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Intracellular Degradation of Ribosomes in a Mesophilic and a Thermophilic Species of *Bacillus*

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INTRACELLULAR DEGRADATION OF RIBOSOMES
IN A MESOPHILIC AND A THERMOPHILIC
SPECIES OF BACILLUS

by

Joe William Davis

A Dissertation
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Doctor of Philosophy
Department of Chemistry

Western Michigan University
Kalamazoo, Michigan
December 1990

INTRACELLULAR DEGRADATION OF RIBOSOMES
IN A MESOPHILIC AND A THERMOPHILIC
SPECIES OF BACILLUS

Joe William Davis, Ph. D.

Western Michigan University, 1990

The rates of intracellular degradation of ribosomal proteins were compared in a mesophilic and a thermophilic species of Bacillus. Cells were grown under various incubation conditions in the presence of a ^{14}C -protein hydrolysate in order to label the ribosomes. Rates of ribosome degradation were evaluated in terms of changes in the specific activity of in vivo ribosomes as a function of incubation time. The results indicated that, under most conditions, ribosomes were degraded (in vivo) at similar rates in both the mesophile and the thermophile. In some studies, ribosome degradation rates were slightly faster for the thermophile than for the mesophile.

In limited in vitro investigations, the thermostability of isolated, purified ribosomes was compared. The results showed a slightly higher stability for thermophilic ribosomes under the conditions studied.

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thermophilic species of *Bacillus***

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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Jochanan Stenesh, for his generous guidance and direction during the course of the research and also in the preparation of this dissertation. I also thank Drs. Lin Foote, Michael McCarville, Susan Stapleton, Stephen Friedman, and James Howell who served on my committee. I thank my supervisor and friend, Dr. Paul Satoh, for his untiring support, advice, counsel, and constructive criticism during the many years we worked together. Appreciations are also due to Drs. John Nappier, Fred Yein, and Terry Gilbertson for their encouragement and advice. I thank Drs. James Ludens and Che-Chen Tomich and Ms. Alma Dietz for the loan of critical equipment and materials required for the research. I thank Dr. Larry Oliver and Mr. Dosh Jackson at The Upjohn Company, Kalamazoo, Michigan, for their support. Lastly, I am deeply grateful to my wife, Suzanne, for her unending support and, most of all, her patience throughout all the years of my academic training.

Joe William Davis

TABLE OF CONTENTS

| | |
|--|-----|
| ACKNOWLEDGEMENTS | ii |
| LIST OF TABLES | vi |
| LIST OF FIGURES | vii |
| CHAPTER | |
| I. INTRODUCTION | 1 |
| Ribosome Structure and Function | 1 |
| Intracellular Protein Degradation | 3 |
| Theories of Thermophily | 8 |
| II. MATERIALS AND METHODS | 12 |
| Biochemicals | 12 |
| Reagents | 14 |
| Buffer A | 14 |
| Agar Slants | 15 |
| 10X Amino Acid Stock Mixture | 15 |
| Growth Media | 15 |
| BCA Protein Assay Reagent (Pierce) | 16 |
| Equipment | 16 |
| Organisms and Growth Conditions | 17 |
| Radioactive Labeling of the Cells | 18 |

Table of Contents--Continued

CHAPTER

| | |
|---|----|
| Isolation of Labeled Ribosomes | 18 |
| Scintillation Counting | 19 |
| Protein Determination | 19 |
| Outline of the General (<u>In Vivo</u>) Experimental Procedure | 21 |
| Outline of the General (<u>In Vitro</u>) Experimental Procedure | 22 |
| Calculations and Data Analysis | 23 |
| III. RESULTS AND DISCUSSION | 25 |
| Growth, Harvest, and Sampling of the Bacterial Cultures | 25 |
| Typical Bacterial Growth Curves | 29 |
| Properties of Isolated Ribosomes | 29 |
| Radioactive Labeling of the Ribosomes | 31 |
| Ribosome Degradation Under Normal Growth Conditions | 33 |
| Ribosome Degradation as a Function of Incubation Temperature | 39 |
| Ribosome Degradation Under Low Temperature (Nongrowth) Conditions | 45 |
| Ribosome Degradation Under High Temperature Conditions (70 °C) | 46 |
| Ribosome Degradation Under Starvation Conditions | 48 |

Table of Contents--Continued

CHAPTER

| | |
|--|----|
| Ribosome Degradation in the Presence of an Amino Acid Analog (Canavanine) | 51 |
| Ribosome Degradation in the Presence of Inhibitors of Protein Synthesis | 53 |
| <u>In Vitro</u> Stability of Isolated, ^{14}C -Labeled Ribosomes | 67 |
| V. SUMMARY | 73 |
| REFERENCES | 75 |

LIST OF TABLES

| | |
|---|----|
| 1. Water Loss From Liquid Bacterial Cultures Grown in Aeration Flasks | 27 |
| 2. Water Loss From Liquid Bacterial Cultures Grown in Shake Flasks | 28 |
| 3. Efficiency of Labeling of Ribosomal Proteins | 32 |
| 4. Ribosome Degradation Under Normal Growth Conditions (Mesophile, 37 °C) | 34 |
| 5. Ribosome Degradation Under Normal Growth Conditions (Thermophile, 55 °C) | 35 |
| 6. Half-Life Values for the Decrease in Specific Activity of Ribosomes Under Normal Growth Conditions | 38 |
| 7. Half-Life Values for the Decrease in Specific Activity of Ribosomes at Different Incubation Temperatures (Mesophile) | 43 |
| 8. Half-Life Values for the Decrease in Specific Activity of Ribosomes at Different Incubation Temperatures (Thermophile) | 43 |
| 9. Half-Life Values for the Decrease in Specific Activity of Ribosomal Proteins in the Presence of Puromycin | 61 |

LIST OF FIGURES

| | |
|--|----|
| 1. Growth Curves for the Mesophile and the Thermophile | 30 |
| 2. Short-Term Changes in the Specific Activity of Ribosomes (Normal Growth Conditions) | 36 |
| 3. Long-Term Changes in the Specific Activity of Ribosomes (Normal Growth Conditions) | 36 |
| 4. First-Order Reaction Plot for the Specific Activity of Ribosomes | 38 |
| 5. Ribosome Specific Activity Changes at Three Incubation Temperatures (Mesophile) | 41 |
| 6. Ribosome Specific Activity Changes at Three Incubation Temperatures (Thermophile) | 41 |
| 7. First-Order Reaction Plots for Ribosome Degradation at Three Incubation Temperatures (Mesophile) | 42 |
| 8. First-Order Reaction Plots for Ribosome Degradation at Three Incubation Temperatures (Thermophile) | 42 |
| 9. Ribosome Degradation Under Low Temperature (Nongrowth) Conditions (4 °C) | 46 |
| 10. Ribosome Degradation Under High Temperature Conditions (70 °C) | 48 |
| 11. Changes in the Specific Activity of Ribosomes Under Starvation Conditions | 50 |
| 12. Changes in Ribosome Specific Activity in the Presence of Canavanine | 52 |
| 13. Effect of Puromycin on Bacterial Growth | 56 |
| 14. Effect of Chloramphenicol on Bacterial Growth | 57 |

List of Figures--Continued

| | |
|---|----|
| 15. Effect of Tetracycline on Bacterial Growth | 58 |
| 16. Changes in Ribosome Specific Activity in the Presence of Puromycin (Mesophile) | 60 |
| 17. Changes in Ribosome Specific Activity in the Presence of Puromycin (Thermophile) | 60 |
| 18. Changes in Ribosome Specific Activity in the Presence of Chloramphenicol | 64 |
| 19. Changes in Ribosome Specific Activity in the Presence of Tetracycline | 65 |
| 20. <u>In Vitro</u> Degradation of Ribosomes at Normal and High Temperatures | 69 |
| 21. <u>In Vitro</u> Degradation of Mesophilic Ribosomes (\pm EDTA) | 70 |
| 22. <u>In Vitro</u> Degradation of Thermophilic Ribosomes (\pm EDTA) | 71 |

CHAPTER I

INTRODUCTION

Ribosome Structure and Function

Ribosomes are complex, multicomponent, cytoplasmic particles whose function is to carry out protein biosynthesis. Littlefield, Keller, and Zamecnik (1) first determined that the functional role played by these ribonucleoprotein particles was to incorporate amino acids into polypeptide chains that ultimately form complete protein molecules. As is now well known, messenger RNA (mRNA), which acts as a template for the synthetic process, contains the required coded information for amino acid incorporation in the form of trinucleotide sequences, or codons, each of which codes for a given amino acid. The ribosomes translate the information contained in mRNA in the 5' to 3' direction, resulting in elongation of the growing polypeptide chain from the amino-terminal to the carboxy-terminal.

In bacteria, ribosomes are generally found dispersed throughout the cytoplasm. Bacterial ribosomes account for about 30% of the cell's dry weight, and are present (in Escherichia coli) in over 15,000 copies per cell (2). The basic functional ribosomal unit is a 70S particle (monomer, or monosome), which is composed of ribonucleic acid (65%) and protein (35%). The 70S monomer, or monosome, consists of two smaller subunits of unequal size. The larger subunit, designated the 50S

subunit, has a molecular weight of about 1.8×10^6 daltons and contains two ribonucleic acid (RNA) molecules (23S and 5S) plus 32 polypeptides. The smaller subunit, designated the 30S subunit, has a molecular weight of about 1.0×10^6 daltons, and contains one 16S RNA molecule plus 20 different polypeptides (3).

The amino acid compositions and amino acid sequences of many of the different ribosomal proteins have been determined. The various ribosomal proteins, isolated from many different organisms (both prokaryotes and eukaryotes) are surprisingly similar in structure. These proteins are primarily basic peptides and, to a lesser extent, neutral and acidic peptides (4). The molecular masses of most ribosomal proteins are in the range of 10,000 to 30,000 daltons. Each individual ribosomal protein appears to be a unique entity, with no known similarities between any two different proteins (5). Many of the proteins contain a high number of lysine and arginine residues, which are sometimes clustered into lysine/arginine-rich segments. These structures may be required for the RNA-binding properties of the ribosomal proteins and result in the net positive charge that most of the molecules have (6).

The primary structures of ribosomal proteins appear to be highly conserved in evolution (7), as evidenced by the extensive sequence similarities of the amino acids in corresponding ribosomal proteins from different species within a given taxonomic group. For example, in the case of the prokaryotes, extensive sequence similarities of ribosomal proteins from the gram-negative E. coli and the gram-positive Bacillus stearothermophilus have been demonstrated. Reportedly, as much as 50-70% of the

amino acid sequence is identical in the two organisms (8). A similarly extensive homology of the amino acid sequences has been observed in corresponding ribosomal proteins from diverse species among the eukaryotes (e.g., comparing the fungus with animals).

The control and regulation of the biosynthesis of ribosomal proteins has been studied extensively in E. coli. Gausing and Kjeldgaard (9) suggest that ribosomal proteins are synthesized only in amounts that are required for ribosome assembly, depending on the available supply of RNA. According to Nomura and Engback (10), the coordinated and stoichiometric production of ribosomal proteins and the prevention of their overproduction are accomplished through a process that represses excess protein translation (translational feedback control).

Intracellular Protein Degradation

Because of its fundamental importance to the normal functioning of the living cell, the regulation and physiological significance of intracellular protein degradation has been studied extensively in the last 30 years. However, the understanding of protein degradation is still incomplete in many areas. Most of the existing information has been obtained from research with bacteria, primarily E. coli. Studies of various eukaryotic cells have shown remarkable similarities with prokaryotic cells; however, there are also some differences between the two (11). Most of the following discussion refers to prokaryotes, specifically bacterial cells from either Bacillus or E. coli.

It is now known that a major physiological function of protein degradation in living cells is the removal of abnormal, defective, or potentially harmful polypeptides and proteins from the internal cell environment. These defective proteins include those resulting from mutations, translation errors, chemical modification, spontaneous denaturation, or from proteins containing amino acid analogs. Furthermore, intracellular protein degradation is generally considered to be a critical physiological response of cells to poor nutritional conditions (12). Consequently, protein degradation in bacteria most likely provides a mechanism for the cell to adapt readily to potentially harmful changes in its external environment (i.e., to help the cell withstand "hard times"). Protein breakdown, or catabolism, in bacteria has been found to depend significantly on the growth rate of the cells, which is often a function of the quantity and availability of external nutrients (13). Under conditions of low nutrient supplies, the cell is able to degrade unneeded or noncritical proteins in order to resynthesize proteins more essential for its survival.

Since the earliest studies of protein degradation in bacteria (13,14), results have indicated that protein degradation rates are quite heterogeneous and variable, depending on the particular proteins under study and the culture conditions of the cells. Most of the earliest work used cells grown in the presence of radioactive amino acids or precursors for varying, short periods of time. This practice preferentially labeled those proteins with the shortest half-lives or turnover times. Because some proteins have half-lives as short as a few minutes while many others have half-lives as long as several hours, it is probable that many of the early estimates of protein

degradation rates are too low. However, one interesting offshoot of these studies has been the definition of two distinct classes of bacterial proteins: (1) a short-lived, labile protein fraction; and (2) a longer-lived, stable protein fraction (15, 16).

Mandelstam (14) proposed that the rates of protein degradation are generally lowest in exponentially growing cells, with an average rate of 1-4% per hour for most intracellular proteins. Others have estimated general protein degradation rates to amount to 2-8% per hour (17, 18). These rates increased dramatically (and reversibly) under starvation conditions to 2-5 times the normal levels of degradation (16, 19). Starvation promotes the selective, limited breakdown of proteins that are normally stable without affecting the degradation of proteins that turn over rapidly during normal growth.

The regulation of intracellular protein degradation rates in bacteria represents another aspect of cell metabolism that is also not fully understood. It has been demonstrated (11, 20), that proteins resulting from deletion mutations and other mutations, proteins containing amino acid analog substitutions, and proteins that have been chemically modified, are hydrolyzed and degraded up to 20 times faster than normal proteins in bacterial cells (18, 20-23). Goldberg and St. John (11) also reported dramatically increased protein degradation rates when bacterial cell growth was slowed or inhibited; when cells entered a stationary growth phase, the rates of protein breakdown approached those seen during starvation. The increase in cellular proteolysis during starvation, as indicated by an enhanced degradation of normally stable proteins, may indicate an acquired susceptibility of these proteins to

intracellular proteases (17). These findings have prompted investigators (21) to theorize that there exist two protein degradation systems in bacteria: (1) one, present in all cells, that serves to routinely protect the cells from accumulation of defective or denatured proteins; (2) an adaptive system that is activated upon deprivation of required nutrients.

Another intriguing aspect of the intracellular protein degradation process is the apparent requirement of a biochemical energy source. Pine (23) suggested that conditions causing moderate decreases in the cellular ATP (adenosine triphosphate) concentrations also enhanced protein degradation (see also 24). However, Goldberg and St. John (11) described two opposing effects of ATP levels on protein catabolism. They showed that, in the presence of various inhibitors of cell respiration, limited reductions in the ATP levels (25-60% of the normal levels) caused twofold or threefold increases in degradation rates of average cell proteins. The breakdown of abnormal proteins apparently was not significantly affected. However, when ATP levels were reduced by 80-90%, the degradation of all cell proteins was almost totally inhibited. From these studies they concluded that (a) a moderate reduction in ATP levels in the cell causes an enhanced rate of protein degradation, and (b) a low level of metabolic energy is absolutely required for the degradation of all cellular proteins.

Further investigations (25) into the mechanisms regulating protein degradation have suggested a connection between RNA synthesis, ATP, and a newly discovered compound, guanosine tetraphosphate (guanosine-5'-diphosphate-3'-diphosphate, ppGpp). The compound ppGpp is reportedly synthesized on ribosomes by a

pyrophosphate transfer from ATP to the 3'-position of guanosine diphosphate (26). Data described by Maruyama and Mizuno (27) showed that those experimental conditions that enhanced rates of protein catabolism in E. coli also decreased the net synthesis of ribosomal RNA (rRNA) and transfer RNA (tRNA). Reportedly, the rates of RNA synthesis were inversely proportional to the levels of ppGpp; the latter accumulates in the cells in large quantities under conditions of amino acid starvation. It was also postulated (27) that changes in the ATP levels, as would occur under starvation for glucose or inorganic nutrients, or after treatment with respiratory inhibitors, stimulates proteolysis by the production of an excess of ppGpp. The accumulation of ppGpp is postulated to lead to increased protein catabolism by some unknown mechanism.

