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The Effect of Puromycin on Protein Synthesis of Cell Free Systems from Mesophilic and Thermophilic Bacteria

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THE EFFECT OF PUROMYCIN ON PROTEIN SYNTHESIS OF CELL FREE SYSTEMS FROM MESOPHILIC AND THERMOPHILIC BACTERIA

by

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The author also wishes to dedicate this thesis to his beloved parents, Mr. & Mrs. T. C. Shen.

Philip Y. Shen

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INTRODUCTION

Mesophiles and Thermophiles

Certain organisms have been known to live at temperatures at which cellular constituents are ordinarily destroyed. These organisms, called thermophiles, live at much higher temperatures (about 55-80 C) than mesophiles which live at more moderate temperatures (about 20-45 C). In 1879, the first thermophilic bacterium was isolated by Miquel (1). Since this time much work has been done in studying such bacteria, isolated from many sources in nature. Attempts to explain the phenomenon of thermophily have resulted in widespread investigation during the past two decades.

Three major theories have been advanced to account for the ability of thermophiles to proliferate at high temperatures. The first of these theories, proposed by Gaughran (1), considers thermophily to be due to the protective action of lipids. The second theory, developed by Allen (2), views thermophily as a phenomenon characterized by rapid rates of synthesis and degradation. The third theory ascribes thermophily to physical-chemical differences between the macromolecules of the thermophiles and those of the mesophiles.

The latter theory has received the most support thus far. The evidence comes largely from recent comparative studies of proteins and ribonucleic acids (RNA) from mesophilic and thermophilic bacteria. Unusual thermal stability of cytoplasmic proteins from

thermophilic organisms has been reported by Koffler (3). The α- amylase isolated from Bacillus coagulans grown at 55 C showed greater thermal stability than the same enzyme isolated from the same organisms grown at 37 C (4). A marked difference in the heat stability of flagella from thermophilic and mesophilic bacteria has been demonstrated by Stenesh and Koffler (5). Stenesh and Holazo (6) showed that the ribosomal RNA from thermophilic strains of Bacillus was more heat stable and had a higher guanine plus cytosine (G + C) content than that from mesophilic strains of Bacillus. The ribosomes of the above strains of Bacillus differed likewise in their heat stability; Stenesh and Yang reported that the ribosomes from the thermophilic strains were more stable than the ribosomes from the mesophilic ones (7). Recently, Stenesh, Roe and Snyder (8) reported that the (G + C) content was also higher in the deoxyribonucleic acid (DNA) from thermophilic strains of Bacillus than in the DNA from mesophilic strains of the same genus. The DNA of the thermophiles was also more heat stable than that of the mesophiles. These comparative studies of mesophilic and thermophilic strains of Bacillus aimed at ruling out the possibility of intergeneric differences that are not related to the property of macromolecular thermostability.

Cell free amino acid incorporating systems from thermophilic bacteria have been investigated quite extensively. However, most studies have been limited to a comparison of protein synthesizing systems from mesophilic strains of Escherichia coli with thermophilic strains of Bacillus (9, 10). In our laboratory, we have

recently made comparative studies of cell free amino acid incoporating systems from the mesophile B. licheniformis (NRS 243) and the thermophile B. stearothermophilus 10 (11). These studies showed that the thermophilic system was more active and was more heat stable. To further compare the two systems an antibiotic, puromycin, is employed in the present investigation because of its usefulness in studying specific mechanisms in protein synthesis.

Puromycin and Protein Synthesis

Puromycin (PM) is an antibiotic which is highly toxic to mammalian cells. It is isolated from culture filtrates of Streptomyces alboniger (12) and has an unusually broad concentration range of growth-inhibitory activity. It is a substituted aminonucleoside, made up of three components (Figure 1): 6-dimethylaminopurine (dimethyladenine), 3-deoxy-3-amino-D-ribose, and p-methoxy-L-phenylalanine. The complete structure has been established both by degradative studies and by total synthesis and is as follows: 6-dimethylamino-9-(3'-deoxy-3'-p-methoxy-L-phenylalaninamido-α-D-ribofuranosyl)-purine (13, 14).

