Protein synthesis in an in vitro system of an extreme thermophile
by Clark Labach Gross

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Chemistry
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Abstract:
The in vitro protein synthesizing system of T2, an unclassified gram-negative extremely thermophilic bacteria, was investigated. The ribosomes of this organism are comparable to those of E. coli in sedimentation constant. The incorporation of 14C-lysine was followed using a synthetic messenger, polyadenylic acid. The optimal conditions for this incorporation of 14C-lysine have been determined.

This in vitro system has a temperature optimum for activity at 55°C. The level of incorporation of 14C-lysine with this system is similar to that of E. coli at 37°C. At higher temperatures, the Tg system is much more efficient in synthesizing 14C-lysine peptides than the E. coli system.

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Signature Clark L. Gross

Date October 2, 1973

PROTEIN SYNTHESIS IN AN IN VITRO SYSTEM OF AN EXTREME THERMOPHILE

by

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ABSTRACT

The *in vitro* protein synthesizing system of T_2, an unclassified gram-negative extremely thermophilic bacteria, was investigated. The ribosomes of this organism are comparable to those of *E. coli* in sedimentation constant. The incorporation of ^{14}C-lysine was followed using a synthetic messenger, polyadenylic acid. The optimal conditions for this incorporation of ^{14}C-lysine have been determined.

This *in vitro* system has a temperature optimum for activity at $55^{\circ}C$. The level of incorporation of ^{14}C-lysine with this system is similar to that of *E. coli* at $37^{\circ}C$. At higher temperatures, the T_2 system is much more efficient in synthesizing ^{14}C-lysine peptides than the *E. coli* system.

1 INTRODUCTION

Reproduction and growth of living organisms are influenced by a variety of environmental factors. Temperature is one of the easiest variables to measure. The high temperature environments exemplified by hot springs in Yellowstone National Park, Iceland, New Zealand, Japan, and Russia provide unique opportunities to study the effect of temperature on the distribution and metabolism of living organisms.

Procaryotic organisms, such as blue-green algae and bacteria, inhabit these thermal springs. The temperature of the environment, as well as pH, salinity, nutrients, and light intensity determine which organism will populate a given spring. Brock (1) has shown that the temperature optimum for blue-green algae has a definite upper limit of $75^{o}C$. However, bacteria can be found growing in pools in Yellowstone National Park where the water is actually superheated.

There are many theories to explain the existence of organisms living in such extreme environments and they can be divided into two general categories (2).

The first category concerns the reputed ability of the organism to rapidly resynthesize any damaged component of the cell and therefore, remain essentially undamaged. This general theory does not seem to hold since an organism growing at $70^{o}C$ should theoretically be able to synthesize cellular components at a rate of 16 times that at $30^{o}C$ assuming a doubling of reaction rate for every 10^{o} rise in temperature. This acceleration of life processes has never been shown.

The second general category infers that the cellular components of a thermophilic organism are inherently more heat stable than the components of mesophilic organisms. Most of the work concerning this category dealt with isolation and study of cellular components.

Gaughran (3) postulated that the composition of lipids in a thermophilic bacteria may dictate the temperature limit of growth since animals live at temperatures below their lipid melting points. This theory has been investigated by a number of authors with conflicting results. Some of this work will be briefly mentioned below.

Bauman and Simmonds (4) investigated the distribution of lipids in a Flexibacteria, an extreme thermophile which grows in filamentous bacterial masses. Although the greatest percentage of fatty acids were C_{18} compared to C_{15} in mesophilic bacteria, they concluded that the distribution was not remarkable.

Daron (5) investigated the distribution of lipids in a thermophilic strain of Bacillus. He concluded that the proportion of unsaturated fatty acids varied inversely with the temperature and the greatest percentage of these fatty acids was 14%. However, the proportion of unsaturated fatty acids depended greatly on the energy source.

Unfortunately, there has not been enough experimentation in lipid research to determine if the membrane is responsible for the thermal resistance of these organisms.

Another plausible explanation for thermophily is that the DNA of

these organisms are more resistant to heat. Since the melting point of DNA is related to the G-C content, it is conceivable that thermophiles could have a higher G-C content than mesophiles. Brock and Freeze (6) have shown that an extreme thermophile, T. aquaticus, has a G-C content of 65%. On the other hand, B. stearothermophilus has a G-C content of 44%. Mesophilic bacteria have G-C contents ranging from 26.5% to 74% (7). Therefore, the G-C content is unlikely to be responsible for thermal stability.

A current field of intensive study has been thermophilic proteins and their protein synthesizing machinery. Most of the experimentation has been limited to the spore-forming thermophilic species of Bacillus.

Much of the early work was restricted to structural proteins such as the flagella of Bacillus (2). These flagellae showed much more heat stability than flagellae from mesophilic strains and were more resistant to classical denaturing agents such as urea, acetamide, and sodium dodecyl sulfate.

Later experiments compared the physical properties of purified enzymes from thermophilic and mesophilic bacteria. Manning and Campbell (8) crystallized α-amylase from B. stearothermophilus and determined the optimum temperature for activity to be in the 55°C to 70°C range. The molecular weight was determined as 15,600 daltons (9) compared to 48,650 daltons for α-amylase isolated from a mesophilic species, B. subtilus. Another study (10) showed that the thermophilic

α-amylase contained a 4.5 fold excess of proline compared to the mesophilic enzyme. The disordered structure resulting from the high proline content was postulated as the reason for the thermal stability.

