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Polysomes from Mesophilic and Thermophilic Bacteria

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POLYSOMES FROM MESOPHILIC AND THERMOPHILIC BACTERIA

by

Philip Y. Shen

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

Western Michigan University
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Polysomes were isolated from mesophilic Bacillus licheniformis grown at 37 and 46° and thermophilic Bacillus stearothermophilus grown at 46 and 55°. It was observed from density gradient centrifugation that the amount of larger polysomes isolated from B. licheniformis grown at 37° was higher than that from B. licheniformis grown at 46°. On the other hand, the amount of larger polysomes isolated from B. stearothermophilus grown at 46° was lower than that from B. stearothermophilus grown at 55°.

In B. licheniformis, an increase in temperature from 37 to 46° resulted in essentially the same number of active ribosomes but the specific activity of ribosomes amounted to only about 10% of the specific activity of ribosomes from cells grown at 37°.

In B. stearothermophilus, an increase in temperature from 46 to 55° resulted in a doubling of the number of active ribosomes but the specific activity of the ribosomes amounted to only about 70% of the specific activity of ribosomes from cells grown at 46°.
ACKNOWLEDGEMENTS

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INTRODUCTION

Certain organisms live at high temperature at which cellular constituents are ordinarily destroyed. These organisms, called thermophiles, live at temperatures between 55 and 80° whereas mesophiles live at more moderate temperatures between 20 and 45°. In 1879, the first thermophilic bacterium was isolated (1). Since then much work has been done in studying such bacteria, isolated from a variety of sources in nature. Attempts to explain the phenomenon of thermophily have resulted in widespread investigation during the past two decades.

Three major theories have been advanced to explain the phenomenon of thermophily. The first of these theories considers thermophily to be due to the protective action of lipids and attempts to correlate heat stability of the organism with the melting point of cell lipid material (2). The second theory views thermophily as a special type of metabolic state characterized by high rates of breakdown and resynthesis (3). The third theory ascribes thermophily to the structure and function of macromolecules and emphasizes physical-chemical differences of macromolecules from mesophiles and thermophiles (4). This latter theory has received the most experimental support so far.
Previous papers from this laboratory in support of this theory have dealt with demonstrated differences in fatty acids (5), ribosomes (6), ribosomal ribonucleic acids (rRNA; 7), deoxyribonucleic acids (DNA; 8), DNA polymerase (9), and cell-free amino acid-incorporating systems (10, 11) from preparations of mesophilic and thermophilic strains of the genus Bacillus.

The work done in this laboratory on cell-free amino acid-incorporating systems from these organisms showed that the thermophilic preparation was more heat stable and more active than the mesophilic one. Specifically, the mesophilic system (from B. licheniformis) was more active at 37 than at 55° whereas the thermophilic system (from B. stearothermophilus) was more active at 55 than at 37°. Furthermore, the overall activity of the thermophilic system was greater than that of the mesophilic system at either temperature. It was also noted that the amount of ribosomal material present in the cell-free extract was generally greater in the case of the thermophilic preparation. In addition, when the ribosomes from the two organisms were interchanged in the amino acid-incorporating systems, the temperature dependence of the systems was shown to be a function of the type of ribosomes involved. Based on these various
observations, it was concluded that, aside from actual physical differences of macromolecules, kinetic considerations may also play a role in bringing about the unique properties of thermophiles. In particular, the kinetic aspects may relate to differences in the number of "active" ribosomes and/or the activity of active ribosomes. Such finding would provide some support for the "kinetic theory of thermophily" mentioned above.

The present work was undertaken with the aim of determining both the number and the activity of active ribosomes in polysome preparations from the mesophilic and thermophilic species of Bacillus discussed above. For the purpose of this work, an active ribosome is defined as one to which a growing polypeptide chain is attached. The specific activity of an active ribosome is defined as the number of amino acid molecules incorporated per active ribosome per unit time.

Both of these kinetic parameters can be determined by means of the puromycin reaction. Puromycin is used routinely to inhibit cell-free amino acid-incorporating systems (12, 13). In this process, puromycin mimics the action of transfer RNA (tRNA, 14) and binds to the growing polypeptide chain which is then released as peptidyl puromycin (15). Under conditions where no recycling of
ribosomes occurs (e.g. by omission of supernatant enzymes and tRNA from an amino acid incorporating system), each active ribosome will lead to the formation of only one amide bond between puromycin and a polypeptide chain. By using $^3$H-labelled puromycin and isolating the peptidyl puromycin (on Millipore filters), the number of molecules of puromycin linked to polypeptides can be determined. This in turn, gives the number of active ribosomes.

The number of amino acids that can be polymerized over a certain time interval can be calculated from an amino acid-incorporating experiment. This experiment is done concurrently with the previous one, and including supernatant enzymes and tRNA in the reaction mixture using an aliquot of the same polysome sample. One can either use a mixture of $^{14}$C-labelled amino acids or a mixture of unlabelled amino acids with the addition of $^{14}$C-labelled phenylalanine. The specific activity is then calculated from the extent of incorporation of $^{14}$C-labelled amino acids.

