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Protein-synthesizing Machinery of Thermophilic Bacteria

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INTRODUCTION

Thermophilic bacteria are among the truly unique microorganisms inhabiting our unicellular kingdom. Attempts to explain their ability to proliferate at temperatures over 70 C have resulted in widespread investigation during the past two decades. (The precise upper temperature limit for bacterial growth is a controversial issue. For a recent discussion of this problem, see reference 10.) The literature in this field has been previously reviewed by Gaughran (27), Allen (3), and Koffler (30). Many of the early studies were focused on the enzymes and structural proteins (flagella) isolated from thermophilic bacteria. With a few exceptions (which may not be valid because of the impure preparations used), the results demonstrated that thermophile proteins were more thermostable than comparable preparations obtained from mesophiles. These findings implied that thermophily was achieved by virtue of inherent properties of thermophile cellular constituents, and was not merely the result of a favorable equilibrium existing between rapidly resynthesized and heat-denatured molecules. The argument for the presence of stabilizing factors or the absence of labilizing factors was not seriously challenged until Campbell and his coworkers isolated and studied a crystalline, heat-stable α-amylase from *Bacillus stearothermophilus* (13, 14, 34, 35). In addition, this important work provided the first clue to a molecular basis for thermostability. The amino acid composition data revealed an unusually high proline content (14), resulting in a large negative optical rotation (35). The suggestion has been made that the thermostability of the protein molecule may be a consequence of the fact that the enzyme exists in an unfolded configuration in the native state, and therefore heat has relatively little further effect upon denaturation (35). This could not serve as a universal mechanism, however, since heat-stable, highly purified flagellar proteins obtained from thermophiles did not contain large amounts of proline (D. Abram and H. Koffler, Abstr. Proc. Intern. Congr. Microbiol., 8th, Montreal, 1962, p. 21).

During the past 10 years there has been a concentrated and fruitful research effort directed toward elucidating the molecular events occurring during protein synthesis and deciphering the genetic code. Once again, thermophilic bacteria have provided a novel and valuable source of experimental material for the investigator. It is the purpose of this review to summarize these recent results with the protein-synthesizing machinery of thermophiles. Most of the studies to be reported were performed with the obligate thermophile, *B. stearothermophilus*, although several experiments utilizing facultative thermophiles have also been included.

MESSENGER RIBONUCLEIC ACID (MRNA)

Saunders and Campbell (44) isolated the mRNA of *B. stearothermophilus* strain 10 after a 30-sec pulse of ^{32}P. The base composition (Table 1) was determined after hydrolysis by alkali and chromatography on a Dowex-1-8x formate column. The guanosine plus cytosine (G + C) ratio (50.4%) of the mRNA agreed well with the G + C ratio of the deoxyribonucleic acid (DNA) isolated from the same strain (50.5% based on the bouyant density value of 1.713 g cc; or 52.4% based on the T_m value of 90.8 C). [Throughout this review, T_m will be used to indicate the tem-

TABLE 1. *Base composition of messenger RNA from Bacillus stearothermophilus*[a]

Base	Mole per cent
Guanine	29.3
Adenine	26.8
Cytosine	21.1
Uracil	22.8

[a] From Saunders and Campbell (44).

perature at the midpoint of total hyperchromicity for DNA or RNA melting curves. As pointed out by Pace and Campbell (43), however, inflection point would be a more appropriate designation in the case of single-stranded nucleic acids.]

The turnover of labile RNA in an unclassified strain of *B. stearothermophilus* was measured by using both kinetic data and the time-interval method after actinomycin D treatment (11). The turnover time for labile RNA was 1 min at both 40 and 63 C, compared to 5 to 6 min for *Escherichia coli* at 40 C. The authors suggested that the very rapid turnover of what is probably mRNA in the thermophile is a mechanism for providing a high rate of protein synthesis to enable replacement of cellular constituents when the organism is growing at 63 C. They also found that the turnover time for proteins, when the thermophile was grown at 40 C, was 10 min, whereas this value was 1 to 2 min when the same culture was incubated at 63 C. To reconcile these results, one could hypothesize the presence of a proteolytic enzyme having a high heat of activation and a messenger-degrading enzyme which does not have an elevated heat of activation. Although other models based upon regulatory mechanisms may also be invoked, the above possibility could be readily tested.

SOLUBLE RIBONUCLEIC ACID (sRNA) AND AMINOACYL sRNA SYNTHETASES

I have grouped these two classes of molecules into one section, since many of the studies with heat-stable synthetases are directly related to the conformational properties of sRNA.

The base composition of sRNA from *B. stearothermophilus* strain 10 has been determined (44), but the same analytical techniques were not simultaneously applied to *E. coli* sRNA, thereby limiting the accuracy of direct comparison. The nucleotide composition of sRNA from *B. stearothermophilus* strain B and *E. coli* strain B was determined in a single study by Mangiantini et al. (33). The data are shown in Table 2. Pseudouridylic acid was present in thermophile sRNA at about 10% the concentration of uridylic acid, which was lower than that reported for *E. coli* sRNA (18). Other than this exception, the nucleotide composition of the two preparations was very similar. In addition, sRNA from *B. stearothermophilus* strain 2184 was found to contain approximately the same amount of 4-thiouracil as *E. coli* sRNA, namely 1 in 120 to 140 bases (M. N. Lipsett, *personal communication*). Similarities between thermophile sRNA and *E. coli* sRNA have also been reflected in virtually identical thermal denaturation profiles of the two preparations under a wide variety of ionic conditions (26, 33, 44). It has been suggested that maintenance of the proper secondary structure of sRNA in the intact thermophile (a necessary prerequisite for biological activity; see discussion which follows) could be achieved by polycation stabilization rather than by alterations in the primary structure (26, 33).

