Ribosomes, Polyribosomes, and Deoxyribonucleic Acid from Thermophilic, Mesophilic, and Psychrophilic Clostridia

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Analysis of deoxyribonucleic acid (DNA) from four species of *Clostridium*, including two thermophiles, a mesophile, and a psychrophile, revealed no obvious relationship between growth temperature and DNA base composition. The melting temperatures (*T*<sub>m</sub>) of the DNA from the four species varied no more among the thermophilic, mesophilic, and psychrophilic species than among many related mesophilic species. Characterization of ribosomes from the clostridia by means of optical rotatory dispersion yielded similar spectra in common with other unrelated organisms. Only small differences were noted in the base composition of ribosomal ribonucleic acid (RNA) and in the amino acid composition of ribosomal proteins, including half-cystine content, as determined by cysteic acid analysis, and accessible sulfhydryl groups, as determined by titration with dithiobis (2-nitrobenzoic acid). Except for the two thermophiles, the ribosomal protein electrophoretic patterns were dissimilar. No unusual thermal stability was manifested in the *T*<sub>m</sub> values of thermophile ribosomal RNA. However, thermophile ribosome *T*<sub>m</sub> values (69 C) were higher than were mesophile and psychrophile *T*<sub>m</sub> values (64 C). Ribosomes from the four clostridial species were also examined in regard to the effect on their functional integrity, measured by their activity in poly U-directed <sup>14</sup>C-phenylalanine incorporation, and their gross physical integrity, measured by sucrose gradient analysis. The *T*<sub>a</sub>,<sub>s</sub> values (temperature which produces 50% inactivation after 5 min) was found to be 70 and 72 C for the two thermophiles *C. tartaricoribum* and *C. thermosaccharolyticum*, respectively; 57 C for a mesophile, *C. pasteurianum*; and 53 C for a psychrophile, *Clostridium* sp. strain 69. At 55 C, little effect was seen on the thermophile ribosomes, but the mesophile ribosomes lost 90% of their activity in 1 hr, and psychrophile ribosomes lost 100% of their activity within 10 min. According to sucrose gradient profiles, heating at 55 C results in dissociation of mesophile ribosomes and aggregation of psychrophile ribosomes. Thermophile S-100 fractions were also more thermostable than were mesophile or psychophile S-100 fractions. The *T*<sub>a</sub>,<sub>s</sub> values were 69 C for *C. tartaricoribum* and *C. thermosaccharolyticum*, 100 and 41 C for *C. pasteurianum*, and *Clostridium* sp. strain 69 S-100. The effect of heat on the endogenous incorporation of <sup>14</sup>C-valine by polysomes was also examined. In the case of thermophile polysomes, the extent of incorporation at 55 and 37 C was about equal. In the case of mesophile and psychrophile polysomes, the extent at 55 C was 44 and 39%, respectively, of the value at 37 C. The initial rates of incorporation in all four cases were greater at 55 C than at 37 C.

Accumulating evidence for unique thermostability of macromolecules from thermophilic organisms has thus far not implicated deoxyribonucleic acid (DNA) insofar as melting temperatures (*T*<sub>m</sub>) and base composition are concerned. For example, the *T*<sub>m</sub> and related guanine plus cytosine (%G + C) values of DNA from the obligate thermophile, *Bacillus stearothermophilus*, fall within the range characteristic of *Bacillus* values in general (9). Studies with *Actinomyces* (7) and *Micromonospora* (11) indicated lower, rather than higher, %G + C in thermophilic than in mesophilic species.

*Thermophile* ribosomes, on the other hand,
are unusually stable in terms of melting characteristics. However, studies have largely been limited to comparisons of only a few thermophilic aerobes, primarily *B. stearothermophilus* (15, 20, 26, 30) and, more recently, *Thermus aquaticus* (41) with unrelated mesophiles such as *Escherichia coli*. Ribosomes from certain aerobic thermophilic bacteria are thermostable not only in their physical resistance to melting, but in their retention of biological activity after heating (13). In vitro protein synthetic systems from *B. stearothermophilus* have been compared with systems from *E. coli* and mesophilic bacilli with regard to the influences of temperature, ionic characteristics, artificial messenger ribonucleic acid (RNA) composition, and source of transfer RNA on the incorporation of amino acids into polypeptides (13, 36). Efforts have also been made to determine the relative contributions to heat stability by individual ribosome components: ribosomal subunits, RNA, and proteins (1, 14, 25).