The present work involved the determination of the number and activity of active ribosomes of B. licheniformis and B. stearothermophilus grown at various temperatures.
MATERIALS AND METHODS

Organisms, Growth and Harvest Conditions

Cells of the mesophile B. licheniformis (NRS 243) and the thermophile B. stearothermophilus 10 were grown in an incubator shaker (New Brunswick Scientific Co., model G-25) with a medium consisting of 1% (w/v) Trypticase (BRL) and 0.2% (w/v) yeast extract (Difco); the medium also contained 0.2 ml of antifoam (Union Carbide Corp., SAG-471) per liter. Cells of B. licheniformis were grown at either 37 or 46° and cells of B. stearothermophilus were grown at either 46 or 55°. Twelve one liter batches of cells were grown at a time. The cells were harvested in the logarithmic phase (absorbance of 0.45 at 540 nm). Growth was terminated by quickly pouring each liter of cells simultaneously with 400 ml of liquid nitrogen, over 2 volumes of ice. The mixture was stirred immediately and then collected by centrifugation at 0° at 30,000 x g using a continuous-flow system (Ivan Sorvall, Inc., RC-2) with a flow rate of about 300 ml/min. The cells were immediately frozen in liquid nitrogen and stored in liquid nitrogen until use.
Lysis of Cells

Lysis of the four bacterial samples (B. licheniformis grown at 37 and 46°; B. stearothermophilus grown at 46 and 55°) was performed concurrently using an identical procedure. All operations for lysis of cells, preparation of subcellular fractions and subsequent experiments were performed at 4° unless otherwise specified.

Frozen cells obtained from 9 liters of culture grown to the logarithmic phase as described above, were thawed for 1 1/2 hours in the cold. The cells were then suspended in 3.0 ml of 0.02 M tris(hydroxymethyl)aminomethane (Tris) at pH 8.6 containing 25% (w/v) sucrose (Mann Research; ribonuclease free), and 3.0 ml of an aqueous lysozyme solution (Calbiochem; 16 mg/ml). After 15 minutes, the following solutions were added in succession: 3.0 ml of a solution containing magnesium acetate, potassium chloride and spermidine trihydrochloride (52; 26; and 600 mM respectively); 3.0 ml of polyvinylsulfuric acid potassium salt (PVS; Eastman Kodak; 10 mg/ml); 2.0 ml of 0.02 M Tris at pH 7.4 containing 25% (w/v) sucrose (Mann Research; ribonuclease free), and stored at room temperature; and 3.0 ml of Tris (0.1 M, pH 7.4). The mixture was gently and occasionally stirred. At the end of 2 hours, 1.0 ml of deoxyribonuclease
(DNase; electrophoretically purified; Worthington; 0.15 mg/ml) was added and the mixture was incubated in the cold room for 15 minutes.
Preparation of Subcellular Fractions

The lysate obtained after the DNase treatment was centrifuged at 10,000 x g for 5 minutes to remove cell debris. The supernatant was collected by pipetting to about 1 cm above the pellet. The pellet was discarded and the supernatant was centrifuged at 81,000 x g for 35 minutes. The pellet obtained in this centrifugation represents the polysomes and the supernatant contains the enzymes and cofactors required for protein synthesis. The supernatant was again collected by pipetting to about 1 cm above the polysome pellet. The remaining supernatant was decanted and discarded, and the polysome pellet was gently rinsed with 1 ml of standard buffer (Tris 22 mM, pH 7.4; magnesium acetate, 8.66 mM; and spermidine trihydrochloride, 4.33 mM) to remove supernatant adhering to the pellet. The polysomes were suspended gently with a rubber policeman in 0.4 ml of standard buffer and 0.1 ml of this polysome suspension (absorbance about 200-300 at 260 nm) was used for sucrose density gradient centrifugation. The remaining suspension was further diluted to an absorbance of 100 or 0.2 at 260 nm.
Density Gradient Centrifugation

A 15-30% sucrose gradient (w/v in standard buffer) was prepared using a Buchler linear gradient maker and a Buchler Polystaltic pump. A volume of 2.3 ml of 15% sucrose was pipetted into one chamber of the gradient maker and 2.2 ml of 30% sucrose was delivered into the other, mixing chamber. The mixed sucrose solution was pumped into a 5 ml Beckman Polyallomer centrifuge tube. Linearity of the resulting gradient was checked by means of a refractometer. The linearity of the gradient was undisturbed by fractionation in the density gradient fractionator.

The sample (0.1 ml of polysome suspension having an absorbance of about 200-300 at 260 nm) was applied onto the gradient at 4° and centrifuged in a Spinco SW 39 rotor at 35,000 rpm for 90 minutes. This was followed by fractionation and analysis for the distribution of the material absorbing at 254 nm with the aid of a continuous fractionating, recording and collecting system (ISCO density gradient fractionator, model D; ISCO ultraviolet analyzer, model UA-2; and ISCO fraction collector, model 270). The sedimentation coefficients of the components were established by analytical ultracentrifugation (Spinco, model E) and density gradient centrifugation with standards of known sedimentation coefficients. The monosome, or 70S ribosomal monomer, peak was further
identified by RNase treatment of polysomes followed by density gradient centrifugation.
Measurement of $^3$H-Peptidyl Puromycin Formation