Studies of E. coli ribosomes have suggested that these bacterial organelles are relatively stable under normal physiological conditions, but are degraded to various extents under less than optimal conditions. Maruyama and Mizuno (28) and others have reported variable degradation of ribosomes when the latter were exposed to a variety of conditions, such as starvation of phosphate, Mg^{+2} (29), K^{+} (30), or glucose (31); treatment with certain drugs (32); and physical damage to cells, such as with pressure or high temperature (33). The observed ribosome breakdown apparently provided an adaptive process to generate new nucleotides and amino acids, as well as energy in the form of nucleoside diphosphates (from rRNA) for the continued survival of the stressed bacterial cells. In E. coli there are reportedly two possible enzyme systems that function in the RNA degradation process in vivo (34). One system

involves a ribonuclease I (EC 2.7.7.16) pathway; and the second involves two enzymes, ribonuclease II (EC 3.1.4.1) and also, polynucleotide phosphorylase (EC 2.7.7.8). It has been proposed that the second pathway is the most critical one for rRNA degradation in most bacteria (35, 36).

Theories of Thermophily

Thermophilic bacteria (thermophiles) possess the ability to grow at elevated (50-90 °C) temperatures. Mesophiles grow at more moderate (25-45 °C) temperatures (37-39). Over the past 75 years, three major theories have been advanced to explain the phenomenon of thermophily. The first theory (40, 41) proposes that thermophily results from the protective action of lipids in the cell membrane and attempts to correlate thermostability with both the amounts of saturation and the melting points of cell lipids. The second theory, known as the kinetic theory (38, 44), proposes that thermophily is due to a very active metabolic state, resulting from high rates of both protein breakdown and synthesis. The third theory, the macromolecular theory (42, 43), ascribes thermophily to differences in the physical-chemical structure and properties of physiologically important macro-molecules from thermophiles as compared to those from mesophiles.

Several studies (45-48), involving either empirical results or theoretical calculations, have investigated the molecular basis for the thermostability of various thermophilic proteins. Results from some studies have implicated subtle differences in thermophilic protein structures, derived from substitutions of a few critical amino

acids, as an important basis for thermophily. For instance, when the amino acid sequences of selected enzymes from mesophiles and thermophiles were compared, the following findings were reported (47): (a) a decrease of aspartic acid, asparagine, and serine, and an increase of arginine, lysine, glutamic acid/glutamine, and glycine in thermophilic proteins compared with mesophilic proteins; (b) an increase in hydrogen bonding, in hydrophobic bonding, and ionic interactions in thermophilic proteins compared with mesophilic proteins (48). According to Argos et al. (48), the results suggested that these and other subtle changes contributed to "small improvements" in the primary structures and provided a stabilization of the secondary and tertiary structures of the protein molecules of the thermophiles. This stabilization effect apparently resulted from increased internal and decreased external hydrophobicity, as well as from increased helical structure in the affected molecules. Ironically, the few proteins that did not fit the experimental criteria for thermostability in the above mentioned studies were various ribosomal proteins. For these proteins, no differences were found when comparing mesophiles and thermophiles (49). Results from other studies (50), which presented evidence minimizing the significance of the above findings, have suggested that there are also many exceptions to the macromolecular theory of thermophily. However, despite these exceptions, the macromolecular theory is still widely considered to be the key factor in thermophily. Thermostability is generally attributed to various cytoplasmic stabilization factors (in vivo), or to various critical, structural differences in important macromolecules. However, in spite of extensive investigations into the molecular basis for thermophily, no single mechanism

or attribute has been discovered that, by itself, conclusively explains the phenomenon of thermophily.

Most of the work done previously in this lab has supported the macromolecular theory of thermophily. This work involved a comparison of the properties of macromolecules from mesophilic and thermophilic strains of the genus Bacillus. These studies demonstrated a higher heat stability for thermophilic DNA (51) and rRNA (52) than for the corresponding mesophilic molecules. Other studies have evaluated differences in the physical character of DNA (53), the fidelity of DNA replication (54-56), fatty acid content (41), properties of ribosomal proteins (57), cell-free amino acid incorporating systems (58, 59), DNA polymerase properties (60-62), and the melting-out temperatures (T_m) of tRNA (63).

Studies performed in this lab and elsewhere have also compared the in vitro thermostability of bacterial ribosomes under several different conditions (57, 59, 64, 65). In one such study (66), thermostability was assessed by measuring the increase in absorbance at 260 nm (increase in acid soluble nucleotides) of isolated ribosomes upon heating. Another measure of ribosome thermostability was the measurement of the ability of the ribosomes to carry out protein synthesis in cell-free amino acid incorporating systems after heating at various temperatures. Pace and Campbell (67) provided data that correlated maximum growth temperatures of several microorganisms with the in vitro melting-out temperature (T_m) of their ribosomes. Results from these studies suggested that many of the macromolecules from thermophiles were slightly or greatly more thermostable than the corresponding macromolecules

from mesophiles.

In other studies from this laboratory, Stenesh and Yang (68) compared the in vitro thermostability and properties of ribosomes from three mesophilic and three thermophilic strains of Bacillus. The results suggested that thermophilic ribosomes were slightly more heat stable than mesophilic ones. More recently, Hoover (69) from this lab, compared the rates of intracellular degradation of total cell protein in B. licheniformis and B. stearothermophilus. The results indicated that the rate of protein degradation in the mesophile was much higher than in the thermophile.

The present investigation involved a comparison of the rates of ribosome degradation (specifically, of ribosomal proteins) in a mesophile and a thermophile of the genus Bacillus. Most of the experiments dealt with the in vivo rate of ribosomal protein degradation; a few experiments dealt with the in vitro rate. The rate of degradation was assessed by measuring the loss of ^{14}C -labeled amino acids from labeled ribosomes (e.g., a loss of radioactive label per unit mass of ribosomal protein) as a function of time. Degradation rates were determined for cells grown and incubated under various culture conditions. This study represented the first comparisons of the in vivo rates of ribosomal protein degradation in a mesophile and a thermophile of the genus Bacillus.

CHAPTER II

MATERIALS AND METHODS

Biochemicals

The following is a listing of biochemicals used in these studies:

Growth media

(a) Liquid Medium

Trypticase, 1% (BBL)
Yeast extract, 0.2% (Difco)

(b) Agar Slants

Trypticase, 1%
Yeast extract, 0.2%
Bacto-Agar, 2% (Difco)

Buffer A (Ribosome Isolation)

0.01 M Tris (tris-
[hydroxymethyl]-aminomethane)
0.01 M Magnesium acetate
0.06 M Ammonium chloride, pH 7.4

Buffer B (Ribosome Assay)

0.01 M Tris
0.01 M Magnesium acetate, pH 7.4

Amino acids (Radioactively labeled)

L-(U-¹⁴C)-Leucine, Code CFB.67, 342
mCi/mmole, (Amersham)

L-(U-¹⁴C)-Arginine monohydrochloride, Code
CFB.63, 344 mCi/mmol, (Amersham)

(U-¹⁴C)-Protein hydrolysate, Code
CFB.25, 57mCi/milliatom carbon,
(Amersham).

A typical analysis of the hydrolysate was as follows:

| | |
|-----------------|-----------------|
| L-Alanine | L-Lysine |
| L-Arginine | L-Methionine |
| L-Aspartic acid | L-Phenylalanine |
| L-Glutamic acid | L-Proline |
| L-Glycine | L-Serine |
| L-Histidine | L-Threonine |
| L-Leucine | L-Tyrosine |
| L-Isoleucine | L-Valine |

Amino acid (AA) Mixture (10X, each 0.005M, unlabeled). The AA mixture
was diluted 1:10 in the final culture medium. The composition of the mixture was
as follows:

| | |
|-----------------|-----------------|
| L-Alanine | L-Lysine |
| L-Arginine HCl | L-Methionine |
| L-Aspartic acid | L-Phenylalanine |
| L-Glutamic acid | L-Proline |
| L-Glycine | L-Serine |
| L-Histidine | L-Threonine |
| L-Leucine | L-Tyrosine |
| L-Valine | L-Tryptophan |
| L-Cysteine HCl | |

Antibiotics

Puromycin dihydrochloride, P7255 (Sigma)

Tetracycline, T-3258, (Sigma)

Chloramphenicol, C-0378 (Sigma)

Miscellaneous

Perchloric acid, 69-72% (JTBaker)

Manganese sulfate monohydrate (Mallinckrodt)

Deoxyribonuclease I (Type IV), DNase I, (EC 3.1.21.1), from Bovine Pancreas, D-5025 (Sigma)

Antifoam (SAG 471), (Union Carbide)

Ready Safe™, Liquid Scintillation Cocktail (Beckman)

Bovine Serum Albumin (BSA), Standard Solution, 2 mg/ml, (Sigma).

BCA Protein Assay Reagent (Pierce):

Reagent A: Reagent A consisted of the following components: sodium carbonate, sodium bicarbonate, sodium tartrate, BCA, and sodium hydroxide.

Reagent B: 4% Copper Sulfate Solution.

Reagents

Buffer A

Buffer A consisted of 0.01M Tris, plus 0.01 M magnesium acetate and 0.06 M ammonium chloride. Two (2) liters of 10X Buffer A Stock were prepared, and the pH was adjusted to 7.5 with 5N hydrochloric acid (HCl). The stock solution was filtered in sterile fashion through a 0.22 micron Nalgene vacuum filter unit and stored under refrigeration until used. Buffer B was prepared exactly as Buffer A, except that

ammonium chloride was omitted.

Agar Slants

Slants were prepared using 2% agar, 1% trypticase, and 0.2% yeast extract. For growth of stock cultures, slants contained, in addition to the above ingredients, 10 ppm of manganese sulfate. Slants were autoclaved and stored at 4 °C until used.

10X Amino Acid Stock Mixture

A mixture of unlabeled L-amino acids was prepared in sterile distilled water. The solution was heated gently at 60 °C for ten minutes to bring materials into solution. The mixture was autoclaved and then divided into 110 ml aliquots, which were stored at -80 °C until used.

Growth Media

The normal, liquid growth medium contained 1% trypticase and 0.2% yeast extract in distilled water. The medium was autoclaved immediately after preparation, cooled to room temperature, and used the same day. A specially fortified medium, which contained the above ingredients plus a 1/10 dilution (v/v) of the 10X stock amino acid mixture, was used to prevent reincorporation of labeled amino acids. This medium was prepared by the addition of 100 ml of the 10X amino acid mixture to 900 ml of the normal growth medium. The final concentration of each amino acid in the mixture was 0.5mM.

BCA Protein Assay Reagent (Pierce)

To prepare the Working Reagent, 50 parts of Reagent A were added to 1 part of Reagent B. The solution was mixed thoroughly and had a slightly turbid, light green color. The Working Reagent was stable for 1 week at room temperature.

Equipment

Spectrophotometric measurements were made with a Kontron Spectrophotometer (Model 820). Sorvall RT6000 and RC5 Superspeed refrigerated centrifuges were used for low and medium speed centrifugations, respectively. Ultracentrifugation was performed with a Beckman Model L5-65 Preparative Ultracentrifuge using a Type 65 or a Type 40 Fixed Angle Rotor. Scintillation counting was done using a Beckman Model LS 5801 Scintillation Counter. Incubations were performed in thermostated, shaking water baths (Gyrotory Water Bath Shaker, Model G-76 [New Brunswick Scientific] and American Optical Water Bath Shaker, Model 2156). Gilson and Eppendorf Pipettors were used for most studies. Sterile, conical polypropylene centrifuge tubes (15 ml, #25330 and 50 ml, #25310, Corning) were routinely used for culture sampling, centrifugation and storage of cells. Cells were sonicated by means of a Branson Sonifier Cell Disruptor 200. Absorbances for the BCA Protein determinations were measured by means of a Perkin Elmer Automated MicroPlate Reader (LAMBDA READER). A French Pressure Cell Press (Aminco/SLM Instruments, Inc., Model FA-073) fitted with a Rapid-fill Kit

(FA-020) was used to disrupt bacterial cells.

Organisms and Growth Conditions

The bacteria used in these studies were Bacillus licheniformis (NRS 243) and Bacillus stearothermophilus 10. The mesophile, B. licheniformis, was grown overnight at 37°C and the thermophile, B. stearothermophilus, was grown at 55 °C. Bacteria from stock cultures were grown on agar slants. The slants were stored at 4 °C and used to initiate each set of experiments. For the initial protein degradation studies, the bacterial growth from a slant was scraped off and suspended in 6 ml of sterile distilled water. The suspension obtained from three slants was used to inoculate 2 L of sterile culture medium in a 4 L reagent bottle equipped with a rubber stopper and fritted glass aeration tube (sparger). In order to isolate adequate amounts of radioactively labeled ribosomes from this culture, it was necessary to add relatively large amounts of labeled amino acids. The prohibitive cost of such quantities of labeled compound, as well as the fact that significant evaporation of the medium occurred (especially above 55 °C), made the use of this apparatus undesirable. Consequently, all subsequent work was done by scaling down the volume of the culture and using Erlenmeyer flasks, fitted with cotton plugs. Specifically, 1 L Erlenmeyer flasks were used; 400 mL of medium were introduced into each flask, followed by an inoculum from one slant. The shake flasks were fitted with cotton plugs and shaken at 150 RPM using thermostated water baths or shakers. In later experiments, the inoculum from the slants was replaced by a frozen seed inoculum

consisting of about 1 mL of frozen liquid culture (see Radioactive Labeling of Cells below). The bacterial cells were harvested during the late log-phase (absorbance at 540 nm about 0.8 to 1.2). Cells were collected by centrifugation at 11,000 x g for 20 minutes using a Sorvall RC-5 Centrifuge with a GSA rotor. The collected cells were then frozen at -20 °C and stored at -80 °C.

Radioactive Labeling of the Cells

Initially, ^{14}C -leucine and ^{14}C -arginine were used to radiolabel the bacterial cell proteins. When results showed that higher specific activities of the ribosomal proteins could be obtained by using smaller amounts of a ^{14}C -labeled mixture of amino acids, the labeling procedure was changed, and a ^{14}C -labeled Algal Protein Hydrolysate (Amersham) was used. The final procedure for preparing radioactively labeled ribosomes involved inoculating a 1 mL aliquot of a frozen seed inoculum into 400 mL of liquid medium containing 0.1 $\mu\text{Ci/mL}$ of the ^{14}C -protein hydrolysate. Bacterial cells were allowed to grow overnight at 37 °C (mesophile) and 55 °C (thermophile).

Isolation of Labeled Ribosomes

Frozen, pelleted cells from duplicate aliquots (10 mL) of liquid culture were thawed on ice, suspended in 10-15 mL of cold (0-4 °C) Buffer A, washed once by centrifugation (0 °C), and resuspended in about 10 mL of fresh, cold Buffer A. This solution of cells was pumped into the Rapid-fill cell of the prechilled French pressure cell. The cells were disrupted after exposure to 18,000 psi (one minute) by the

release of the pressure outlet valve. The broken-cell suspension was then recovered by collection of the effluent. The broken-cell suspension was treated with DNase (RNase-free; 1 ug per mL of cell suspension for at least 15 minutes), and the mixture centrifuged at 30000 x g for 40 minutes. The supernatant (S-30 fraction) was centrifuged at 105,000 x g for 2.25 hours in a Beckman L5-65 Preparative Ultracentrifuge. The resulting pellet was resuspended in 1 mL of fresh Buffer A by very gentle swirling overnight at 4 °C.

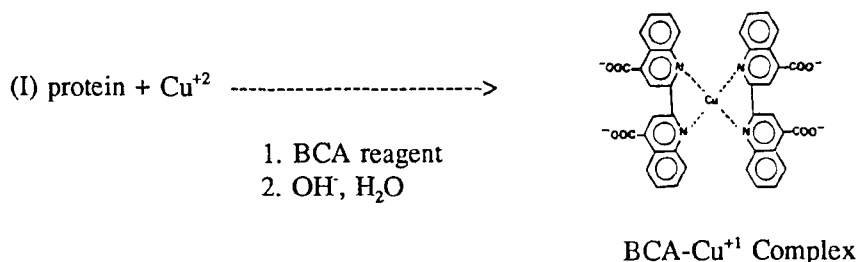
Scintillation Counting

Approximately 0.5-1.0 mL aliquots of the resuspended ribosomes or of the sonicated cells (for total count estimates, the pellet from 2 mL of culture medium was disrupted by sonication at 5000 Hz for one minute) were mixed with 15 mL of scintillation fluid (ReadySafe) in 20 mL glass scintillation vials. After thorough shaking (five minutes), the vials were counted in a Beckman LS 5801 Scintillation Counter. Vials were counted for at least 10 minutes and corrections for quenching were made using the External Standards Channel Ratio Method and also an Automatic Quench Compensation (AQC) program.