In the present study, we sought to determine the thermostability of DNA and ribosomes from two thermophilic anaerobes, *Clostridium tar-tarivorum* and *C. thermosacharolyticum*, in comparison with the same constituents from a related mesophile, *C. pasteurianum*, and psychrophile, *Clostridium* sp. strain 69. We have characterized the ribosomes from these organisms by a variety of chemical and physical techniques and have examined the effect of temperature on the components of the in vitro amino acid-incorporating system. Ribosomes, polysomes, and S-100 from the thermophilic clostridia were found to be significantly more thermostable than the same components from mesophilic and psychrophilic clostridia. The greater stability of thermophile ribosomes was observed by comparing *Tm* values, the effect of heating on sucrose gradient centrifugation profiles, and the effect of heating on the ability of ribosomes to function in amino acid incorporation reactions.

**MATERIALS AND METHODS**

**Cultivation of organisms.** *Clostridium* sp. strain 69, a psychrophile (33), and *C. tartarivororum* strain T9-0 were gifts from J. L. Stokes and R. H. Vaughan, respectively. The media for *C. thermosaccharolyticum* strain 3814 (39), *C. tartarivororum* (39), *Clostridium* sp. strain 69 (33), *E. coli* A19 (16), *C. pasteurianum* W5 (5), and *C. pasteurianum* ATCC 6013 (28) have been described. The thermophiles were grown at 55 °C, the mesophiles at 37 °C, and the psychrophile at 18 °C. Cells were harvested at the mid-log phase of growth, which corresponded to an absorbancy (A260) value of about 1.0 to 1.5, with the exception of *C. pasteurianum* ATCC 6013, which was collected at an *A*260 value of 3 to 4. Cells were frozen, or in some cases lyophilized, and stored at −80 °C.

**General assay procedures.** Protein was assayed by a modification of the Lowry method (29). DNA and RNA were determined by methods of the diphenylamine and orcinol assays described by Schneider (31).

**Isolation of DNA.** DNA was prepared by the method of Miura (22) with a slight modification, i.e., the freeze-thawing steps were omitted, and an initial Pronase treatment was added. Before extraction of the nucleic acids with phenol, the cells were incubated at 37 °C for 1 hr in a buffer which contained 0.1 M tri(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 9), 0.1 M NaCl, 0.1% dodecyl sulfate, and 0.1% Pronase. Using this procedure, 1 mg of DNA was isolated from 1 g of wet cells. This is 4 to 10 times the yield reported by Tonomura et al. (38) for several clostridial species. Protein was not detectable in the DNA preparations. The absence of uracil after acid hydrolysis indicated that the DNA was also free of RNA.

**Tm determination.** The hyperchromic increase of absorbance of DNA, ribosomes, and ribosomal RNA (rRNA) upon heating was followed in a Zeiss spectrophotometer equipped with a Gilford recorder and Lauda K-2/R temperature-controlled circulator. *Tm* values were determined as described by Marmur and Doty (21). The melting of DNA was done in 0.015 M sodium citrate (pH 7.3) containing 0.15 M NaCl. The *Tm* values of ribosomes and rRNA were determined in 0.01 M Tris-hydrochloride (pH 7.4) containing 0.01 M MgCl2.

**Base composition of DNA and rRNA.** Perchloric acid hydrolysis of DNA and paper chromatography of purine and pyrimidine bases were carried out by the procedure of Wyatt (40). The method of Katz and Comb (17) was used for the hydrolysis of RNA and the separation of 2′-3′-ribonucleotides.

**Isolation of ribosomes and S-100 fractions.** Two procedures were used to isolate ribosomes from the clostridia. Wet (1 g/10 ml) or lyophilized (1 g/25 ml) cells were suspended in TM buffer (0.01 M Tris-hydrochloride, pH 7.4; 0.01 M MgCl2). The cells were disrupted with a French pressure cell (15,000 psi), and ribosomes were purified by differential centrifugation. Cell debris was removed by centrifugation at 30,000 × g for 20 min. The supernatant fluid containing the ribosomes was centrifuged at 100,000 × g for 2.5 hr, and the translucent ribosome pellet was suspended in a small volume of TM buffer, either by gradual resuspension overnight in the cold or by brief homogenization. Low- and high-speed centrifugation sometimes revealed a slowly sedimenting contaminant which corresponded to a thin, white layer on the top of the ribosome pellet after centrifugation at 100,000 × g. This was effectively removed by adding a small amount of TM buffer, allowing the pellet to resuspend for about 2 hr in the cold, agitating the tube gently, and discarding the suspended material. The second procedure involves the use of lysozyme and has already been reported (16).