The Millipore method as described by Wool and Nurihara (16) was used for the measurement of $^3$H-peptidyl puromycin formed. Polysomes, diluted in standard buffer to appropriate concentrations, were incubated at various temperatures in a standard incubation mixture of 0.250 ml that contained the following: 0.100 ml Mix II; 0.050 ml of standard buffer; 0.020 ml of a solution containing magnesium acetate and spermidine trihydrochloride (65.0 and 32.5 mM respectively); 0.050 ml of polysomes (absorbance of 0.2 at 260 nm); 0.010 ml of PVS (10 mg/ml); and 0.020 ml of $^3$H-puromycin (nominally labelled; specific activity 1.11 c/mmole; New England Nuclear Corp.). Mix II was prepared fresh prior to each experiment and consisted of the following: 0.250 ml of Mix I; 0.001 ml of 2-mercaptoethanol (Eastman); 0.250 ml phospho(enol)pyruvate (according to Nirenberg (17)); 0.005 ml of pyruvate kinase (Sigma); and 0.502 ml of doubly distilled deionized water. Mix I was prepared in advance and was frozen in aliquots until use. It contained Tris (pH 7.4), adenosine 5'-triphosphate (disodium salt; Sigma), and guanosine 5'-triphosphate (trisodium salt; Sigma) at 967, 9.4, and 28.8 mM respectively. At the end of the incubation period,
which was usually 60 minutes, the reaction was terminated by the addition of 3 ml of cold 10\% trichloroacetic acid. The sample was heated at 90-95° for 20 minutes to hydrolyze RNA, cooled, passed through a Millipore filter (type HA, pore size 0.45 u), and washed ten times with 5 ml of cold 5\% trichloroacetic acid. The Millipore filter containing the sample was then dried under an infrared heat lamp for 30 minutes and was placed in a counting vial. Scintillation fluid (10 ml), which consisted of 42 ml of liquid scintillator (Nuclear Equip. Chem.) per liter of dioxane (Matheson) containing 10\% naphthalene, was then added to the vial and the radioactivity was measured in a Nuclear Chicago Mark I liquid scintillation counter for 10 minutes. The efficiency of counting was approximately 30\%.
Measurement of $^{14}$C-Phenylalanine Incorporation

The measurement of radioactive phenylalanine incorporation was similar to that of peptidyl puromycin, with the following modifications: The standard incubation mixture contained 0.100 ml of polysomes (absorbance of 100 at 260 nm) instead of 0.050 ml of dilute polysomes and 0.050 ml of standard buffer; 0.020 ml of supernatant enzymes was used instead of 0.020 ml of puromycin; and Mix II contained 0.126 ml of a mixture of 19 unlabelled amino acids (2 mM with respect to each amino acid and containing no phenylalanine), 0.341 ml of doubly distilled deionized water, and 0.035 ml of $^{14}$C-L phenylalanine (uniformly labelled; specific activity 10 mC/m mole; New England Nuclear Corp.) instead of 0.502 ml of water. The sample was washed with five 5 ml portions of cold 5% trichloroacetic acid and counted in the scintillation counter as above. The efficiency of counting was approximately 75%.
Calculation of the Number of Active Ribosomes

The observed dpm for each reaction mixture were first normalized to dpm per 1 ug of ribosomal RNA. The concentration of RNA was determined from a phosphorus analysis according to the procedure of Fiske and Subbarow (18). These phosphorus data were corrected for DNA phosphorus (amounted to less than 1% correction) by first determining the amount of DNA using the diphenylamine reaction (19). The phosphorus content of the DNA was then calculated using an average conversion factor for \textit{B. licheniformis} and \textit{B. stearothermophilus} which is 9.98 ug DNA/ug P (8). The corrected phosphorus data were then converted to ug of RNA on the basis of the known base composition of the ribosomal RNA (7).

The molecular weight of puromycin is 544.4; therefore, applying Avogadro’s number, 1 ug of puromycin contains 1.1 x 10^{15} molecules. Since the specific activity of the puromycin was 1.11 Ci/mole, or 1.52 x 10^6 dpm/ug of puromycin (using \( 1 \text{ C} = 2.22 \times 10^{12} \text{ dpm} \)), it follows that the number of active ribosomes (equal to the number of molecules of \(^3\)H-puromycin incorporated) would be equal to the dpm times 1.1 x 10^{15} and divided by 4.52 x 10^6 (16).
Calculation of the Number of Amino Acids Incorporated per Active Ribosome

The observed dpm were normalized to dpm per 1 ug of ribosomal RNA as outlined above. Likewise, again using Avogadro's number, it follows that 1 ug of phenylalanine (molecular weight 165.2) contains $3.65 \times 10^{15}$ molecules. Since the specific activity of the phenylalanine used was 10 mC/mole, it follows that $1.34 \times 10^5$ dpm are equivalent to 1 ug of phenylalanine incorporated. The number of phenylalanine molecules incorporated is, therefore, equal to the dpm times $3.65 \times 10^{15}$. On the assumption that the 19 unlabelled amino acids are incorporated to the same extent as phenylalanine, the total number of amino acid molecules incorporated (or, essentially, the number of peptide bonds formed) is equal to 20 times the number of phenylalanine molecules incorporated. The number of amino acid molecules incorporated by each active ribosome is then obtained by dividing the total number of amino acid molecules incorporated per ug of ribosomal RNA by the number of active ribosomes per ug of ribosomal RNA.
RESULTS AND DISCUSSIONS

Growth of Organisms

As indicated in the Methods section, one of the growth temperatures for *B. licheniformis* and *B. stearothermophilus* was 37 and 55°C, respectively. These temperatures were chosen for two reasons. First of all, most of the studies performed in this laboratory with these organisms involved cells grown at these temperatures. Secondly, these particular temperatures are within the optimal range of growth temperatures which are 37-45°C for *B. licheniformis* and 55-65°C for *B. stearothermophilus*. These experiments using cells grown at 37 and 55°C, respectively, are useful for correlation with other data and for an assessment of protein synthesis when cell growth is optimal.