Many other isolated enzymes have been studied and although there may be some exceptions, most of the enzymes from thermophiles are much more resistant to heat than the corresponding mesophilic enzymes.

Since α-amylase is such a small protein, it had been thought that larger enzymes would not show heat resistance. Studies of glyceraldehyde 3-phosphate dehydrogenase (11) (M.W. = 130,000 daltons) from B. stearothermophilus still show resistance to heat. The comparison of this enzyme to mesophilic enzymes in amino acid composition does not shown any significant differences although the primary sequence is not known.

The literature in this field is becoming replete with examples of thermostable enzymes. Although the reason for this stability is unknown, the application of sequence analysis and X-ray crystallography will prove valuable in determining the primary structure to indicate the types of interactions necessary to confer thermal stability.

With the interest generated in thermophilic proteins, it was only natural that the protein synthesizing machinery should be investigated on a subcellular level. The first reports (12) of thermostable amino acid-activating enzymes suggested that thermophilic systems could be utilized in the study of coding properties of messenger RNA and in

temperature-induced modifications of this messenger.

Friedman and Weinstein (13) used a subcellular system from B. stearothermophilus. They were able to demonstrate that incorporation of ^{14}C-labelled phenylalanine, lysine, and proline was optimal at 55°C to 60°C using a native messenger RNA. They also showed that incorporation of ^{14}C-lysine was greater at 65°C than at 37°C using synthetic polyadenylic acid as messenger. This greatly increased incorporation was postulated as the enhanced ability of attachment of messenger to the ribosomes because of the loss of secondary structure of the messenger at the higher temperature. By comparison, an E. coli system showed only 10% incorporation at 65° relative to base line levels at 37°C. These authors also demonstrated that the thermal denaturation profile of t-RNA from B. stearothermophilus was not significantly different from that of E. coli.

Algranati and Lengyel (14) also investigated a subcellular system from B. stearothermophilus but employed a random copolymer of adenylic and uridylic acid. They were interested in determining how much incorporation would occur at higher temperature because of the high degree of secondary structure in this copolymer. They found that this synthetic messenger was completely inactive in the E. coli system but had some activity at 37°C in the thermophilic system. This activity was increased 3-fold when incubated at 65°C.

In addition to heat resistant amino acid-activating enzymes,

thermophilic bacteria also possess heat resistant ribosomes. Friedman (15) preheated E. coli and B. stearothermophilus ribosomes for 15 minutes at 65°C. He then tested the ability of these ribosomes to incorporate ^{14}C-phenylalanine at 37°C using polyuridylic acid as messenger. With this pretreatment, E. coli ribosomes could incorporate only 5% of that observed at 37°C with no pretreatment. The ribosomes of B. stearothermophilus were virtually unaffected since they incorporated 80% of that observed at 37°C with no pretreatment.

Extensive study of thermal denaturation profiles of ribosomes by Pace and Campbell(16) demonstrated that the ribosomes of thermophiles had a much higher melting point than those of mesophiles. This observation led the authors to speculate that the melting point of the ribosome may set the upper limit of growth.

The vast majority of the research on thermophilic organisms has utilized spore-forming thermophilic species of Bacillus. Recently, a non-sporulating extreme thermophile which is gram-negative has been described by Brock and Freeze (6). This organism was originally isolated from Yellowstone National Park although it can be found in such diverse spots as the hot water heaters of commercial laundromats (17). This organism has been classified Thermus aquaticus and it is an obligate aerobe with an optimum growth temperature of 70°C.

Zeikus et al. (18) have investigated some of the physical and chemical properties of the ribosomes and RNA from this organism. The

ribosomes contain 59% protein and 41% RNA compared to 59% RNA and 41% protein in B. stearothermophilus but this finding may reflect different cultural conditions. The thermal denaturation profile of ribosomes exhibited a melting temperature of 86°C and the authors correlate the temperature limit of growth with this melting point.

Further investigation by Stellwagen (19) has shown that this organism possesses extremely thermostable enzymes. He studied enolase from T. aquaticus and found that the enzyme was composed of 8 equal polypeptide chains and had optimal activity at 90°C. Above 90°, the enzyme rapidly denatured.

Freeze (20) has also shown that fructose diphosphate aldolase from T. aquaticus has a temperature optimum of 90°C but he worked with a partially purified enzyme.

Although enzymes and ribosomes from T. aquaticus have been isolated, there have been no reports of protein synthesis in a subcellular system. This system will undoubtedly be clarified in a few years.

2 EXPERIMENTAL RATIONALE

Most investigations on the protein synthesizing machinery of thermophilic bacteria have been limited to spore-forming, gram-positive organisms such as B. stearothermophilus. The results of these investigations have been compared to E. coli, a gram-negative bacteria common to the lower digestive tract of man.

Recently, an extremely thermophilic bacteria designated as T_2 was isolated by Uhlrich (21). This organism, although unclassified, resembles T. aquaticus which was isolated by Brock (6). It is gram-negative and non-sporulating and forms long filaments at high temperatures.