Using crude extracts of *B. stearothermophilus* strain 2184, Friedman and Weinstein (26) studied the effect of temperature on leucyl and phenylalanyl sRNA synthetases. The reaction mixtures were incubated for 1 min at *p*H 7 in order to minimize nonenzymatic deacylation. When the thermophile enzymes were tested with thermophile sRNA, both synthetases were more active at 55 to 65 C than at 37 C. Quantitatively similar results were obtained with thermophile enzymes and *E. coli* sRNA, suggesting that the high temperature optima were primarily a function of the thermophile enzymes.

Bubela and Holdsworth (12) studied the temperature optimum of aminoacyl sRNA synthetases from an unclassified strain of *B. stearothermophilus*. These workers employed a *p*H 5 fraction obtained from the 105,000 $\times$ *g* supernatant fluid. They assayed for both aminoacyl adenylate formation (activation) and the attachment of amino acids to sRNA molecules. Similar

TABLE 2. *Nucleotide composition of soluble RNA from Bacillus stearothermophilus and Escherichia coli*[a]

Determination	*B. stearothermophilus*	*E. coli*
Adenylic (mole %)	20.5 ± 0.57	20.6 ± 0.75
Guanylic (mole %)	31.5 ± 1.60	32.0 ± 1.33
Cytidylic (mole %)	26.5 ± 1.37	28.0 ± 1.67
Uridylic[b] (mole %)	21.1 ± 0.26	19.3 ± 1.98
Purines/Pyrimidines	1.09	1.11
6 Amino/6 Keto	0.89	0.94
A + U/G + C	0.72	0.66

[a] From Mangiantini et al. (33).
[b] Includes pseudouridylic.

results were obtained in both instances. The reactions were not rapid until a temperature of 35 to 40 C was reached, and rose to a maximum at 59 to 61 C. When the *p*H 5 fraction was heated at various temperatures, it was found that the amount of amino acid-activating activity remaining in the solution decreased above 40 C. After 10 min at 60 C, only 50% of the initial activity was recovered.

These results emphasize the precautions which must be exercised in evaluating enzyme data. A high temperature optimum for enzymatic activity does not necessarily imply the presence of a heat-stable enzyme per se, but may reflect an elevated heat of activation for the substrate or cofactor-stabilized enzyme complex. For example, calculations based upon the Arrhenius equation revealed the heat of activation for protein synthesis in *B. stearothermophilus* to be 13,700 cal, whereas this value was 9,150 cal for *E. coli* (11). It must be emphasized, however, that the demonstration of heat stability is never conclusive unless highly purified, or preferably crystalline, proteins are tested.

In contrast to the studies with the *p*H 5 fraction, it was found that an amino acid-activating system in a membrane fraction prepared from protoplasts retained approximately 85% of the initial activity after heating at 63 C for 10 min. When a soluble activating system was prepared by ultrasonic treatment of the membranes, it was observed that the enzymes were relatively heat-labile. The authors suggested that the organization of enzymes in membranes could be an important factor in conferring thermostability.

Crude extracts of *B. stearothermophilus* strain B were used to test the temperature range for isoleucyl and leucyl sRNA synthetase activities (5). Leucine-dependent adenosine triphosphate (ATP)-pyrophosphate exchange still occurred at 80 C, whereas leucyl sRNA formation did not take place at this temperature. Similarly, isoleucine-dependent ATP-pyrophosphate exchange occurred at 85 C, but the synthesis of isoleucyl sRNA ceased abruptly at 80 C. The results could not be attributed to heat denaturation of the enzyme or to cleavage of sRNA, since preincubation for 10 min at 80 C of either the enzyme fraction or the complete assay mixture did not effect the attachment of isoleucine at 50 C. In addition, the possibility that isoleucyl sRNA formation ceased at 80 C because of nonenzymatic cleavage was ruled unlikely on the basis of the following experiments. The activity of the synthetase was decreased only very slightly in the presence of 3 M urea, whereas the T_m of unfractionated *B. stearothermophilus* sRNA in the presence of 3 M urea was 8 C lower than in the absence of urea. Correspondingly, in the presence of urea, isoleucylacylation of sRNA stopped at 72 C, while, in the absence of urea, the reaction stopped at 80 C. Furthermore, isoleucine-dependent ATP-pyrophosphate exchange still occurred at 75 C in the presence of urea. The authors concluded that a certain degree of loss of sRNA secondary structure prevents interaction with the aminoacyl sRNA synthetase. Unfortunately, the results are somewhat ambiguous, owing to the impurity of both the synthetase and sRNA preparations.

Fresco and his associates (22) have purified a heat-stable valyl sRNA synthetase from the facultative acidophilic thermophile, *B. coagulans* ATCC 7050, grown at 50 to 55 C. The enzyme was apparently capable of acylating two of three valine acceptor ($sRNA^{val}$) components of *E. coli* at 37 C, and catalyzed valine-dependent ATP-pyrophosphate exchange at temperatures up to 75 C. The authors studied the effect of temperature on valylacylation of unfractionated *E. coli* sRNA by the enzyme. Since the synthetase had an acid *p*H optimum, the reactions were carried out at *p*H 6, thereby minimizing nonenzymatic deacylation and allowing the course of acylation of sRNA to be followed to completion at each temperature. Figure 1 shows the results in the form of plots of the amount of valyl sRNA formed at equilibrium

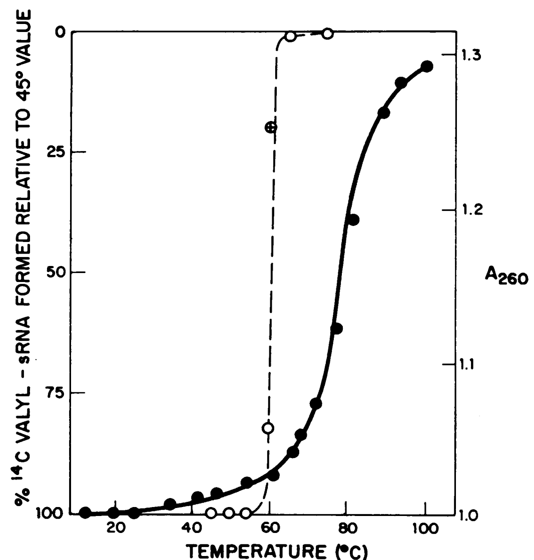

FIG. 1. *Valine acceptor activity* (○) *and absorbance* (●) *of unfractionated Escherichia coli sRNA as a sunction of temperature. Assays for valine acceptor activity included a purified valyl sRNA synthetase from Bacillus coagulans and were made in the presence of of 1.0 mM valine, except for one experiment* (⊕), *where 0.1 mM valine was used. From Fresco et al.* (22).

