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Characterization and Stability of Ribosomes from Mesophilic and Thermophilic Bacteria

J. STENESH AND C. YANG
Department of Chemistry, Western Michigan University, Kalamazoo, Michigan

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Ribosomes were isolated from three mesophilic and three thermophilic strains of *Bacillus*. The ribosomes consisted of about 55% protein and 45% ribonucleic acid. Average ratios for the absorbance at 260/235 and 260/280 mμ were 1.77 and 1.92 for the mesophiles and 1.63 and 1.84 for the thermophiles. Ultracentrifugation revealed mainly components with sedimentation coefficients of about 30, 50, 70, 100, and 120*S*. All the preparations were shown to contain a ribonuclease which, in the presence of ethylenediaminetetraacetic acid, led to ribosome breakdown as measured by the increase in acid-soluble nucleotides. The stability of the ribosomes from the thermophiles was consistently greater than that of the ribosomes from the mesophiles. After 5 hr at 37 C, the breakdown was about 80% for the ribosomes from the mesophiles and 55 to 70% for those from the thermophiles. At 60 C, the ribosomes from the mesophiles were broken down slightly more and at a faster rate than those from the thermophiles. At temperatures above 60 C, the breakdown was again more pronounced for the ribosomes from the mesophiles.

The ability of thermophiles to grow at relatively high temperatures (about 55 to 80 C) as compared to mesophiles which grow at temperatures of about 20 to 45 C (4) has been ascribed to chemical and physical differences of their macromolecules. Evidence for this theory comes mainly from studies of proteins, especially of purified enzymes (6, 19) and of bacterial flagella (14). Further evidence for uniquely heat-stable macromolecules has been reported in studies of amino acid-activating enzymes (3), a thermophilic bacteriophage (25), and a subcellular protein-synthesizing system from a thermophile (11).

Studies of nucleic acids have been less conclusive in their support of this theory. Thus, the deoxyribonucleic acid (DNA) from thermophilic bacteria and a thermophilic bacteriophage (20, 25), and the soluble ribonucleic acid (RNA) from a thermophile (2, 3), exhibited no unusual heat stability. On the other hand, the ribosomes (3, 18) and the ribosomal RNA (3, 18) from thermophilic strains were more heat-stable when compared to similar components of *Escherichia coli*.

Heretofore, the heat stability of these various macromolecules has usually been assessed by a comparison of thermophilic strains of *Bacillus* with mesophilic strains of *E. coli*. Since these organisms represent different genera, the possibility of intergeneric differences cannot be ruled out and may have affected the results quoted above. With this in mind, we decided to examine the stability of ribosomes in a comparative study of mesophilic and thermophilic strains from one genus, namely *Bacillus*. Since ribosomes from some of the strains used here have not been isolated previously, a characterization of all the preparations is given first. This is followed by an assessment of ribosome stability through measurements of the breakdown of the ribosomal RNA due to the action of a ribonuclease which is associated with the ribosomes.

MATERIALS AND METHODS

Medium and growth conditions. Three mesophilic and three thermophilic strains of the genus *Bacillus* were used. The former included *B. pumilus* (NRS 236), *B. licheniformis* (NRS 243), and an unclassified *Bacillus* sp. (X1). The thermophiles were strains of *B. stearothermophilus* (FJW, 10, 2184).

The cells were grown in large petri dishes on a solid medium consisting of 1% Trypticase (BBL), 0.2% yeast extract (Difco), and 2% agar (Difco). The cells were harvested in the logarithmic phase (approximately 6 hr at 37 C for the mesophiles and 5 hr at 55 to 60 C for the thermophiles).

Isolation of the ribosomes. A buffer consisting of 0.01 M tris(hydroxymethyl)aminomethane (Tris) and 0.01 M magnesium acetate (*p*H 7.4) was used throughout.