Both types of organisms were, however, also grown at a second, common temperature, namely 46°C. In this fashion, the polysomes can be compared in the absence of the temperature variable. On the other hand, these data, in conjunction with those on cells grown at 37 and 55°C, allow an evaluation of the effect of temperature on polysome activity.
Figure 1. Growth Curves of the Organisms

Ordinate: Absorbance (540 nm)
Abscissa: Time (minutes)

( △ ) *E. licheniformis*, grown at 37°
( ▲ ) *E. licheniformis*, grown at 46°
( ○ ) *E. stearothermophilus*, grown at 46°
( ● ) *E. stearothermophilus*, grown at 55°
**B. licheniformis** grew well at both 37 and 46° as expected. It is of interest to note that 46° is generally a fairly high temperature as far as mesophilic organisms are concerned. **B. licheniformis** is somewhat more heat stable than many other mesophiles, a fact that is consistent with studies in our laboratory on the fatty acid distribution in various strains of Bacillus (5).

**B. stearothermophilus** grew best at 55° but also reasonably well at 46°. When the cells grown at 55° were centrifuged after harvesting, a sediment having a characteristic pink color was obtained. When the cells were grown at 46°, the sediment lacked this color and had a more milky appearance much like the sediment obtained with **B. licheniformis** cells.
Harvest Conditions

In order to prepare undegraded polysomes, it is essential to perform chilling and harvesting very rapidly. Otherwise, polysomes in whole unlysed cells degrade rapidly to ribosomal monomers prior to cell lysis (20), chiefly due to run-off of the ribosomes from the messenger RNA. In our experiments, a degraded polysome sample could be detected by the increase in the ribosomal monomer peak and the decrease in the polysome peaks obtained by density gradient centrifugation. Since a comparison of the number and activity of active ribosomes only has meaning if one compares undegraded polysomes, several methods of cilling and harvesting were investigated.

All existing methods had their shortcomings. Initially, cells were grown in 1 or 2 liter batches which were then either poured over 2 volumes of ice, or chilled in an acetone-dry ice bath with stirring. The cold culture was centrifuged immediately at 11,700 x g for 20 minutes in polyethylene bottles. Although the results were satisfactory, as indicated by the polysome profile from density gradient centrifugation, it was difficult to scale up this procedure for larger volumes of cells.

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The capacity of this procedure could be increased greatly by collecting the cells in a continuous-flow system at 4°. However, when more than 6 liters of cells were chilled in 1 liter batches and fed through the continuous system, some ribosome run-off was observed. No improvement was gained with the addition of chloramphenicol (final concentration of 0.1-0.5 mg/ml; Parke Davis) either 1 minute prior to harvesting or during lysis. Chloramphenicol is known to prevent polysome degradation by "freezing" the polysomal structure (21).

The method finally adopted involved the chilling of the culture simultaneously with ice and liquid nitrogen. The combined effect resulted in the chilling of the culture to 0-2° within 10 seconds. Continuous-flow centrifugation was started immediately and the temperature was maintained at near 0° by keeping the inflowing culture in a large volume of ice and by using a maximum flow rate. The time required to centrifuge 1 liter of culture was approximately 2 minutes. The cells were then frozen in liquid nitrogen and stored in liquid nitrogen. Cells could be stored for at least 4 weeks without a noticeable change in the polysome profile obtained from density gradient centrifugation.
Lysis of Cells

At the time this research was started, many procedures were available for the lysis of various bacteria but there was no information available on the lysis of either *B. licheniformis*, a gram-variable organism, or *B. stearothermophilus*, a gram-positive organism. Moreover, gram-positive and gram-negative *Bacilli* had been shown to have different requirements for lysis (22). Since it was essential for the present study to lyse both organisms using an identical procedure, the search for a method applicable to both *B. licheniformis* and *B. stearothermophilus* was a tedious but necessary task.

Various known procedures and their combinations were tried. Lysis was originally performed in the classical manner, in the presence of ethylenediaminetetraacetic acid (EDTA) and sodium deoxycholate (DOC). Since it was later observed that lysis could be obtained equally well in the absence of these components, and since DOC was known to interfere with the polysomal size distribution while EDTA might cause polysome breakdown (23, 24), both components were henceforth omitted from the lysis mixture.

A step-by-step procedure was designed to better study the phases of cell lysis. After the cells were thawed...
they were suspended in 25% sucrose and were lysed in the presence of Tris and lysozyme, as outlined in "Materials and Methods". The sucrose and Tris were used to provide proper osmotic and ionic conditions for the maintenance of the spheroplasts. The Tris was at a basic pH (8.6) and at a low ionic strength (0.02 M), both of which were favorable for lysozyme action (23). The formation of spheroplasts was followed by phase contrast microscopy.