TABLE 3. *Aminoacyl sRNA formation at 50 and 75 C by purified isoleucyl sRNA synthetase from Bacillus stearothermophilus*[a]

Amino acid	Counts per min per μg of sRNA (50 C)	Counts per min per μg of sRNA (75 C)
Isoleucine	715	550
Valine	45	500
Leucine	200	180
Serine	60	182
Threonine	0	162
Phenylalanine	59	44

[a] From Arcà et al. (7).

as a function of temperature and an absorbance-temperature profile of unfractionated *E. coli* sRNA. It can be seen that valylacylation was quantitative up to 55 C, and then decreased sharply so that no valylacylation was observed at 65 C. Moreover, the abrupt change was manifest when a relatively small amount of the total hyperchromic change had occurred. The activity lost at 65 C was fully recovered on lowering the temperature. The enzyme was able to acylate homologous sRNA from *B. coagulans* and also to catalyze valine-dependent ATP-pyrophosphate exchange at the temperature where *E. coli* sRNA could no longer be acylated. The results were interpreted to indicate that native sRNA possesses a conformation which is essential for synthetase recognition, and that this conformation can be reversibly lost on raising the temperature and regained when the temperature is lowered. A comparison of these results with the data of Nishimura et al. (40) serves to emphasize that the loss of biological activity of the $sRNA^{val}$ occurs at a temperature where this sRNA has not begun to melt, since these workers have obtained a melting profile on purified $sRNA^{val}$ rather than unfractionated sRNA.

In addition to the obvious importance of these studies to the problem of molecular requirements for synthetase-sRNA interaction, the data also raise another interesting question. The fact that thermophile sRNA can still react with the synthetase at 65 C might indicate differences in the secondary structures between thermophile sRNA and *E. coli* sRNA. Although melting curves of unfractionated mesophile and thermophile sRNA appeared identical (26, 33, 44), physical measurements might not be as sensitive a probe as testing acceptor capacity. Furthermore, recent studies (40) have demonstrated significant differences in melting curve profiles between several purified amino acid-specific sRNA molecules and unfractionated sRNA from *E. coli*. Therefore, to obtain meaningful data, it would be essential to compare purified amino acid-specific sRNA molecules prepared from mesophiles and thermophiles. On the other hand, these results could also be explained by the stabilizing effect of a tightly bound cationic molecule present in the thermophile sRNA preparation. Alternatively, the thermophile enzyme preparation might be contaminated with a heat-stable pyrophosphatase, which would tend to drive the reaction and thus alter the equilibrium between native and denatured sRNA. The resolution of this problem awaits future investigation.

A purified isoleucyl sRNA synthetase prepared from *B. stearothermophilus* strain B had an optimum temperature for the formation of isoleucyl sRNA at 60 C, which decreased sharply at 75 C (7). This preparation, when tested at 50 C, also catalyzed valine-dependent ATP-pyrophosphate exchange (indicating valyl adenylate formation), but not valyl sRNA synthesis. In this respect, the enzyme performed exactly as did its counterpart from *E. coli* (8, 42). At temperatures above 70 C, however, the thermophile enzyme started to catalyze the formation of valyl sRNA. In addition to valyl sRNA, seryl sRNA and threonyl sRNA were also formed at 75 C (Table 3). The purified isoleucyl sRNA synthetase, when tested at 50 C, also contained a significant amount of leucyl sRNA synthetase activity.

Further studies (6) showed that the formation of valyl sRNA at 75 C was greatly reduced by adding excess cold isoleucine, thus suggesting that valine was being charged onto an isoleucine acceptor sRNA ($sRNA^{ileu}$), rather than to $sRNA^{val}$. More convincing support for this thesis was obtained by methylated albumin-kieselguhr (MAK) column separation of ^{14}C-valyl sRNA synthetized at 75 C by purified isoleucyl sRNA synthetase and of ^{3}H-valyl sRNA synthesized at 50 C by crude extract. In addition, periodate oxidation of unfractionated sRNA charged with isoleucine resulted in the formation of specifically active $sRNA^{ileu}$, which could accept valine at 75 C but not at 50 C.

Two possible mechanisms occurring at elevated temperatures were advanced. Either there is a conformational change in the sRNA molecule, thereby allowing valine to bind to $sRNA^{ileu}$, or there is a conformational change in the synthetase which enables the enzyme-valyl adenylate complex to bind to $sRNA^{ileu}$. The first possibility was considered more likely, since anomalous charging began at temperatures corresponding to the onset of hyperchromic changes in the sRNA melting curve.