The cells were harvested with buffer and collected by centrifugation for 20 min at 12,000 × *g* in a Sorvall (model RC-2) refrigerated centrifuge. The cells were ground with three parts by weight of alu-

mina (Merck 71707) and extracted with eight volumes of buffer; deoxyribonuclease (Worthington Biochemical Corp., Freehold, N.J.; electrophoretically purified; final concentration, 1 μg/ml) was added to the mixture. Cell debris and alumina were removed by centrifugation for 15 min at 12,000 $\times$ g in the Sorvall, and the supernatant fluid was centrifuged in a Spinco (model L) ultracentrifuge for 65 min at 105,000 $\times$ g or for 90 min at 78,500 $\times$ g to sediment the ribosomes. The ribosomes were purified by cycles of low- and high-speed centrifugation (20 min at 12,000 $\times$ g followed by 65 min at 105,000 $\times$ g and resuspension of the pellet). After two such cycles, the solution was centrifuged for 20 min at 12,000 $\times$ g to give the final preparation.

Analytical methods. For chemical analyses, the ribosome solution was treated with three volumes of cold 10% trichloroacetic acid and the precipitate was collected by centrifugation. RNA was separated from the protein by the method of Schneider (27), by heating the precipitate for 15 min at 90 C in three volumes of 5% trichloroacetic acid. The residual protein was washed once with three volumes of 5% trichloroacetic acid, and the combined acid extracts were used for RNA and DNA determinations. The protein precipitate was washed once with three volumes of cold ethyl alcohol and redissolved in 0.017 N NaOH. Protein was assayed in this solution by the method of Lowry et al. (16), with bovine serum albumin as standard, and by a nitrogen determination with a nesslerization procedure (J. S. Lovett, *personal communication*).

RNA was determined by ultraviolet absorption (10) and by phosphorus analysis (15). DNA was determined according to the method of Dische (8).

All spectrophotometric measurements were done in a Zeiss (model PMQ-II) spectrophotometer.

Ultracentrifugation. All ultracentrifugal analyses were conducted in a Spinco (model E) analytical ultracentrifuge, equipped with rotor temperature indicator control (4 to 8 C) and with schlieren optics. Sedimentation coefficients were measured with a Nikon (model 6C) microcomparator and were corrected to 20 C for the viscosity of water. They are expressed in Svedberg units (S).

Ribosome breakdown. The basic incubation mixture included 1 ml of a solution containing ribosomes (absorbance of 5 at 260 mμ), 10 μmoles of Tris (pH 7.4), and 10 μmoles of magnesium acetate. Other components were added as specified in the figures. The total volume was 2.5 ml. Tightly closed tubes were incubated at a given temperature for various lengths of time. The reaction in each tube was stopped by the addition of 2.5 ml of 5% (v/v) perchloric acid (Mallinckrodt). After 30 min in ice, the solution was centrifuged for 10 min at 10,000 $\times$ g in the Sorvall. The supernatant fluid was collected, and its absorbance was measured at 260 mμ versus appropriate zero-time controls.

RESULTS AND DISCUSSION

Characterization of the ribosomes. The chemical composition of the ribosomes is shown in Table 1. Protein determinations by the Lowry et al. method (16) and RNA determinations by phosphorus analysis (15) yielded comparable results. The proportions of protein and RNA are similar to those reported for the ribosomes from *B. cereus* (13) and *B. stearothermophilus* B (18). No DNA was detected in any of the preparations (i.e., DNA contamination, if any, was less than 0.5%). The ultraviolet absorption spectra are shown in Fig. 1. All the data have been corrected for Rayleigh scattering by measuring the scattering as a function of wavelength and extrapolating a double logarithmic plot into the region of consumptive absorption (5, 26).

TABLE 1. *Chemical composition of the ribosomes*[a]

Organism	RNA	Protein
	%	%
Bacillus pumilus	41	59
Bacillus sp.	45	55
B. licheniformis	48	52
B. stearothermophilus FJW	46	54
B. stearothermophilus 10	43	57
B. stearothermophilus 2184	45	55

[a] Protein is based on nitrogen determinations, and the RNA is calculated from the absorbance at 260 and 290 mμ. All the data are averages of duplicate determinations performed on two or three different ribosomal preparations. The percentages are based on the combined weight of protein and RNA. The average values for the three mesophiles were: RNA, 45%; protein, 55%. The average values for the three thermophiles were also 45 and 55%, respectively.