The Alumina grinding method of Nirenberg (24) was used in the growth of *E. coli* A19 ribosome and S-100 fractions. The final solutions of ribosomes had *A*260 values of 150 to 250. Ribosome concentra-
tions were calculated from the extinction coefficient for E. coli ribosomes: $E_{260}^{\text{nm}} = 157$ (12). The yield of ribosomes was about 10 mg/g of wet cells or 25 mg/g of lyophillized cells. The spectrum of purified ribosomes had a maximum at 259 to 260 nm and a minimum at 235 to 237 nm. Absorbance ratios ranged from 1.6 to 1.8 for $A_{260}/A_{280}$ and from 1.5 to 1.7 for $A_{260}/A_{235}$. Purity of the ribosome preparations was further verified in a Spinco model E analytical ultracentrifuge equipped with schlieren optics. A purified preparation usually gave three to four peaks, the largest corresponding to the 70S monosome. In addition to the 30S and 50S peaks, there was a smaller peak corresponding to a 100S component, and occasionally there was a small amount of heavier aggregates. Ribosomes and S-100 fractions were stored in 0.5-ml volumes at $-80\,^\circ$C.

**Isolation of RNA.** RNA was extracted from partially purified ribosomes by the method of Kurland (19). The RNA contained less than 1% protein.

**Preparation of polyosomes.** The preparation of polyosomes from *C. pasteurianum* in the presence of the detergents Br3 and sodium deoxycholate has been reported fully elsewhere (16). This procedure with minor modifications was used in this study for the four species of *Clostridium*. In endogenous incorporation assays, the P-30 fractions of *C. tartarivorum* and *C. pasteurianum* and the P-100 fractions of *C. thermosaccharolyticum* and *Clostridium* sp. strain 69 were used.

**Sucrose gradient analysis.** Ribosomes or poly- somes (0.01 ml containing about two $A_{260}$ units) were diluted to 0.2 ml in TMK buffer (0.01 M Tris-hydrochloride, pH 7.6; 0.1 M NaCl; 0.004 M MgCl2; 0.08 M KCl) and layered on 5-ml to 15% linear sucrose gradients. The gradients were prepared in ribonuclease-free sucrose (Mann Research) in TMK buffer. The tubes were centrifuged at 45,000 rev/min for 90 or 120 min (ribosomes) or 75 min at 35,000 rev/min (polyosomes) in an SW50.1 rotor in a Spinco model L-2 preparative ultracentrifuge. After centrifugation, the contents were forced out the top of the tubes and through an ISCO ultraviolet analyzer by pumping a 50% sucrose solution into the bottom of the tubes.

Approximate sedimentation values of ribosome peaks in unheated preparations were estimated based on earlier analytical centrifuge studies of ribosomes prepared under similar conditions. Relative percentages of particles under the peaks were estimated planimetrically.

**Amino acid composition of ribosomal protein.** Solutions of ribosomes containing 1.5 to 3.0 mg of protein were made 6 N in HCl, sealed in vials under vacuum, and hydrolyzed at 110 C for 24 hr. After the vial was cooled and opened, the sample was dried over NaOH in a desicator under vacuum. Amino acids were analyzed with a Beckmann-Spinco model 120 amino acid analyzer. One-half cystine was determined as cystic acid after performic acid oxidation (23).

**Isolation of ribosomal protein and disc gel electrophoresis.** Extraction of ribosomal proteins from purified ribosomes by 2-chemical and analytical acrylamide gel electrophoresis in 8 M urea were carried out as described by Fogel and Sypherd (12) except that sample and spacer gels were not used. To achieve better resolution of the large number of proteins, long gel columns (5 by 100 mm) were prepared.

**Titration with dithiothreitol (2-nitrobenzoic acid) DTNB.** Free sulfhydryl groups of ribosomal proteins were determined with dithiothreitol (2-nitrobenzoic acid) (DTNB; 10). Ribosomes were added to 1 ml of either TMK buffer or 5 M urea such that the final concentration was 0.5 to 1.0 mg of protein/ml. After measuring the initial absorbance at 412 nm, excess DTNB (0.02 ml of a 4% solution) was added, and the absorbancy was measured until no further increase was observed. The time required for the solutions to reach maximal absorbance varied from 30 min for ribosomes in urea to 1 hr for ribosomes in TMK buffer.

**4C-amino acid incorporation assay.** Uniformly labeled 4C-amino acids (Phe and Val) were purchased from New England Nuclear. Polyuridylic acid (poly U) was obtained from Miles Chemicals. *E. coli* B sRNA (stripped) was obtained from General Biochemicals. Phosphono-pyruvate, 4C-amino acids, pyruvate kinase, adenosine triphosphate (ATP), and guanosine triphosphate (GTP) were purchased from Sigma Chemical Co.