The authors proposed that coding errors brought about through anomalous charging of sRNA at temperatures above 70 C could be

TABLE 4. *Nucleotide composition of ribosomal RNA from Bacillus stearothermophilus and Escherichia coli*[a]

Determination	*B. stearothermophilus*	*E. coli*
Adenylic (mole %)	25.9 ± 1.07	27.6 ± 0.47
Guanylic (mole %)	34.5 ± 0.72	26.5 ± 1.84
Cytidylic (mole %)	22.0 ± 0.68	22.5 ± 1.36
Uridylic[b] (mole %)	17.5 ± 0.64	23.3 ± 0.72
Purines/Pyrimidines	1.53	1.30
6 Amino/6 Keto	0.94	0.89
A + U/G + C	0.78	0.86

[a] From Mangiantini et al. (33).
[b] Includes pseudouridylic.

instrumental in defining the upper temperature limits for the growth of thermophilic bacteria. Another interesting aspect of this study was the fact that all ot the amino acids which participated in the anomalous reaction exhibited structural similarities. This may indicate that the active site on the synthetase responsible for amino acid recognition cannot be indiscriminantly deceived as a result of conformational changes in sRNA.

RIBOSOMAL RIBONUCLEIC ACID (rRNA)

Mangiantini et al. (33) analyzed the nucleotide composition of rRNA from *B. stearothermophilus* strain B and *E. coli* B (Table 4). The data reveal that thermophile rRNA had a slightly higher G content and a markedly lower uridine (U) content. The base composition of the two rRNA fractions from *B. stearothermophilus* strain 10 was determined by Saunders and Campbell (44). The 16*S* rRNA had a G + C mole per cent ratio of 61%, compared to 54% reported for *E. coli* (28). The 23*S* rRNA had a G + C ratio of 56%, compared to 53% reported for *E coli* (28). These differences in G + C composition were consistent with elution patterns obtained with cochromatograms of thermophile rRNA and *E. coli* rRNA on a MAK column.

Stenesh and Holazo (47) have studied the rRNA obtained from mesophilic and thermophilic strains belonging to the same genus, namely, *Bacillus*. This approach was aimed at ruling out the possibility of intergeneric differences that are not related to the property of macromolecular thermostability. Their base composition data revealed an average mole per cent G + C value of 55.1% for the three mesophiles tested (*B. pumilus*, *B. licheniformis*, and an unidentified species), and 59.8% for three thermophiles (*B. stearothermophilus* strains FJW, 10, and 2184). The average mole per cent A + U value for mesophiles was 44.9%, and the corresponding value for thermophiles was 40.3%. Ribonuclease digests of the various rRNA preparations yielded nucleotide maps with essentially identical patterns. This result suggested that rRNA molecules isolated from mesophiles and thermophiles may have similar nucleotide sequences, but differ in the relative frequency of these sequences.

In an extensive study of 19 organisms, Pace and Campbell (43) found that with a few exceptions, the G + C content of the rRNA molecules increased and the A + U content decreased with increasing maximal growth temperature.

The presence of a higher G + C:A + U ratio in thermophile rRNA than in mesophile rRNA predicts a more stable structure achieved through more extensive hydrogen bonding. This has been verified by several laboratories through the use of thermal denaturation profiles. Thus, greater T_m values for thermophile rRNA than for *E. coli* rRNA have been reported (23, 33, 44). One of these studies (44) demonstrated that both the 16*S* and 23*S* fractions of thermophile rRNA were more heat stable than the corresponding components from *E. coli*. In addition, the rRNA molecules from thermophilic bacilli were shown to have higher T_m values than rRNA molecules from mesophilic bacilli (47). Finally, melting curves of rRNA molecules from a psychrophile (*Vibrio marinus*), a mesophile (*Spirillum itersonii*), and a thermophile (*B. stearothermophilus* strain 10) revealed increasing T_m values, respectively (43).

A differential effect of polyamines on thermophile and mesophile rRNA has been reported (23). When thermal denaturation profiles were run in a magnesium-free buffer, putrescine stabilized the rRNA of *B. stearothermophilus* strain 2184, but had no effect on *E. coli* rRNA. Spermidine, on the other hand, stabilized both types of nucleic acid. The possible relevance of these results to the thermostability of thermophile ribosomes is discussed in the Ribosome section of this review. In addition to the polyamine studies, the same authors reported the separation of the two rRNA components from the thermophile on a sucrose density gradient. The results demonstrated that the amount of the 16*S* component exceeded that of the 23*S* component, whereas *E. coli* rRNA was separated into 23*S* and 16*S* peaks with ratios of approximately 2:1. When thermophile rRNA was separated on a sucrose density gradient immediately after isolation, the 23*S* to 16*S* ratio was 1.6:1. The change in ratio, which occurred after brief storage at −20 C, suggested preferential degradation of the 23*S* fraction and its conversion to 16*S* rRNA. Mangiantini et al. (33) studied the rRNA from *B. stearothermophilus*

TABLE 5. *Amino acid composition of ribosomes from Bacillus stearothermophilus*[a] *and Escherichia coli*[b]

Amino acid	Mole per cent	
	B. stearothermophilus	*E. coli*
Lysine	6.30	9.01
Histidine	1.93	1.91
Arginine	5.01	7.30
Aspartic acid	9.16	8.30
Threonine	5.53	5.22
Serine	4.44	4.38
Glutamic acid	13.33	10.08
Proline	4.44	3.67
Glycine	9.29	8.18
Alanine	10.51	10.98
Valine	9.07	9.63
Methionine	2.33	2.40
Isoleucine	6.64	5.51
Leucine	8.34	7.40
Tyrosine	2.16	1.78
Phenylalanine	3.55	3.03
One-half cystine	0.82	0.53

[a] From Saunders and Campbell (44).
[b] From Spahr (46).

strain B in the analytical ultracentrifuge and found a major component with a sedimentation coefficient of 16*S*, a small but variable amount of 21*S* material, as well as 10–11*S* RNA. Similar results with rRNA obtained from *B. cereus* were described by Takai and Kondo (50). Apparent break-down products of rRNA derived from higher organisms have also been recently reported (4, 29, 38). In reference to bacterial species, further work is needed to establish whether instability of 23*S* rRNA is a general characteristic of gram-positive bacilli and to clarify the possible relationship between instability and the molecular structure of rRNA.

