extracts cooled and 3.0 ml water was added to each. The supernatants of the extracts obtained by centrifugation were used for color developement.

From each extract 0.5 ml, 1.0 ml, 1.5 ml, and 2.0 ml were used for color reaction. Volume of each extract was made upto 2 ml with 0.5 N PCA and 4 ml DPA reagent was added to it. Readings were taken at 600 mµ in a Beckman Spectrophotometer Model DB1402 after 18 hr. DOR values were determined by comparing with the color values of identically treated standard series and were converted to DNA equivalents by multiplying by 4.62.

4.1.4 Total Nucleic acid Estimation by U.V. absorption method (122)

Chilled growing culture (equivalent to about 5 mg of dry cells) was spun down and washed with 0.85 percent saline and pellet was resuspended in 6 ml of 0.5 N PCA. Optimal temperature and time for total nucleic acid extraction was found out to be 90° C and 20 min. respectively. PCA suspension extracts (90° C, 20 min) were cooled, centrifuged. Supernate was used for U.V. absorption measurements as stated below.

U.V. absorption measurement: To each 10 ml of 0.5 N PCA extract of the sample 5 ml of 0.5 N PCA was added. Absorption readings were taken at 270 mμ and 290 mμ in a Beckman Spectrophotometer Model DU4800. Total nucleic acid was determined by using following equation:

$$\text{Total nucleic acid content (microgram)} = 10.3 \times \frac{D270 - D290}{0.19}$$

4.1.5 Protein Estimation (119)

Reagents used:

Reagent A: i) Sodium potassium tartrate - 3.38 gm was dissolved in water and volume made upto 100 ml.

ii) $CuSO_4.5H_2O$ 1.25 gm was dissolved in 100 ml of water.

2 ml of (i) and 2 ml of (ii) were mixed and the final volume made upto 200 ml with 2.5 percent sodium carbonate solution.

Reagent B: Folin Ceacalteu reagent - Commercially available. Folin Ceacalteu reagent was found to be 1.8 N in acetic acid. This was diluted with water to make 0.5 N in acetic acid.

Preparation of standard curve of B.S.A.: To 4 mg of crystalline bovine serum albumin (BSA, Sigma Chemical Co., U.S.A.) 4 ml of 0.5 N NaOH was added and mixed to obtain 1000 μg/ml BSA solution. 1.0 ml of this solution was diluted with 0.5 N NaOH to have 100 μg/ml BSA solution. Tubes were prepared containing 0 μg to 100 μg BSA and volume in each case was made upto 1.0 ml with 0.5 N NaOH. Reagent A, 4 ml was added to each tube and the contents were mixed over vortex and allowed to

stand for 100 min. Then 1.0 ml of freshly prepared Reagent B was added and mixed thoroughly over vortex for few seconds and the whole was allowed to stand for 30 min. blue color was developed and color readings were taken at 760 mμ in a Beckman Spectrophotometer Model DB1402.

Experimental procedure: Growing cultures (equivalent to 1 - 2 mg of dry cells) were chilled, spun down and washed twice with same volume of saline. To each of the pellet was added 0.1 ml of 20 μg/ml lysozyme. Lysozyme incubation was followed at 38° C for 24 hr. From the final result of protein values protein added as lysozyme was deducted. To each tube containing the lysozyme-digest was added 2.0 ml of 0.5 N NaOH. To 1.0 ml of the extract thus obtained 9.0 ml of 0.5 N NaOH was added. Final extracts, 0.0 ml to 1.0 ml, were taken in different tubes and volume in each was made upto 1.0 ml with 0.5 N NaOH. Identical procedures were followed for color developement and OD measurements as in the standard series. Protein values of the samples were calculated from the standard curve of BSA.

4.1.6 Viable counting (pour plate method)

One ml of chilled cultures harvested at different AOD levels was diluted with sterile 0.85 percent saline to AOD at about 100. These 100-AOD cultures were then diluted 10^2, 10^4, 10^5, 10^6 times serially with the sterile saline. Then 1.0 ml of the diluted cultures from each of 10^{-5} and 10^{-6} dilutions was plated (in triplicate). The finished complete synthetic medium sterilized by filtration and solidified with sterile agar (at a final level of 1.5 percent) was used for plating. Counting of the colonies was done after 24 hr. incubation (incubation upto

48 hr did not increase the counts) at 38° C. Count per ml was determined from each of the triplicate plates of different dilutions and the average was calculated for plotting the results (Fig. 4.3).