The effect of puromycin on the synthesis of macromolecules has been studied in intact bacteria, in mammalian cells and in whole animals. Creaser (15), in a survey of several antibiotics, first observed the inhibition by puromycin of induced enzyme synthesis in bacteria. The specific susceptibility of bacterial protein synthesis to puromycin was shown by measurements of protein synthesis, and of RNA and DNA synthesis. It was found

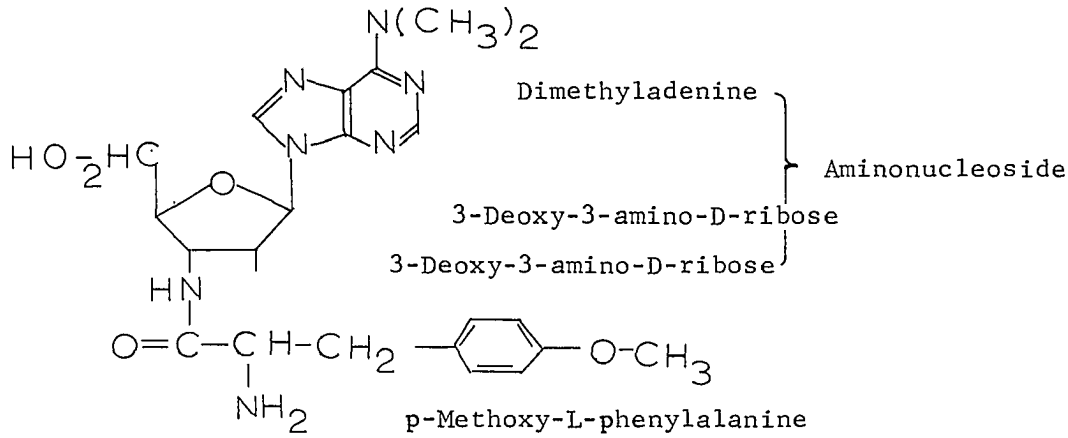


Figure 1. Structure of Puromycin

that protein synthesis is inhibited immediately, while DNA and RNA synthesis continue for a time at normal or near-normal rates. This pattern of macromolecular synthesis has come to be recognized as characteristic of the inhibition of protein synthesis at a step subsequent to the formation of aminoacyl-soluble RNA (sRNA) (16). It seems that the effect of puromycin on DNA and RNA synthesis is secondary to the inhibition of protein synthesis.

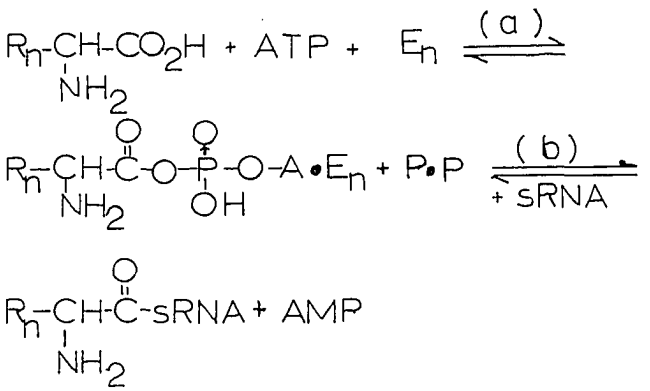
After studies with intact cells showed that inhibition of protein synthesis was the principal effect of puromycin, extensive investigations were carried out on the effect of puromycin in cell-free systems engaged in protein synthesis. Before presenting these results, however, it is necessary to review briefly what is now known about the process of protein synthesis.

The synthesis of proteins begins with the activation of amino acids by adenosine-triphosphate (ATP) and a specific enzyme for each amino acid (aminoacyl-sRNA synthetase). The immediate product of this reaction is an enzyme-bound aminoacyl-

adenylate (Figure 2, reaction 1a). The same enzymes have binding sites for specific sRNA's, to which the activated aminoacyl residue is transferred, resulting in an aminoacyl-sRNA in which the amino acid is esterified to the 2' or 3' hydroxyl of the terminal adenosine of sRNA (reaction 1b). Aminoacyl-sRNA retains the activated aminoacyl group and serves as the adaptor for positioning the amino acid in the polypeptide chain in accordance with the sequence of codons in messenger RNA (mRNA).