RIBOSOMAL PROTEIN

Mangiantini et al. (33) reported that only about 50% of the total protein could be extracted with acid from *B. stearothermophilus* strain B ribosomes, whereas, under the same conditions, this value was 85% for *E. coli* ribosomes. In the case of thermophile ribosomes, 6 M urea was effective in dissociating the protein fraction from the RNA. Ultracentrifuge analysis of acid-extracted proteins showed a single peak with a sedimentation coefficient of about 3*S*. Starch-gel electrophoresis of the acid extract in the presence of 6 M urea demonstrated the presence of 12 distinct bands, 10 migrating to the cathode and 2 to the anode. Comparison with *E. coli* ribosomal proteins examined under the same conditions revealed only minor differences. The ribosomal proteins from *B. stearothermophilus* strain 2184 and *E. coli* have also been separated by the currently preferred technique of polyacrylamide gel electrophoresis in urea, and no obvious differences were detected (J. G. Flaks, *personal communication*).

The quantitative amino acid composition of ribosomal protein hydrolysates from *B. stearothermophilus* strain 10 was determined by Saunders and Campbell (44). When compared to data obtained for *E. coli* (Table 5), no marked differences were observed. The thermophile protein had a slightly higher half cystine content than *E. coli* protein, raising the possibility of disulfide bridge stabilization. This seems unlikely, however, because of the very small amounts present in both preparations. It must be emphasized that these similarities in quantitative amino acid composition do not rule out the possibility of differences in primary structure between the two proteins.

RIBOSOMES

The ribosomes of *B. stearothermophilus* strain 2184 were found to consist of 59% RNA and 41% protein (26). These data were similar to those reported for *E. coli* ribosomes (52). Stenesh and Yang (48), on the other hand, reported average values for ribosomes from three mesophiles and three thermophiles belonging to the genus *Bacillus* to be 45% RNA and 55% protein. The chemical analysis of ribosomes from *B. stearothermophilus* strain B showed an average content of 0.85 (range: 0.60 to 0.95) mg of RNA per mg of protein (33). It remains to be seen whether the discrepancies observed in the composition of thermophile ribosomes are due to differences in growth conditions, analytical procedures, purity of ribosome preparation, or strain differences.

When purified ribosomes of *B. stearothermophilus* strain 10 were suspended in 10^{-2} M $MgCl_2$-10^{-2} M tris(hydroxymethyl)aminomethane (Tris)-chloride (*p*H 7.4), ultracentrifuge analysis revealed peaks of 4*S*, 72.5*S*, 101*S*, and 135*S*, with the 101*S* peak being the most prominent (44). Dialysis against 10^{-3} M $MgCl_2$-10^{-2} M Tris-chloride (*p*H 7.4) resulted in peaks with sedimentation coefficients of 40*S*, 31*S*, and 54*S*. Further breakdown resulting in peaks of 33*S* and 24*S* was obtained after dialysis of thermophile ribosomes into 10^{-4} M $MgCl_2$-10^{-2} M Tris-chloride, *p*H 7.4. The sedimentation values of ribosomes from an unclassified strain of *B. stearothermophilus* in 10^{-1} M NaCl-10^{-2} M $MgCl_2$ were 31*S*, 50*S*, and 73*S*; in 10^{-1} M NaCl-10^{-3} M $MgCl_2$, these values

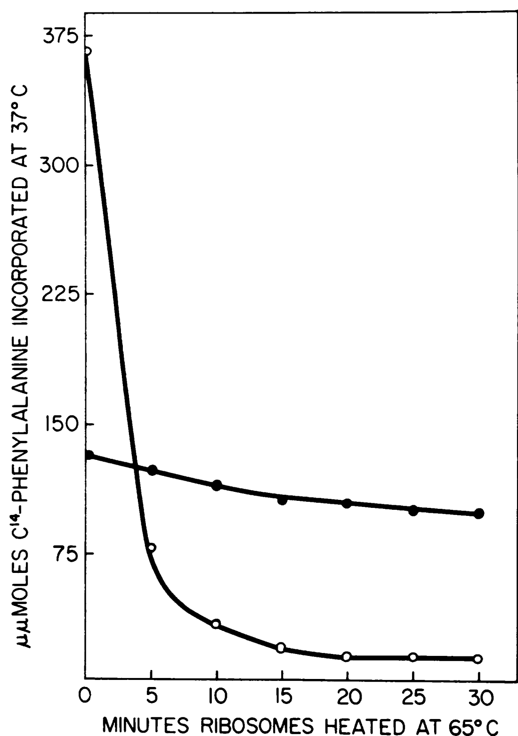

FIG. 2. *Heat stability of ribosomes from Escherichia coli* (○) *and Bacillus stearothermophilus* (●). *From Friedman et al.* (*23*).

were 28*S*, 48*S*, and 65*S*. The major fractions were 73*S* and 65*S* peaks. No polysomes were detected (12). When prepared in 10^{-2} M phosphate buffer-10^{-3} M Mg acetate, ribosomes from *B. stearothermophilus* strain B revealed two main components of 33*S* and 48*S* in addition to minor constituents of 8*S*, 70*S*, and 85*S*. Peaks with sedimentation coefficients of 45*S*, 52*S* 73*S*, 84*S*, and 103*S* were obtained in 5 × 10^{-3} M Mg acetate-10^{-2} M Tris-chloride, *p*H 7.5 (*see* 33).