This organism was found to be inducible for β-galactosidase (21), an inducible enzyme which has been well characterized. This enzyme was induced by lactose and repressed by glucose in the classic manner (22). However, this enzyme was thermostable and exhibited maximal activity at $70^{o}C$.

Since this extreme thermophile shares some of the common attributes possessed by E. coli, it was of interest to determine if the protein synthesizing machinery of this organism is similar to that of E. coli.

The primary purpose of this study is to determine whether a subcellular protein synthesizing system isolated from T_2 is capable of incorporating ^{14}C-lysine using polyadenylic acid as messenger. The temperature dependence on this incorporation was to be studied and the

conditions for maximum activity were to be determined. Sedimentation velocity experiments were to be performed utilizing purified ribosomes to indicate the size of these ribosomes.

3 MATERIALS AND METHODS

(a) Bacterial strains: T_2, an unclassified thermophilic bacteria resembling Thermus aquaticus, was obtained from Dr. J. Terry Uhlrich (21) as a frozen culture. This bacteria was used as a source of ribosomes and enzymes employed in this study. Ribosomes and enzymes from Escherichia coli MRE-600 utilized in parallel studies were a generous gift from Mr. Charles A. Roessner of the Zoology and Entomology Department, Montana State University.

(b) Bacterial growth media: Regular studies employed a growth medium containing 0.2% tryptone (Difco) plus 0.2% yeast extract (Difco) in a basal salts solution containing: 0.04 g $(NH_4)_2SO_4$, 0.07 g KCl, 0.22 g NaCl, 0.7 g $Na_2HPO_4 \cdot 7H_2O$, 0.01 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.01 g $MgSO_4 \cdot 7H_2O$, and 5.0 mls of Hutner's trace element solution (23) per liter of singly distilled water. The pH was adjusted to 8.2 with 0.8 N NaOH and autoclaved for 45 minutes at 15-20 lbs of steam pressure.

(c) Incubation conditions: A Research Specialties tube heater (Model #2128A) was used for incubation of unshaken tube cultures.

Batch cultures were grown in a New Brunswick Gyrotory Shaker (Model #625) which was adapted to accommodate 12 2-liter culture flaks. A constant air temperature ($70^{\circ} \pm 2^{\circ}C$) was used. Agitation provided the only means of aerating these cultures with a concommitant increase in generation time. Growth was monitored by withdrawing

aliquots and determining the turbidity of 640 nm in a Bausch & Lomb Spectronic 20 (24).

When turbidity reached a level corresponding to mid-logarithmic phase (A_{640} of 0.40 to 0.50) the culture flasks were removed from the shaker and transferred to the cold room (4°C). After 1-2 hours of cooling, the cultures were harvested with a Sharples Super Centrifuge, Type T-1. The cells were removed from the rotor, resuspended in an appropriate buffer, and recentrifuged in the Sorvall RC-2 at 7,000 xg, 0°C, for 30 minutes. The clear supernatant was decanted and the cells were stored at -20°C until use.

(d) Ribonuclease removal: Any contaminating RNAase activity was removed by the following methods. (1) Glassware was heated at 240°C overnight before use. (2) Buffers were bentonite treated for 4 hours by adding 2 drops of a bentonite suspension. They were then millipore-filtered and stored in the cold. (3) Nalgene or polycarbonate tubes were soaked in 5% H_2O_2 for 30 minutes and then rinsed with copious amounts of doubly distilled water. (4) Other material was autoclaved for 20 mintues at 15 lbs of steam pressure.

(e) Buffers: All buffers were prepared with doubly-distilled water which had a minimum specific resistance of 670 $K\Omega cm^{-1}$. This doubly-distilled water was prepared from house distilled water with a Corning AG-2 distillation apparatus.

Necessary pH adjustments were made with constant boiling hydrochloric acid (the constant boiling portion of a distilled 1:2 mixture of a reagent hydrochloric acid with distilled water). The pH measurements were made with a Corning Model 12 pH Meter after prior standardization with the appropriate Coleman pH standards.

All buffers were treated with bentonite to selectively remove any contaminating ribonuclease activity. Two drops of a bentonite suspension were added to a liter of buffer, allowed to stand at 4°C for at least four hours, and then removed by filtration through a 50 mm Millipore nitrocellulose filter, into a heat treated reagent bottle using a sterile Millipore suction filtration apparatus. After filtration, the buffers were stored at 4°C.

Buffer OA^-

0.01 M $Mg(OAc)_2$

0.01 M Tris

The buffer was adjusted to pH 7.8 at 4°C with constant boiling HCl, bentonite treated for 4 hours, and then filtered.

Buffer OB^-

0.5 M NH_4Cl

0.01 M $Mg(OAc)_2$

0.01 M Tris

The buffer was adjusted to pH 7.8 at 4°C with constant boiling HCl,

bentonite treated for 4 hours, and then filtered.

Buffer OC^-

0.25 M NH_4Cl

0.01 M $Mg(OAc)_2$

0.01 M Tris

The buffer was adjusted to pH 7.8 at 4° C with constant boiling HCl, bentonite treated for 4 hours, and then filtered.