The assay was similar to that described by Nirenberg (24). The 4C-amino acid reaction mixture contained in a final volume of 0.25 ml: 0.1 M Tris-hydrochloride, pH 7.8; 0.05 M KCl; 1 mM ATP; 0.04 mM GTP; 6 mM 2-mercaptoethanol; 9 mM phosphoenolpyruvate; 24 mM magnesium acetate; 400 $\mu$g of *E. coli* B sRNA; 5 $\mu$g of pyruvate kinase; 0.25 mm each of 19 4C-amino acids; and 0.04 mM of a labeled amino acid (15 Ci/m mole). With *E. coli* and *Clostridium* sp. strain 69 ribosomes or polyesomes, the optimal concentration of Mg**2** was 14 mm. The other clostridial species require a higher Mg**2** as previously described for *C. pasteurianum* (16). Ribosomes or polysomes, supernatant fraction (S-100), and poly U were included in the reaction mixture as indicated. Incubation was usually for 30 min at 37 C; otherwise, the time and temperature of incubation are described for each experiment. Three milliliters of 10% trichloroacetic acid was added to stop the reaction. The tubes were heated at 90 C for 20 min and then placed in ice for 30 min. The precipitates were collected on glass-fiber filter discs (Whatman GF/A, 24 mm) with a Millipore multi-filter vacuum apparatus and washed with 25 ml of cold 5% trichloroacetic acid. The discs were placed in scintillation vials containing 10 ml of Bray solution (3) and counted in a Packard 500 Tri-Carb scintillation counter.

**RESULTS**

$T_m$ and base composition of DNA. Thermal denaturation curves of DNA from the four clostridial species showed that transitions occurred at similar temperatures. The base compositions determined from the $T_m$ values and from chemical analyses are indicated in Table 1.
**Table 1. Base composition of clostridial DNA**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Guanine (%)</th>
<th>Cytosine (%)</th>
<th>Adenine (%)</th>
<th>Thymine (%)</th>
<th>(% G + C )</th>
<th>(T_m) (°C)</th>
<th>(% G + C )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridium sp. strain 69</td>
<td>13.5 ± 1.1</td>
<td>16.4 ± 0.7</td>
<td>32.5 ± 0.7</td>
<td>37.5 ± 0.6</td>
<td>29.9 ± 1.0</td>
<td>83.0 ± 0.2</td>
<td>33.4 ± 0.4</td>
</tr>
<tr>
<td>C. pasteurianum W5</td>
<td>17.5 ± 1.2</td>
<td>14.9 ± 1.2</td>
<td>34.1 ± 2.1</td>
<td>33.5 ± 0.2</td>
<td>32.4 ± 3.0</td>
<td>82.2 ± 0.1</td>
<td>31.5 ± 0.1</td>
</tr>
<tr>
<td>C. thermosacharolyticum</td>
<td>18.2 ± 1.5</td>
<td>17.8 ± 1.4</td>
<td>33.6 ± 1.1</td>
<td>30.6 ± 1.1</td>
<td>36.0 ± 1.6</td>
<td>84.2 ± 0.1</td>
<td>36.3 ± 0.3</td>
</tr>
<tr>
<td>C. tartarivorum</td>
<td>18.1 ± 2.8</td>
<td>18.2 ± 3.8</td>
<td>34.5 ± 1.9</td>
<td>29.2 ± 2.7</td>
<td>36.3 ± 2.9</td>
<td>85.8 ± 0.1</td>
<td>40.2 ± 0.1</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation for 3 to 5 determinations.

**Table 2. \(T_m\) values for clostridial ribosomes and rRNA**

<table>
<thead>
<tr>
<th>Species</th>
<th>(T_m) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clostridium thermosacharolyticum</td>
<td>68.7 ± 1.4</td>
</tr>
<tr>
<td>C. tartarivorum</td>
<td>69.6 ± 2.4</td>
</tr>
<tr>
<td>C. pasteurianum</td>
<td>63.8 ± 0.7</td>
</tr>
<tr>
<td>Clostridium sp. strain 69</td>
<td>64.4 ± 2.4</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation for number (n) = 8 to 11.

**Bacterial ribosomes and rRNA.**

Ribosomes from the two thermophiles, *C. thermosacharolyticum* and *C. tartarivorum*, have similar melting temperatures (68.7 and 69.6 °C, respectively) as do ribosomes from the mesophile, *C. pasteurianum* (63.8 °C), and the psychrophile, *Clostridium* sp. strain 69 (64.4 °C) (Table 2). The greater thermostability of thermophile ribosomes in terms of \(T_m\) is not reflected in the \(T_m\) values obtained from isolated rRNA (Table 2). Qualitatively, differences existed in the melting curves of both ribosomes and rRNA from the thermophiles, mesophile, and psychrophile. Before the largest increases in absorbancy were observed, some "melting out" occurred at much lower temperatures (40 to 60 °C). This is more pronounced with *C. pasteurianum* and *Clostridium* sp. strain 69 ribosomes and rRNA than with components from the two thermophiles.