After the lysozyme treatment, the spheroplasts were broken by the combined effect of a non-ionic detergent (Brij) and osmotic shock (lowering of sucrose concentration). The ionic strength was increased to proper levels by the addition of ions such as magnesium and potassium, to give a final concentration of 8.66 and 100 mM respectively. Spermidine trihydrochloride was added to give a final concentration of 4.33 mM. (These concentrations are only approximate since the mass of the cell pellet and the amount of water carried with it was somewhat variable).

Earlier work from our laboratory had indicated the presence of high ribonuclease activity in these bacteria, particularly in *B. licheniformis*. This observation was corroborated in the present study since *B. licheniformis* consistently resulted in polysome profiles inferior to those from *B. stearothermophilus*. Spermidine, which is a
nuclease inhibitor, was evidently insufficient to protect the polysomes from nuclease attack. Of the two other nuclease inhibitors tested, namely, bentonite (25) and PVS (26), only the latter resulted in a significant improvement of the polisome profiles, particularly in the case of *B. licheniformis*.

*B. stearothermophilus* cells were lysed at a faster rate than those of *B. licheniformis*. A difference in rate of cell lysis is not unexpected since the two organisms respond differently to the gram stain and hence differ in the structure of the cell wall. In an attempt to achieve lysis with the more refractory cells of *B. licheniformis*, the Brij concentration was increased by 30% but this led to a degradation of polysomes and an increase of ribosome monomers and trimers. While an increase in temperature during lysis would probably have resulted in better lysis of *B. licheniformis* cells, this was impractical because of the expected polisome degradation due to increased nuclease activity.

Because of these considerations, lysis was carried out at 40°C for both bacteria for an equal and sufficient length of time until an acceptable degree of lysis was obtained. The degree of lysis was judged visually by the increase in viscosity due to the cellular DNA. The lysis
of *B. stearothermophilus* was essentially complete within 45 minutes after the addition of Brij; the lysis of *B. licheniformis*, however, was complete only after about 120 minutes. Hence, both types of cells were lysed for 120 minutes at 4°C. The prolonged standing of the *B. stearothermophilus* caused no change in the polysome profile. As a matter of fact, the polysomes isolated from both bacteria by the above method were stable at 4°C for at least 6 hours. The stability can probably be attributed to the combined effect of spermidine trihydrochloride and the high concentration (1.6 mg/ml) of PVS present in the lysis mixture.

Another interesting observation was that the rate of lysis of *B. stearothermophilus* grown at 46°C appeared to be intermediate between that of *B. stearothermophilus* grown at 55°C and that of *B. licheniformis* grown at either 37 or 46°C. Whether the relative ease of lysis of these various organisms was related to the type and/or amount of lipid material (acted upon by Brij) in the membrane, or to the difference in susceptibility of the cell wall to lysozyme action, or both, awaits further investigation.

Recently, Van Dijik-Salkinoja and Planta (27) isolated polysomes from both the membrane and the soluble
fraction of *E. licheniformis* (S244) under conditions somewhat similar to ours. They extracted polysomes in the presence of chloramphenicol and macaloid (a nuclease inhibitor used instead of PVS). The cells were treated for 10 minutes at 0°C with lysozyme at a relatively high Tris concentration (0.25 M at pH 7.5) and at a high ionic strength. This was followed by treatment with Brij for only 10 minutes at 0°C. The soluble fraction was then separated from the membrane fraction by centrifugation. The membrane fraction was subsequently treated with additional Brij, DNase, and lipase and then incubated at 37°C for 15 minutes. They found that under those conditions of lysis, 96% of the total ribosomal material was membrane-bound; the remaining 4% was found in the soluble fraction.

According to our results, lysis of *E. licheniformis* cells under those conditions (20 minutes at 0°C) was probably incomplete. Incomplete lysis would lead to an apparent low polysome yield in their so-called "soluble fraction" and contamination of the "membrane fraction" with unlysed cells. On the other hand, incubation of the "membrane fraction" at 37°C for 15 minutes, even in the absence of lipase, is probably equivalent to at least one hour of lysis in the cold, and would most likely bring about complete lysis. Thus, while the recovery of polysomes
in their experiments is probably complete, their conclusions regarding the relative amounts of soluble and membrane bound polysomes may not be justified.
Density Gradient Centrifugation

Density gradient centrifugation of the polysomes resulted in a profile consisting of, at the most, twelve peaks which were numbered consecutively in order of their recovery from the tube (i.e. from the top to the bottom of the tube). The first peak (No. 1) corresponds to low molecular weight UV absorbing compounds (nucleotides, amino acids, peptides etc.). The last peak (No. 2) did not represent a polysome fraction, but rather was due to membrane fragments which had collected at the bottom of the tube during centrifugation and were stirred up by the initial injection of the dense sucrose solution. Since the appearance of this peak with reference to peak 1 was reproducible (same distance on the recorder chart), it was useful as a marker. Different profiles could then be compared by determining the relative distance of a peak from peaks 1 to 12. Peaks 2, 3, 4 and 5 represent 30S, 50S, 70S and 100S ribosomes, respectively. Subsequent peaks represent classes of actual polysomes, that is messenger RNA molecules with an increasing number of attached ribosomes. Thus peak 6, 7, and 8 represent polysomes of 3, 4, and 5 ribosomes per messenger RNA, respectively.
Identification of peaks obtained in these profiles was achieved in five ways. First, a sample of ribosomes was prepared from *E. stearothermophilus* according to the procedure used routinely in this laboratory (11). Aliquots of this sample were then analyzed by density gradient centrifugation and by analytical ultracentrifugation (Spinco, model E). Since the patterns obtained were identical in both cases, the sedimentation coefficients calculated from analytical ultracentrifugation defined peaks 2-5 obtained by density gradient centrifugation. The second method of peak identification involved the analysis of a polysome sample from *E. licheniformis* before and after treatment with pancreatic ribonuclease (Sigma; final concentration of 0.01 mg/ml and incubated at 37° for 15 minutes). Since this results in degradation of messenger RNA an increase in the ribosome monomers (70S) can be observed. In our case this resulted in an increase of peak 4 which therefore must represent the ribosome monomer. The third approach of peak identification involved the collection of peaks 2-5 from density gradient centrifugation (*E. stearothermophilus*) and measurement of the sedimentation coefficient of this sample by means of analytical ultracentrifugation. Peaks 2-5 were shown to be 30, 50, 70, and 100S ribosomes, respectively. The fourth method consisted of the addition of a sample of
Figure 2. Density Gradient Identification of Polysome Fractions