4.1.7 Total Microscopic count (modified Breed's method)

When we failed to count the cells directly by using Haemocytometer because of the very small size and motility of the cells we used Breed's method with some modifications. A definite small volume of the culture was spread carefully and uniformly over a small area on a clean slide. After fixing, staining and washing, the cells were counted under oil-immersion microscope using a standardized eye-piece micrometer. We found among various dyes Loeffler's methylene blue (113) to be most satisfactory for the counting of the cells. Details followed are stated below.

Chilled cultures harvested at different AOD levels were diluted quantitatively with saline to AOD about 100. With a micro-capillary pipette 20 μ of the diluted culture was spread on 4 sq.cm. area of the glass slide. Spreading was made as uniform as possible with the inoculating needle. The cells were then fixed by drying the slide under an electric lamp (bulb 100 watt, from a distance of 15 cm for 2 - 3 min.). The slide was next allowed to cool to room temperature. Methylene blue dye (123) was used to stain the smear for 2 - 3 min. Excess dye was washed off carefully by dropwise addition of tap water from a pasteur pipette. After air drying counting was done with a Karl-zeiss Microscope under oil-immersion lense (x100) and an eye-piece (x15) using a standardized eye-piece micrometer disc.

4.3 RESULTS* AND DISCUSSION

Results of DNA, RNA and protein content (per unit weight of dry cells) of growing cells of different types are given in Fig. 4.1. It would be apparent from Fig. 4.1-a that the content of DNA was not affected by pantothenate deficiency (in both pantothenate-limited and pantothenate-starved cultures) throughout the AOD levels observed. RNA content (Fig. 4.1-b) of pantothenate-starved cells was not significantly different upto about 600 AOD, later on, however, it showed some tendency to fall gradually. In pantothenate-limited culture RNA content was not affected significantly upto 450 AOD and later on, however, it showed somewhat downward tendency. Results (Fig. 4.1-c) further showed that cellular protein content was not at all affected by pantothenate starvation. In case of pantothenate limitation protein content did not seem to alter significantly upto about 200 AOD. Later, this gradually diminished and reached a value of about 16 percent lesser (around the peak AOD) than that of normal culture

Growth rates and rates of macromolecular synthesis of the above three types of cultures are shown in the Fig. 4.2. It appears from Fig. 4.2 that the rates of DNA synthesis of the pantothenate-deficient (Pantothenate-limited and pantothenate-starved) cultures did not seem to be significantly different from that of the normal. Rates of RNA synthesis (Fig. 4.2-b) in pantothenate-limited cultures was not significantly changed upto about 2½ generations (at 600 AOD) but later on the synthesis rate began to slow down. In case of pantothenate-limited culture the rate of RNA synthesis appeared to be slightly slower and this further

*As similar results were obtained with both the cultures used, results of *S. faecalis* R ATCC NO. 8043 are only presented here.