Buffer OD^-

1.0 M NH_4Cl

0.01 M $Mg(OAc)_2$

0.01 M Tris

The buffer was adjusted to pH 7.8 at 4°C with constant boiling HCl, bentonite treated for 4 hours and then filtered.

Buffer N^-

0.08 M NH_4Cl

0.011 M $Mg(OAc)_2$

0.1 M Tris

The buffer was adjusted to pH 8.1 at 4°C (pH 7.8 at 37°C because of Tris) with constant boiling HCl, bentonite treated for 4 hours, and then filtered.

BC-0$^-$

0.1 M NH_4HCO_3

No adjustment was necessary; the pH was 7.88 at room temperature (20°C). It was bentonite treated for 4 hours and then filtered.

BC-10$^-$

0.1 M NH_4HCO_3

0.01 M $Mg(OAc)_2$

No adjustment was necessary; the pH was 7.88 at room temperature, (20°C). It was bentonite treated for 4 hours and then filtered.

BC-50$^-$

0.1 M NH_4HCO_3

0.05 M $Mg(OAc)_2$

No adjustment was necessary; the pH was 7.88 at room temperature (20°). It was bentonite treated for 4 hours and then filtered.

50-30

0.1 M KCl

0.01 M Tris

0.001 M $MgCl_2 \cdot 6H_2O$

The buffer was adjusted to pH 7.2 at 4°C with constant boiling HCl, bentonite treated for 4 hours and then filtered.

pH 5'

0.01 M Tris

0.05 M KCl

0.011 M $Mg(OAc)_2$

The buffer was adjusted to pH 5.2 at 4°C with glacial acetic acid, bentonite treated for 4 hours, and then filtered.

When a sulfhydryl protector was desired, 2-mercaptoethanol was added to give a final concentration of 0.006 M. This addition to the buffer was made just prior to use. These buffers are designated (+).

(f) Reagents and chemicals

DNAase: Ribonuclease free. Obtained from Worthington Biochemicals.

^{14}C-Lysine: Specific activity of 318 mCi/m mol. Purchased from the Radiochemical Centre, Amersham

Soluble RNA: Contains "stripped" E. coli t-RNA. Purchased from Calbiochem, cat. #557112.

Spermidine: Purchased from Calbiochem, cat. #56766.

Tungstate reagent (α_3): Prepared from equal volume mixture of α_1 and α_2 (see below).

α_1: 0.6 N trichloroacetic acid. Adjusted to pH 2 with solid pellets of NaOH.

α_2: 0.02 N sodium tungstate. Adjusted to pH 2 with solid trichloroacetic acid.

(g) Ribosomal preparation: Laboratory materials and buffers used in this preparation were made ribonuclease-free by heat treatment, bentonite washing, or equivalent procedures. This procedure is a slight modification of Stanley, Salas, Wahba and Ochoa (25).

Seventy-five grams of frozen T_2 cells were thawed and buffer OA^- (or N^-) was added (1 ml/g of wet cells). The resulting suspension was stirred until a consistent slurry was obtained. (These operations and subsequent operations were carried out at 0^oC unless otherwise specified).

The slurry was passed through a French Pressure Cell (Aminco Power Laboratory Press) at a pressure of 15,000 lbs/in^2 for 3 complete passes. After the first pass, 100 μg of DNAase I were added. The broken cell suspension was incubated for an additional 30 minutes to allow complete digestion of DNA.

The cell lysate was centrifuged at 18,000 rpm for 45 minutes in the 30 rotor of the Spinco L-2 ultracentrifuge. The supernatant was transferred into clean, pretreated tubes and recentrifuged at 18,000 rpm (30,000 xg) for 2 hours.

The top 90% of S-30 supernatant was transferred into treated 50 Ti tubes and spun at 50,000 rpm (170,000 xg) for 3 hours. The top 90% of this supernatant was removed with a sterile syringe and recentrifuged 50,000 rpm for 3 hours. The upper 75% of this supernatant (purified S-100) was removed and divided into 3.0 ml aliquots. These aliquots

were stored at -90°C in the REVCO freezer until use.

The pellets resulting from the first 50,000 rpm centrifugation were covered with 1.5 mls of buffer B^+ and allowed to solubilize overnight in the refrigerator with occasional stirring. The tubes were then filled with buffer B^+ and centrifuged at 25,000 rpm (Low Speed Wash) in the 50 Ti rotor.

The top 90% of the supernatant was removed with a sterile syringe and transferred into treated tubes. It was then centrifuged at 40,000 rpm for 4 hours. The supernatant was removed with a sterile syringe and the pellets were covered with 1.5 mls of buffer OC^+ and allowed to solubilize overnight.

The ribosomal suspension was diluted to ~50 mls with buffer OC^+ and applied to diethylaminoethane (DEAE)-cellulose column (see Materials and Methods: DEAE column preparation) which had been previously equilibrated with OC^+. Approximately 1 liter of OC^+ was passed through the column and the eluate monitored.

The column was then eluted with 1 liter of buffer OD^+. Fractions were collected and the A_{260} was determined. The fractions containing the greatest amount of A_{260} material were pooled and centrifuged at 40,000 rpm for 4 hours in the 50 Ti rotor. The pellets from this centrifugation were covered with 1.0 mls of buffer $BC\text{-}10^+$ and allowed to solubilize for 4 hours. The concentration was estimated by A_{260} measurements, the ribosomes were bottled in 0.4 ml aliquots and stored

in the REVCO freezer at -90°C until use.