It is seen from Fig. 1 that the spectra are essentially identical, with some variation at lower wavelengths. The minima of the curves were at 235 mμ and the maxima were at 258 to 260 mμ for all the preparations. The average ratios for the absorbance at 260/235 and 260/280 mμ were 1.77 and 1.92 for the mesophiles and 1.63 and 1.84 for the thermophiles. These values are similar to those found for other ribosomes from *Bacillus* (13, 18).

The ultracentrifuge patterns of the ribosomes are shown in Fig. 2. The components had sedimentation coefficients of approximately 30, 50, 70, and 100S, which are similar to those reported for other ribosomes from *Bacillus* (18, 23, 24). In all cases, the monomers (about 70S), or larger ribosomes, were predominant. The sedimenting components dissociated and aggregated as a function of magnesium ion concentration in a manner that is typical of ribosomes in general.

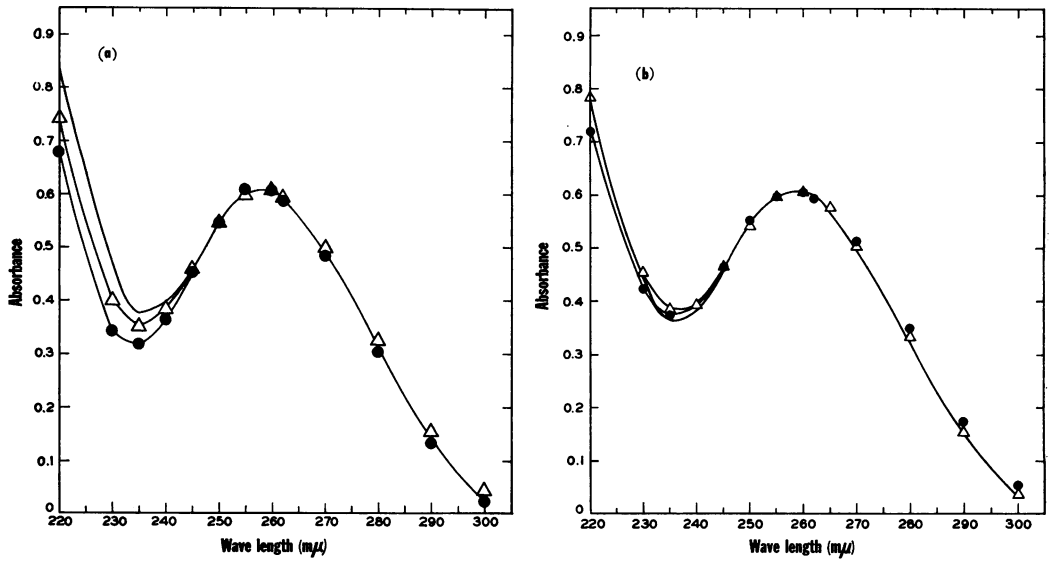

FIG. 1. *Absorption spectra of the ribosomes. All curves have been normalized to give the same absorbance at 260 mμ. (a) Mesophilic strains. (—) Bacillus licheniformis; (●) Bacillus sp.; (△) B. pumilus. (b) Thermophilic strains. (—) B. stearothermophilus 10; (●) B. stearothermophilus FJW; (△) B. stearothermophilus 2184.*