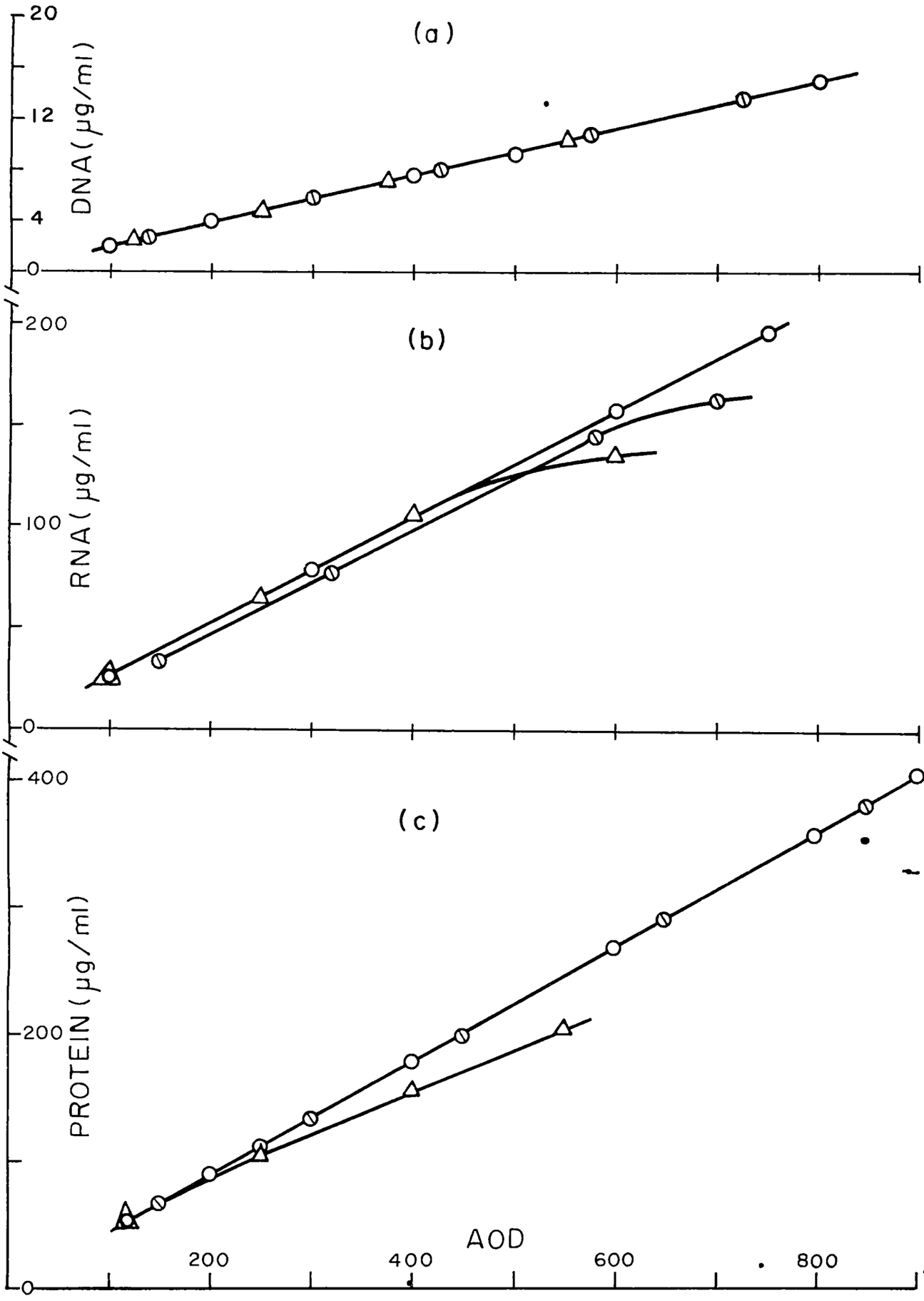
(a)
DNA (µg/ml)
20
12
4
0
(b)
RNA (µg/ml)
200
100
0
(c)
PROTEIN (µg/ml)
400
200
0
AOD
200
400
600
800

Fig. 4·1

LEGEND OF FIG. 4.2

Growth response, synthesis of DNA, RNA and Protein of differ cell types of *S. faecalis* R ATCC NO. 8043. 'a', represents growth c 'b', synthesis of DNA; 'c', synthesis of RNA; 'd', synthesis of pro For clarity the curves have been separated by spaces corresponding 25 min. in 'a' and 'c', and 10 min. in 'b' and 'd' on the time scal Symbols: O , represents normal cells; ϴ, represents pantothenate-st cells; and Δ , represents pantothenate-limited cells. Times recorde after three different cultures attained AOD 100 (approx.). Average four identical samples were calculated in each case for plotting the graph.