(h) DEAE column preparation: (1) One hundred grams of DEAE-cellulose (Biorad cellex-D) were soaked in 2 liters of doubly distilled water for one hour. (2) The suspension was stirred and left to settle for 20 minutes and the fine particles poured off. This process was repeated twice. (3) the DEAE was washed with 1 liter of 0.1 N NaOH for 20 minutes and the slurry was vacuum filtered in a Buchner funnel. This procedure was repeated twice. (4) The DEAE was washed to neutrality with water and again suction filtered. (5) One liter of 95% ethanol was added and the suspension was stirred and left standing for 20 minutes. The DEAE cellulose was filtered and this process was repeated. (6) Ethanol traces were removed by washing with distilled water and filtration. (7) A second washing with 1 liter of 0.1 N NaOH was perfored. (8) The DEAE was washed to neutrality with doubly distilled water and suction filtered. (9) The DEAE was resuspended in the appropriate buffer and a fine slurry was made. (10) The resin was poured into a Biorad Type D column (65 cm x 3.5 cm) and packed under 10 psi nitrogen pressure at room temperature. The column was washed with 2 liters of OC^+ at 4°C and stored in the cold room until use. The final dimensions of the packed column were 45 x 3 cm.

(i) Preparation of pH 5' fraction: A 3.0 ml aliquot of the purified S-100 was thawed and applied to a G-50 Sephadex column (2 cm x 12 cm)

equipped with an extra coarse porosity sintered glass disc. The pH 5' fraction was prepared by eluting with pH 5' buffer and the yellowish precipitate was collected in a 12 ml conical centrifuge tube. The precipitate was centrifuged at top speed in a clinical centrifuge for 15 minutes and the supernatant was discarded. The precipitate was washed twice by resuspension in pH 5' and centrifugation. The final precipitate was resuspended in 0.5-1.0 mls of BC-10^{+} and used as the pH 5' fraction.

(j) In vitro incorporation procedures: Incubations were performed in 12 ml ribonuclease-free conical centrifuge tubes. Glassware and chemicals were also made ribonuclease free by methods already described. Each tube contained the following materials and all reagents were made up in BC-10^{-}. The standard incubation volume was 0.5 mls.

0.025 mls of 0.08 M ATP

0.025 mls of 5×10^{-4} M GTP

0.025 mls of 4 mg/ml E. coli t-RNA

0.025 mls of 10 μCi/ml ^{14}C-Lysine

0.025 mls 0.4 M Spermidine

0.010-0.50 mls of 2 mg/ml poly A

0.010-0.50 mls of T_2 ribosomes (12 mg/ml)

0.010-0.50 mls pH 5' fraction

The final volume and Mg^{++} concentration was adjusted to 0.5 mls by adding the appropriate amounts of BC-0^{+} and BC-50^{+} buffers.

All additions of the reagents were done in an ice bath. The reaction was initiated by the addition of the energy source and radioactivity. The tubes were briefly mixed and placed in a water bath at the appropriate temperature. Upon completion of the incubation, the reaction was stopped by adding ice-cold tungstate reagent.

(k) Counting of total acid-precipitable counts: The reaction was terminated by the addition of 3.0 mls of ice-cold α_3 and 0.1 mls of 10% trichloroacetic acid and the tube was placed in an ice bath. Flocculation was continued for 30 minutes and the tubes were centrifuged in the clinical centrifuge for 10 minutes at top speed. The supernatant was aspirated and the precipitate was dissolved in 0.5 mls of 0.2 N KOH and let stand for 30 minutes at room temperature.

The solution was acidified by the addition of 3.0 mls of α_3 and 1 drop of 6 N HCl. It was allowed to stand for 2-4 hours and then suction filtered through a Millipore filter manifold using Millipore nitrocellulose filters. The empty tube was rinsed with α_3 and the rinse was added to the filters. This step was performed 3 times.

The funnels of the Millipore manifold were also rinsed 3 times with α_3. The nitrocellulose filters were placed on planchets and dried at 70°C for 15 minutes. The samples were counted for 1 minute in a Nuclear-Chicago Model 4312 3 cm windowed gas flow counter.

(1) Sedimentation velocity measurements: Ribosomes were thawed and diluted to the appropriate concentration with 50-30 buffer. They were dialyzed against 2-500 ml changes of 50-30 buffer with the forced flow dialyzer.

Approximately 0.5 mls were placed in the AN-H rotor of the Beckman Model E Ultracentrifuge. After the rotor reached speed at 36,000 rpm, photographs were taken every 4 minutes. The glass slide was developed and measurements were made with a Nikon Microcomparator. The sedimentation constants were caluclated by use of the following equations:

$$(1) \quad S_{OBS} = \frac{2.303 \times \text{slope}}{(2\pi \times \frac{\text{rpm}}{60})^2}$$

$$(2) \quad S_{20,w} = (\frac{\eta_T}{\eta_{20}})(S_{OBS})$$

4 EXPERIMENTAL RESULTS

(a) Requirement of 2-Phosphoenolpyruvic acid (PEP) and pyruvate kinase in ^{14}C-lysine incorporation: Most in vitro protein synthesizing systems require PEP and pyruvate kinase as an ATP generating system since high levels of ATP are found to be inhibitory (26). Some thermophilic systems (13) do not have this requirement. It was of interest to determine whether an ATP generating system was obligatory for ^{14}C-lysine incorporation or if increased concentrations of ATP could be substituted. The results of this investigation are shown in Table 3.