Protein Determination

Spectrophotometric determinations of ribosomal protein concentrations were performed using a microtiter plate-modified version of the BCA Protein Assay (Pierce). BCA, bicinchoninic acid, is a highly specific reagent that utilizes features

of the well-known biuret reaction (protein reacting with Cu^{+2} in alkaline solution to produce Cu^{+1}). The overall reaction scheme (Equation I) is outlined below.



The concentration of protein in the solution is directly proportional to the resulting purple BCA- Cu^{+1} complex; the latter is measured spectrophotometrically at 562 nm. Two different protocols were used for the protein determinations. For samples containing high quantities of protein, the Standard protocol (sample protein concentration range = 100-1200 $\mu\text{g/mL}$) was used. This procedure involved an incubation at 37 °C for 30 minutes. For samples containing low quantities of protein, the Enhanced Protocol (sample protein concentration range 0-350 $\mu\text{g/mL}$) was used (incubation at 60 °C for 30 minutes).

The assay was performed as follows. A set of duplicate 12 x 75 mm glass test tubes was prepared containing 0.1 mL of the bovine serum albumin (BSA) standards or of the unknown samples. Two mL of the working BCA reagent (see Reagents, above) was added to each tube. The tubes were mixed well and then incubated at each of the prescribed temperatures. After incubation, the tubes were allowed to cool to room temperature. Duplicate aliquots of 250 μL from each tube were pipetted into corresponding wells of a 96-well, plastic microtiter plate (Corning) using a suitable

micropipettor. The absorbances of the solutions in the plate were then measured (all 96 simultaneously) at 562 nm versus a reagent blank using a Perkin-Elmer ELISA plate reader. The mean absorbance of the blank was subtracted from the mean absorbances for the standards and the unknowns. A standard curve was prepared by plotting the corrected absorbances of the standards as a function of their protein concentrations.

Outline of the General (In Vivo) Experimental Procedure

Cells were grown initially in liquid medium containing the ^{14}C -Protein hydrolysate (0.1 $\mu\text{Ci/mL}$) at 37 °C for the mesophile and at 55 °C for the thermophile. They were grown overnight to the late log-phase (7-8 hours). The cells were harvested the next day by centrifugation at 11,000 x g (Sorvall RC5 Centrifuge, GS3 Rotor) and resuspended in fresh, sterile liquid medium (prewarmed to the prescribed growth temperature) containing the mixture of cold amino acids (0.5 mM). The cells were then allowed to resume growth at a specific temperature. At various time intervals, a 25 mL sampling aliquot was removed from the culture medium. From the sampling aliquot, duplicate 2 mL aliquots (for estimation of total cell radioactivity incorporation) and duplicate 10 mL aliquots (for ribosome isolation) were immediately pipetted into plastic centrifuge tubes. The bacterial cells in these aliquots were quickly pelleted using a Sorvall RT-6000 centrifuge at 4 °C (the supernatant was discarded). The pelleted cells were immediately frozen at -20 °C and stored under liquid nitrogen until used. Subsequently, the cells of the 10 mL aliquots were

disrupted by means of the French press, the ribosomes were isolated, and the specific activity of the ribosomes was determined.

In other studies, the mesophile was grown at 27, 37 (growth temperature optimum), and 47 °C, while the thermophile was grown at 45, 55 (growth temperature optimum) and 65 °C. Rates of degradation of ribosomal proteins were measured by the decrease in ^{14}C -labeled amino acids in the ribosomal protein as a function of time. Mean values for total cell label (the total label incorporated into all the cellular proteins), ribosomal protein counts, ribosomal protein concentration, and the specific activity of the ribosomes were determined as a function of time. The degradation of ribosomal proteins was also studied under several other growth conditions:

- (a) Low temperature (nongrowth) conditions,
- (b) High temperature (70 °C) conditions,
- (c) Starvation conditions,
- (d) Growth in the presence of an amino acid analog (canavanine),
- (e) Growth in the presence of various inhibitors of protein synthesis.

Outline of the General (In Vitro) Experimental Procedure

Ribosomes were labeled and isolated as described for the in vivo procedure. The labeled ribosomes were then suspended in Buffer B to a concentration of about 20,000 cpm/mL. The solution was placed in Sorvall centrifuge tubes (#3269), the

tubes were tightly capped, and the closed tubes were incubated in a thermostat controlled water bath at various temperatures. Tubes were removed as a function of time, the reaction was stopped by the addition of 2 mL of 5 % (v/v) perchloric acid, and the tubes were kept in ice for 30 minutes. After 30 minutes, the solutions were centrifuged for 10 minutes at 10,000 x g in a Sorvall RC-5 centrifuge (SM 24 Rotor). A one milliliter aliquot of the supernatant was counted for 10 minutes in the scintillation counter. The rate of degradation was measured by the relative increase of acid soluble ^{14}C -labeled amino acids and peptides in the supernatant.

Calculations and Data Analysis

Duplicate sampling was used in all the experimental studies. For each set of duplicates, the specific activity of the ribosomal proteins was determined as the mean counts per minute (cpm) per mg of ribosomal protein (i.e., cpm/mg). If the duplicate samples did not agree within 10-20 % (coefficient of variation, C.V.), they were not used in the calculations. The specific activity was plotted as a function of time. Curve fitting was performed using SLIDEWRITEPlus™ (Vers.3, Advanced Graphics Software, Inc.). Because the degradation process under certain conditions appeared to follow first-order reaction kinetics, the data for protein degradation were also plotted as the natural log (ln) of the specific activity as a function of time. A linear regression analysis of these data was carried out by means of the EZSTATS Statistics Software Program (Upjohn Laboratories Statistics Software Library). A straight line was derived to fit the data, and the slope of the line (k) was determined. The slope

of the line corresponded to the rate constant of a first order reaction. For such a reaction, the half-life ($t_{1/2}$) is given by: $t_{1/2} = (\ln 2)/k = 0.639/k$ (42, 72).

CHAPTER III

RESULTS AND DISCUSSION

Growth, Harvest, and Sampling of the Bacterial Cultures

The bacterial cells were initially grown in sterile 2 L reagent bottles, custom fitted with a rubber stopper, a pressure release port, and a glass gas-dispersion tube (sparger). Air pumped through sterile glass-wool filters attached to the gas-dispersion tube provided agitation for mixing the cultures. The bottles were incubated in thermostat controlled water baths without shaking (mesophile, 37 °C, and thermophile, 55 °C). An antifoam, SAG-471 (Union Carbide 471) was added (0.2 mL per liter) to prevent foaming.

Initially this system was used for growth and radioactive labeling of the cells, and subsequently for degradation studies. However, in preliminary studies, it was noted that extensive evaporation losses occurred after prolonged incubation of the cells (24-48 hours), especially at temperatures at or above 55 °C. Consequently, a comparison was made of the rates of water loss from the media as a function of time using this aeration system and a conventional shake flask (an Erlenmeyer flask with a cotton plug). In this study, identical bacterial cell preparations were grown at 37 °C and 55 °C for up to 48 hours in order to compare the decrease of liquid volume in both types of flasks. At indicated times, 25 mL aliquots were removed from the

culture, and the remaining volume was determined. The results (Tables 1 and 2) indicated that evaporation losses could be minimized by using the shake flasks. Consequently, it was decided to use the shake flasks for all subsequent work. Additionally, in order to minimize the cost of the radioactive materials required for labeling the ribosomes, the total volume of the incubation mixture was reduced from 1-2 L down to about 400 mL (see also Optimum Conditions For Radioactive Labeling of Ribosomes, below).

It was observed that bacterial cultures propagated on agar slants showed poorer growth characteristics after a few weeks of storage, even when transferred repeatedly onto fresh agar slants. The bacterial cells often displayed longer lag periods before exponential growth began. Therefore, to avoid these problems, the possibility of using a frozen aliquot of a stock culture as an inoculum (as opposed to using the agar slants) was investigated. The inocula were obtained from log-phase cultures (the mesophile grown at 37 °C, and the thermophile grown at 55 °C). Several 1-2 mL aliquots of each culture were dispensed (in sterile fashion) into sterile glass vials, and the vials were then frozen and stored in liquid nitrogen (vapor phase) at -80 °C. A series of brief studies showed that the growth irregularities were prevented through the use of the such frozen inocula and henceforth, this format was used for all of the subsequent work. The sizes of the aliquots of each culture medium used for sampling analyses (total cell label incorporation and for ribosome isolation) were chosen so that a significant number of counts would be measured and so that a clearly visible ribosome pellets would be obtained.

Table 1
 Water Loss From Liquid Bacterial Cultures
 Grown in Aeration Flasks

| | | <u>Mesophile</u> | | <u>Thermophile</u> | |
|------------------------|----------|------------------|------|--------------------|------|
| | | Volume (mL)* | | | |
| Incubation Time (hrs.) | Expected | Observed | Loss | Observed | Loss |
| 0 | 300 | 300 | 0 | 300 | 0 |
| 2 | 275 | 274 | -1 | 275 | 0 |
| 4 | 250 | 245 | -5 | 230 | -20 |
| 8 | 225 | 213 | -12 | 188 | -37 |
| 20 | 200 | 158 | -42 | 122 | -78 |
| 24 | 175 | 125 | -50 | 91 | -84 |
| 32 | 150 | 95 | -65 | 57 | -93 |
| 48 | 125 | 61 | -125 | 0 | -125 |

*At the indicated times, 25 mL aliquots were removed from the culture, and the remaining volume was determined.

Table 2
Water Loss From Liquid Bacterial Cultures
Grown in Shake Flasks

| Incubation Time (hrs.) | <u>Mesophile</u> | | | <u>Thermophile</u> | |
|------------------------------|------------------|----------|------|--------------------|------|
| | Volume (mL)* | | | | |
| | Expected | Observed | Loss | Observed | Loss |
| 0 | 300 | 300 | 0 | 300 | 0 |
| 6 | ND | ND | ND | ND | ND |
| 12 | 275 | 270 | -5 | 265 | -10 |
| 18 | 250 | 240 | -10 | 238 | -12 |
| 24 | 225 | 214 | -11 | 209 | -16 |
| 32 | 200 | 186 | -14 | 175 | -25 |
| 48 | 175 | 158 | -17 | 138 | -37 |

*At the indicated times, 25 mL aliquots were removed from the culture, and the remaining volume was determined.

ND = Not Done

Typical Bacterial Growth Curves

Typical growth curves for B. licheniformis, grown at 37 °C, and B. stearothermophilus, grown at 55 °C, are shown in Figure 1. To obtain these data, 1 mL of the frozen, liquid stock culture was added in sterile fashion to 400 mL of sterile liquid medium (prewarmed to 37 or 55 °C). At hourly intervals, a small aliquot (5 mL) was removed, and the absorbance at 540 nm was measured. For samples having high absorbances (>1.3), the culture medium was diluted and remeasured to insure the accuracy and linearity of the absorbance readings. The mesophile appeared to require a slightly longer lag-period than the thermophile before exponential growth ensued. In other experiments, it was determined that the thermophile failed to grow at temperatures below 37 °C, while the mesophile grew very slowly at 55 °C. Pelleted bacterial cells from the mesophile had an ivory-colored appearance and were more difficult to resuspend in fresh buffer than cells from the thermophile. The cells of the thermophile had a distinctive reddish-brown appearance.

Properties of Isolated Ribosomes

Isolated ribosomes from both organisms had an opaque, brown appearance, and could only be resuspended by either gentle stirring with a rubber policeman or by gentle swirling at 4 °C. The average absorbance ratios of the ribosomes at 260/235 and 260/280 nm were 1.71 and 1.98 for the mesophile and 1.65 and 2.01 for the

thermophile respectively. These values are similar to those previously reported for ribosomes from Bacillus (68).

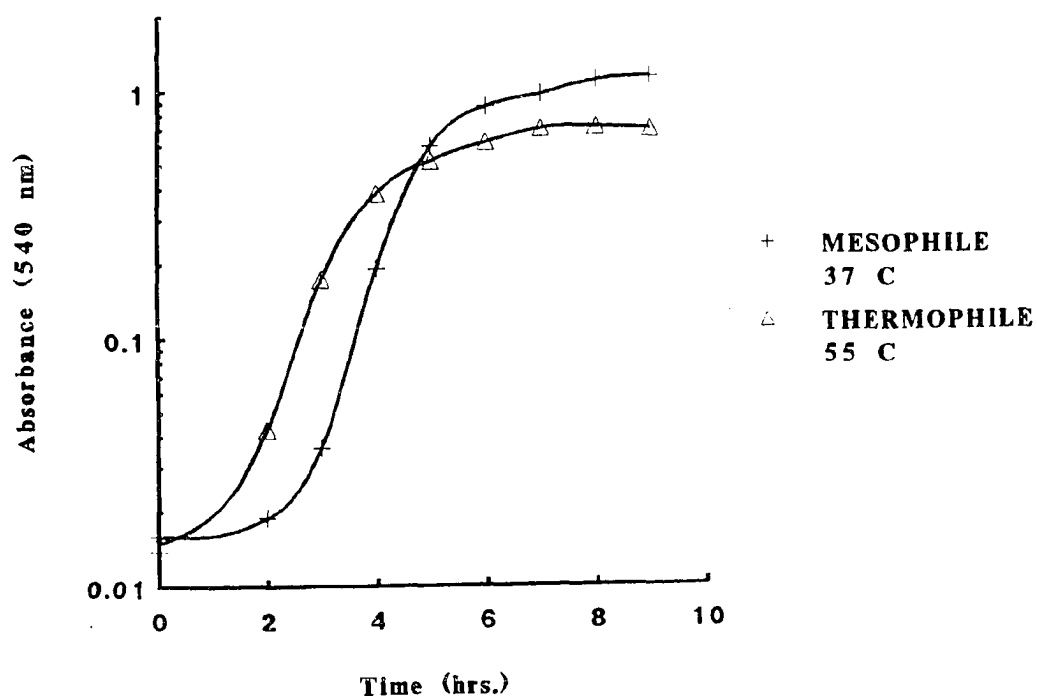


Figure 1. Growth Curves for the Mesophile and the Thermophile.

Radioactive Labeling of the Ribosomes

According to available amino acid sequence information reported in the literature for ribosomal proteins (70, 71), ribosomes contain a large number of moderately basic proteins, and several of the ribosomal proteins contain significant amounts of arginine, lysine, leucine, glutamic acid, and valine (70). Based on these data, ^{14}C -labeled leucine and arginine were initially selected for labeling of the ribosomes. In these studies, about 0.05-0.1 μCi of ^{14}C -labeled amino acid were used per mL of growth medium, and the culture was incubated overnight. Because of the fairly rich culture medium, a long labeling period was required in order to obtain sufficient incorporation of the label into the ribosomes. While a reasonable amount of label was incorporated into the ribosomes by using ^{14}C -leucine or ^{14}C -arginine, it was found that much higher levels of incorporation could be achieved by using a ^{14}C -protein hydrolysate. These results are shown in Table 3. Accordingly, the ^{14}C -labeled protein hydrolysate at a level of 0.1 $\mu\text{Ci/mL}$ of liquid medium was used henceforth in all studies. An additional bonus from using the protein hydrolysate was the fact that it was much less expensive than the individual ^{14}C -labeled amino acids.

The percentage of added counts that became incorporated into total cellular proteins under the conditions of these experiments was about 5-6% for the mesophile and 6-8% for the thermophile. The percentage of added counts that became incorporated into ribosomal proteins was between 0.3 - 0.8% for both the mesophile and the thermophile.

Table 3
Efficiency of Labeling of Ribosomal Proteins

| Label | Concentration (μ Ci/mL Medium) | Specific Activity of Ribosomes (cpm/mg protein) |
|----------------------------------|--|---|
| Mesophile | | |
| 14 C-Leucine | 0.05 | 2441 |
| | 0.1 | 3911 |
| 14 C-Arginine | 0.05 | 3588 |
| 14 C-Protein Hydrolysate | 0.05 | 5228 |
| | 0.1 | 10032 |
| Thermophile | | |
| 14 C-Leucine | 0.05 | 3084 |
| | 0.1 | 6177 |
| 14 C-Arginine | 0.05 | 3780 |
| 14 C-Protein Hydrolysate | 0.05 | 7379 |
| | 0.1 | 15304 |

As can be seen from Table 3, the incorporation of ^{14}C -counts into ribosomal proteins was generally 1.5 to 2.5 times higher for the thermophile than the mesophile. The reasons for these differences in incorporation levels are unknown. It is possible that the different incorporation levels resulted from different rates of cross-membrane transport of the labeled amino acids in the two organisms.