**Base composition of rRNA.**

The base composition of rRNA from the thermophilic, mesophilic, and psychrophilic clostridia are essentially identical (Table 3). This is consistent with the lack of difference in the \(T_m\) values. It appears, then, that the greater thermal stability of ribosomes from thermophilic clostridia is not due to differences in rRNA, at least as thermal stability is manifested as resistance to melting or differences in \(\% G + C\) values, or both.

**ORD of ribosomes.**

The optical rotary dispersion (ORD) spectra of ribosomes from *C. thermosacharolyticum*, *C. pasteurianum*, and *Clostridium* sp. strain 69 are nearly superimposable, with prominent positive Cotton effects at about 266 nm (Fig. 1). The value of \(\alpha\) at 233 to 235 nm, which is due primarily to ribosomal protein, is not as great for *Clostridium* sp. strain 69 as for *C. pasteurianum* and *C. thermosacharolyticum*, but is still comparable to values for *E. coli* ribosomes (19). In general, the similarities of the spectra of clostridial ribosomes are consistent with the absence of large differences among such phylogenetically diverse sources as *E. coli*, yeast, and rabbit reticulocyte ribosomes (19).

**Amino acid composition of ribosomal proteins.**

The similarity of the amino acid composition of ribosomes from the clsostridia species can be seen in Fig. 2. The apparently large lysine content of the psychrophile protein was obtained from widely varying values (12 to 27 mole percent), so that the difference from the other clostridia is probably not significant. For comparison, the reported amino acid composition of *E. coli* ribosomal protein (27) has also been included in Fig. 2. The congruence of the composition of clostridial ribosomes with that of *E. coli* is extensive, although it differs from *E. coli* and also some plant and animal compositions in the larger amount of glycine and somewhat smaller amount of alanine. In general, however, the distribution of acidic, basic, hydrophobic, aromatic, and sulfur-containing amino acids in clostridial ribosomal protein is similar to other ribosomal protein from a large number of bacterial, plant, and animal sources.

**Total and free sulphydral content of ribosomal protein.**

The half-cystine content of clostridial ribosomal protein ranged from 0.68

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Vol. 113, 1973 RIBOSOMES, POLYRIBOSOMES, AND DNA FROM CLOSTRIDIA 255
presumably present as disulfides. DTNB reacts more slowly with sulfhydryl groups in TMK buffer, and only to the extent of \( \frac{1}{4} \) to \( \frac{1}{2} \) the total present in ribosomal protein. Therefore, under conditions of ribosome stabilization, from \( \frac{1}{4} \) to \( \frac{3}{4} \) of protein sulfhydryl groups are buried in the ribosome structure, unavailable for reaction with DTNB. There is no apparent correlation of the disulfide, or free and buried sulfhydryl content with thermostability of the ribosomes.

**Disc gel electrophoresis of ribosomal protein.** Although certain general features of the electrophoretic patterns of ribosomal protein were similar (for example, the presence of a number of proteins near the center of the gels forming a broad polydisperse stained region), there was a wide variation in the distribution and intensity of the bands. A large difference was even seen between the two strains of *Clostridium pasteurianum*. However, the two thermophiles gave almost identical patterns when corrected for the migration of tracking dye.

**Effect of heat on the activity and structure of ribosomes.** The thermal stability of clostridial ribosomes in terms of their activity in protein synthesis in vitro is indicated in Fig. 3. Unheated ribosomes, with the exception of *Clostridium* sp. strain 69 ribosomes (which incorporated 0.5 nmoles of \(^{14}C\)-Phe/mg of ribosomal protein), incorporated about 2.0 nmoles of \(^{14}C\)-Phe/mg of ribosomal protein in the standard assay. After preincubation for 1 hr at 37 C, none of the ribosomes lost more than 20% of the original activity. At 55 C, the growth temperature of the thermophiles, ribosomes from *C. tartarivororum* and *C. thermosaccharolyticum* lost 0% and 25% activity, respectively, after preincubation for 1 hr. On the other hand, ribosomes from *C. pasteurianum* lost activity gradually (92% after 1 hr at 55 C). The psychrophile ribosomes lost all activity within 10 min (Fig. 3A).