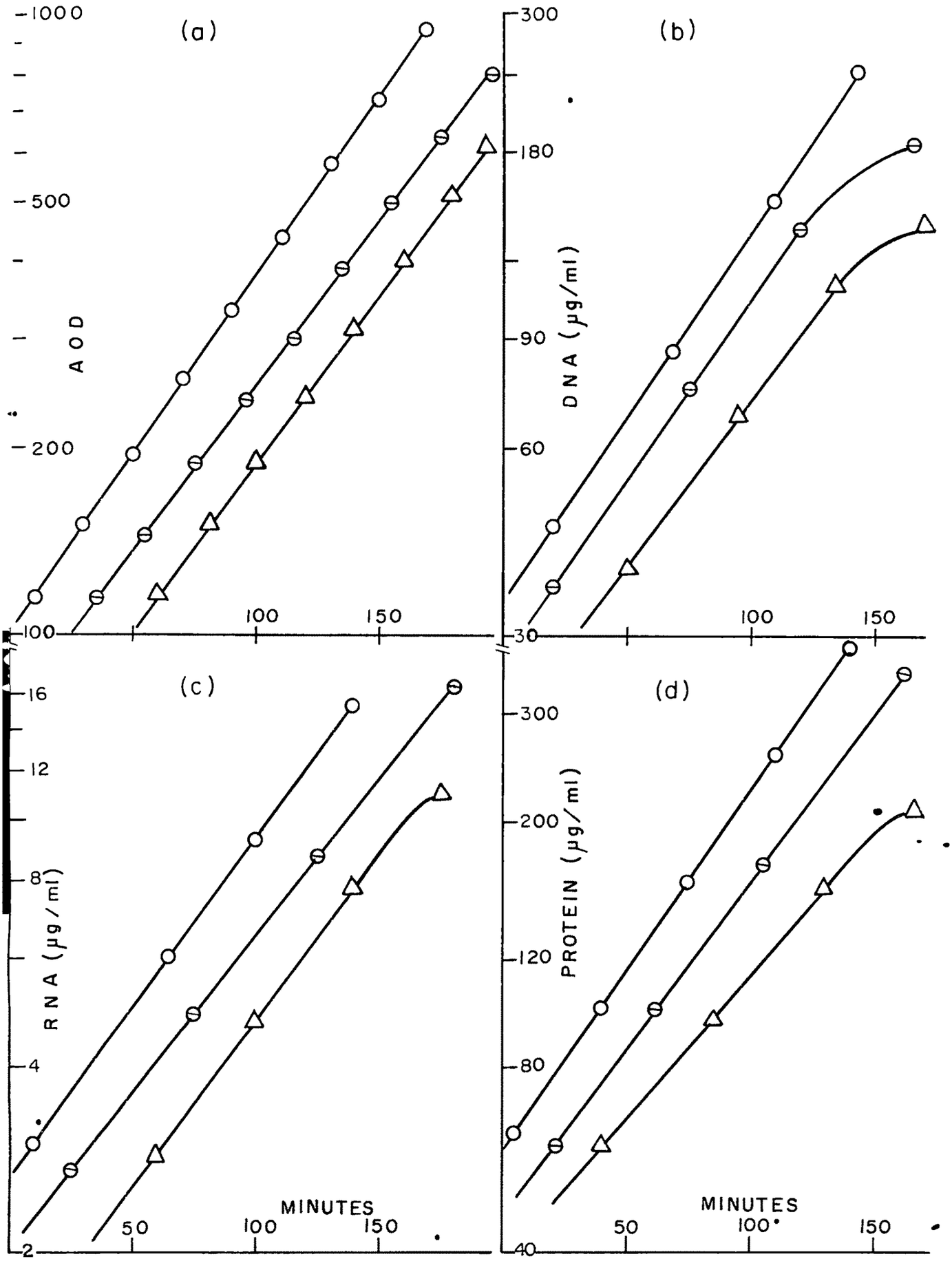
(a)
AOD
1000
500
200
100
150
(b)
DNA (µg/ml)
300
180
90
60
30
100
150
(c)
RNA (µg/ml)
16
12
8
4
2
MINUTES
50
100
150
(d)
PROTEIN (µg/ml)
300
200
120
80
40
MINUTES
50
100
150

FIG. 4·2

lowered down towards the peak-growth. Pantothenate starvation did not appear to make significant difference in the rate of protein synthesis compared to that of normal. Pantothenate-limited culture, however, showed significantly lowered rate of protein synthesis (Fig. 4.2-c).

Importance of bacterial cytoplasmic membrane in its growth, developement, metabolism, transport, division (74,108) and survival (110) is already known. Observations of chapter 2-experiments of this thesis showed that inspite of the membrane biosynthesis being severely curtailed or much diminished in pantothenate deficiency, the cells still grew further exponentially to a considerable extent. Observations of the growth under defective membrane biosynthesis naturally raised some questions. Was this growth normal? Were these cells still dividing? Was the viability of the deficient cells affected during the exponential growth? In an attempt to answer these questions counting experiments (Fig. 4.3) were undertaken with pantothenate-deficient cells.

Results (Fig. 4.3-a) indicated lower microscopic counts in case of pantothenate-deficient cells throughout the exponential growth. This was more marked (about 80 percent lower microscopic counts compared to that of the normal)in case of pantothenate-limited cells. This drop in counts, perhaps was due to lysis of cells caused by the defective membrane (and also of wall) biosynthesis in such cultures. It is further to be noted that in case of pantothenate-limited culture, microscopic count, after about 250 - 300 AOD levels of growth, tended to come to a plateau indicating thereby significant inhibition of cell divisions in such culture.