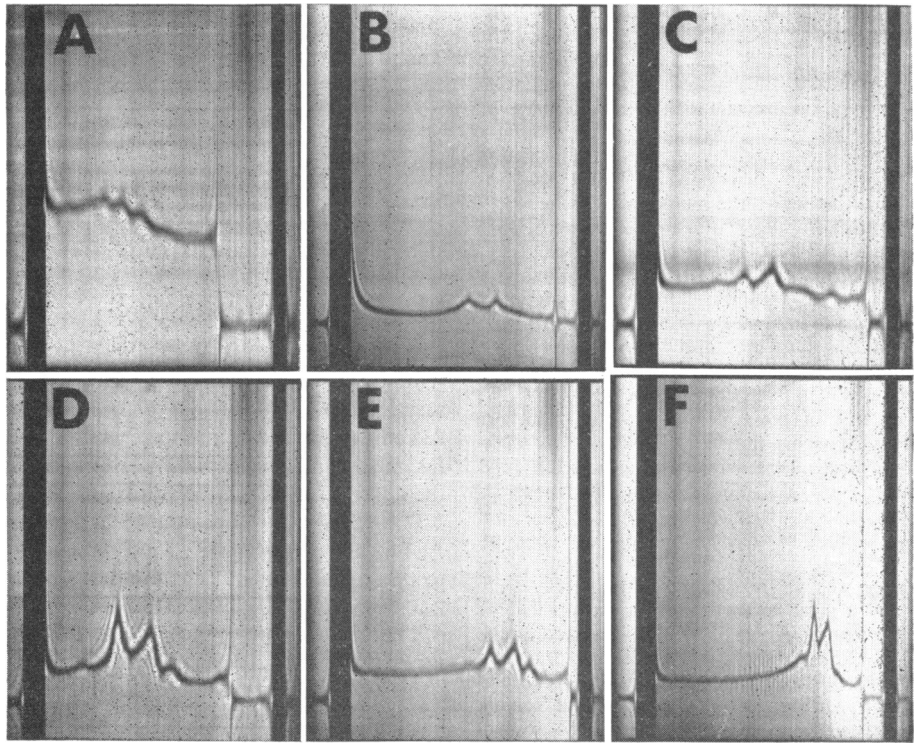

FIG. 2. *Ultracentrifuge patterns of the ribosomes. Sedimentation is from right to left. Centrifugations were at 29,500 rev/min (15,220 rev/min for Bacillus sp. and 23,150 rev/min for B. stearothermophilus FJW). Concentration, 5 to 10 mg/ml. Numbers in parentheses are sedimentation coefficients. Mesophilic strains: (A) B. pumilus (72, 93, 102); (B) Bacillus sp. (127, 194); (C) B. licheniformis (27, 63, 75, 97, 113). Thermophilic strains: (D) B. stearothermophilus FJW (47, 64, 90, 119); (E) B. stearothermophilus 10 (49, 66, 97); (F) B. stearothermophilus 2184 (55, 68, 94, 108).*

Thus, for example, the 70*S* and 100*S* ribosomes of *B. licheniformis* and of *B. stearothermophilus* FJW, in 0.01 or 0.005 M Tris (*p*H 7.4), were dissociated into 30*S* and 50*S* particles when the magnesium ion concentration was lowered from 0.005 to 0.001 M. These particles reassociated when the magnesium ion concentration was raised to 0.005 M.

A functional test was performed on the ribosomes by measuring the incorporation of ^{14}C-labeled phenylalanine in a cell-free amino acid-incorporating system (22). The ribosomes were active under these conditions.

Ribosome breakdown. All the ribosome preparations were shown to contain a ribonuclease activity similar to the one found in association with many other ribosomes (9, 23). This enzyme, in the presence of ethylenediaminetetraacetic acid (EDTA), led to a breakdown of the ribosomes as judged by the increase in acid-soluble nucleotides. Figure 3 shows the results obtained upon incubation of the ribosomes at 37 C. The percentage of ribosome degradation was calculated from the absorbance (at 260 mμ) of the ribosomal RNA. There was always an initial rapid degradation, after which the rate of breakdown for the thermophiles was slower than that for the mesophiles. At the end of 5 hr, the extent of breakdown was about 55 to 70% for ribosomes from the thermophiles and about 80% for ribosomes from the mesophiles. Similar results have been reported for the ribosomes from *E. coli* (30).