(b) Sedimentation profile of T_2 ribosomes: The sedimentation profile of ribosomes was investigated to estimate the size of T_2 ribosomes. Two concentrations of ribosomes were used and the values were corrected to $S_{20,w}$. The sedimentation profile is represented in Figure 1 and the sedimentation values are shown in Table 2.

(c) Optimal conditions for incorporation of ^{14}C-lysine using polyadenylic acid as messenger at 37°, 45°, 55°, and 65°C: The conditions necessary for obtaining maximum incorporation were investigated. These experiments were limited to the determination of optimum concentrations of Mg^{++}, spermidine, and polyadenylic acid required for maximum biosynthesis of lysine peptides. No attempt was made to investigate the concentration of ribosomes and enzymes necessary for maximum incorporation since these variables were standardized in every incorporation.

Mg^{++} titration: The concentration of Mg^{++} necessary for maximum incorporation of ^{14}C-lysine in an in vitro protein synthesizing system was investigated. The concentration of Mg^{++} was varied by using the appropriate amounts of $BC\text{-}0^{+}$ and $BC\text{-}50^{+}$ buffer. These studies were performed in the presence and absence of spermidine. An arbitrary concentration of 4 mM spermidine was used. All incubations were continued for 30 minutes. The results at each temperature are presented in Figures 2-5 and summarized in Table 1.

Spermidine titration: The arbitrary concentration of 4 mM spermidine was utilized in determining the optimum concentration of Mg^{++} required for maximum incorporation. It was necessary to determine the optimum concentration of spermidine required for maximum synthesis of ^{14}C-lysine peptides. The results of these investigations are depicted in Figures 6-9 and summarized in Table 1.

Polyadenylic acid titration: The concentration of polyadenylic acid required for maximum biosynthesis was investigated using the previously determined optimum concentrations of Mg^{++} and spermidine. These results are presented in Figures 10-13 and summarized in Table 1.

(d) Incorporation of ^{14}C-lysine compared at 37^{o}, 45^{o}, 55^{o}, and $65^{o}C$:

These studies were performed simultaneously at each temperature using the same preparation of pH 5' precipitable protein fraction to preclude the possibility of variation in enzyme concentration. All incu-

bations were perfomed for 30 minutes. The results of these experiments are shown in Figure 14.

(e) Time study of incorporation of ^{14}C-lysine at 37°, 45°, 55°, and 65°C using the pH 5' precipitable protein fraction and ribosomes from T_2:

The incorporation of ^{14}C-lysine was followed as a function of time using the pH 5' protein fraction and ribosomes isolated from T_2. Aliquots were removed every 15 minutes for a total incorporation time of 120 minutes. The results of this investigation are shown in Figures 15-18. The 15 and 30 minute values were selected for comparison to the E. coli system and these values can be found in Figures 20-23.

(f) Time study of incorporation of ^{14}C-lysine at 37°, 45°, 55°, and 65°C using the pH 5' precipitable protein fraction and ribosomes from E. coli:

The incorporation of ^{14}C-lysine was followed as a function of time using the pH 5' precipitable protein fraction and ribosomes isolated from E. coli. The concentrations of the pH 5' protein fraction and ribosomes from E. coli were approximately the same as the pH 5' protein fraction and ribosomes from T_2. The same optimal concentrations of spermidine, polyadenylic acid, and Mg^{++} were used. The total incorporation time was 30 minutes and aliquots were removed every 5 minutes. The results from this investigation are shown in Figure 19. The 15 and 30 minute incorporation values were used in comparison with those obtained with the T_2 system and can be found in Figures 20-23.

5 DISCUSSION

The use of synthetic homoribopolynucleotides as messenger RNA in the study of *in vitro* protein synthesis in *E. coli* was initiated by Nirenberg and Matthaei (27). Their classic experiments showed that polyuridylic acid in this *in vitro* system could direct the incorporation of ^{14}C-phenylalanine into a polyphenylalanine peptide. This discovery was an elegant confirmation of the messenger hypothesis proposed by Jacob and Monod (28).

Later experiments involving random and non-random copolymers (29) showed that this *in vitro* system could promote the incorporation of the remaining amino acids. These investigations were responsible for the eventual elucidation of the genetic code. Recent experiments (30) have shown that an *in vitro* protein synthesizing system from *E. coli* can synthesize an active enzyme using the appropriate conditions.

The requirements for an *in vitro* protein synthesizing system have been well defined (31). The complexity of the system depends upon the type of messenger employed. Natural messengers require a number of factors in addition to those required with synthetic messengers.

Many of the synthetic messengers studied in an *in vitro* system from *E. coli* possess considerable secondary structure and are inactive as messengers. Thermophilic bacteria possess heat-stable ribosomes and enzymes which should allow the investigation of these normally inactive messengers at higher temperatures. These higher temperatures

would cause partial denaturation of the messengers, allowing their attachment to ribosomes and their subsequent translation. The coding properties of these messengers could then be studied.