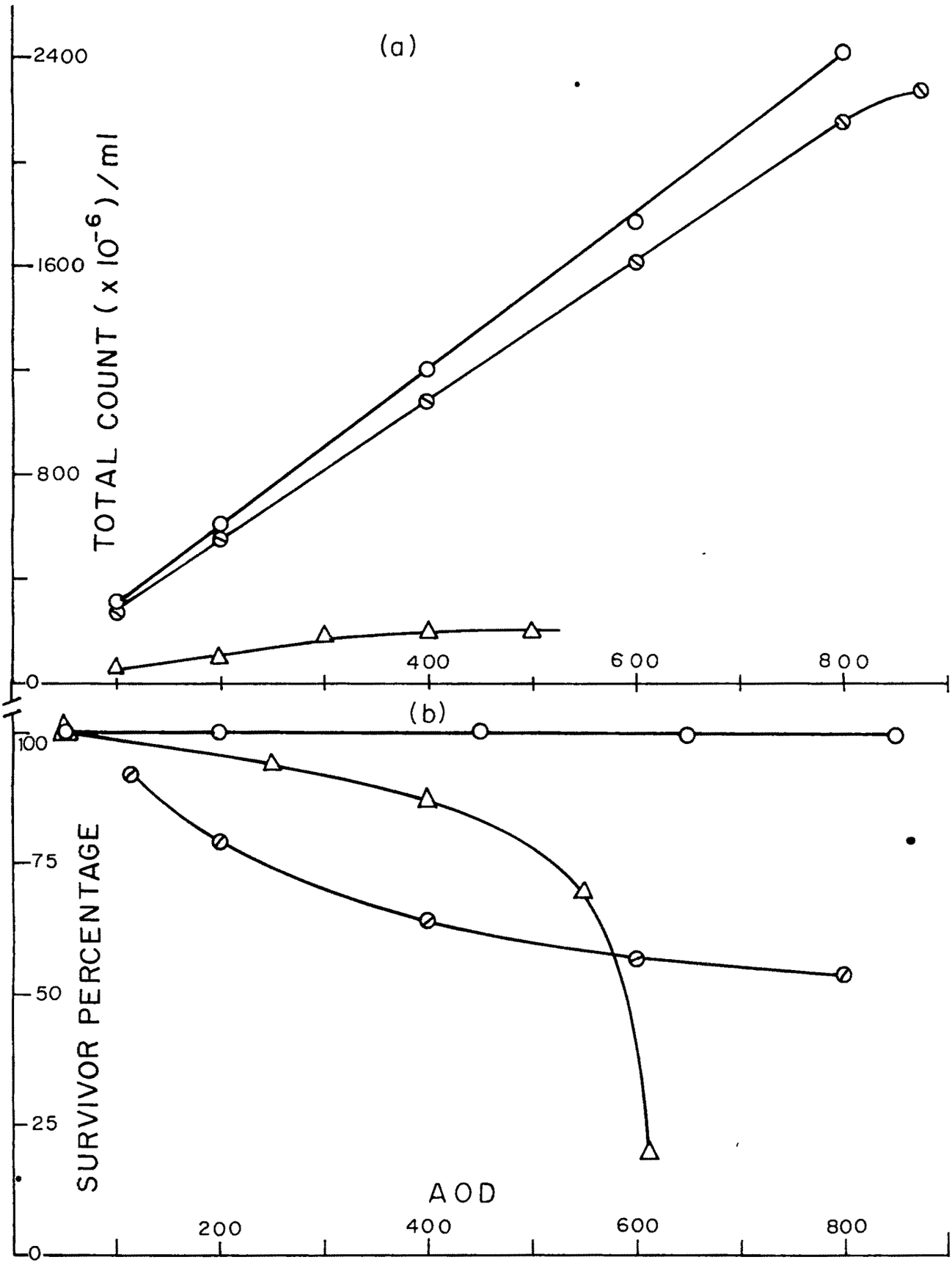
(a)
2400
1600
800
0
TOTAL COUNT (x 10⁻⁶) / ml
400
600
800
(b)
100
75
50
25
0
SURVIVOR PERCENTAGE
AOD
200
400
600
800

FIG. 4·3

Results (Fig. 4.3-a) would indicate that pantothenate deficiency caused significant inhibition of the cell division. Division-inhibition effect showed to be very marked in case of pantothenate-limited cells unlike the pantothenate-starved cells. Pantothenate-limited cells seemed to divide upto about 300 AOD, but later on the cell division activity almost came to a plateau.