The next step of protein synthesis, in which the aminoacyl residue is inserted into peptide linkage, is more complex and is not as well understood. As shown diagrammatically in Figure 2 (reaction 2a), a molecule of aminoacyl-sRNA becomes bound to a ribosome-mRNA complex by hydrogen bonding with the appropriate codon and attaching to a site on the ribosome (site 1, the acceptor site), adjacent to the sRNA to which the growing peptide chain is attached (site 2, the donor site). This carboxyl-activated peptide is then transferred to the amino group of the aminoacyl-sRNA, with displacement of the sRNA from site 2 (reaction 2b). As a result of this reaction the polypeptide is lengthened by one residue and the peptidyl-sRNA now occupies the acceptor site. Movement of the ribosome with respect to the peptidyl-sRNA-mRNA unit returns the peptidyl-sRNA to the donor site (reaction 2c) in preparation for the next incoming aminoacyl-sRNA and repetition of the reaction sequence. At least two enzymes are involved in the overall reaction, one of which probably catalyses peptide-bond formation (reaction 2b) and the other, the movement of the ribosome

Reaction 1



Reaction 2

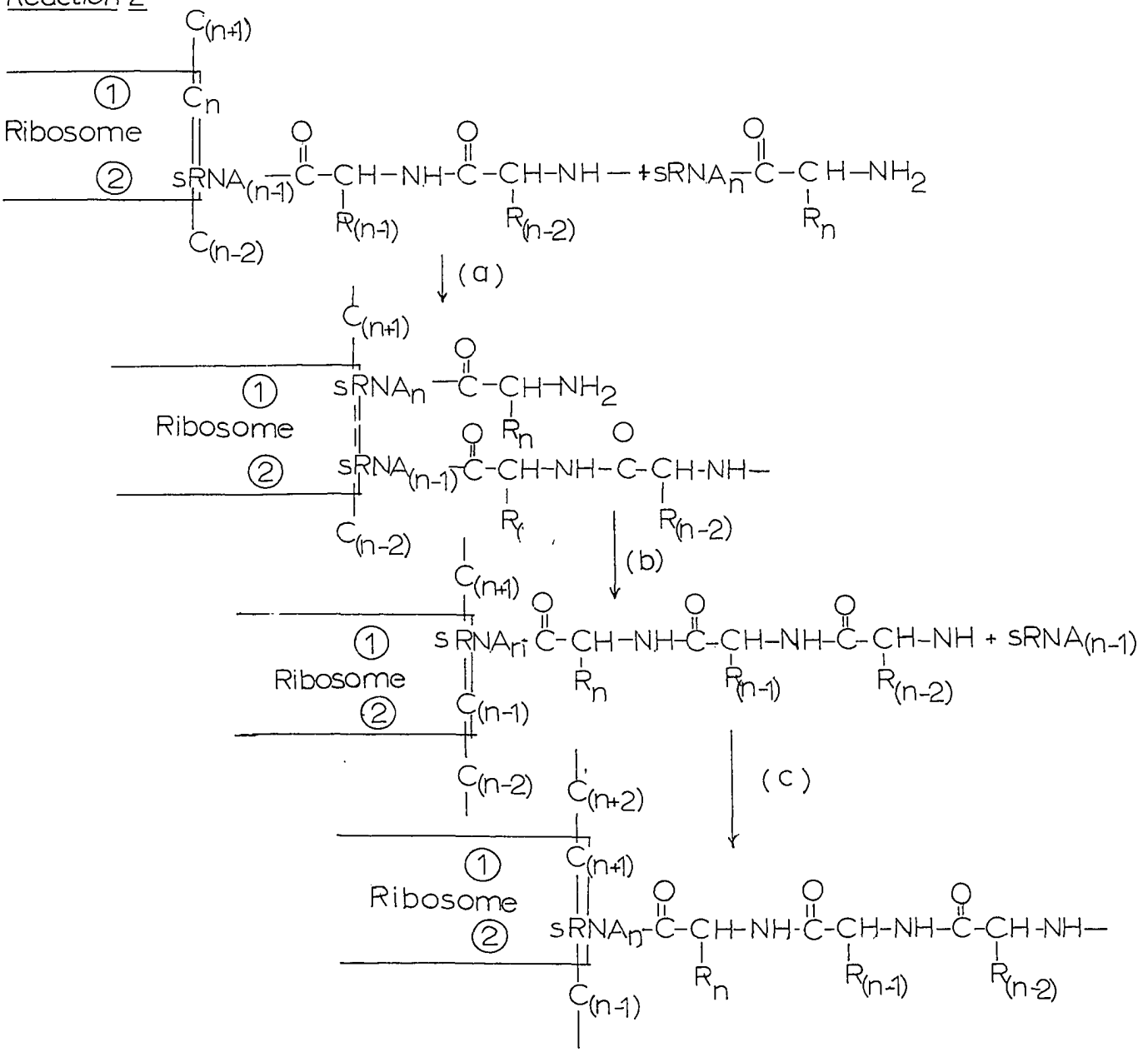


Figure 2. Reactions of Protein Synthesis (17).
[(-C-C-C) sequence of codons in messenger-RNA;
(1) acceptor, (2) donor site]