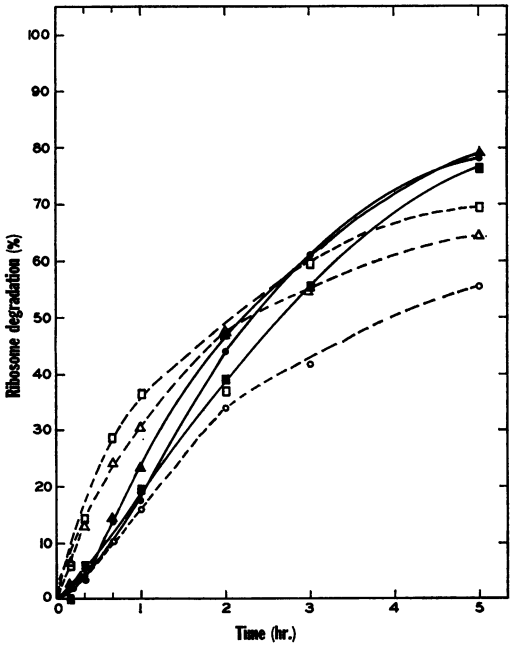

FIG. 3. *Ribosome breakdown at 37 C. Basic incubation mixtures plus 25 μmoles of EDTA (pH 7.4). Mesophilic strains:* (■) *B. pumilus;* (▲) *Bacillus sp.;* (●) *B. licheniformis. Thermophilic strains:* (□) *B. stearothermophilus 2184;* (△) *B. stearothermophilus 10;* (○) *B. stearothermophilus FJW.*

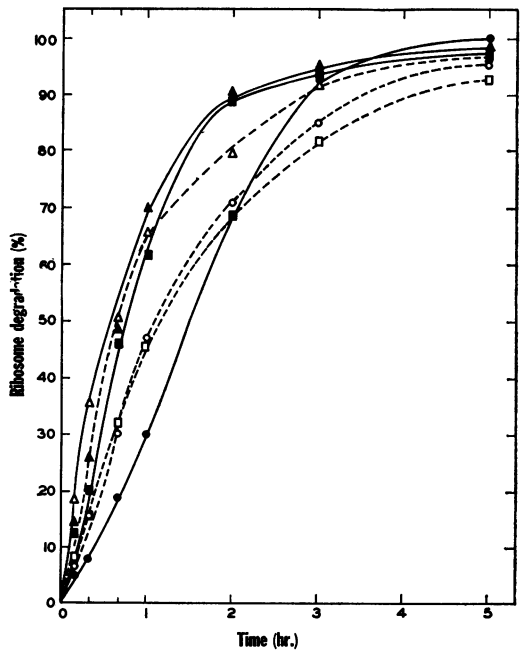

FIG. 4. *Ribosome breakdown at 60 C. Basic incubation mixtures plus 25 μmoles of EDTA (pH 7.4). Mesophilic strains:* (■) *B. pumilus;* (▲) *Bacillus sp.;* (●) *B. licheniformis. Thermophilic strains:* (□) *B. stearothermophilus 2184;* (△) *B. stearothermophilus 10;* (○) *B. stearothermophilus FJW.*

In Fig. 4 are shown comparable experiments to those in Fig. 3, but conducted at 60 C. Again, after a rapid initial degradation, ribosome breakdown proceeded at a slower rate for the thermophiles than for the mesophiles. At the end of 5 hr, the ribosomes from the thermophiles were degraded to about 95%, whereas those from the mesophiles were completely degraded. Control experiments, conducted in the absence of EDTA, gave the following 1-hr and 5-hr average breakdown values: for the mesophiles, 2 and 7% at 37 C, 6 and 18% at 60 C; for the thermophiles, 1 and 6% at 37 C, 1 and 14% at 60 C.