Residual activity of ribosomes preincubated for 5 min at various temperatures was measured to determine the temperature of 50% inactivation, or \( T_{1/2} \) (2). The thermophile ribosomes
Fig. 2. Amino acid composition of ribosomal proteins. (1) C. pasteurianum ATCC 6013; (2) C. pasteurianum W5; (3) Clostridium sp. strain 69; (4) C. tartarivorum; (5) C. thermosaccharolyticum; (6) E. coli (24).

Table 4. Sulfhydryl content of ribosomal protein

<table>
<thead>
<tr>
<th>Organism</th>
<th>nMoles of SH/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>By cysteic acid</td>
</tr>
<tr>
<td>Clostridium sp.</td>
<td>69</td>
</tr>
<tr>
<td>strain 69</td>
<td>75</td>
</tr>
<tr>
<td>C. pasteurianum ATCC 6013</td>
<td>47</td>
</tr>
<tr>
<td>C. tartarivorum</td>
<td>64</td>
</tr>
<tr>
<td>C. thermosaccharolyticum</td>
<td>47</td>
</tr>
</tbody>
</table>

*Mean ± standard deviation for number = 5 or 7.

With Tₘₐ values of 72°C (C. thermosaccharolyticum) and 70°C (C. tartarivorum) required considerably higher temperatures to be inactivated than did mesophile and psychrophile ribosomes which had Tₘₐ values of 57 and 53°C, respectively (Fig. 3B).

Sucrose gradient analysis of the mesophile and psychrophile ribosomes after incubation at 55°C indicated that structural alterations occurred (Table 5). The major changes which occurred to the C. pasteurianum ribosomes was a decrease in both the 70S peak and material sedimenting greater than 70S, with a corresponding increase in the 30S and 50S fractions. In the case of the ribosomes from Clostridium sp. strain 69, aggregation apparently occurred, resulting in an increase in >70S material. A decrease in the 50S peak was also observed. Essentially no changes occurred at 37°C, and in the case of the thermophile profiles no change was observed after 1 hr at 55°C. It should be emphasized that the S values are approximate, and small changes would not have been detected. The gross changes which took place in the mesophile and psychophile at 55°C occurred within 2 to 5 min. Further incubation did not result in more obvious changes. Under the conditions used no ribonuclease activity was observed with the ribosome preparations, indicating that this enzyme is not responsible for the changes seen.

Effect of temperature on S-100 fractions. The effect of heating on the S-100 fractions is shown in Fig. 4. The absolute activity of E. coli ribosomes with the various S-100 preparations ranged from about 2.5 nmoles of 14C-Phe incorporated/mg of ribosomal protein with C. tartarivorum, C. thermosaccharolyticum, and Clostridium sp. strain 69 S-100, to about 4 nmoles with C. pasteurianum and 6 nmoles with E. coli, respectively.

The stability of S-100 preparations at 55°C is shown in Fig. 4A. The thermal inactivation of S-100 from the two thermophiles was rather abrupt, beginning at about 60°C and reaching 50% inactivation at 66°C in both cases. No activity remained above about 75°C (Fig. 4B). Up to the temperature of transition, C. tartarivorum S-100 appeared to be somewhat activated by preincubation. Inactivation of the mesophile and psychophile S-100 fractions was gradual, without the sharp transition of thermophile S-100. The curves of C. pasteurianum and Clostridium sp. strain 69 virtually overlap, with gradual inactivation beginning above 30°C, reaching 50% inactivation at 40°C, but not losing complete activity until the temperature reached about 67°C. The E. coli S-100 had a higher Tₘₐ (53°C), but loss of activity also occurred gradually above 30°C. The higher stability of the thermophile S-100 fractions is consistent with the general finding that obligate thermophiles contain enzymes which are very thermostable.

Effect of heat on the endogenous incorporation by polysomes. Polysomes were obtained from the four species of Clostridium with varying degrees of efficiency. Sucrose gradient profiles of the polysome preparations from the mesophile and psychophile showed the presence of distinct peaks up to hexamers. The profiles of thermophile preparations were not as well delineated.
FIG. 3. Functional stability of ribosomes to heat. Ribosomes (6 mg of protein/ml) were incubated in TMK buffer at the temperatures indicated. After preincubation, equal samples containing 0.2 to 0.3 mg of protein were removed and added to the remaining components of the complete assay mixture, which included 125 µg of poly U, E. coli S-100 (0.78 mg of protein), and ¹⁴C-Phe. The tubes were then incubated at 37 C for 30 min by the standard procedure (Materials and Methods). (1) C. tartarivorum; (2) C. thermosaccharolyticum; (3) C. pasteurianum ATCC 6013; (4) Clostridium sp. strain 69. A, Preincubation at 55 C for the times shown; B, Tₘ values. Ribosomes were preincubated for 5 min at various temperatures.