Ohrinate: Absorbance (254 nm) from 0 to 50
Abscissa: Effluent (Fractionated at the rate of 0.2 ml/min.)

(a) *E. licheniformis*, grown at 37°
(b) *E. stearothermophilus*, grown at 55°
(c) Equal amounts of (a) + (b)
ribosomes from *B. stearothermophilus* to a sample of polysomes from the same organism and analysis of the combined samples by means of density gradient. An increase in peaks 2-5 was observed. The final way of peak identification was achieved by placing on the density gradient a mixture of polysomes from *B. licheniformis* (grown at 37°) and *B. stearothermophilus* (grown at 55°). The results are shown in Figure 2 and it can be seen that there was complete overlap of peaks 1 through 7 (concentration of the remaining peaks was too high to be within the range of the recorder scale). This shows clearly that peaks having the same number do represent ribosomes or polysomes of the same size in preparations from *B. licheniformis* and *B. stearothermophilus*.

Analysis by means of sucrose density gradient centrifugation was performed on every freshly prepared polysome preparation in order to ensure that the proper polysome profile was obtained and that there had been no polysome degradation. This analysis was performed concurrently with the puromycin and the phenylalanine experiments. Typical polysome profiles obtained from *B. licheniformis* grown at 37 and 46° and *B. stearothermophilus* grown at 46 and 55° are shown in Figure 3. While roughly similar there are some important differences between the various profiles.
Figure 3. Density Gradient Profile of Polysomes.

Ordinate: Absorbance (254 nm) from 0 to 4.5
Abscissa: Effluent (Fractionated at the rate of 0.2 ml/min.)

(a) *B. licheniformis*, grown at 37°
(b) *B. licheniformis*, grown at 46°
(c) *B. stearothermophilus*, grown at 46°
(d) *B. stearothermophilus*, grown at 55°
A total of 12 peaks were observed in the polysome profiles of *B. licheniformis* grown at 37° and of *B. stearothermophilus* grown at 55°; however, only 11 peaks were observed in the polysome profiles of *B. licheniformis* grown at 46° and of *B. stearothermophilus* grown at 46°. This indicated that, since each peak represented a class of polysomes containing a certain number of ribosomes, there was one additional class of polysomes of a particular size in *B. licheniformis* grown at 37° and in *B. stearothermophilus* grown at 55°.

When *B. licheniformis*, which normally is grown in our laboratory at 37°, was grown at 46°, some distinct changes in the polysome profile could be noted. The relative amounts of 30, 50, 70, and 100S ribosomes, as indicated by the areas (or heights) under peaks 2, 3, 4, and 5 were essentially unchanged. However, there was a noticeable decrease in peak height commencing with peak 6. Furthermore peak 11 was absent. Thus, when *B. licheniformis* was grown at the higher temperature, one class of polysomes appeared to be missing and all the remaining classes of polysomes were relatively smaller in amounts. Another point of interest is that, when *B. licheniformis* was grown at 46° instead of at 37°, peak 8 became the highest peak instead of peak 9. In other words, the population of ribosome
pentamers became the greatest, instead of ribosome hexamers. Since the growth rate of *B. licheniformis* at 46°C was essentially like that at 37°C (Figure 1), this could mean that even at 46°C there was still a sufficient number of polysomes per cell or that cells grown at higher temperatures are somewhat different in their content of certain proteins.

A similar observation was made in the case of *B. stearothermophilus*. When this organism was grown at the lower temperature of 46°C instead of at the usual temperature of 55°C, the relative heights of the peaks commencing with peak 6, were also decreased substantially. Peak 9, instead of peak 11, became the most abundant polysomal peak. This observation was consistent with the fact that *B. stearothermophilus* grew only very sluggishly at 46°C (Figure 1).

It should be noted that these interpretations were based on relative peak heights, for unless one assumes that the extent of lysis in all cases was exactly the same, it would be invalid to compare individual peak heights.
TABLE 1. Effect of Time on the Formation of
$^3$H-Peptidyl Puromycin.*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Temp.</th>
<th>40 min.</th>
<th>60 min.</th>
<th>80 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. licheniformis</td>
<td>37°</td>
<td>5,041</td>
<td>4,644</td>
<td>5,297</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>46°</td>
<td>7,287</td>
<td>6,749</td>
<td>7,371</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>45°</td>
<td>5,921</td>
<td>6,609</td>
<td>5,212</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>55°</td>
<td>5,777</td>
<td>6,077</td>
<td>6,165</td>
</tr>
</tbody>
</table>

* Based on experiments using 10 ul of puromycin. At time zero, a typical reaction mixture showed only background counts.