The T_2 *in vitro* system was investigated to determine whether this system could be applied to the study of inactive messengers. Polyadenylic acid, which does not possess extensive secondary structure, was used as messenger to determine the temperature optimum of the *in vitro* system.

Most *in vitro* protein synthesizing systems require an ATP generating system. However, Friedman (13) has shown that increased concentrations of ATP and GTP will substitute for this systme in *B. stearothermophilus*. Since the PEP-pyruvate kinase ATP generating system was used in early studies of ^{14}C-lysine incorporation in the T_2 system, it seemed likely that higher temperatures could inactivate this mesophilic enzyme system with consequent loss of incorporation.

This problem was investigated in the T_2 system at 37°C. The ATP and GTP concentrations were increased and a PEP-pyruvate kinase system was used as a control. The results from Table 3 indicate that twice the normal concentration of ATP and GTP will substitute for the ATP generating system in the T_2 *in vitro* system.

The sedimentation profile of the T_2 ribosomes was investigated to determine whether these ribosomes differed significantly from those of *E. coli*. This estimation at two concentrations of ribosomes showed

there was no significant difference (Table 2) when the sedimentation values were corrected to $S_{20,w}$. The variations noted in these two determinations may be due to the differences in concentration. A mathematical expression (see Materials and Methods) providing approximate values was used in the calculation.

Crude ribosomes from $\underline{T}_2$ were prepared by differential centrifugation and were completely inactive in supporting ^{14}C-lysine incorporation. Accordingly, ribosomes were purified by DEAE-cellulose chromatography (25) and were only partially active at 37°C. A similar situation exists with B. stearothermophilus ribosomes when purified by the same method. Algranati and Lengyel (14) overcame this problem by adding spermidine, a natural component of ribosomes, to their incorporation mixtures. This procedure was tried with the in vitro system of $\underline{T}_2$.

The addition of 4 mM spermidine to the $\underline{T}_2$ system at 37°C resulted in a lowering of the Mg^{++} concentration from 12 mM without spermidine to 10 mM (see Figure 2). There was also a 5-fold increase of incorporation. Earlier studies without spermidine showed optimum incorporation at 12 mM Mg^{++} and a gradual decrease of incorporation to 20 mM Mg^{++}.

At 45°C, the addition of 4 mM spermidine resulted in a lowering of the Mg^{++} concentration to 6 mM. There was also another 5-fold increase of incorporation. Without spermidine, optimal incorporation of ^{14}C-lysine occurred at 12 mM (see Figure 3).

At 55°C, the addition of 4 mM spermidine again resulted in an

optimal Mg^{++} concentration of 6 mM and a 6-fold increase of incorporation (see Figure 4). At this temperature, there was a more pronounced requirement for Mg^{++} in the presence of 4 mM spermidine. Without spermidine, the optimal requirement of Mg^{++} was 12 mM as previously noted.

At 65°C, the addition of 4 mM spermidine resulted in a slight increase of the Mg^{++} requirement to 8 mM and a more pronounced peak of incorporation (see Figure 5). Without spermidine, hardly any incorporation of ^{14}C-lysine took place.

The reason for the variation in Mg^{++} concentration as a function of temperature is unclear. This phenomenon may be due to partial denaturation of ribosomal sites or heat-induced conformational changes in the messenger which may affect the binding of this messenger to the ribosomes.

The effect of spermidine in promoting higher levels of incorporation is also unknown. Polyamines, such as putrescine, stabilize the r-RNA of thermophilic ribosomes but have no effect on the r-RNA of E. coli. Spermidine, on the other hand, stabilizes both types of r-RNA (13) using thermal denaturation profiles as a measure of stability.

Weiss (32) and Kimes (33) have shown that the addition of small amounts of spermidine or putrescine to extensively purified 30s and 50s subunites of E. coli ribosomes causes structural changes. These alterations make the subunit more susceptible to attack by pancreatic

ribonuclease and a decrease in sedimentation constant. However, a detailed study of thermophilic ribosomes using these methods has not been done.

Since the use of spermidine resulted in a change of Mg^{++} concentration dependence at higher temperatures, it was conceivable that higher temperatures may change the requirement for spermidine. Accordingly, spermidine titrations were performed at each temperature.

At 37°C, the level of ^{14}C-lysine incorporation with 10 mM Mg^{++} reached a plateau at 4 mM spermidine (Figure 6). At 45°C, optimum incorporation of ^{14}C-lysine was obtained at 4 mM spermidine in the presence of 6 mM Mg^{++} (Figure 7). When the temperature was increased to 55°C with 6 mM Mg^{++}, there was a slight increase in the optimum spermidine concentration to 5 mM (Figure 8). At 65°C, the optimum spermidine concentration was again 5 mM in the presence of 8 mM Mg^{++} (Figure 9).

The reason for this varying spermidine concentration is also unclear. It is tempting to speculate that the spermidine may be substituting for Mg^{++} as the temperature is increased but the results at 37°C do not support this notion.