### TABLE 5. Effect of temperature on ribosomal subunit distribution

<table>
<thead>
<tr>
<th>Organism</th>
<th>Fraction</th>
<th>Percent of total*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No incubation</td>
</tr>
<tr>
<td>Clostridium tartarivorum and C. thermosaccharolyticum</td>
<td>30S</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>50S</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>50-70S</td>
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<tr>
<td></td>
<td>70S</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>&gt;70S</td>
<td>5</td>
</tr>
<tr>
<td>C. pasteurianum ATCC 6013</td>
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<tr>
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</tr>
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<td></td>
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<td></td>
<td>&gt;70S</td>
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<td>Clostridium sp. strain 69</td>
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<tr>
<td></td>
<td>70S</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>&gt;70S</td>
<td>11</td>
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* Values are given as percentage of ultraviolet-absorbing material.

**Ribosomes (6 mg of protein/ml) were incubated in TMK buffer at 55 C after which samples were centrifuged in sucrose gradients as described in Materials and Methods. The times of incubation are 1 hr for C. tartarivorum and C. thermosaccharolyticum, 5 min for C. pasteurianum ATCC 6013, and 2 min for Clostridium sp. strain 69.

However, the extent of protein synthesis by thermophile polysomes was about equal at 55 and 37 C, whereas incorporation at 55 C by mesophile polysomes was only 44%, and by psychrophile polysomes only 39% of that at 37 C. Thus, the latter two systems behave as a typical enzyme reaction, i.e., as the temperature is increased the rate measured over short periods of time increases, but the period of time over which the system is active decreases.

**DISCUSSION**

From the various procedures used here to characterize clostridial ribosomes physically and chemically, only one obvious difference emerges upon comparison of the thermophiles, mesophile, and psychrophile: based on the higher Tₘ values and the greater thermostability of the biological activity, thermophile ribosomes are more heat stable than ribosomes from clostridia which grow at lower temperatures.

The differences in Tₘ values of ribosomes observed here is consistent with results which have been obtained in virtually all studies comparing ribosomes from thermophilic and mesophilic organisms. Since identical buffer systems were used, the Tₘ values of clostridial ribosomes can be compared directly with values obtained in studies of B. stearothermophilus and E. coli ribosomes (30) and several unrelated organisms including thermophiles, mesophiles, and psychrophiles (26). As in the case of clos-
Fig. 4. Functional stability of S-100 to heat. S-100 (15 mg of protein/ml) was preincubated at the temperatures shown. Small portions containing 0.8 to 1.0 mg of protein were removed and added to the remaining components of the incorporation assay which included 100 μg of poly U and 0.84 mg of E. coli ribosomes. (1) Clostridium sp. strain 69; (2) C. pasteurianum ATCC 6013; (3) E. coli; (4) C. thermosaccharolyticum; (5) C. tartarivorum. A, Preincubation at 55 C for the times shown; B, Td, s values. S-100 was preincubated for 5 min at various temperatures.

Fig. 5. Endogenous incorporation of 14C-Val by clostridial polysomes at 37 and 55 C. Rates were determined in the absence of poly U as described in Materials and Methods. E. coli S-100 (0.78 mg of protein) and polysome preparations (0.2–0.6 mg of protein) were included in each reaction mixture.
tridial ribosomes, B. stea rothermophilus and E. coli ribosomes differed in Tm values by about 5 C. The ribosomes from several organisms varied in thermal stability (Tm values ranged from 69 to 79 C) which correlated with the maximal growth temperature (18 to 73 C) of the organism (26). Similar differences between thermophilic and mesophilic ribosomes have also been found under widely varying solvent conditions (4, 15, 20, 41).

Our finding that the Tm value of ribosomes from C. pasteurianum, which grows optimally at 37 C, is not higher than the Tm value of the ribosomes from Clostridium sp. strain 69, which grows at 18 C, is inconsistent with the conclusion of Pace and Campbell (26), whose correlation of ribosome stability with growth temperature included data from psychrophilic organisms. However, the difference in ribosome Tm values between psychrophiles and mesophiles was small (1–3 C) as compared with the differences between mesophiles and thermophiles (5–7 C) and would be difficult to measure accurately. Moreover, there is evidence which indicates that Clostridium sp. strain 69 is really a mesophile which contains a temperature-sensitive enzyme (32).