Ribosome Degradation Under Normal Growth Conditions

Cells were grown and analyzed following the general (*in vivo*) method described above. Two sets of typical results are given in Tables 4 and 5. The identical data are also depicted graphically in Figure 2. Results from a similar experiment, but carried out for 72 instead of 24 hours, are shown graphically in Figure 3. In general, the duplicate samples used in the data analysis agreed within 10-20% (C.V.), or they were not used. The initial specific activity of the ribosomal proteins was usually 30-60% higher for the thermophile than for the mesophile (see Figures 2 and 3). This difference in the specific activity probably reflected variations between the two organisms in the rate of transport of amino acids across the cell membrane, and/or the balance between protein biosynthesis and protein degradation.

As can be seen from both Figures 2 and 3, the specific activity of the ribosomal proteins changed rapidly during the first hours of incubation, and more slowly subsequently. The curves leveled off after 12-24 hours of incubation. Because of these observations and because it is probable that after 24 hours, the cells enter a

Table 4
Ribosome Degradation Under Normal Growth Conditions
(Mesophile, 37 °C)

| Incubation Time (hrs.) | Radioactivity ¹ (cpm) | | Ribosomes | |
|------------------------------|-------------------------------------|-----------|------------------------------|---|
| | Cells | Ribosomes | Protein ² (mg) | Specific Activity ³ (cpm/mg r-protein) ⁴ |
| 0 | 102680 | 17224 | 2.22 | 7752 |
| 0.5 | 96020 | 20298 | 3.60 | 5638 |
| 1.0 | 94240 | 15292 | 2.76 | 5541 |
| 1.5 | 89160 | 19204 | 3.58 | 5364 |
| 2.0 | 85060 | 15388 | 3.56 | 4323 |
| 2.5 | 84600 | 17352 | 4.36 | 3966 |
| 3.0 | 81710 | 12332 | 3.28 | 3760 |
| 4.0 | 74620 | 8988 | 2.60 | 3457 |
| 10.0 | 71180 | 9908 | 5.56 | 1998 |
| 20.0 | 57660 | 8328 | 6.96 | 1197 |

1. Total cpm (Mean) in both the cell and ribosome pellets, obtained from 10 mL of culture.
2. Total ribosomal protein (r-protein) in 10 mL of culture.
3. Ratio of columns 3 and 4.
4. cpm/mg of ribosomal protein.

Table 5
Ribosome Degradation Under Normal Growth Conditions
(Thermophile, 55 °C)

| Incubation Time (hrs.) | Radioactivity ¹ (cpm) | | Ribosomes | |
|------------------------------|-------------------------------------|-----------|------------------------------|---|
| | Cells | Ribosomes | Protein ² (mg) | Specific Activity ³ (cpm/mg r-protein) ⁴ |
| 0 | 104710 | 62896 | 5.86 | 10751 |
| 0.5 | 102310 | 53344 | 5.68 | 9408 |
| 1.0 | 111146 | 42880 | 4.98 | 8597 |
| 1.5 | 100180 | 39168 | 4.88 | 8026 |
| 2.0 | 98220 | 47064 | 6.68 | 7046 |
| 2.5 | 98050 | 41324 | 6.80 | 6665 |
| 3.0 | 97600 | 22808 | 3.92 | 5818 |
| 4.0 | 95120 | 21400 | 4.68 | 4573 |
| 10.0 | 75810 | 21768 | 9.44 | 2306 |
| 20.0 | 57870 | 8580 | 6.58 | 1316 |

1. Total cpm (Mean) in both the cell and ribosome pellets obtained from 10 mL of culture.
2. Total ribosomal protein (r-protein) obtained from 10 mL of culture.
3. Ratio of columns 3 and 4.
4. cpm/mg of ribosomal protein.

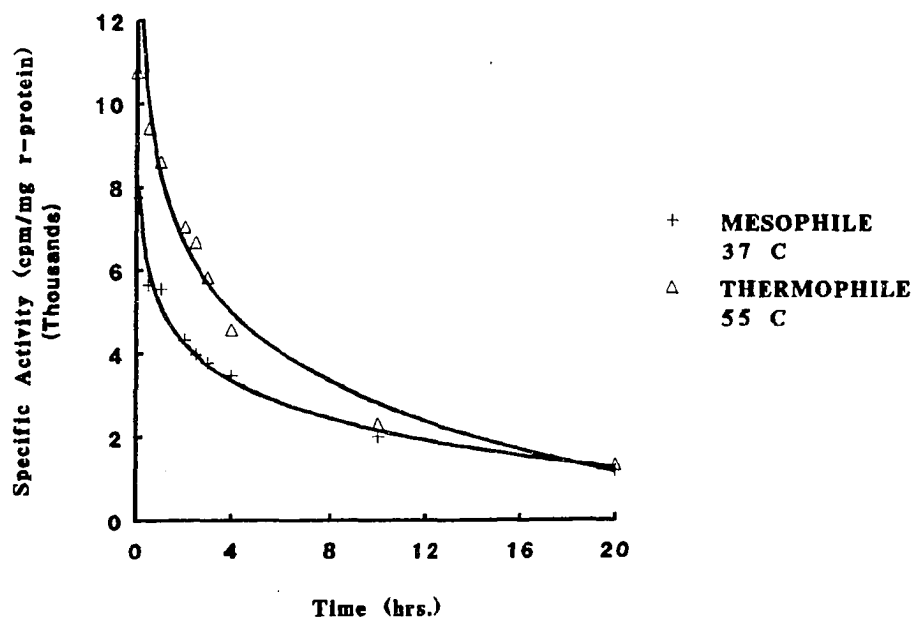


Figure 2. Short-Term Changes in the Specific Activity of Ribosomes. (Normal Growth Conditions).

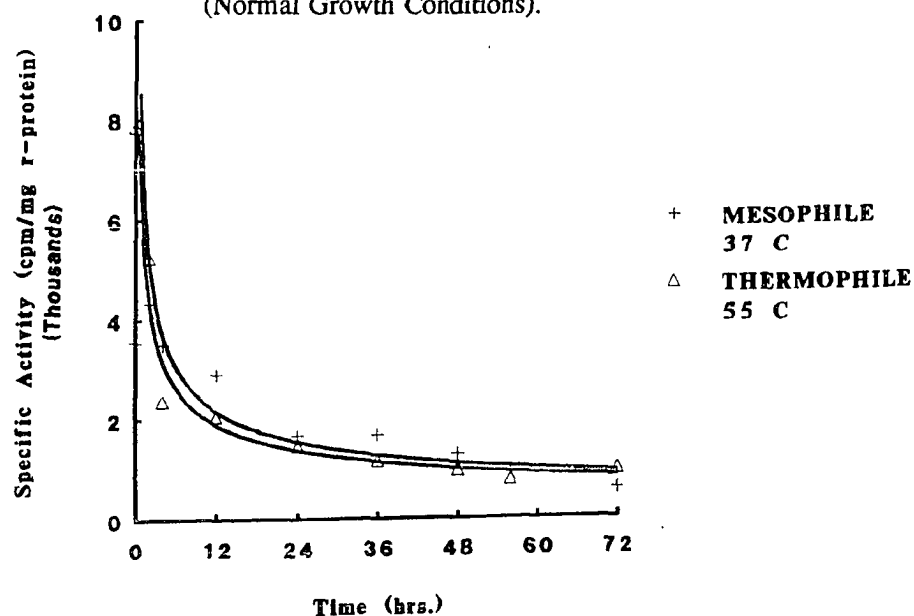


Figure 3. Long-Term Changes in the Specific Activity of Ribosomes (Normal Growth Conditions).

sporulation stage, all subsequent experiments were carried out only for a period of 24 hours. Typically, over a 24-hour period, the specific activity of the ribosomes decreased by about 40% for the mesophile and about 60% for the thermophile.

The data indicated that the changes in specific activity were very similar in the two organisms and appeared to be described by an exponential curve. This suggested that the observed changes reflected a first-order rate process. In that case, one could determine the half-life ($t_{1/2}$) of the process by plotting the natural logarithm of the specific activity as a function of time. Such plots are shown in Figure 4. The initial 12 hours of incubation provided the best fit for these data and were, therefore, used throughout this work. The half-life values obtained from this plot are listed in Table 6.

In view of the calculated values and their standard deviations, one can conclude that the half-life values for the mesophile and the thermophile were not significantly different ($p < 0.122$).

It is important to stress that a measured change in the specific activity of ribosomes could actually reflect a number of different intracellular conditions. It could, of course, simply represent the progressive breakdown of ribosomes, release of ribosomal proteins, and proteolytic digestion of these released proteins. These processes by themselves would not lead to a decrease in the specific activity of the ribosomes unless there were a preferential loss of ^{14}C -labeled proteins from the ribosomes.

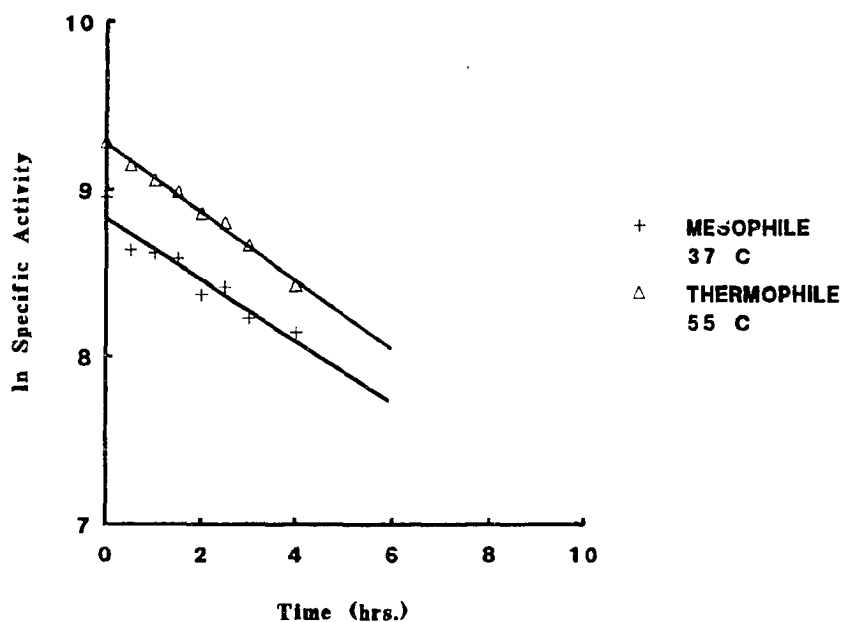


Figure 4. First-Order Reaction Plot for the Specific Activity of Ribosomes.

Table 6

Half-Life Values for the Decrease in Specific Activity
of Ribosomes Under Normal Growth Conditions

| | Number of Experiments | Mean Half-Life (hrs.) | Standard Deviation |
|------------------------|--------------------------|--------------------------|-----------------------|
| Mesophile (37 °C) | 3 | 7.73* | ± 1.87 |
| Thermophile (55 °C) | 4 | 5.85* | ± 0.77 |

* ($p < 0.1222$), not significant.

The decrease in specific activity could also have resulted from synthesis of new, unlabeled ribosomal proteins and their assembly into new, unlabeled ribosomes in the absence of any breakdown of ribosomes. Furthermore, the observed decrease in the specific activity of the ribosomes could reflect the overall balance of both of the above intracellular conditions. In other words, the observed decrease in specific activity was probably a result of both breakdown of labeled ribosomes and synthesis of new, unlabeled ribosomes. Changes in ribosome specific activity reflect, in this case, the intracellular steady state of ribosome breakdown and synthesis. Most likely, this latter interpretation comes closest to describing the true basis for the observed decrease in specific activity. Work done in this study was not designed to pinpoint the source of specific activity changes any further.

With these qualifications in mind, and with reference to the data shown in Table 6, one can conclude that the ribosomes are organelles that are degraded and synthesized in vivo at comparable rates in both the mesophile at 37 °C and the thermophile at 55 °C. This was an interesting result in light of some previously reported data (65-69), which showed that, in vitro thermophilic ribosomes were slightly more stable than mesophilic ones.

Ribosome Degradation as a Function of Incubation Temperature

In these studies, cells were again grown and analyzed following the general (in vivo) method described above, except that the incubation temperature of the cells

was varied. Cells of the mesophile were incubated at 27, 37, and 47 °C, and those of the thermophile were incubated at 45, 55, and 65 °C. Thus, in both cases, the cells were incubated at the optimum growth temperatures of the organisms, and at a temperature 10 °C above and 10 °C below the optimum temperature. The results of this study are shown in Figures 5-8 and Tables 7-8. The changes in the specific activity of the ribosomes for the mesophile are shown in Figure 5, and those for the thermophile are shown in Figure 6. It can be seen that the changes in specific activity were very similar for each organism at all three temperatures. Furthermore, there were no significant differences in both the shapes of the curves and the extents of the changes between the two organisms.

Assuming, as before, that these changes can be described by a first-order reaction, the corresponding regression plots are shown in Figures 7 (mesophile) and 8 (thermophile). The half-lives, computed from these plots, are given in Tables 7 and 8. As can be seen from these tables, the half-life for the decrease in specific activity had the smallest value at the lowest incubation temperature. That was true for both the mesophile and the thermophile. In other words, at the lowest temperatures, the specific activity decreased most rapidly. This may have been due to the fact that at these low temperatures ribosome degradation was largely prevented, and that decrease in specific activity reflected continued protein synthesis at some basal level. If this interpretation is correct, one would conclude that protein synthesis for the thermophile at 45 °C exceeds that of the mesophile at 27 °C.

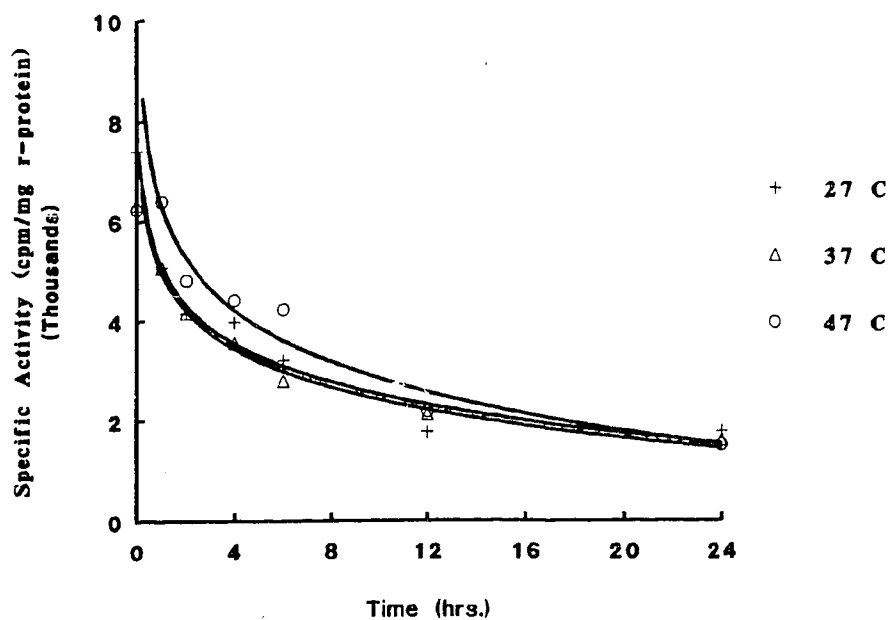


Figure 5. Ribosome Specific Activity Changes at Three Incubation Temperatures (Mesophile).