(reaction 2c). In addition, guanosine-triphosphate (GTP) is required, and it has been suggested that this nucleotide triphosphate is needed for the movement of the ribosome. Although in Figure 2 only the ribosome is shown attached to mRNA, in the cell many ribosomes function simultaneously with the same messenger molecule, the aggregate being termed a polysome.

On examining the structures of the various molecules involved in the protein biosynthetic pathway, Yarmolinsky and De La Haba (18) noted the similarity between the 3' end of aminoacyl-sRNA and puromycin (Figure 3).

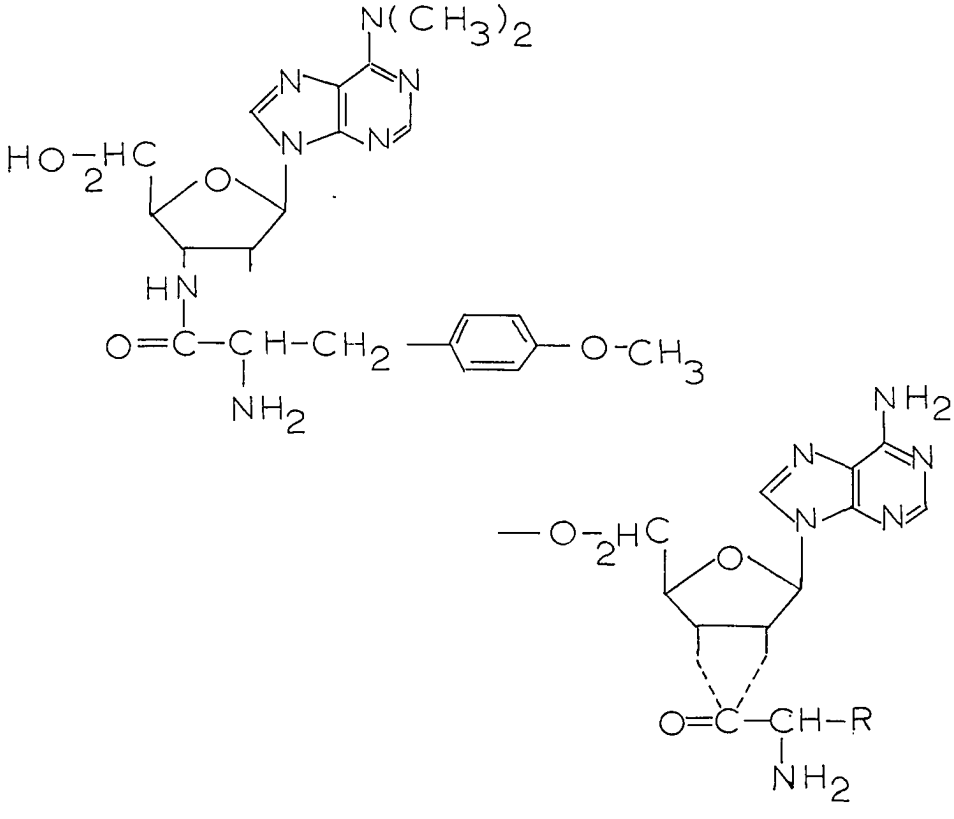


Figure 3. Comparison of Puromycin (above) and the Aminoacyl-adenosine End of sRNA (below)