The differences in the thermostability of the clostralid ribosomes were more pronounced when the biological activity was measured (Fig. 3). The fact that the mesophile and psychrophile ribosomes are inactivated at temperatures significantly lower than their Tm values implies that melting of the ribosomes is not required for the inactivation. Although structural changes in the mesophile and psychrophile ribosomes upon heating at 55 C were noted by sucrose gradient centrifugation (Table 5), these changes probably did not directly cause the loss in activity. For example, the structural changes occurred within 5 and 2 min in the case of C. pasteurianum and Clostridium sp. strain 69, respectively (Table 5). The loss in biological activity was more gradual (Fig. 3). It should be emphasized however that small changes in S values would not have been observed by our techniques.

The effect of heating on C. pasteurianum and Clostridium sp. strain 69 ribosomes was somewhat different from that which Bodley found with E. coli ribosomes under similar conditions (2). After heating E. coli ribosomes in TMK buffer at temperatures ranging from 58 to 66 C, he found that the gradual disappearance of the 70S ribosome peak was accompanied by an increase in 50S and a loss of 30S material. The destruction of the 30S subunit was correlated with the loss of activity in poly U-directed 14C-Phe incorporation. We found that in the case of C. pasteurianum ribosomes the decrease in 70S peak was accompanied by an increase in both the 30S and 50S material. The aggregation of 50S ribosomal subunits from the psychrophile, Clostridium sp. strain 69, after heating in TMK buffer, is similar to the case of E. coli ribosomes (2) heated under slightly different conditions (TM buffer). In the latter case, Bodley also suggested that aggregation accompanied functional destruction (2).

On the basis of the virtually identical melting temperature curves, Tm values, and base composition of the rRNA from the different clostralid ribosomes, it would appear that the nucleic acid component does not contribute to the difference in the thermal stability of the ribosomes. The results of other investigators on this point vary. Comparative studies between B. stea rothermophilus and E. coli indicated that the thermophile rRNA had higher Tm values in most (15, 20, 34), but not all, cases (30). The 23S rRNA, but not 16S rRNA, from Thermus aquaticus had a higher Tm value than its counterpart from E. coli (41). Other results (26) indicate that the Tm values of bacterial rRNA may correlate with the growth temperature of the organisms. In some studies, however, the Tm values of ribosomes and rRNA have not been compared in identical solvents. It is well known that the ionic environment has a pronounced effect on Tm values.

On the basis of spectral differences between adenine-uracil (AU) and guanine-cytosine (GC) base-paired RNA segments observed by Cox (6), Stenesh and Holazo (34) suggested that additional GC base pairing might account for unusually high Tm values of rRNA from thermophilic bacilli. However, the absence of differences in Tm values or GC content of rRNA from thermophilic and mesophilic clostridia, as well as rRNA from other species (30), indicates that this cannot be a universal explanation for thermostability of ribosomes.

Ribosomal protein probably is the important factor in determining the stability of ribosomes. It is well known that, in general, proteins from obligate thermophiles show great thermostability. However, comparison of the amino acid composition and free sulfhydryl groups to total half-cystine content of the ribosomal proteins from the different species failed to show substantial differences. The ribosomal proteins from the clostralid species show quite different electrophoretic mobilities, but there was no obvious relationship to the growth temperature of the organisms.

The effect of the proteins must be to produce
protein-RNA complexes of a more stable conformation. The functional thermostability of hybrid 30S ribosomal subunits, reconstituted from thermophile and mesophile components, has been examined, and although the results indicate that RNA must contribute to thermal stability of thermophile ribosomes, the proteins may have an even greater role (25). Because basic polypeptides are known to stabilize helical regions of rRNA to heat, the lack of additional stability of intact E. coli ribosomes when compared to isolated rRNA has been interpreted to indicate that E. coli ribosomal proteins bind primarily to nonhelical regions (8). Since thermophile ribosomes do exhibit additional stability relative to their rRNA, it is possible that more protein binding to helical regions occurs. The differences in the protein-RNA interaction within the ribosome which might contribute to thermostability must be rather subtle, since differences between thermophile and mesophile or psychrophile ribosomes are not readily apparent in ORD spectra.

The melting curves of DNA from the four clostridial species were found to be similar. The Tm values and %G+C values of the thermophile DNA fell within the range of 30 to 40%, characteristic of clostridial species (9). This finding is in substantial agreement with Stenes et al. (35) who found that DNA from thermophilic bacilli had %G+C values in the upper regions of the range characteristic for this genus, but was not uniquely thermostable when compared with certain other mesophilic species (9). The DNA from the two thermophilic clostridia did appear to have slightly higher Tm and %G+C values than the mesophile and psychrophile; however, other mesophilic clostridia are known which have higher values (9, 38).

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LITERATURE CITED

26. Pace, B., and L. L. Campbell. 1967. Correlation of maximal growth temperature and ribosome heat sta-
IRWIN, AKAGI, AND HIMES

262 J. Bacteriol.