Results in Fig. 4.3-b showed that pantothenate deficiency caused very significant lowering of the percentage of survivors (i.e., colony formers). The percentage of colony formers dropped gradually upto about 50 percent in case of pantothenate-starved cells and 20 percent in case of pantothenate-limited cells at about 500 AOD. The fall in percentage survivors in case of pantothenate-limited culture was very steep later (after about 500 AOD),

The significant drop in the viability (survival) of deficient cells starting from about 100 AOD and continuing till late log phase is evidently to be correlated more with the significant drop in the membrane biosynthesis than that of the wall. King and Grula et al (124,125) also stressed the fact that cell wall and cell membrane, both are intimately involved in cell division process. They held cell membrane to be more important of the two structures for the purpose.

Grula et al (126,127) reported that some agents like penicillin, vancomycin, D-serine, UV, etc. which affect mucopeptide synthesis in Enterobacteriaceae including Erwinia species caused inhibition of septum formation (inducing filament formation in such cells), but did not affect significantly macromolecular synthesis of DNA, RNA and protein.

Our present observations with gram-positive organism (i.e., S. faecalis), pantothenate deficiency appeared to affect mucopeptide synthesis and cellular division but with no significant effect upon DNA synthesis. Whether this observation is true for other pantothenate-auxotroph gram-positive organisms remains to be seen.

It would be evident from the above discussions that deficiency of pantothenate caused more pronounced effect in regard to viability and cell division-inhibition than its effect upon the rates of macromolecular synthesis. These effects seem to run parallel with the severe curtailment or diminution of membrane phospholipid biosynthesis (Ch. 2, Fig. 2.1). This, it appears, might have led to defective de novo membrane synthesis (quantitatively and qualitatively). Henning et al (110) also reported defective membrane biosynthesis to be responsible for inhibition of macromolecular biosynthesis and death in case of oleate (one class of building block for the cell membrane) starvation in a fatty acid auxotroph of E. coli K-12. They further noted that the oleate-starved cells tended to lyse in an attempt to synthesise cytoplasmic membrane with altered phospholipid composition or during phospholipid deficiency.

The somewhat lowered rates in RNA and protein synthesis as observed in case of low-pantothenate (Figs. 4.2-a and 4.2-b) seemed to be due to defective membrane biosynthesis. Pantothenate-deficient cells of Lactobacillus plantarum have been reported by Holden et al (128) to cause defective amino acid accumulation and transport within the cells, marked reduction of cellular lipid constituents and thereby altering

the membrane structure and functions.

Similar defective amino acid accumulation (transport) systems as well as other transport systems of some important cellular nutrients and ATP-generation system could be very likely to occur in our pantothenate-deficient S. faecalis cells. Restriction of amino acids are already known to have significant metabolic consequences. This restriction in bacteria has been reported (129), to cause decrease in the overall rate of protein biosynthesis, reduction in soluble, ribosomal and messenger RNA and restricted initiation of DNA replication. In our experimental conditions protein synthesis was possible as we used all the essential amino acids, but restriction of amino acid pool as suggested by Holden et al (128), could have occurred due to defective amino acid accumulation.

4.1.3 SUMMARY

Studies of the synthesis of macromolecules and survival of pantothenate-sufficient and pantothenate-deficient S. faecalis R ATCC NO. 8043 and S. faecalis ATCC NO. 9790 cells have been made and the following observations have been noted.

(i) Cellular DNA-synthesis rate and DNA content were not affected by pantothenate deficiency.

(ii) Cellular RNA content and RNA synthesis rate of pantothenate-deficient cells did not change till about the mid-log-phase of growth. Thereafter, there was a gradual tendency towards decrease

in the values as the peak-growth (stationary phase) was reached.

(iii) Protein content was not at all affected by pantothenate starvation, but in case of pantothenate-limited culture, this gradually diminished after 200 AOD. Pantothenate-limited cultures showed significantly lowered rate of protein synthesis.

(iv) Pantothenate deficiency caused significant inhibition of cell division. Division-inhibition effect was found to be more marked in case of pantothenate-limited cells. Pantothenate-limited cells seemed to divide upto about 300 AOD, but later on cell division activity came to a plataeu.

(v) Pantothenate deficiency caused very significant lowering of percentage of survivors (i.e., colony formers). The percentage of colony formers dropped gradually upto about 50 percent in case of pantothenate-starved cells and 20 percent in case of pantothenate-limited cells. The fall in percentage of survivors in case of the latter was very steep later.