If the ribosomes are becoming partially denatured at higher temperatures, the amount of messenger able to attach may increase. Since higher temperature may also cause loss of secondary structure of messenger, more messenger could bind to the ribosomes and result in

a greatly enhanced incorporation. Accordingly, a polyadenylic acid titration was performed at each temperature.

At 37°C, the amount of messenger required for optimal incorporation seems to be 100 μg (Figure 10). The system is saturated after addition of this amount of messenger. At 45°C, optimal incorporation of ^{14}C-lysine takes place at 100 μg and the system is saturated (Figure 11). When the temperature is increased to 55°C, saturation is again reached at 100 μg (Figure 12). At 65°C, the system is again saturated with 100 μg (Figure 13). Accordingly, subsequent investigations were performed using these concentrations of polyadenylic acid.

Since the optimum concentrations of spermidine, Mg^{++}, and polyadenylic acid had been determined at each temperature, a comparison of incorporations of ^{14}C-lysine was investigated at these temperatures. These studies attempted to determine the optimal temperature of this in vitro protein synthesizing system.

Incorporations of ^{14}C-lysine were performed simultaneously at each temperature and the same pH 5' precipitable protein fraction was used to preclude any variation in enzyme concentration. The results of this investigation (Figure 14) show optimal activity at 55°C. The reduced incorporation of ^{14}C-lysine at 65°C was not expected since the B. stearothermophilus system has enhanced activity at 65°C (14). However, incorporation values at 65°C are approximately the same as at 37°C.

Since the incubation time in the above studies was limited to 30 minutes, the rate and extent of incorporation of ^{14}C-lysine was investigated for 120 minutes. Samples were removed every 15 minutes. At 37°C, the rate of incorporation was much slower than at 45°C or 55°C (Figures 15, 16 and 17) but approximately the same as at 65°C. However, the extent of incorporation is much greater at 37°C than at 65°C (Figures 15 and 18). This result seems to indicate that denaturation of some component of the system may be proceeding at this high temperature.

Most studies involving in vitro protein synthesis in thermophilic systems are compared to the E. coli system under the same conditions. Similar concentrations of ribosomes and pH 5 precipitable protein fractions from E. coli were incubated at the various temperatures under identical conditions previously determined for T_2. These incubations were performed for 30 minutes and samples were removed every 5 minutes.

At 37°C, the initial rate of ^{14}C-lysine incorporation with the E. coli system (Figure 19) was slower than at 45°C although the extent of incorporation was approximately 10% higher. At 55°C, the initial rate of incorporation was much slower than at 37°C or 45°C. The extent of ^{14}C-lysine incorporation was approximately 38% of that occurring at 37°C. At 65°C, the level of incorporation was only a few hundred counts above background.

It was desirable to compare both the E. coli and T_2 systems

directly. For this comparison, previous data were extracted and plotted. In Figure 20, both systems are fairly comparable at 37°C. At 45°C, the T_2 system has approximately the same initial rate but the incorporation of ^{14}C-lysine continues to a greater extent (Figure 21). At 55°C, the T_2 system has a much greater initial rate (Figure 22) and approximately 3 1/2-fold as much incorporation of ^{14}C-lysine. At 65°C, the T_2 system has 20 times the amount of ^{14}C-lysine incorporated as the E. coli system (Figure 23).

The results presented in this study have shown that the in vitro protein synthesizing system from this extremely thermophilic gram-negative bacteria does support polyadenylic acid directed ^{14}C-lysine incorporation. Although gram-negative thermophilic bacteria have been isolated and the ribosomes studied, no one has reported a successful in vitro protein synthesizing system.

The sedimentation constants of the thermophilic ribosomes are comparable to those of E. coli. This system has an optimal temperature for activity at 55°C. The reason for reduced incorporation at 65°C is unclear since it was hoped that this thermophilic system would have a higher temperature optimum than obtained with B. stearothermophilus.

TABLE I

OPTIMUM CONDITIONS for INCORPORATION of ^{14}C-LYSINE

Temperature (°C)	Mg^{++} ($\times 10^{-3}$ M)	Spermidine ($\times 10^{-3}$ M)	Poly-A (μg)
37	10	4	100
45	6	4	100
55	6	5	100
65	8	5	100

TABLE 2

T_2 RIBOSOMAL SEDIMENTATION CONSTANTS

CONCENTRATION	SEDIMENTATION CONSTANTS*	
2.0 mg/ml	32.9 ± 0.6	50.2 ± 0.4
5.8 mg/ml	32.2 ± 0.4	45.9 ± 0.7

* expressed as Svedbergs (10^{-13} sec.)

TABLE 3

% ^{14}C-LYSINE INCORPORATION

SYSTEM	% INCORPORATION
PEP Pyruvate kinase 2.0 μM ATP 0.012 μM GTP	100
2.0 μM ATP 0.012 μM GTP	83
2.0 μM ATP 0.025 μM GTP	94
4.0 μM ATP 0.012 μM GTP	101
4.0 μM ATP 0.025 μM GTP	102

Figure 1. Sedimentation profile of ribosomes

T_2 ribosomes at the designated concentrations were dialyzed overnight against 50-30 buffer. This solution was then centrifuged at 36,000 rpm in the Beckman Model E analytical ultracentrifuge equipped with Schlieren optics. After the rotor attained speed, photographs were taken at 4 minute intervals.