The heat stability of ribosomes from *B. stearothermophilus* has been demonstrated in comparative studies with *E. coli* ribosomes utilizing thermal denaturation profiles. Thus, several laboratories have reported higher T_m values for thermophile ribosomes than for *E. coli* ribosomes (23, 33, 44). These physical measurements are limited in their scope, however, since they do not necessarily relate to the biological role of the ribosome in protein synthesis. With this in mind, Friedman et al. (23) heated ribosomal suspensions from *B. stearothermophilus* strain 2184 and *E. coli* strain B at 65 C, removed samples at various time intervals, and tested their ability to support poly U-directed phenylalanine incorporation at 37 C (*see* Fig. 2). After 5 min at 65 C, 94% of the initial activity was retained by the thermophile ribosomes, whereas only 21% was retained by *E. coli* ribosomes. When the time interval at 65 C was extended to 15 min, incorporation values were 80 and 5% for thermophile and *E. coli* ribosomes, respectively. Similar results on the heat stability of mesophile and thermophile ribosomes were also reported by Algranati and Lengyel (2).

The greater resistance of thermophile ribosomes than *E. coli* ribosomes to heat denaturation suggested a possible correlation with the maximal growth temperature of these organisms (33). Pace and Campbell (43) made an extensive study of the thermal denaturation profiles of 70*S* ribosomes from 19 organisms, spanning the range from psychrophiles to thermophiles. A psychrophile (7E-3) with a maximal growth temperature of 20 C had ribosomes with a T_m of 69 C, whereas *B. stearothermophilus* strain 10, with a maximal growth temperature of 73 C, had ribosomes with a T_m of 79 C (*see* Fig. 3). Two mesophilic bacteria, *E. coli* strain B and *Spirillum itersonii*, with maximal growth temperatures of 45 C, had ribosomes with intermediate T_m values of 72 and 73 C, respectively. The results indicated that the thermostability of ribosomes could be the limiting factor in determining the upper growth temperature for organisms. As has been pointed out previously, coding error brought about through anomalous charging of sRNA at elevated temperatures might also be instrumental in defining the maximal growth temperatures for thermophiles. The relative importance of these two factors to the problem of thermobiosis remains to be determined.

The unusual heat stability of thermophile ribo-

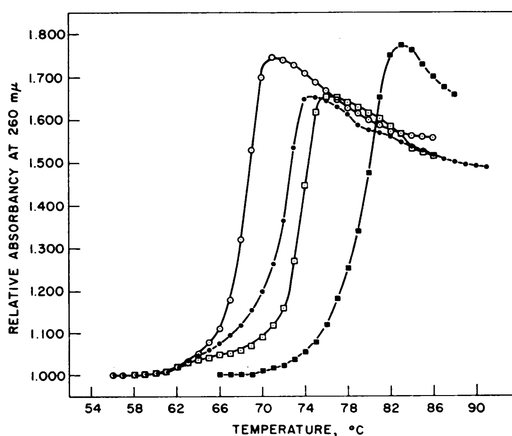

FIG. 3. *Thermal denaturation profiles of psychrophile 7E-3* (○), *Escherichia coli B* (●), *Spirillum itersonii* (□), *and Bacillus stearothermophilus 10* (■) *ribosomes. From Pace and Campbell* (*43*).

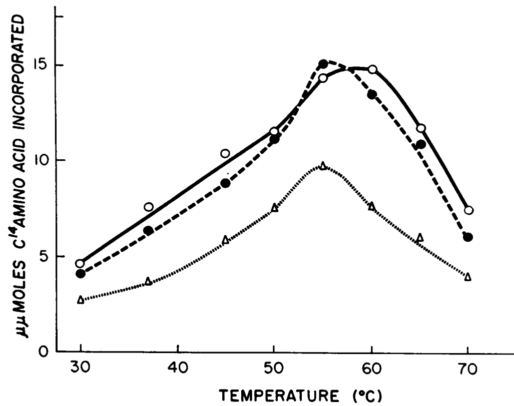

FIG. 4. *Endogenous incorporation of lysine* (△), *phenylalanine* (●), *and proline* (○) *by the S-30 fraction of Bacillus stearothermophilus. From Friedman and Weinstein* (*26*).

somes has been established both by thermal denaturation profiles and amino acid incorporation studies. In this connection, we note that hybrid ribosomes consisting of ribosomal subunits derived from *B. stearothermophilus* and *E. coli* have been shown to be active in protein synthesis (15). Such heterologous complexes should continue to prove useful in determining specific subunit properties and functions related to protein synthesis.

The molecular basis for the heat stability of ribosomes from thermophilic bacteria remains to be elucidated. The ribosomal proteins of thermophiles appear similar to those of *E. coli*, although the possibility of differences in the primary structure relevant to the property of thermostability has not been ruled out. Since the ribosomes employed for studies on thermostability have not been extensively purified, thermostability could be a consequence of a stabilizing factor which is tightly bound to the ribosome. Friedman et al. (23) found that the polyamine, putrescine, was more effective in stabilizing thermophile ribosomes and rRNA than in stabilizing *E. coli* ribosomes and rRNA. When the total polyamine content of *B. stearothermophilus* strain 2184 was analyzed, however, the value obtained was less than that reported for *E. coli* by Dubin and Rosenthal (17). In addition, the major components in both the thermophile and mesophile were putrescine and spermidine. Similar results were reportea by Mangiantini et al. (33).

Another possible mechanism related to ribosome thermostability could be the activity of the ribosome-bound ribonuclease. Two laboratories (33, 48) have reported that rRNA in thermophile ribosomes was more resistant to attack by ribosomal ribonuclease than rRNA in mesophile ribosomes. In one of these studies (48), this difference was observed across a wide temperature range.