In each case there is a D-ribosyl (or aminoribosyl) group glycosidically linked in the α configuration to the position 9 of adenine (or an adenine derivative) and substituted in the 2' or 3' position with an amino acid. Puromycin has a 3' amino acid substituent, whereas aminoacyl-sRNA is thought to be an equilibrium mixture of the 2' and 3' aminoacyl esters (19, 20). The nature of the amino acid linkage to the nucleotide is thus different in the two cases. In aminoacyl-sRNA the linkage is an ester bond of special lability owing to the vicinal hydroxyl, whereas in puromycin the amino acid is linked by the more stable amide bond to the 3' amino group of the amino-ribose moiety. On the basis of the similarity in structure, Yarmolinsky and De La Haba proposed that puromycin acts as an analogue of aminoacyl-sRNA, and this has been the guiding hypothesis for most subsequent studies of its mode of action. As a structural analogue of aminoacyl-sRNA, puromycin accepts growing peptide chains intended for transfer to the next aminoacyl-sRNA, thus terminating further extension of the polypeptide. Using the polyuridylate-directed synthesis of polyphenylalanine in E. coli extracts, Gilbert (21) observed the accumulation of polyphenylalanyl-sRNA attached to 50S ribosomes. In the presence of puromycin and a soluble cell fraction, polyphenylalanine chains no longer linked to sRNA were released from ribosomes. This finding indicates that puromycin leads to cleavage of the peptidyl-sRNA intermediate of protein synthesis, thus releasing incomplete protein chains. Allen and Zamecnik also observed that rabbit reticulocyte ribosomes, which

contain incomplete globin peptides, when incubated with puromycin (labelled in the amino acid portion), release peptides which are radioactive. This indicates that all or part of the puromycin is linked to the released peptides.

Puromycin was used in the present study for several reasons. Because of its antitumor activity, it was of interest to compare its effect on the protein synthesizing system of a mesophile and a thermophile since both a cancer cell and a thermophile exhibit a degree of abnormality in protein synthesis. Furthermore, since the mode of action of puromycin is fairly well understood, the use of this compound allows for a direct comparison between mesophiles and thermophiles with respect to certain steps in protein synthesis. In this way, such studies may contribute to our understanding of protein synthesis in general and to our understanding of the phenomenon of thermophily in particular.

MATERIALS AND METHODS

Apparatus

The following is a list of apparatus used in this study:

(a) centrifuges : low speed - Sorvall model RC-2 refrigerated centrifuge
high speed - Spinco model L refrigerated preparative ultracentrifuge

(b) fermentor : New Brunswick Scientific Co., model MF-128S Microferm fermentor

(c) French press : Aminco model 5-590

(d) Homogenizers : Potter-Elvejehm homogenizers

(e) microliter pipettes : Eppendorf microliter pipettes, Brinkman

(f) Millipore filters : HA Millipore filter, 25 mm in diameter, o.45 u pore size, Millipore Corporation