** Temperature of bacterial growth and peptidyl-puromycin formation.
Formation of $^3$H-Peptidyl Puromycin

The reaction was carried out as described in "Materials and Methods", using polysomes diluted with standard buffer to an absorbance of 0.2 at 260 nm.

In order to ensure that no recyclization of ribosome would occur, the supernatant enzyme fraction, which contained enzymes and aminoacyl-tRNA, was omitted from the reaction mixture. The data were corrected for blanks which consisted of all components except polysomes, and amounted to approximately 1,000 dpm.

It was essential that the formation of peptidyl-puromycin be carried out under conditions where all the active ribosomes in all of the bacterial samples would form the peptidyl-puromycin. This required a determination of both reaction time and puromycin concentration. It can be seen in Table 1 that the reaction was complete after 40 minutes and remained essentially unchanged after 60 and 80 minutes. On the basis of this experiment, an incubation time of 60 minutes was chosen. The puromycin concentration was then determined for the four bacterial preparations using an incubation time of 60 minutes. It can be seen from Figure 4 that the various preparations required different amounts of puromycin for saturation.
Figure 4. Effect of Puromycin Concentration.

Ordinate: dpm (x 1,000) per ug RNA
Abscissa: ul Puromycin per Reaction Mixture.

( △ ) B. licheniformis, grown at 37°.
( ▲ ) B. licheniformis, grown at 46°.
( ○ ) B. stearothermophilus, grown at 46°.
( ● ) B. stearothermophilus, grown at 55°.
but that with either 15 or 20 ul of puromycin there was an excess of puromycin so that all the growing polypeptide chains could be converted to the \(^3\)H-peptidyl puromycin. Under these conditions, the number of active ribosomes per ug RNA was calculated (Table 2).
TABLE 2. Formation of Peptidyl-Puromycin

<table>
<thead>
<tr>
<th></th>
<th>Temp.*</th>
<th>dpm per reaction mixture</th>
<th>ug RNA per reaction mixture</th>
<th>dpm per ug RNA</th>
<th>No. of active ribosomes per ug RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. licheniformis</em></td>
<td>37°</td>
<td>5,517</td>
<td>0.81</td>
<td>6,811</td>
<td>1.66 x 10^{12}</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>45°</td>
<td>7,420</td>
<td>1.05</td>
<td>7,068</td>
<td>1.72 x 10^{12}</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>45°</td>
<td>6,830</td>
<td>0.64</td>
<td>10,872</td>
<td>2.59 x 10^{12}</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>55°</td>
<td>13,835</td>
<td>0.64</td>
<td>21,619</td>
<td>5.25 x 10^{12}</td>
</tr>
</tbody>
</table>

*Temperature of bacterial growth and peptidyl-puromycin formation.
$^{14}$C-Phenylalanine Incorporation

The reaction was carried out as described in "materials and Methods" using both polysomes and supernatant which contained enzymes and tRNA. The data were corrected for blanks, which consisted of all components except polysomes, and amounted to about 200 dpm.

The results are summarized in Table 3. It can be seen that as the temperature of incubation for the mesophile was increased from 37 to 46°, phenylalanine incorporation was decreased. On the other hand, raising the incubation temperature for the thermophile from 46 to 55° led to an increase in the phenylalanine incorporation. The extent of phenylalanine incorporation in these reaction mixtures was the same at 40°, 60 and 80 minutes of incubation (Table 4).
TABLE 3. $^{14}$C-Phenylalanine Incorporation.

<table>
<thead>
<tr>
<th>Temp.*</th>
<th>Incorpo-</th>
<th>No. of Phenylalanine Molecules Incorporated per ug RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. licheniformis</td>
<td>37°</td>
<td>1,503</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>46°</td>
<td>155</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>46°</td>
<td>1,760</td>
</tr>
<tr>
<td>E. stearothermophilus</td>
<td>55°</td>
<td>2,607</td>
</tr>
</tbody>
</table>

* Temperature of bacterial growth and phenylalanine incorporation.

** Actual dpm per reaction mixture corrected for blank.

Each reaction mixture contained 0.1 ml of polysome solution having an absorbance of 100 at 260 nm.
**TABLE 4.** Effect of Time on $^{14}$C-Phenylalanine Incorporation.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Temp.</th>
<th>40 min.</th>
<th>60 min.</th>
<th>80 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. licheniformis</em></td>
<td>37°</td>
<td>1,771</td>
<td>1,751</td>
<td>1,774</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>46°</td>
<td>356</td>
<td>323</td>
<td>323</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>46°</td>
<td>2,047</td>
<td>1,979</td>
<td>1,919</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>55°</td>
<td>2,735</td>
<td>2,879</td>
<td>2,714</td>
</tr>
</tbody>
</table>

* Temperature of bacterial growth and phenylalanine incorporation.
Number of Active Ribosomes and Number of Amino Acids Incorporated by Each Active Ribosome

The final calculations are summarized in Table 5. In the case of the mesophile, *B. licheniformis*, an increase in temperature from 37 to 46° resulted in essentially the same number of active ribosomes but the activity of the ribosomes amounted to only about 10% of the activity of ribosomes from cells grown at 37°.