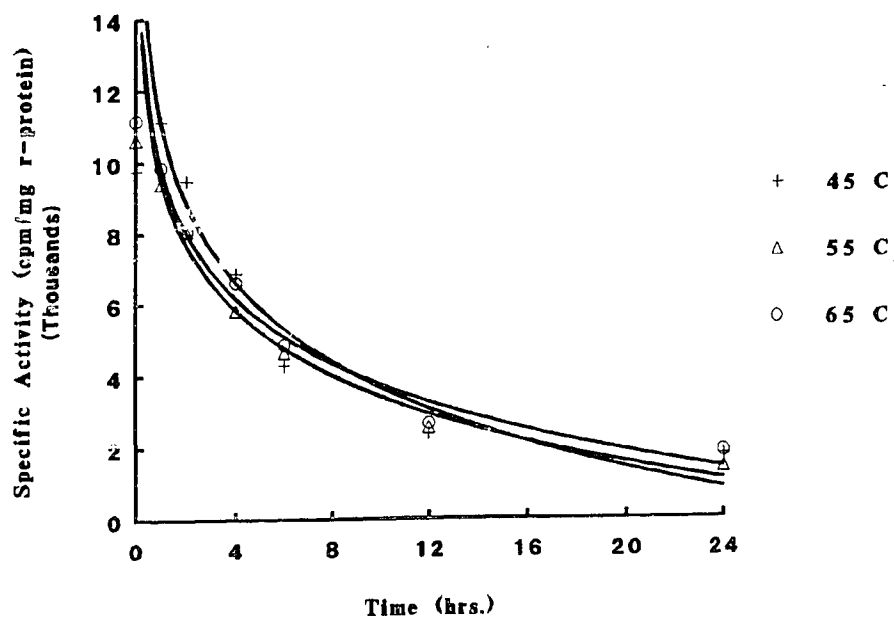


Figure 6. Ribosome Specific Activity Changes at Three Incubation Temperatures (Thermophile).

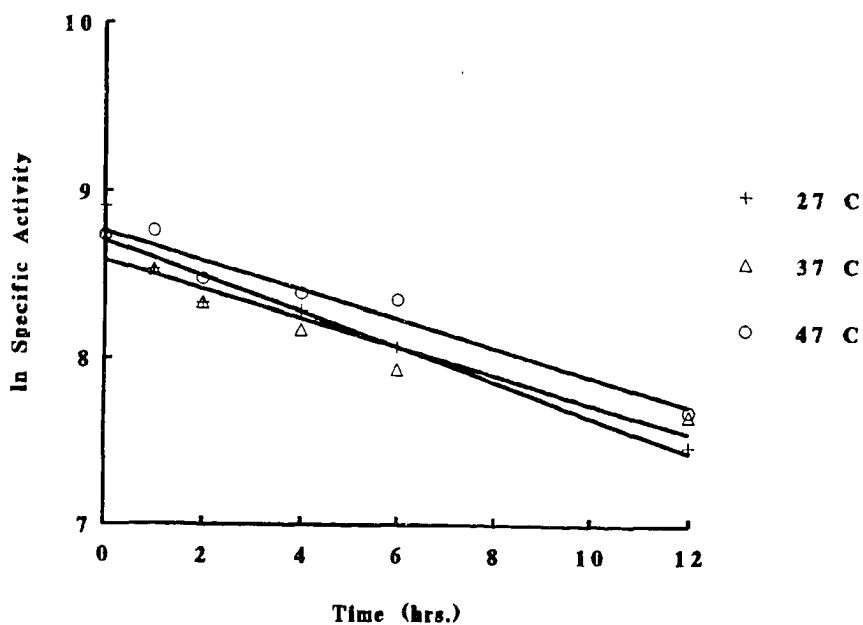


Figure 7. First-Order Reaction Plots for Ribosomal Degradation at Three Incubation Temperatures (Mesophile).

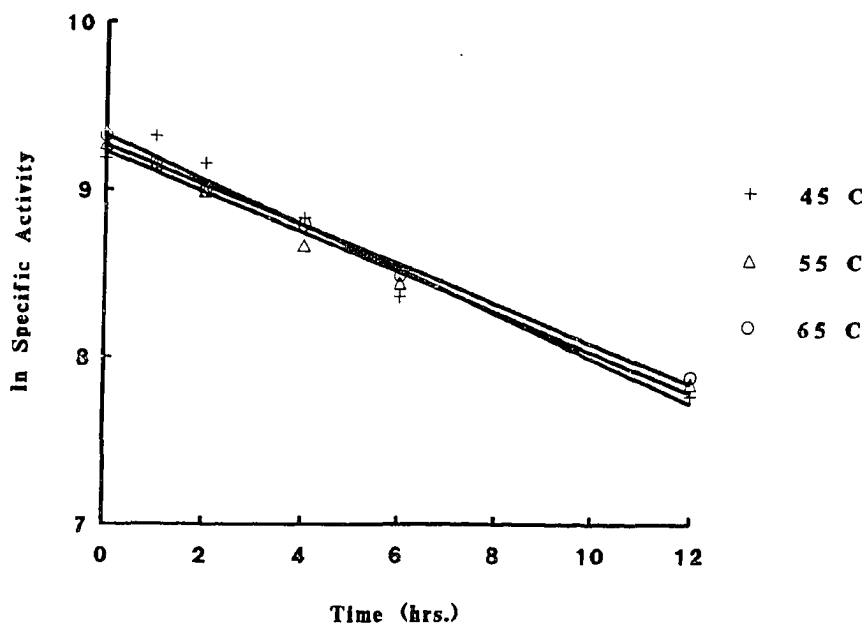


Figure 8. First-Order Reaction Plots for Ribosome Degradation at Three Incubation Temperatures (Thermophile).

Table 7

Half-Life Values for the Decrease in Specific Activity of Ribosomes
at Different Incubation Temperatures (Mesophile)

| | Temperature | | |
|-------------------------|-------------|--------|--------|
| | 27 °C | 37 °C | 47 °C |
| Slope | 0.1056 | 0.0860 | 0.0869 |
| Correlation Coefficient | 0.9670 | 0.9544 | 0.9774 |
| Half-Life (hrs.) | 6.56 | 8.06 | 7.97 |

Table 8

Half-Life Values for the Decrease in Specific Activity of Ribosomes
at Different Incubation Temperatures (Thermophile)

| | Temperature | | |
|-------------------------|-------------|--------|--------|
| | 45 °C | 55 °C | 65 °C |
| Slope | 0.1335 | 0.1203 | 0.1199 |
| Correlation Coefficient | 0.9788 | 0.9941 | 0.9957 |
| Half-Life (hrs.) | 5.19 | 5.76 | 5.78 |

At the higher incubation temperatures, the half-life was increased slightly. This was again true for both organisms. Moreover, the half-life was virtually the same at both the optimum growth temperature and at 10 °C above it for each organism. One might conclude that, within this 10 °C range, there are no significant changes in the steady-state of ribosome breakdown and synthesis for either the mesophile or the thermophile.

The differences in half-life at the lowest temperatures (25 or 45 °C) compared with those at the optimal growth temperatures (37 or 55 °C) were relatively small for both the mesophile and the thermophile. They were certainly much less than the factor of 2 by which the reaction rates of most chemical and enzymatic reactions are typically increased as the temperature is raised by 10 °C (72). Interestingly, this reaction rate does not increase for many enzymes from certain cold-adapting, intertidal species of invertebrates and several microorganisms (73). Apparently, in these organisms, the reaction rates do not change appreciably as a function of the changing environmental conditions (temperature). It appears from the study conducted here, that this is also true for the rates of ribosome breakdown and synthesis. In this respect, ribosomes may be unique and may differ significantly from other cellular proteins. As mentioned previously, most of the studies of intracellular protein degradation in growing bacteria have focused primarily on total cellular proteins. The half-lives of these processes are reportedly very short (several minutes) for about 10% of the proteins. However, most cellular proteins in growing bacteria are relatively stable, and are degraded to amino acids at an average rate of 1-3 % per hour (14, 15).

Ribosome Degradation Under Low Temperature (Nongrowth) Conditions

Growth in many microorganisms is slowed or suspended completely at low temperatures. In order to measure the intracellular degradation of ribosomes under nongrowth conditions, ribosomes were again labeled as previously described. The cells were harvested and then resuspended in fresh, cold (4 °C) medium that contained the mixture of unlabeled amino acids. Cells were allowed to "grow" in this medium for 36 hours while aliquots were removed as a function of time and analyzed as described. Since it was postulated that cell metabolism might be dramatically slowed at the lower temperatures, the longer incubation period was used in this experiment in order to detect any small changes that might occur as a function of time. The results are shown in Figure 9. For both the mesophile and the thermophile, a decrease of about 20% occurred in the specific activity during the first 2 hours of incubation. After that, the specific activity of the ribosomes remained essentially constant for the next 34 hours. It is probable that this initial loss was not due to degradation of intact ribosomes, but to the breakdown of ribosomes under assembly at the time of the transfer to the cold temperature environment. Thus, the response might have been due to a cold-temperature acclimation response. In any case, the data indicated that there were no significant differences between the mesophile and the thermophile, and that, at low temperatures, both ribosome degradation and ribosome synthesis appeared to completely stop in both organisms.

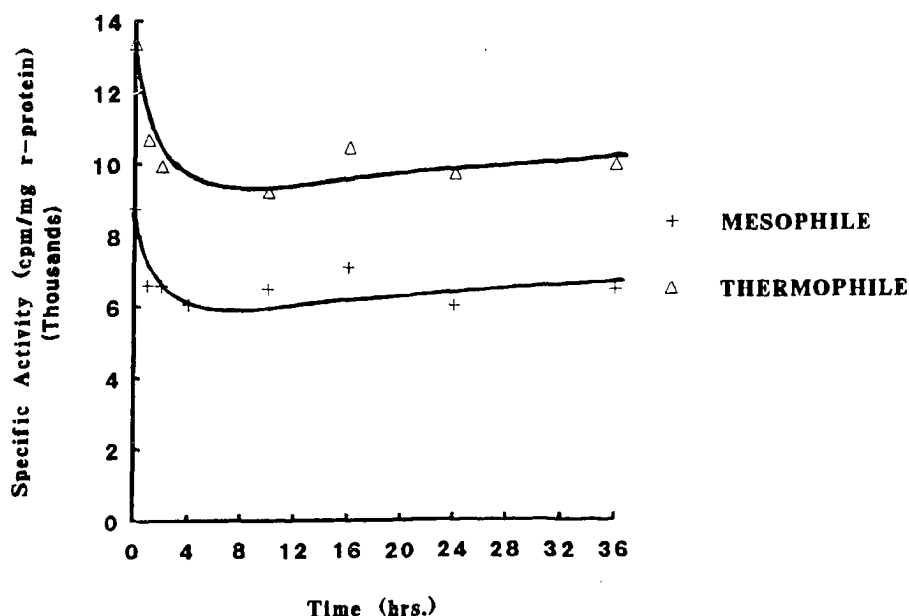


Figure 9. Ribosome Degradation Under Low Temperature (Nongrowth) Conditions (4 °C).

Ribosome Degradation Under High Temperature Conditions (70 °C)

Bacterial growth at high temperatures is apparently dependent on the thermostability of several critical macromolecules, especially those involved in protein biosynthesis (74). In order to investigate the *in vivo* thermostability of bacterial ribosomes under such conditions, the rate of degradation of the ribosomes was measured at 70 °C. As before, the cells were labeled by growing overnight in a medium containing the ^{14}C -protein hydrolysate and at the normal growth temperatures of the organisms (37 °C for the mesophile and 55 °C for the thermophile). The

labeled cells were then resuspended in prewarmed (70 °C) culture medium containing an excess of unlabeled amino acids, and aliquots were removed and analyzed as a function of time. The results are presented graphically in Figure 10. As can be seen, after the first 2 hours there was virtually no loss in specific activity for the mesophile, even after incubation for up to 24 hours at 70 °C. The final specific activity was essentially the same as the initial, zero-time value. These results probably indicate that the high temperature caused a loss of integrity of the bacterial cell membrane plus denaturation and inactivation of the enzymes involved in both the degradation and biosynthesis of the ribosomes for the mesophile. In the case of the thermophile, on the other hand, the change in specific activity was similar to that observed previously at 65 °C and had a half-life value of 5.4 hours. A temperature of 70 °C is at or near the maximum growth temperature for this organism (43, 74).

The data indicated that both ribosome degradation and synthesis occurred at comparable rates for the thermophile at both the maximum growth temperature and at lower temperatures. These data are interesting in that they suggest that, at least at high temperatures, there is a greater relative thermostability (*in vivo*) for certain thermophilic enzymes and macromolecules as compared with mesophilic ones, a finding that is in agreement with the macromolecular theory of thermophily (43).

It should be noted that the curves in Figure 10 and the other figures are drawn with a specific graphic software program. In many cases the fit appears to be a very good one, but in other cases, there could be a different graphical interpretation of the data.

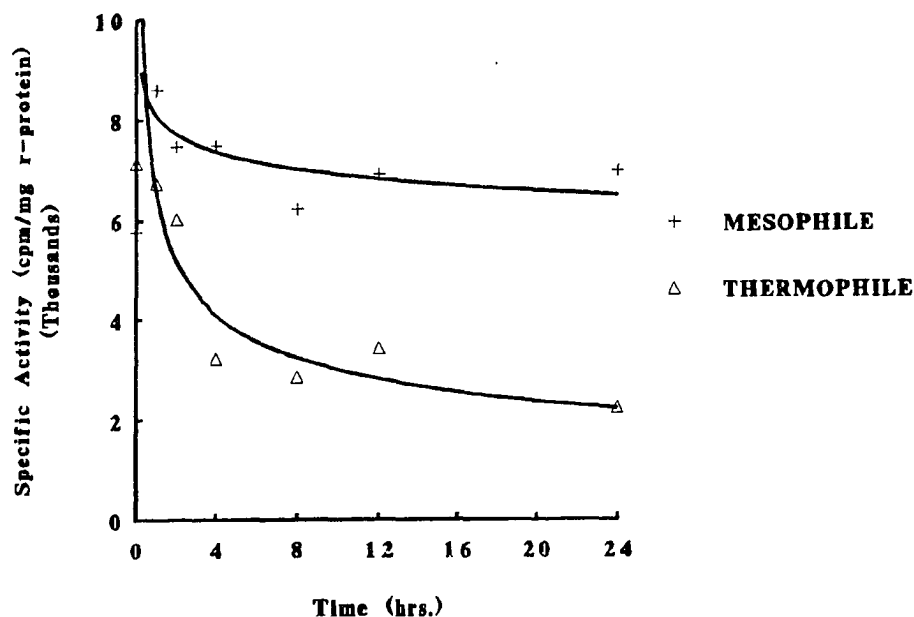


Figure 10. Ribosome Degradation Under High Temperature Conditions (70 °C).

The curve for the mesophile in Figure 10 is a case in point. There, and in certain other experiments, an initial increase in specific activity was observed, usually within the first 1-2 hours. If true, the data demonstrated that under certain conditions, the cells experience a sudden, short-term burst of ^{14}C -label incorporation after resuspension in the final growth medium.

Ribosome Degradation Under Starvation Conditions

For this study the cells were labeled, grown, and collected as previously described. The cells were then washed and resuspended in prewarmed, sterile distilled water (devoid of any labeled amino acids) for further growth and sampling. The

results are presented in Figure 11. In general, the degradation patterns were similar for both organisms, but the half-life for the mesophile was 10.6 hours while that for the thermophile was 6.5 hours. These half-life values were about 10% larger than those observed under normal growth conditions (Table 6). It should be noted that no unlabeled amino acids were added to the incubation mixture so as to prevent reincorporation of the original labeled amino acids. Consequently, the longer half-lives under starvation conditions might have resulted from short-term reincorporation of radiolabeled amino acids, which were derived from endogenous labeled precursors released by surviving cells into the medium (possibly due to partial lysis of some cells [75]).

These results would seem to contradict certain previous reports (11, 14) investigating total cell protein degradation patterns (nonribosomal proteins). In those studies, investigators claimed that the rates of total cellular protein degradation were greatly increased in bacteria under starvation conditions. Further, it was reported (14) that the amounts and types of proteins degraded during starvation were quite different from those degraded normally in exponentially growing cells. The observed acceleration of total cell protein degradation during starvation was readily reversible after the return of nutrients.