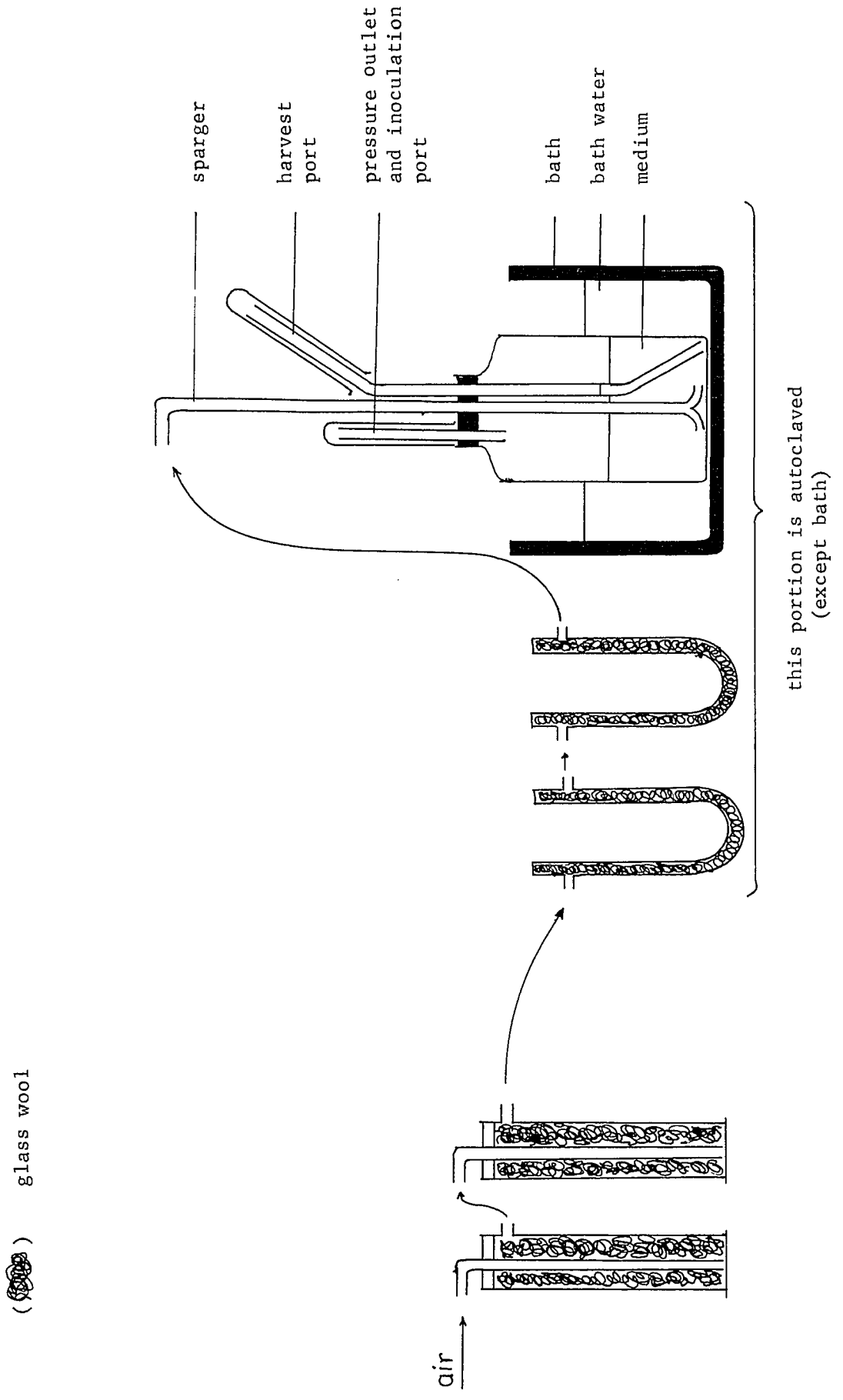
(g) nitrogen tank : Linde model LD-10

(h) planchette counter : Nuclear Chicago model 4338 thin-window gas-flow automatic planchette counter

(i) seedflask : see Figure 4

(j) spectrophotometer : Fermentation use - Bausch & Lomb Spectronic 20
Protein determination - Zeiss model PMQ-II

Figure 4. Design of Seedflask



Chemicals

The following is a list of Chemicals used:

(1) ammonium chloride - Allied Chemical

(2) adenosine-5'-triphosphate (ATP, disodium salt) - Sigma

(3) agar - Difco

(4) antifoam - Union Carbide 471

(5) bovine serum albumin - Sigma

(6) calcium chloride - Baker Chemical

(7) deoxyribonuclease (DNase) - Worthington Biochemicals

(8) ferric chloride - Fisher Scientific

(9) guanosine-5'-triphosphate (GTP, disodium salt) - Schwarz Bioresearch

(10) magnesium acetate - Matheson Coleman & Bell

(11) magnesium sulfate - Mallinckrodt Chemical

(12) manganese sulfate (monohydrate) - Mallinckrodt Chemical

(13) 2-mercaptoethanol - Eastman Organic Chemicals

(14) phenol reagent - Fisher Scientific

(15) phenylalanine-C^{14} (UL) - International Chemical & Nuclear Co. (10 mc/mM, in 0.1 N HCl)

(16) phospho(enol)pyruvate (PEP, tricyclohexylamine salt converted to free acid form by Nirenberg's method (22)) - Sigma

(17) phospho(enol)pyruvate kinase (PEP kinase, 8 mg/ml of a suspension in ammonium sulfate) - Sigma

(18) polyuridylic acid (poly U) - Miles

(19) potassium dihydrogen phosphate - Baker Chemical

(20) puromycin dihydrochloride (M.W. 544.15) - Nutritional Biochemicals

(21) sodium chloride - Mallinckrodt Chemical

(22) soluble-ribonucleic acid (sRNA, B. subtilis) - General Biochemicals

(23) spermidine trihydrochloride - Nutritional Biochemicals

(24) trichloroacetic acid (TCA) - Fisher Scientific

(25) tris(hydroxymethyl)aminomethane (Tris) - Sigma

(26) trypticase - Baltimore Biological Laboratory

(27) yeast extract - Difco

Reagents

Buffer A consisted of 0.01 M tris, 0.01 magnesium acetate, and 0.06 M ammonium chloride. It was adjusted to pH 7.4 with concentrated hydrochloric acid. Buffer B was identical to buffer A except that it was also made 0.006 M in 2-mercaptoethanol just prior to use. Buffer C was identical to buffer B except that it was also 0.006 M in spermidine trihydrochloride.