Although the rRNA from thermophiles has a slightly higher T_m than rRNA from mesophiles, this probably could not fully account for the greater heat stability of thermophile ribosomes. Thermophile ribosomes have a higher T_m than their corresponding rRNA, whereas this effect was not observed with *E. coli* (23, 33). In addition, the slope of the melting curve was less steep with thermophile ribosomes than *E. coli* ribosomes (23, 33). These results suggested that the stacking arrangement of ribosomal protein and rRNA in thermophile ribosomes may be different from that found in mesophile ribosomes (33, 43). Additional support in favor of this theory comes from recent preliminary experiments performed in my laboratory. When equal optical density (260 mμ) units of ribosomes from *B. stearothermophilus* strain 2184 and *E. coli* strain B were treated with pancreatic ribonuclease, approximately equal amounts of acid-soluble nucleotides were found in solution after 5 min of incubation, but at longer time points, considerably more RNA was available for enzymatic digestion and release from *E. coli* ribosomes than from thermophile ribosomes (S. M. Friedman and A. Ricca, *unpublished data*). Further work on the topography of thermophile ribosomes will be needed to clarify the specific nature of the protein-RNA interaction.

IN VITRO PROTEIN-SYNTHESIZING SYSTEMS

A subcellular protein-synthesizing system prepared from *B. stearothermophilus* strain 2184 was studied extensively by Friedman and Weinstein (26). When directed by native mRNA molecules (endogenous reaction), the optimal temperature for the incorporation of ^{14}C-phenylalanine, lysine, and proline into polypeptides was 55 to 60 C. Incorporation of all three amino acids occurred across a temperature range from 30 to 70 C. In each case, incorporation at 65 C was greater than at 37 C (Fig. 4). In contrast to the high temperature optimum of the thermophile system, parallel studies with the *E. coli* system showed that endogenous incorporation of ^{14}C-phenylalanine at 65 C was about 10% that at 37 C.

When assayed in standard buffer containing 0.01 M magnesium, poly U-directed incorporation of phenylalanine was greater at 37 C than at 65 C (Fig. 5A). The significant incorporation occurring at 37 C was unexpected, because *B.*

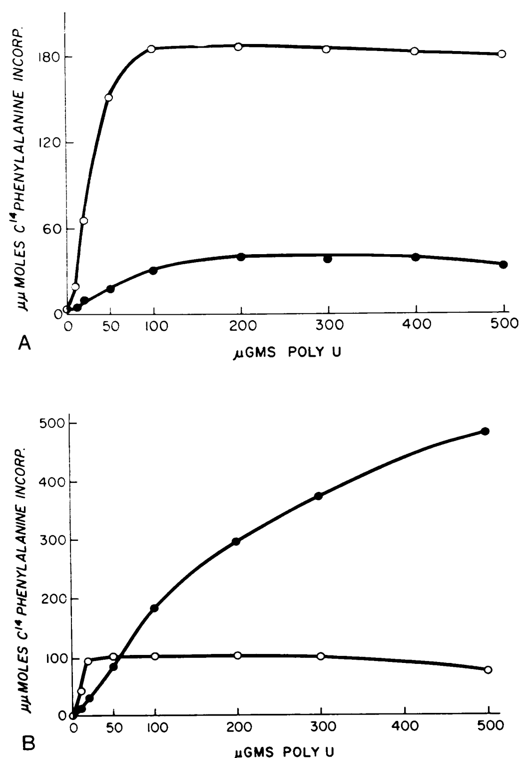

FIG. 5. *Effect of poly U on the incorporation of phenylalanine at 37 C* (○) *and 65 C* (●) *by the preincubated S-30 fraction of Bacillus stearothermophilus. Magnesium acetate was added at a final concentration of 0.01 M in* (A) *and 0.018 M in* (B). *From Friedman and Weinstein* (*26*).

stearothermophilus is an obligate thermophile and did not grow at 37 C in the growth medium employed in these studies. Therefore, the lesion which prevented growth at 37 C did not involve the components of the protein synthetic apparatus of this organism. Presumably, the in vitro system supplied an essential component not available at this temperature in vivo, or the inability to grow at 37 C resided in an aspect of cell metabolism not directly related to protein synthesis. In this connection, the work of Bubela and Holdsworth (11) may be relevant. They showed that the uptake of amino acids and uracil by an unclassified strain of *B. stearothermophilus* was very low at 35 to 38 C, but increased rapidly, reaching an optimum at 60 C. Their conclusion was that the inability of obligate thermophiles to grow below 40 C was a result of the very low rate of entry of essential metabolites into the cell. They cited unpublished work which indicates that the supply of energy required for the transport mechanism is defective at low temperatures.

The interaction of sRNA with mRNA is presumed to be by means of Watson-Crick base-pairing. Therefore, the thermolability of the poly U-phenylalanine reaction could be a reflection of stresses on hydrogen bonds existing between phenylalanyl sRNA molecules and UUU codons. Since cations are known to stabilize helical regions (32, 37, 41), the effect of increasing the magnesium concentration of the incubation mixture was investigated (Fig. 5B). When the magnesium concentration was raised to 0.018 M, poly U-directed phenylalanine incorporation at 65 C was greater than at 37 C for polymer concentrations higher than 50 μg. Under these conditions, the 65 C reaction had a high requirement for poly U and was not saturated at 500 μg per assay system.

It was known from early studies with *E. coli* extracts (9, 39) that poly U, in addition to coding for phenylalanine, could also stimulate the incorporation of leucine to a lesser extent. The thermophile subcellular system was utilized to study the effects of temperature and magnesium concentration on leucine-phenylalanine ambiguity (24, 26). The results demonstrated that low temperature and high magnesium greatly enhance the incorporation of leucine in the presence of poly U. In addition, miscoding in the thermophile system was observed with the copolymers, poly UG and poly UC (24).