Very little has ever been reported on the degradation patterns of ribosomes (in vivo). Cohen and Ennis (95), reported that in E. coli cells, starvation resulting in glucose exhaustion or Mg^{+2} depletion caused a rapid breakdown of the polysomes to 70S monosomes. These monosomes accumulate in the cells during the early phases

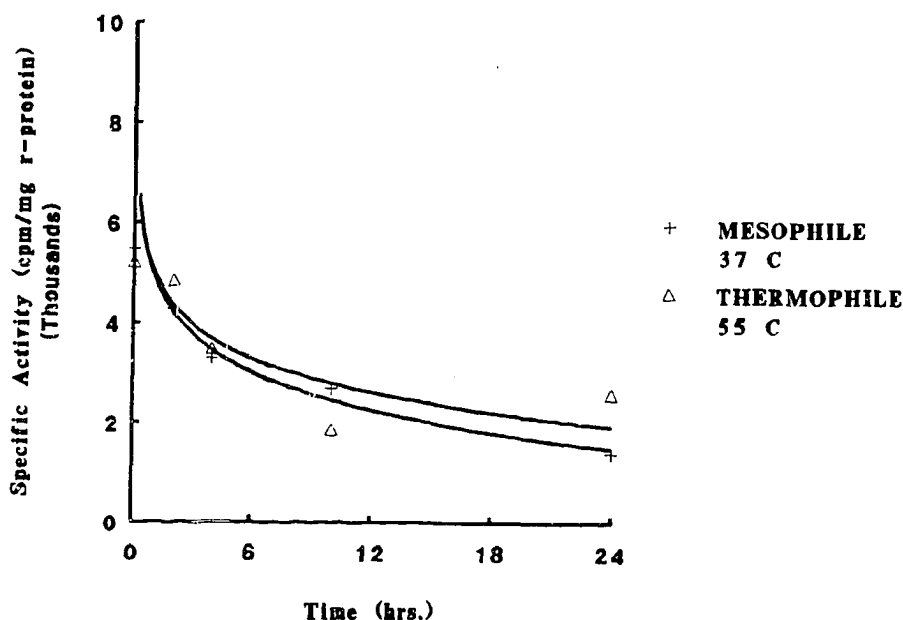


Figure 11. Changes in the Specific Activity of Ribosomes Under Starvation Conditions.

of starvation. According to their studies, the 70S monosomes are eventually broken down to ribonucleoprotein structures of lower Svedberg sedimentation coefficients. Apparently, the RNA is not broken down further to smaller molecules immediately, nor is the degraded RNA reutilized in forming new ribosomes. In contrast, the ribosomal protein from degraded ribosomes is reused in the production of new ribosomes (96). According to Schlessinger and Ben-Hamida (96), the synthesis of ribosomes is markedly reduced during starvation, but is still measurable. They estimated that during starvation the rate of ribosomal protein synthesis is about 5% the rate of soluble protein synthesis. They concluded that in starving bacterial cells,

ribosomal proteins start to degrade at 1% per hour, and this degradation continues for several hours. The slow degradation in starving cells provides, from a metabolically useless excess of ribosomes, a pool of precursors to support synthesis of soluble proteins and energy supplies for subsequent restoration of normal cell growth later.

Ribosome Degradation in the Presence of an Amino Acid Analog (Canavanine)

It is known that the incorporation of certain structural analogs of amino acids into bacterial proteins results in the formation of inactive or defective enzymes and proteins as well as in the inhibition of growth of these organisms (76-78). These defective, or abnormal, proteins are reportedly subject to extremely rapid proteolytic degradation, and are thus rapidly catabolized to amino acids and removed from the cells (21, 79). L-canavanine is an analog of L-arginine which is known to function in this fashion, and is incorporated into intracellular proteins of E. coli. When intracellular arginine concentrations were low, incorporation of canavanine (50-100 ug/mL) in E. coli cells resulted in growth inhibition, and ultimately in cell death (77). Since ribosomes contain significant amounts of arginine, it is likely that this analog could substitute for arginine to a great extent in ribosomal proteins containing this amino acid.

In an attempt to compare the effects of defective or abnormal ribosomal proteins on ribosomal protein degradation rates, cells were grown as before in liquid medium containing the ¹⁴C-protein hydrolysate. Cells were then washed, pelleted, and

resuspended in the prewarmed culture medium containing the unlabeled amino acid mixture (minus arginine) plus 300 ug/mL of canavanine. The cells were allowed to continue to grow at their respective optimal growth temperatures (mesophile 37 °C, thermophile 55 °C). Results are shown in Figure 12. No significant differences were seen in ribosomal protein degradation rates between the mesophile and the thermophile. Calculated values for the half-life of this process were 6.4 hours for the mesophile and 5.2 hours for the thermophile. These values are very similar to those for cells grown under normal growth conditions. It is probable that any possible effect due to the presence of canavanine was obscured by the use of the rich culture medium, which probably contained at least small amounts of arginine derived from other components of the liquid medium.

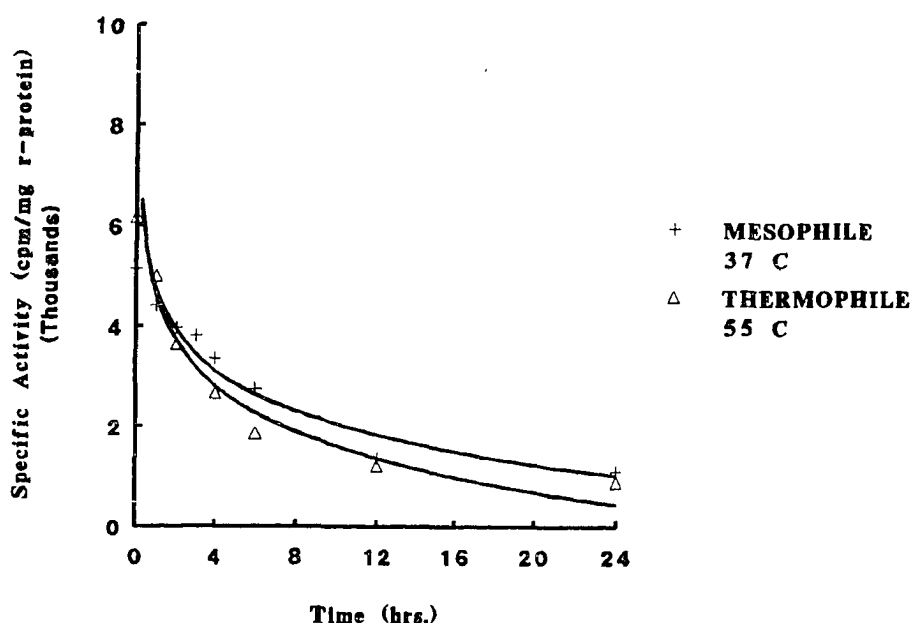
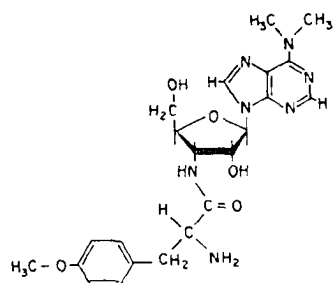


Figure 12. Changes in Ribosome Specific Activity in the Presence of Canavanine.

Ribosome Degradation in the Presence of Inhibitors of Protein Synthesis

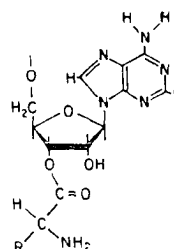
Several antibiotics have been shown to be potent inhibitors of protein synthesis (80-82), and have been used as tools in the study of the macromolecules and processes involved in protein synthesis. Many antibiotics function by binding to bacterial ribosomes and/or by interrupting protein synthesis by various mechanisms. In bacteria grown in the presence of low-moderate levels of such antibiotics (20-200 ug/mL), protein synthesis is partially inhibited, while DNA/RNA synthesis continue for a time at near normal rates. The purpose of our studies was to attempt to shut off protein biosynthesis and then to compare the rates of degradation of the ribosomes in the mesophile and the thermophile. In order to assess the effect of the protein synthesis inhibitors on bacterial ribosome degradation rates, three well-known antibiotics were chosen for study: puromycin, chloramphenicol, and tetracycline.

Puromycin (Structure I), has a structure similar to the aminoacylated 3'-terminal adenosine of an aminoacyl-tRNA molecule (Structure II).



PUROMYCIN

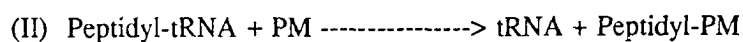
Structure I



AMINOACYLATED 3'-END
OF tRNA

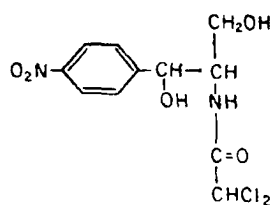
Structure II

Peptidyl transferase activity is the main catalytic activity of the ribosomes in the process of synthesizing the growing polypeptide chain. Puromycin (PM), when added to the intact, translating ribosome (of either prokaryotes and eukaryotes), results in an interruption of peptide-chain elongation (Equation II) by replacing an entering aminoacyl-tRNA (83, 84):



The peptidyl-puromycin cannot react with additional aminoacyl-tRNA molecules; thus, synthesis of the polypeptide chain is terminated, and the incomplete peptide is released from the ribosome.

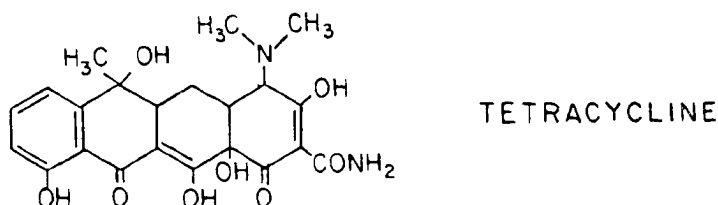
Chloramphenicol (Structure III, also known as chloromycetin) is a well-known inhibitor of prokaryotic ribosomes (85, 86), and also functions by blocking the peptidyl transferase activity of the 50S ribosomal subunit.



CHLORAMPHENICOL

Structure III

Tetracycline (Structure IV) acts by binding to the 30S ribosomal subunit in prokaryotes (87). It blocks the A site on the ribosome and prevents the binding of any additional aminoacyl-tRNAs to the ribosome.



Structure IV

Preliminary studies were carried out to select concentrations of antibiotics that caused a quick and complete cessation of cell growth and of protein synthesis. For those studies, bacterial cells were grown overnight in liquid medium (without labeled amino acids) as previously described (the mesophile at 37 °C and the thermophile at 55 °C). The cells were then harvested and resuspended in fresh, prewarmed medium containing varying, increasing concentrations of a given antibiotic plus the cold amino acid mixture. The continued growth of the cells was monitored by measuring the absorbance at 540 nm as a function of time (as described under Growth Curves, above). Results of these preliminary experiments are shown in Figures 13, 14, and 15. Based on these data, various concentrations of antibiotic were chosen for use in the ribosome degradation studies. The following concentration of antibiotics in the culture medium were used in the final studies: puromycin (0 [Control], 20, 50, 300

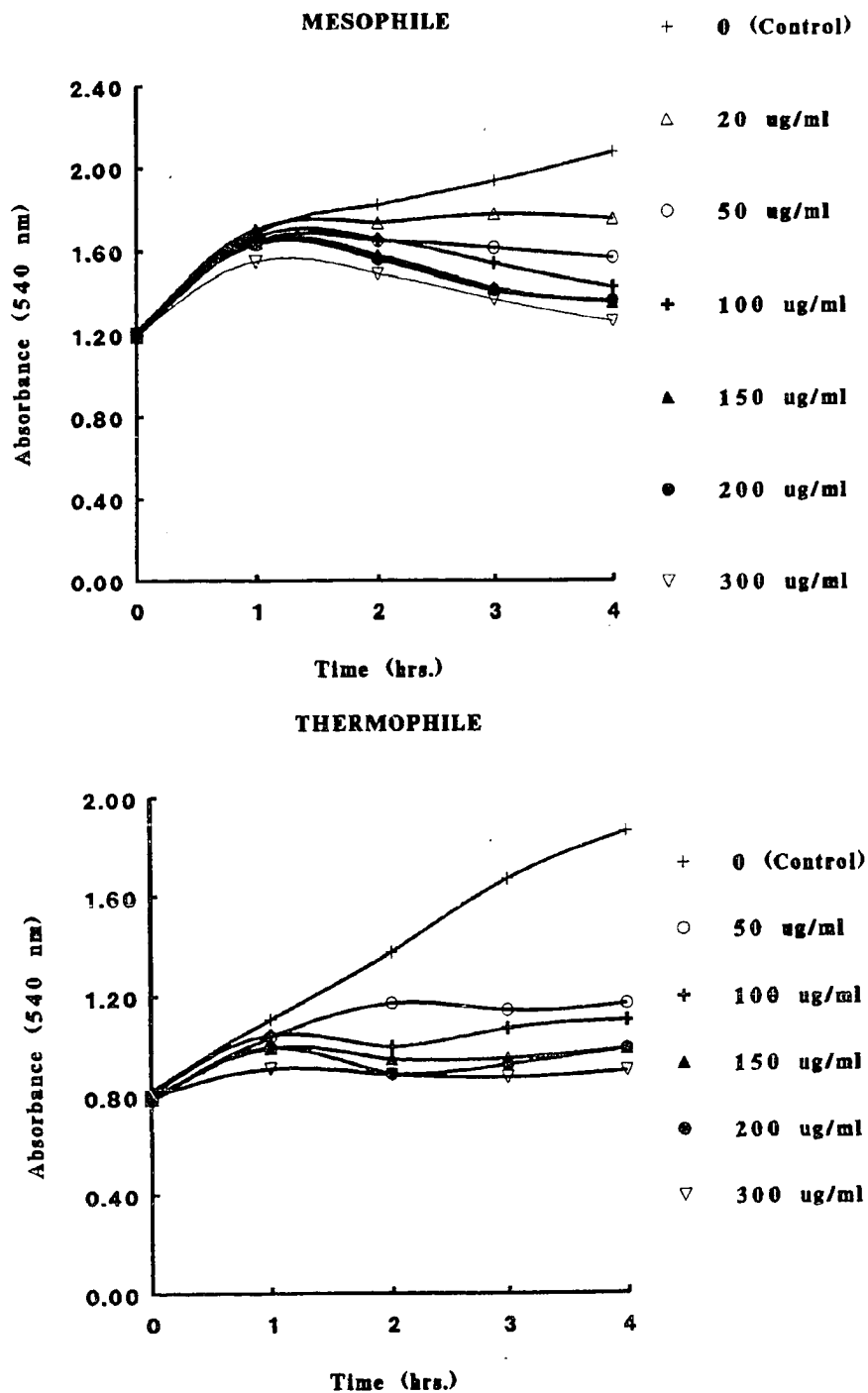
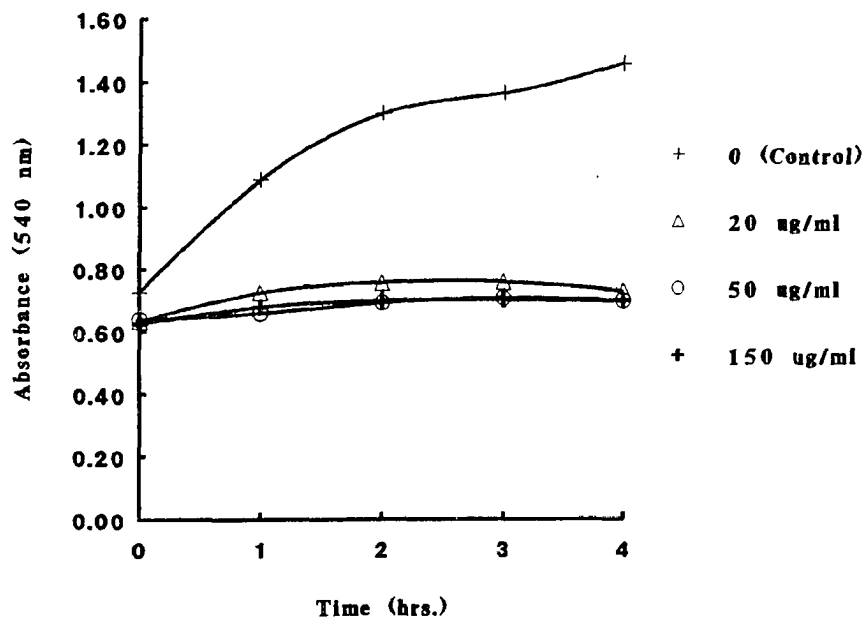


Figure 13. Effect of Puromycin on Bacterial Growth.