In the case of the thermophile, *B. stearothermophilus*, an increase in temperature from 46 to 55° resulted in a doubling of the number of active ribosomes but the specific activity of the ribosomes amounted to only about 70% of the specific activity of the ribosomes from cells grown at 46°.

These results must be interpreted with two considerations in mind. The first of these relates to the preparation of the polysomes. It is assumed that the efficiency of cell lysis and extraction of polysomes is the same in all cases. This is supported by the roughly similar quantities of polysomes and low molecular weight materials (peak 1) obtained in all cases (Figure 3). However, even differences in the efficiency of lysis would not affect the results unless the intracellular distribution of polysomes were not constant. It is
possible, for example, that variable amounts of membrane bound polysomes occur at the different temperatures and that these polysomes, and their activity, somehow compensate for the changes in number and activity of free polysomes investigated in this study. This is especially pertinent in view of the second consideration discussed below.

The second consideration relates to the actual rates of growth of the cells at the various temperatures. These have been presented in Figure 1. It is apparent that the actual rate of growth of *B. licheniformis* was essentially the same at both 37 and 46°. On the other hand, the rate of growth of *B. stearothermophilus* was more than doubled by increasing the temperature from 46 to 55°. It is known that the total number of ribosomes is roughly proportional to the growth rate (28). Our findings that the number of active ribosomes is the same for the two *B. licheniformis* preparations, while it is more than doubled for the two *B. stearothermophilus* preparations is thus in agreement with the known relationship between ribosome number and growth rate.

The growth rate of *B. licheniformis* at 46° was greater than that of *B. stearothermophilus* at 46° but the latter preparation contained a larger number of active
ribosomes. If the first consideration discussed above can be neglected this would indicate that thermophiles have inherently a larger number of active ribosomes (and hence presumably also total ribosomes) per cell than mesophiles. In that case the ability to synthesize proteins at a faster rate becomes a definite kinetic contribution to the unique properties of thermophiles.

That the number of active ribosomes, rather than their activity, is the prime factor is apparent from the fact that the activity of three out of four bacterial preparations was essentially the same (Table 5). Hence the increased growth rate of *B. stearothermophilus* (55 versus 46°) means basically a larger number of active ribosomes per cell with only a relatively small decrease in specific activity. This is consistent with the known stability of thermophilic ribosomes to temperature as compared to that of mesophilic ribosomes.

Since the growth rate of the two *B. licheniformis* cultures was essentially the same it follows that the number of active ribosomes should be the same as indeed it was found to be. Likewise, it would be expected, that the specific activity should have remained constant, however it decreased markedly from 37 to 46°. This is somewhat puzzling since a constant growth rate implies a constant
TABLE 5. Number and Activity of Active Ribosomes

<table>
<thead>
<tr>
<th></th>
<th>Temp.*</th>
<th>No. of Active Ribosomes per ug RNA</th>
<th>No. of Amino Acids Incorporated per Active Ribosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. licheniformis</td>
<td>37°</td>
<td>$1.66 \times 10^{12}$</td>
<td>0.62</td>
</tr>
<tr>
<td>B. licheniformis</td>
<td>46°</td>
<td>$1.72 \times 10^{12}$</td>
<td>0.05</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>46°</td>
<td>$2.59 \times 10^{12}$</td>
<td>0.58</td>
</tr>
<tr>
<td>B. stearothermophilus</td>
<td>55°</td>
<td>$5.25 \times 10^{12}$</td>
<td>0.41</td>
</tr>
</tbody>
</table>

*Temperature of bacterial growth and incubation.
growth rate implies a constant product of (number of active ribosomes) x (activity per active ribosome). Here, however, in spite of drastically reduced ribosomal activity, the *B. licheniformis* cells maintained their constant rate of growth. This may mean that only those proteins essential for growth are being synthesized and that the synthesis of other, somewhat less essential proteins, is decreased. Differences with respect to type and amount of various polysomes (and hence type and amount of various proteins) have already been noted and discussed in connection with the density gradient profiles (Figure 3). Such changes in polysome profiles may also affect the efficiency of protein synthesis. A comparison of actual protein content per cell under these various growth conditions should clarify this point. Alternatively, or in addition, this finding may be attributed to the first consideration discussed above. That is, the decreased activity of free polysomes may be compensated for by the number and activity of membrane bound polysomes. Such polysomes are presumed to be more stable toward heat denaturation than free polysomes. A determination of the distribution and activity of free and membrane bound polysomes should resolve this problem. In our polysome preparation we are studying "total" polysomes, that is those that were
originally free in the cytoplasm plus those that were originally membrane bound and were dissociated from the membrane during the preparation of the polysomal sample.
BIBLIOGRAPHY


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VITA

The author was born to Tse Dzoen Hsia and Ting Chun Shen on March 21, 1944 in Shanghai, China. He received his elementary and secondary education in Hong Kong, and entered Western Michigan University and graduated with a degree of Bachelor of Arts in chemistry in June, 1966 and Master of Science in August, 1968. He then enrolled in the PhD program in biochemistry.