Results obtained in the thermophile system with poly U, poly UG, and poly UC indicated that, when miscoding occurred, it was restricted to groups of amino acids which were characteristic for each polymer (24). The pattern of response suggested that polycations induce miscoding by stabilizing the alignment of sRNA molecules to codons which contain two rather than three complementary nucleotides. A type of

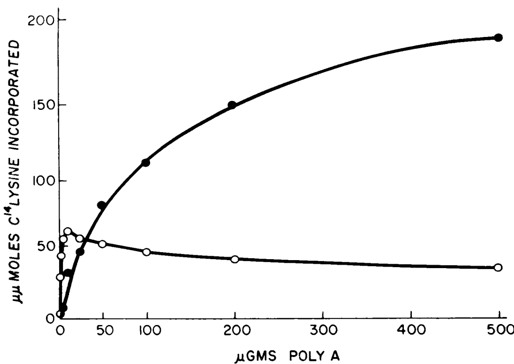

FIG. 6. *Effect of poly A on the incorporation of lysine at 37 C* (○) *and 65 C* (●) *by the preincubated S-30 fraction of Bacillus stearothermophilus. From Friedman and Weinstein* (*26*).

miscoding in the thermophile system which does not obey this "two out of three" rule was described by Friedman and Weinstein (25). At 37 C and in the presence of dihydrostreptomycin, poly UG and poly UGA directed the incorporation of proline to a small but significant extent. Since the codon assignments for proline are CCC, CCU, CCA, and CCG (36), it would be impossible for there to be more than one complementary nucleotide (according to Watson-Crick base-pairing rules) between available triplets in the synthetic mRNA molecules and the anticodon sites on sRNA molecules. A more detailed discussion of miscoding mechanisms is beyond the scope of this review and has been presented elsewhere (53).

The high temperature optimum of the thermophile system has also been used to study the effect of temperature on the coding functions of synthetic polyribonucleotides possessing extensive secondary structure. Thus, studies by Friedman and Weinstein (26) with poly A showed that, at 37 C, there was a sharp optimum at 10 μg for lysine incorporation. The incorporation at 65 C was much more extensive than that at 37 C for polymer concentrations of 50 μg or higher. In addition, the 65 C reaction had a high poly A requirement and was not saturated at 500 μg per assay system (Fig. 6).

The effect of secondary structure on the coding properties of synthetic polyribonucleotides has been studied in the *E. coli* system by Singer et al. (45). Increases in the relative content of guanylic acid residues present in UG copolymers resulted in increased secondary structure and was paralleled by inhibition of amino acid incorporation. Sucrose density gradient studies performed by Takanami and Okamoto (51) showed that polymers with extensive secondary structure were unable to bind to ribosomes. The ability of high temperatures to stimulate poly A-directed lysine incorporation in the thermophile system was thought most likely the result of enhanced complexing between ribosomes and the melted configuration of the polymer. Similar results with poly A were reported by Szer and Ochoa (49) in the *E. coli* system, although the maximum temperature that could be tested with the mesophile extract was 45 C.

Algranati and Lengyel (2) studied the coding properties of a poly AU (1.4:1) preparation in a subcellular system derived from *B. stearothermophilus* strain 1503-4R. The T_m of the polyribonucleotide in a buffer similar to that used in the cell-free system was 58 C. As predicted from this observation, the incorporations of isoleucine, leucine, and lysine were much higher at 65 C than the low levels observed at 37 C. The same authors made an extensive study of the kinetics and optimal environmental conditions for amino acid incorporations directed by poly U, poly A, and poly C. In addition, they found that the nuclease activity of the *B. stearothermophilus* supernatant fluid at 37 C was 10% that at 65 C, and about 13% that of *E. coli* at 37 C. Thus, thermophile extracts, which are active in protein synthesis at 37 C, should be helpful in studying the coding properties of mRNA molecules under conditions which minimize their degradation.

OUTLOOK

The enlistment of thermophilic bacteria in the study of protein synthesis has enhanced our knowledge of some important steps occurring during this complex process. Studies with heat-stable aminoacyl sRNA synthetases have indicated that essential conformations in sRNA are required for accurate participation in this reaction. In addition, thermostable cell-free extracts have been utilized to examine the effects of temperature on the formation and function of the messenger-sRNA-ribosome complex. Finally, although the heat stability of thermophile ribosomes has been firmly established, the precise molecular organization responsible for this unusual property remains a prime target for future research.

I would like to digress somewhat from the main theme at this point and briefly consider some other areas that might contribute to our understanding of thermobiosis. Lest the reader be misled by this text, it should be pointed out that the search for unique thermophile components need not be confined to proteins and ribosomes. Recent studies by Wicken and his coworkers (20, 21, 54) have revealed unusual teichoic acid-like polymers in the cell walls of *B. stearothermophilus* and *B. coagulans* grown at 55 C. The relationship, if any, between this finding and the ability of thermophiles to grow at high temperatures has yet to be determined.

Information concerning lipids in thermophiles is both sparse and conflicting. Dyer (19) claimed that the phospholipid fraction of *B. stearothermophilus* consisted primarily of sphingomyelin, which has a higher melting point than other phospholipids. Long and Williams (31), on the other hand, were unable to detect any sphingomyelin in either thermophile vegetative cells or spores. Clearly then, this subject is in need of careful re-evaluation.

The stability of thermophile cell membranes has proven useful in ultrastructure analysis (1). Although this observation was based solely on morphological considerations, interesting corre-

lations can be drawn from other studies. For example, chemical analysis of membranes from *B. stearothermophilus* revealed large amounts of C_{15} and C_{17} branched-chain fatty acids (16). In addition, it has been proposed that the presence of branched-chain fatty acids should bestow a greater degree of flexibility upon a membrane (E. Kodicek, Abstr. Proc. Intern. Congr. Microbiol. 8th, Montreal, 1962, p. 23). Brock (10) recently suggested that the integrity of the cell membrane might be the limiting factor in thermal death. His hypothesis is based, in part, upon the fact that thermal killing follows simple first-order kinetics, whereas multiple hits would be required for the inactivation of enzymes and ribosomes. These considerations, coupled with the previously described results on enzyme stability in membrane fractions (12), make the role of the cell membrane in thermophily a subject worthy of serious attention.

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