MESOPHILE

57



THERMOPHILE

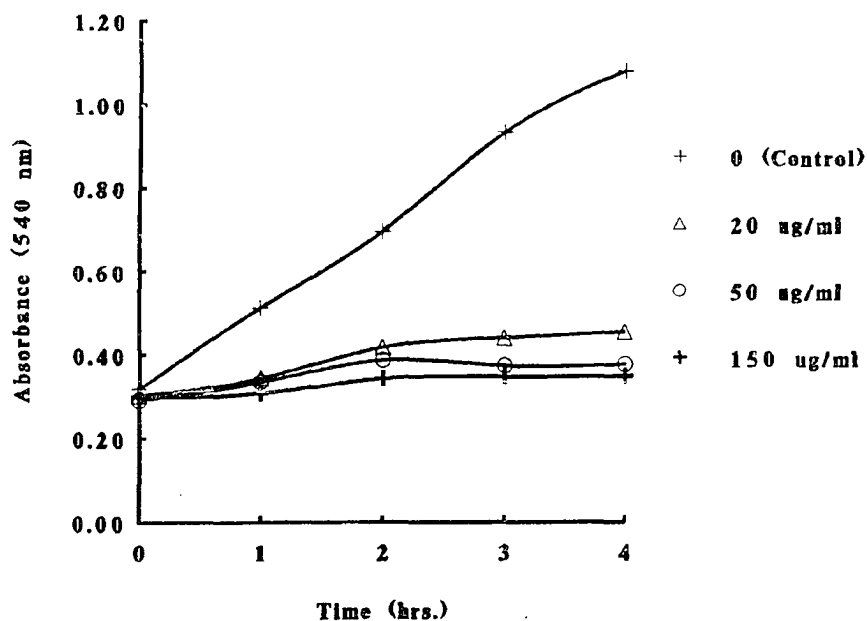


Figure 14. Effect of Chloramphenicol on Bacterial Growth.

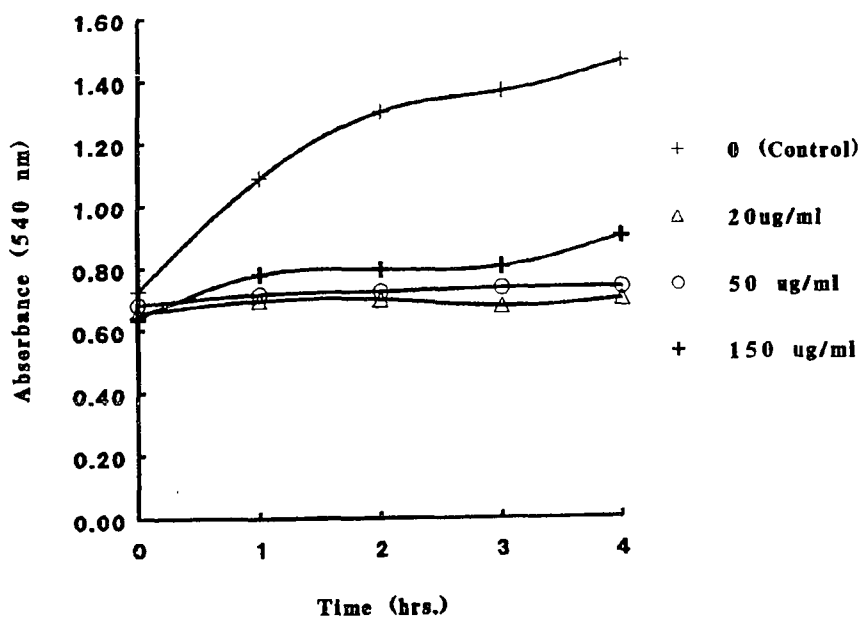
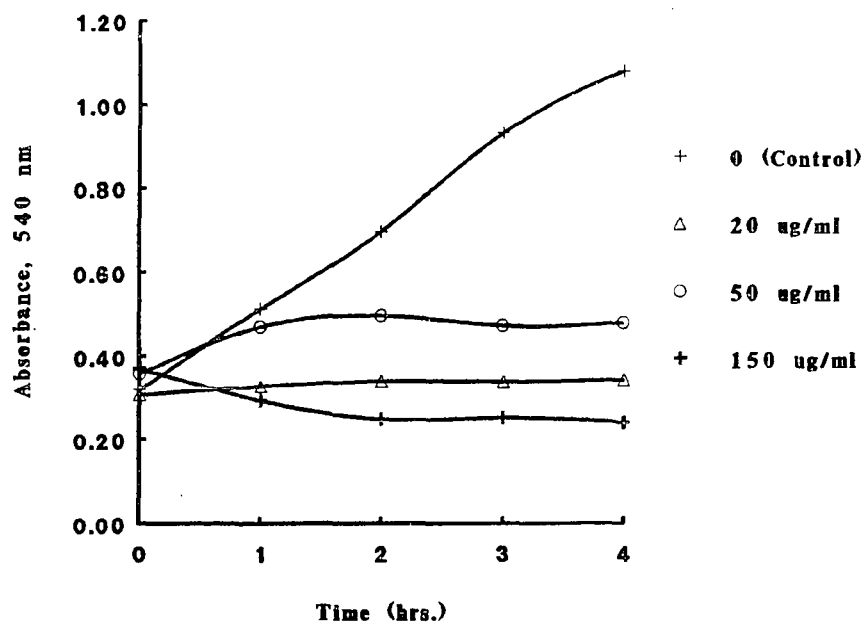
MESOPHILE**THERMOPHILE**

Figure 15. Effect of Tetracycline on Bacterial Growth.

ug/mL), chloramphenicol (0 and 100 ug/mL), and tetracycline (0 and 100 ug/mL). For the ribosome degradation studies, the cells were grown and radioactively labeled at 37 °C for the mesophile and at 55 °C for the thermophile as previously described. After the cells were collected, they were quickly resuspended in prewarmed liquid culture medium containing a given concentration of a specific antibiotic (plus the unlabeled amino acid mixture).

The effect of puromycin on ribosome degradation is shown in Figure 16 for the mesophile and in Figure 17 for the thermophile. It can be seen from these figures that the rate of ribosome degradation leveled off at slightly different times for the different levels of puromycin in the two organisms. It also appeared that for the mesophile, the specific activity actually increased slightly with high levels (300 ug/mL) of puromycin. For the thermophile the ribosome degradation process completely stopped at 300 ug/mL puromycin, giving a totally flat response over time. The half-life values corresponding to the curves of Figures 16 and 17 are shown in Table 9. No attempt was made to calculate the half-life value for the curves corresponding to the highest puromycin concentration (300 ug/mL). At the lowest puromycin concentrations (20 ug/mL), where cell growth (and protein synthesis) were apparently only partially inhibited, the half-life for both the mesophile and the thermophile was longer than in the absence of puromycin. The results possibly indicated either a stabilization of the ribosome or enhanced protein synthesis. At a puromycin concentration of 50 ug/mL, the half-life was, within experimental error, nearly identical to that in the absence of puromycin for both the mesophile and the

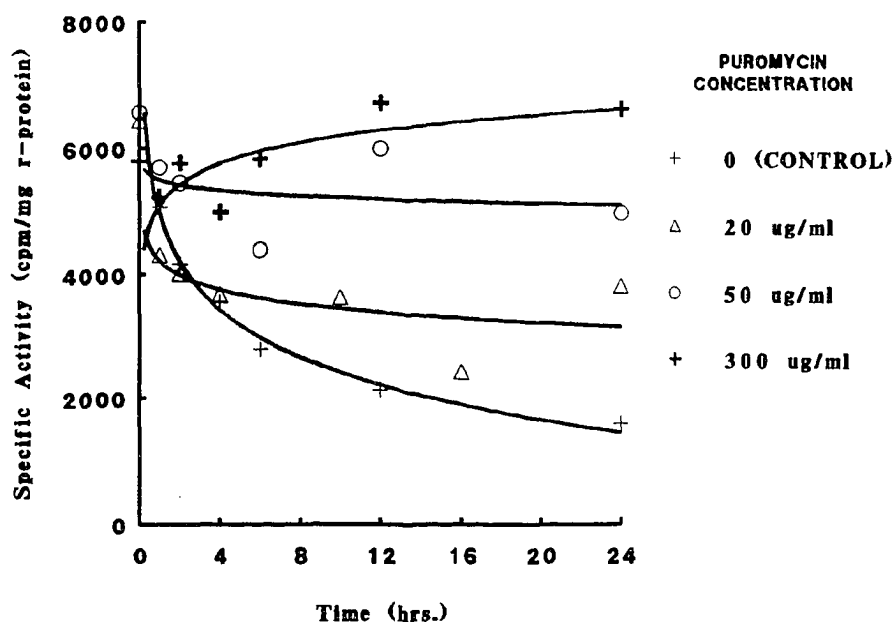


Figure 16. Changes in Ribosome Specific Activity in the Presence of Puromycin (Mesophile).

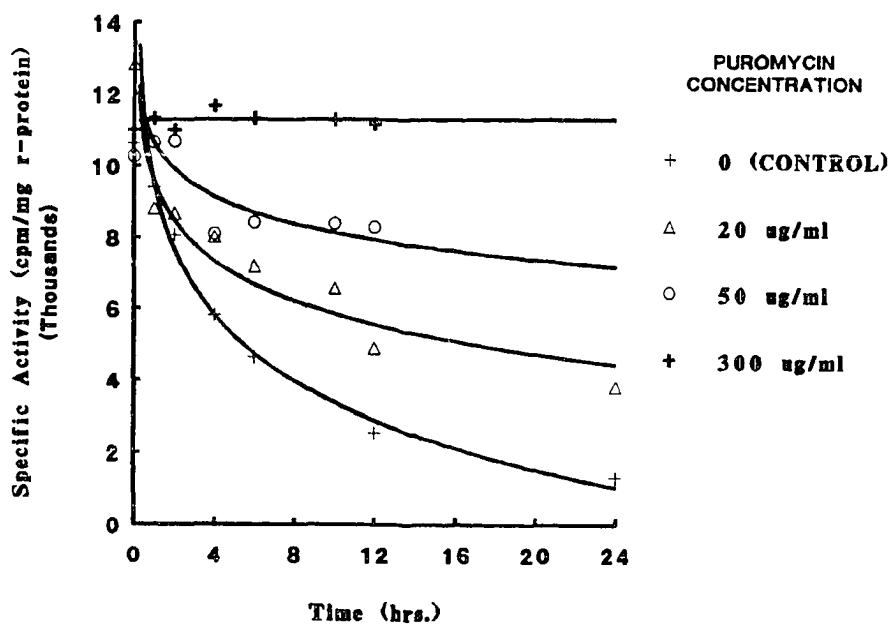


Figure 17. Changes in Ribosome Specific Activity in the Presence of Puromycin (Thermophile).

Table 9

Half-Life Values for the Decrease in Specific Activity of Ribosomal
Proteins in the Presence of Puromycin

| | Puromycin, ug/mL | | | |
|--|------------------|--------|--------|-----|
| | 0 | 20 | 50 | 300 |
| <u>Mesophile</u> <u>(B. Licheniformis)</u> | | | | |
| Slope | 0.0860 | 0.0503 | 0.0736 | ND |
| Correlation Coefficient | 0.9544 | 0.8523 | 0.7692 | ND |
| Half-Life (hrs.) | 8.1 | 13.8 | 9.4 | ND |
| <u>Thermophile</u> <u>(B. stearothermophilus)</u> | | | | |
| Slope | 0.1203 | 0.0812 | 0.1167 | ND |
| Correlation Coefficient | 0.9941 | 0.9288 | 0.8976 | ND |
| Half-Life (hrs.) | 5.8 | 8.5 | 5.9 | ND |

ND, calculation not done.

thermophile. Since under this condition, it appeared that bacterial growth and protein synthesis were more effectively inhibited during the first hour (see Figure 13), this change in specific activity must have represented ribosome degradation. Also, since this half-life value was similar to that observed under normal intracellular conditions, it is likely that simple ribosome degradation was the most appropriate model. At the highest concentration of puromycin tested (300 ug/mL), the growth of the bacterial cells was nearly completely inhibited. Under these conditions, the ribosomes were seemingly "frozen" biochemically, and thus ribosome degradation was slowed to undetectable levels.

The observed variation in the rates of degradation of ribosomes with increasing concentrations of puromycin was interesting. Puromycin is a broad-spectrum, bacteriostatic antibiotic (rather than bacteriocidal [88]). White and White (89) reported that under treatment with high concentrations of puromycin (>500 ug/mL), E. coli ceased to multiply, but the viable counts did not decrease for another 2 hours. However, in studies with B. subtilis (88), cells were killed with as little as 30 ug/mL puromycin, but not with 100 ug/mL as measured by viable counts. The speculation was that this paradoxical observation was caused by the lethality of accumulated, long, nonfunctional peptides expected at low concentrations, but not seen at high concentrations. Earlier studies from this lab (Shen, 90), also demonstrated opposing effects of puromycin on protein synthesis. In studies of cell-free protein synthesis, Shen reported a pronounced stimulatory effect on protein synthesis at low concentrations of puromycin. The thermophile (B. stearothermophilus) was more

sensitive than the mesophile to the effects of the antibiotic. He speculated that the difference in sensitivity was attributable to a higher binding affinity of the drug to thermophilic ribosomes, or that the puromycin binding step in the thermophile was faster than in the mesophile.

It was not possible to definitively demonstrate any such enhancing effect of this antibiotic on either one of the two tested bacterial strains from the data described in our studies. However, the observed results demonstrated an interesting dose-related effect of the antibiotic on the rate of degradation of the ribosomes in the cells. The results for the mesophile and the thermophile were similar and parallel to a great extent. According to Takeda, Hayashi, Nakagawa, and Suzuki (91), one dramatic consequence of the imbalance in RNA and protein synthesis in puromycin-treated bacterial cells is the accumulation of abnormal, RNA-rich ribonucleoprotein particles in these cells. These ribonucleoprotein particles (PM-particles) are apparently aberrant ribosome precursors that are similar to those reported with other antibiotics (92; also see discussion below). These PM-particles are stable and are not readily degraded in cells grown in a rich, supplemented medium. A massive accumulation of PM-particles might explain the virtually undetectable degradation of the ribosomes for both the mesophile and the thermophile at high concentrations of puromycin (300 ug/mL).

The effect of chloramphenicol on ribosome degradation is shown in Figure 18. Results for both the mesophile (solid lines) and the thermophile (dotted lines) are presented on the same plot. As can be seen in the figure, the degradation of the

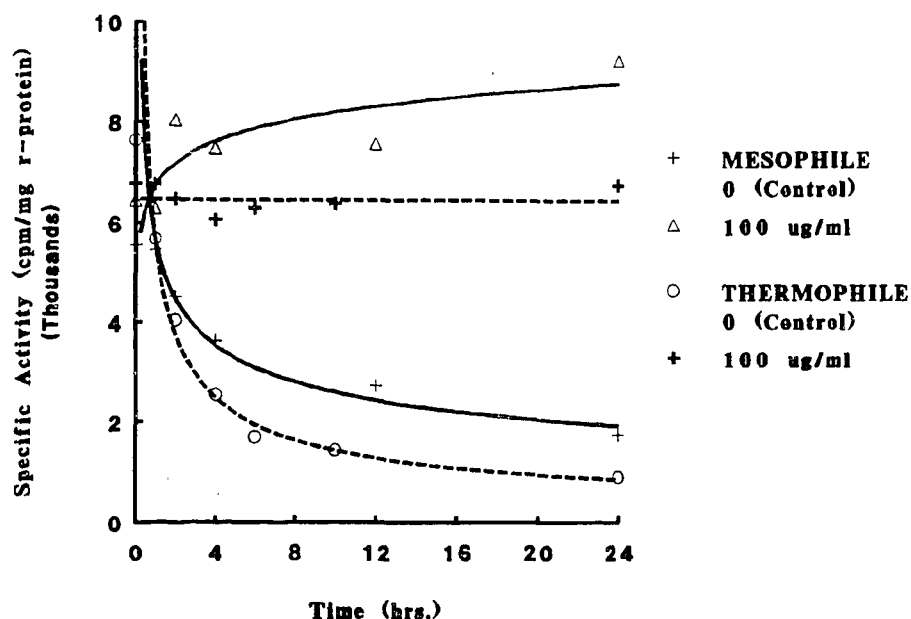


Figure 18. Changes in Ribosome Specific Activity in the Presence of Chloramphenicol.

ribosomes was virtually stopped for both bacteria treated with chloramphenicol (100 ug/mL). Remarkably, cells from the mesophile showed a slight increase in specific activity as a function of time, while there was virtually no change in the specific activity for the thermophile over the entire 24-hour period. These results are qualitatively similar to those with high concentrations of puromycin.