It can be seen (Fig. 3 and 4) that the ribosomes from the thermophiles exhibited a somewhat greater stability than those from the mesophiles at both 37 and 60 C. To determine whether this difference in stability might be more pronounced at temperatures exceeding the maximal growth temperatures (about 55 C) of these mesophiles

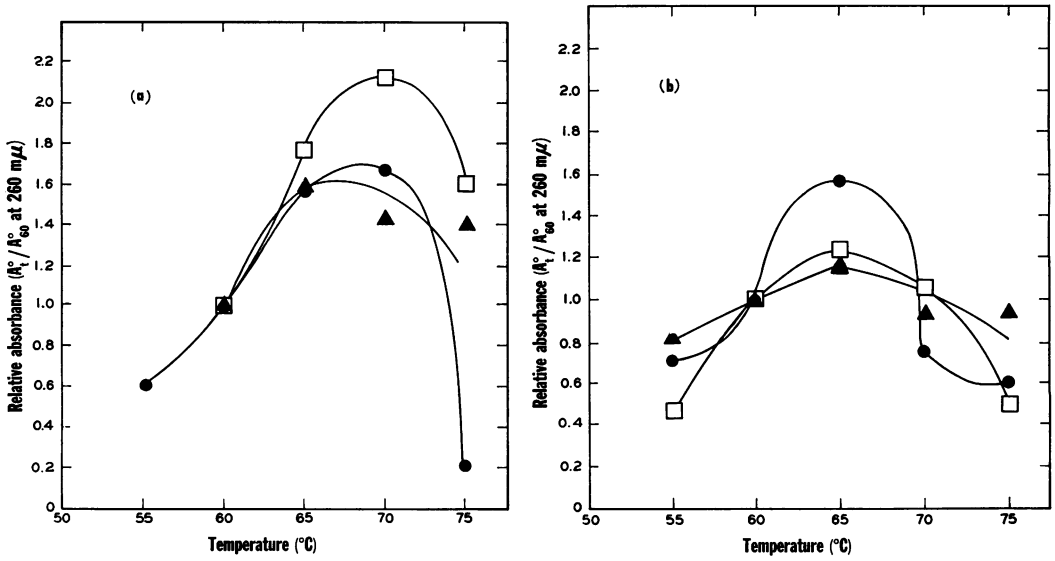

FIG. 5. *Ribosome breakdown at various temperatures. Basic incubation mixtures plus 30 μmoles of EDTA (pH 7.4) were incubated at various temperatures for 1 hr. (a) Mesophilic strains: (▲) B. pumilus; (□) Bacillus sp.; (●) B. licheniformis. (b) Thermophilic strains: (▲) B. stearothermophilus FJW; (□) B. stearothermophilus 10; (●) B. stearothermophilus 2184.*

(Stenesh and Koffler, Federation Proc. **21**:406, 1962), the reaction was measured over a range of temperatures from 55 to 75 C (Fig. 5). The results show that the ribosomes from the mesophiles are, in fact, less stable at temperatures above 60 C than are those from the thermophiles. This refers to both the relative breakdown as depicted in Fig. 5 and to the absolute extent of degradation. These findings support the theory that thermophily is based upon chemical-physical differences on the molecular level.

To evaluate properly the relative stability of the ribosomes, it was important to establish that the ribosome breakdown was due to the same enzyme in all instances. For this purpose, the ribosomes were incubated in buffer with the addition of EDTA, EDTA and phosphate, or EDTA and arsenate (Fig. 6). The shapes of the curves and the magnitudes of the changes observed are very similar. The ribosome degradation in the absence of EDTA is slow, in part because of the inhibition of the enzyme by the magnesium in the buffer (1, 17). In common with other ribosomal ribonucleases (7, 29), this inhibition was removed by chelation of the magnesium with EDTA. The addition of either phosphate or arsenate led to a pronounced activation of the enzyme, with a resultant linear rate of ribosome breakdown. The activation by either phosphate or arsenate was essentially identical, as reported also for rat liver microsomes (21).

These data provide good evidence that the ribosome breakdown was due to the action of a ribonuclease. This conclusion is based on the fact that other enzymes involved in RNA breakdown cannot account for the results obtained here. Phosphodiesterases are inhibited by arsenate and EDTA but are activated by phosphate (21, 28); the potassium-activated phosphodiesterase found in association with *E. coli* ribosomes is inactivated by EDTA (28); and polynucleotide phosphorylase requires magnesium for activity (12, 30). Ribonucleases, on the other hand, are inhibited by magnesium, but are activated by EDTA, arsenate, and phosphate (21).