The effect of tetracycline on ribosome degradation is shown in Figure 19 (mesophile, solid lines; thermophile, dotted lines). Again, rates of ribosome protein degradation were seen to be fairly constant during the time interval under study. For the mesophile, the degradation rates, although somewhat erratic, declined slightly up

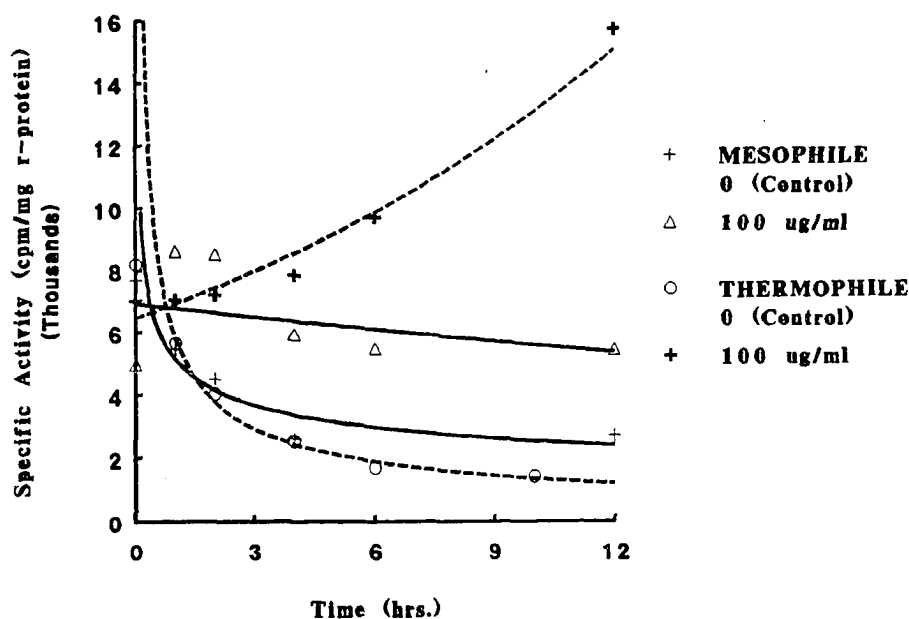


Figure 19. Changes in Ribosome Specific Activity in the Presence of Tetracycline.

to 4 hours, and then reached a plateau after that time-point. For the thermophile, the specific activity was seen to increase up to 2.5 times the initial (t_0) value after 10 hours of incubation. No further measurement was possible, due to partial lysis of the cells, as evidenced by the decline in the size of the cell pellet used for the assay measurements. One possible explanation for the results with the thermophile is that the partial lysis of the cells allowed the reutilization of some of the labeled amino acids (released by lysed cells) in the surviving cells.

Nomura and Hosakawa reported (92) the existence of chloramphenicol particles (CM-particles), that had many similarities to the PM-particles mentioned above, and that were derived from RNA grown in the presence of chloramphenicol. (The

existence of similar tetracycline particles is uncertain). While normal ribosomes are composed of about 65% RNA and 35% protein, in PM- or CM-particles, the proportion of protein is less (PM-particles contained 30%, while CM-particles contained 25% protein). The ribosomal subunits contained in the particles have been characterized and represent various stages in the assembly of the 30S and 50S ribosome units (93).

Several investigators have reported that protein synthesis inhibitors also reduce protein degradation in bacterial cells (11, 14, 96). However, this effect was only demonstrable in cells in poor nutritional states or under starvation conditions. As was previously noted for cells grown under starvation conditions (11, 31), the overall rates of protein degradation are accelerated above basal levels, while the rates of protein synthesis are reduced. Interestingly, the effects of the protein synthesis inhibitors on accelerated protein degradation (in starving cells) were only decreased when protein synthesis was blocked almost completely, (e.g., >80-90% inhibition, at high concentrations of antibiotics). In other studies, the sensitivity of normal cells to the effects of the protein inhibitors was also apparently quite dependent on whether a minimum salts medium was used for growth or whether a fully supplemented medium was utilized (94). When the fully supplemented medium was used, the effect of the protein synthesis inhibitors on protein degradation rates was minimal.

The complexity of the in vivo experimental data reported here made interpretation of the results somewhat difficult. The most logical explanation for the observed results was that these antibiotics all caused a slowdown in the rate of the

degradation of the ribosomes to barely detectable levels. The accumulation of ^{14}C -labeled ribosome particles in the treated cells (puromycin and chloramphenicol) occurred when protein synthesis was terminated. The termination of protein synthesis also prevented the synthesis of the appropriate ribonucleases to degrade the ribosomes. Due to the overall similarities in the results for the mesophile and the thermophile, one might conclude that there were no significant differences in the rates of degradation of the ribosomes in the presence of moderately inhibiting levels of antibiotics.

In Vitro Stability of Isolated, ^{14}C -Labeled Ribosomes

Differences in the in vitro stability of bacterial ribosomes from thermophiles as compared with those from mesophiles have been frequently reported by several investigators (64-66) over the past 40 years. These data have provided a significant share of the evidence substantiating the macromolecular theory of thermophily. As previously mentioned, Stenesh and Yang (68) from this laboratory, reported that the in vitro stability of ribosomes from thermophiles was slightly greater than that of ribosomes from the mesophiles at both 37 °C and 60 °C. They also provided evidence that a similar ribonuclease was involved in the degradation processes in all six Bacillus strains tested (including the two strains studied in the present work). In an attempt to further corroborate their findings, isolated, ^{14}C -labeled ribosomes from the mesophile (B. licheniformis) and the thermophile (B. stearothermophilus) were incubated at several different temperatures over a 6 hour period. In the initial studies,

comparisons were made between ribosomes incubated at their optimal growth temperatures, and at 70 °C. In other studies, the isolated ribosomes from the mesophile were incubated at 27, 37, and 47 °C, while those from the thermophile were incubated at 45, 55, and 65 °C. Incubations were performed with and without 0.01 M EDTA (Edetate, ethylenediaminetetracetate) in the assay buffer in order to evaluate the influence of bacterial ribonucleases on the rates of ribosome degradation. EDTA functions to chelate magnesium ions present in the assay buffer; magnesium is a potent inhibitor of the enzymes. In the initial study, the changes in ^{14}C -counts in the perchloric acid soluble fraction were measured as a function of time and temperature. In subsequent studies, the percent (%) ribosome degradation or breakdown was calculated from the increase in the net acid-soluble counts relative to the zero-time controls.

Comparisons of the rate of in vitro ribosome breakdown at 37 versus 70 °C (mesophile, solid lines) and 55 versus 70 °C (thermophile, dotted lines) over a 6-hour time period are shown in Figure 20. The figure depicts the increase in ^{14}C -labeled amino acids released into the supernatant (relative to the zero-time sample) as a function of time and temperature. The original (zero-time) counts were about 20,000 cpm in the intact ribosome pellet for both organisms. After 6 hours of incubation at the normal growth temperatures (mesophile, 37 °C and thermophile, 55 °C), the levels of acid-soluble counts in the supernatant were similar for the two organisms (about 4000 cpm for the mesophile and about 5000 cpm for the thermophile). At the high incubation temperature (70 °C) however, the level of acid-soluble, ^{14}C -labeled amino

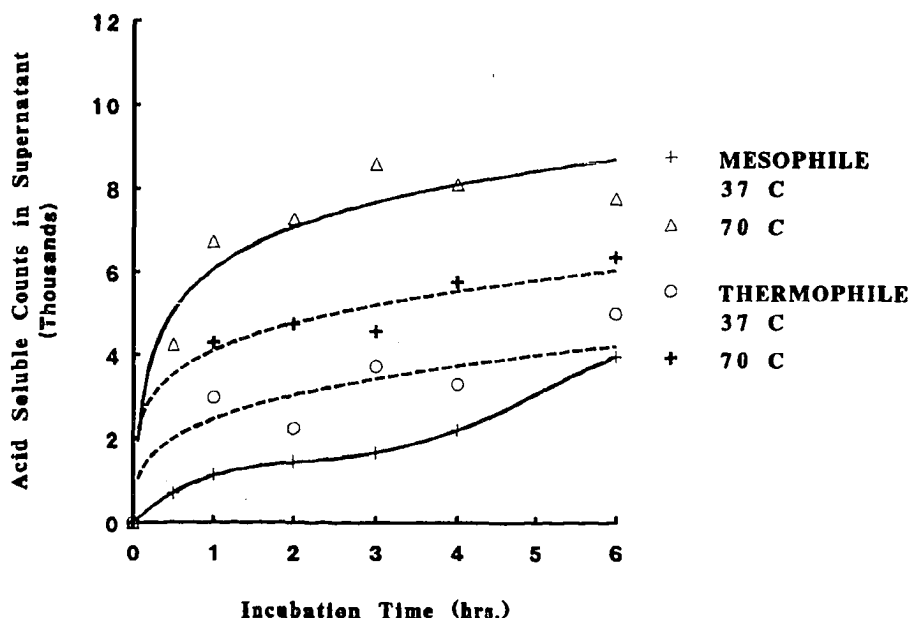


Figure 20. In Vitro Degradation of Ribosomes at Normal and High Temperatures.

acids increased for the mesophile nearly fourfold during an incubation period of 6 hours. For the thermophile, the level was increased also, but was only about twofold higher than control levels over the same incubation period. These data demonstrated a slight thermostability of the thermophilic ribosomes (in vitro) as compared with mesophilic ones. Rates of ribosome degradation for the mesophile at 27, 37, and 47 °C (with and without EDTA) are shown in Figure 21. The rates of ribosome degradation were measured as the net percent increase in ^{14}C -labeled amino acids in the supernatant as a function of time and temperature. (The zero-time point was arbitrarily designated as 0%. Zero-time counts averaged about 1500 cpm for the mesophile and about 1750 cpm for the thermophile). In the plots, the curves

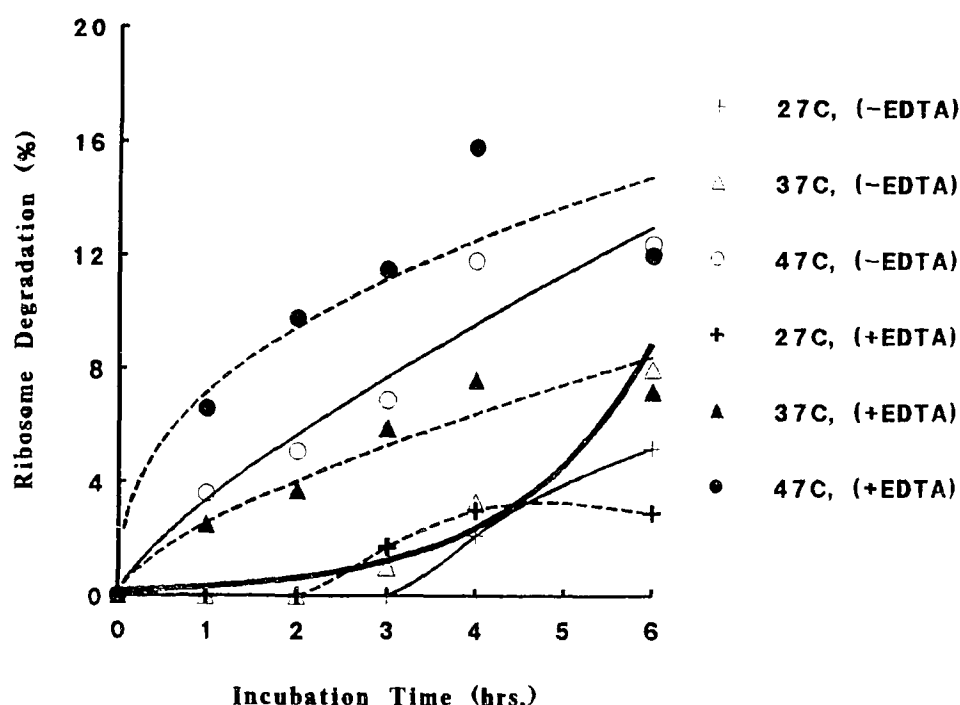


Figure 21. In Vitro Degradation of Mesophilic Ribosomes (\pm EDTA).

depicting treatment with EDTA are represented by dotted lines while those without EDTA added are represented with solid lines. At the lowest temperature (27 °C), little degradation is apparent until 3 hours of incubation. However, in nearly all cases, the rates of ribosome degradation were slightly higher in the presence of EDTA for the mesophile.

Results for the thermophile at 45, 55, and 65 °C (with and without EDTA) are shown in Figure 22. The data indicated that the results for the thermophile were similar in most respects to those described for the mesophile. In general, the in vitro rates of ribosome degradation increased as a function of time as the temperature was increased for both the mesophile and the thermophile. At the temperature that most

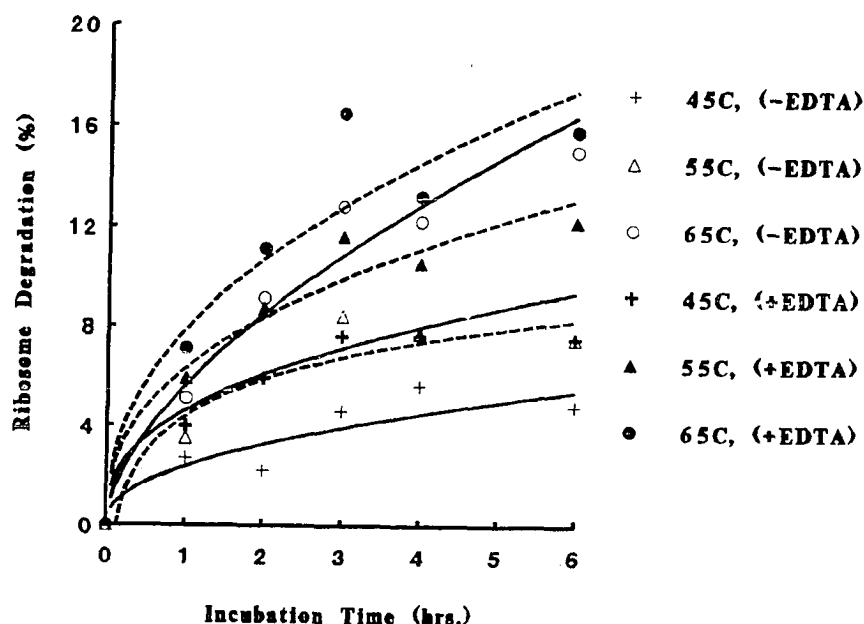


Figure 22. In Vitro Degradation of Thermophilic Ribosomes (\pm EDTA).

closely represented a common sampling temperature (47°C for the mesophile, and 45°C for the thermophile), the rates of ribosome degradation were 12.4% and 12.0% (with and without EDTA) for the mesophile, and 4.8% and 7.5% (with and without EDTA) for the thermophile after 6 hours of incubation. Thus, it appeared that, at this temperature, the rate of in vitro degradation of ribosomes in the thermophile was about half the rate observed in the mesophile. These results were in general agreement with those reported by Stenesh and Yang (68), except that the current degradation rates were about half those previously reported. Therefore, as reported by Stenesh and Yang, our data suggest that thermophilic ribosomes are slightly more stable in vitro.

than mesophilic ribosomes. The differences were not pronounced, however, and thus do not provide a clear clue to the origin of thermophily.

CHAPTER IV

SUMMARY

This study was designed to compare the rates of intracellular degradation of ribosomal proteins (in vivo) in a mesophile (B. licheniformis) and a thermophile (B. stearothermophilus). This study represents the first comparisons of in vivo ribosome stability in bacteria from the same genus (Bacillus). Cells were allowed to grow overnight in the presence of a ^{14}C -protein hydrolysate in order to adequately label the ribosomal proteins. The degradation rates were monitored as changes in the specific activity of the ^{14}C -labeled ribosomes (cpm/mg ribosomal protein) under various growth and incubation conditions as a function of time. In addition, a brief study examining the in vitro stability of ^{14}C -labeled ribosomes was also done using isolated, purified ribosomes.

The results indicated that ribosomes from the thermophile were degraded at rates that were similar or slightly higher than those from the mesophile in vivo. This observation was made consistently using several different culture and incubation conditions, including starvation and high and low temperatures. The results suggested that there were few differences in the in vivo degradation rates of ribosomes between the mesophile and the thermophile. However, the results may have provided a slight indication of support for the kinetic theory of thermophily.

The in vitro measurements of ribosome degradation indicated a slightly higher thermostability for thermophilic ribosomes as compared with mesophilic ribosomes under the assay conditions studied here. The in vitro results were in good general agreement with much of the previously reported in vitro data, and support the macromolecular theory of the thermophily.

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