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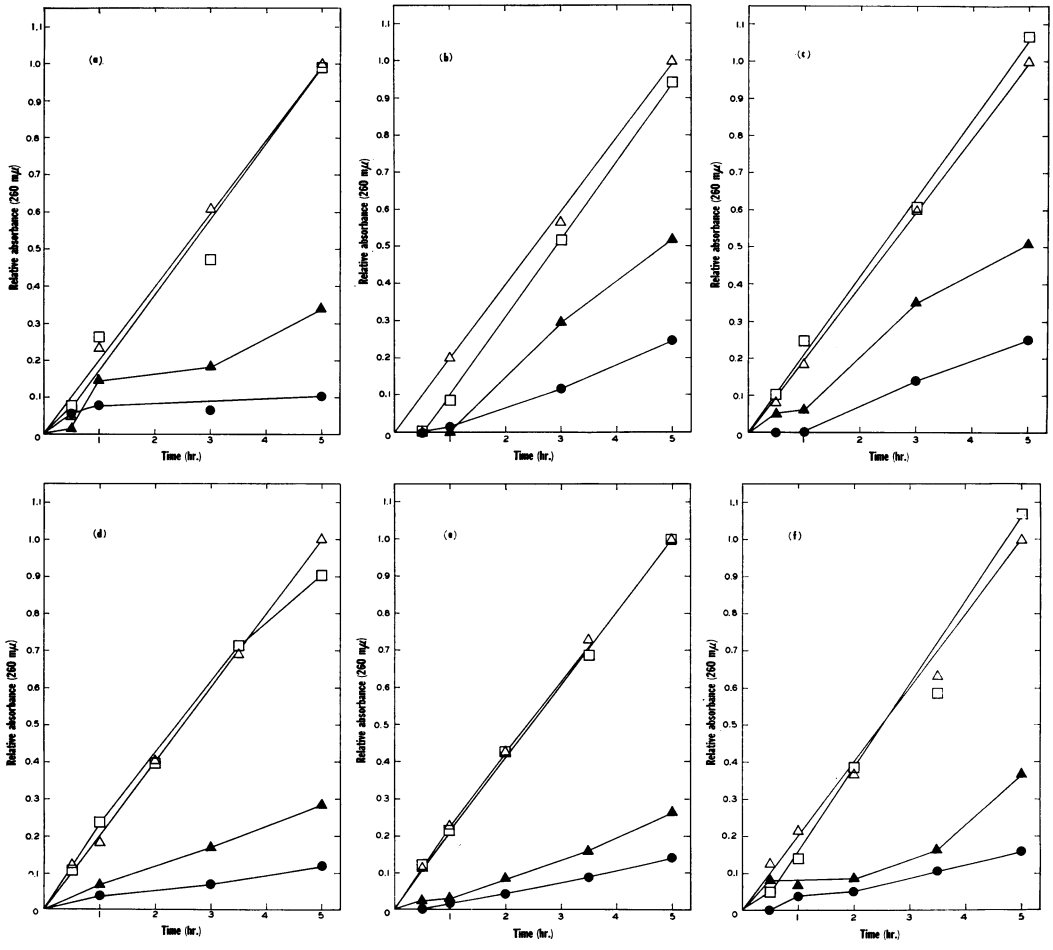

FIG. 6. *Activators and inhibitors of the ribonuclease. Basic incubation mixtures plus one of the following:* (●) *no additions;* (▲) *30 μmoles of EDTA (pH 7.4);* (□) *30 μmoles of EDTA (pH 7.4) and 25 μmoles of* Na_2HAsO_4*;* (△) *30 μmoles of EDTA (pH 7.4) and 25 μmoles of* Na_2HPO_4*. Incubation at 45 C. The relative absorbance is the absorbance of the sample divided by the absorbance (after 5 hr) of the solution containing EDTA and* Na_2HPO_4*. Mesophilic strains: (a) B. pumilus; (b) Bacillus sp.; (c) B. licheniformis. Thermophilic strains: (d) B. stearothermophilus FJW; (e) B. stearothermophilus 10; (f) B. stearothermophilus 2184.*

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