Mix I contained tris buffer (0.967 M at pH 7.4), ATP (9.603×10^{-3} M), GTP (2.882×10^{-4} M), and spermidine trihydrochloride (0.033 M). It was stored in small aliquots at -20 C.

Mix II was made prior to each experiment. It was a mixture of six solutions in the following ratio: 250 ul of Mix I; 1 ul of 2-mercaptoethanol; 250 ul of PEP; 5 ul of PEP kinase suspension; 477 ul of cold doubly distilled water; and 25 ul of phenylalanine-C^{14} solution.

Organisms and Growth Conditions

Cells of B. licheniformis (NRS 243) and B. stearothermophilus 10 were first grown on slants containing a medium of 2% agar, 1% trypticase, and 0.2% yeast extract. Slants which contained in addition to the above 10 ppm of manganese sulfate were used for growing the stock cultures. Slants were incubated from stock cultures and were grown to the logarithmic phase; the mesophilic B. licheniformis was grown at 37 C and the thermophilic B. stearothermophilus 10 was grown at 55 C. The growth was then suspended in about 6 ml of sterile water and inoculated into a 4-liter seedflask containing 2 liters of sterile medium. A total of approximately 18 ml of inoculum was used for one seedflask.

The seedflask (Figure 4) was designed to allow abundant passage of filtered air through the medium, causing vigorous stirring. Positive pressure was maintained constantly in the seedflask to lessen the possibility of outside contamination from entering the seedflask. The seedflask containing the mesophile or the thermophile was incubated in a 37 or 55 C water bath, respectively.

After about four hours of incubation (absorbance of approximately 0.5 at 540 mu), the contents of the seedflask were inoculated into a Microferm fermentor containing 22 liters of sterile medium at 37 or 55 C.

Several different media were tested for use in the seedflask and the fermentor. These included: (1) 1% trypticase and 0.2%

yeast extract at pH 6.9; (2) 2% trypticase and 0.5% yeast extract at pH 6.9; (3) 2% trypticase, 0.5% yeast extract, and 0.5% KH_2PO_4 at pH 6.3; (4) 2% trypticase, 0.5% yeast extract, 0.5% NaCl, 0.15% $CaCl_2$, 0.07% $FeCl_3$ and 0.29% $MgSO_4.7H_2O$ at pH 6.7; and (5) 2% trypticase, 0.5% yeast extract and 0.5% glucose. Growth curves obtained from the seedflask indicated that the first medium gave as good or better growth than the other media. Medium (1) was, therefore, used throughout for both strains. Growth curves, using this medium, are shown in Figure 5. To prevent foaming of the cultures due to the vigorous agitation, a silicone antifoam fluid was included in the medium at a concentration of 1 ml in 22 liters of medium. Because of the presence of this antifoam, reliable absorbance measurements can only be made on chilled samples.

Standard conditions used in the fermentation proces in the Microferm fermentor were as follows: air pressure 28 psi, vessel pressure 10-12 psi, steam pressure 27 psi, air flow 20 liters per minute, agitation speed 200 rpm, growth temperature 37 or 55 C.

Cells were harvested at an absorbance of 1.0 at 540 mu and chilled immediately. The yield from 24 liters of medium was approximately 40 gm for B. licheniformis and 50 gm for B. stearothermophilus 10. The cells were collected by means of the continuous flow system of the Sorvall. This was done at 4 C, at 30,000 x g, and at a flow rate of approximately 250 ml/minute. The cells were suspended in buffer A, collected by centrifugation at 11,700 x g for 20 minutes, frozen in liquid nitrogen, and stored at -20 C.

Figure 5. Growth Curves for B. licheniformis and B. stearothermophilus 10 in the Fermentor

(Growth in the seedflask was similar, although the rate of growth was slower).

Ordinate : Absorbance (540 mu)

Abscissa : Time (minutes)

(△) B. licheniformis

(○) B. stearothermophilus 10

Medium :

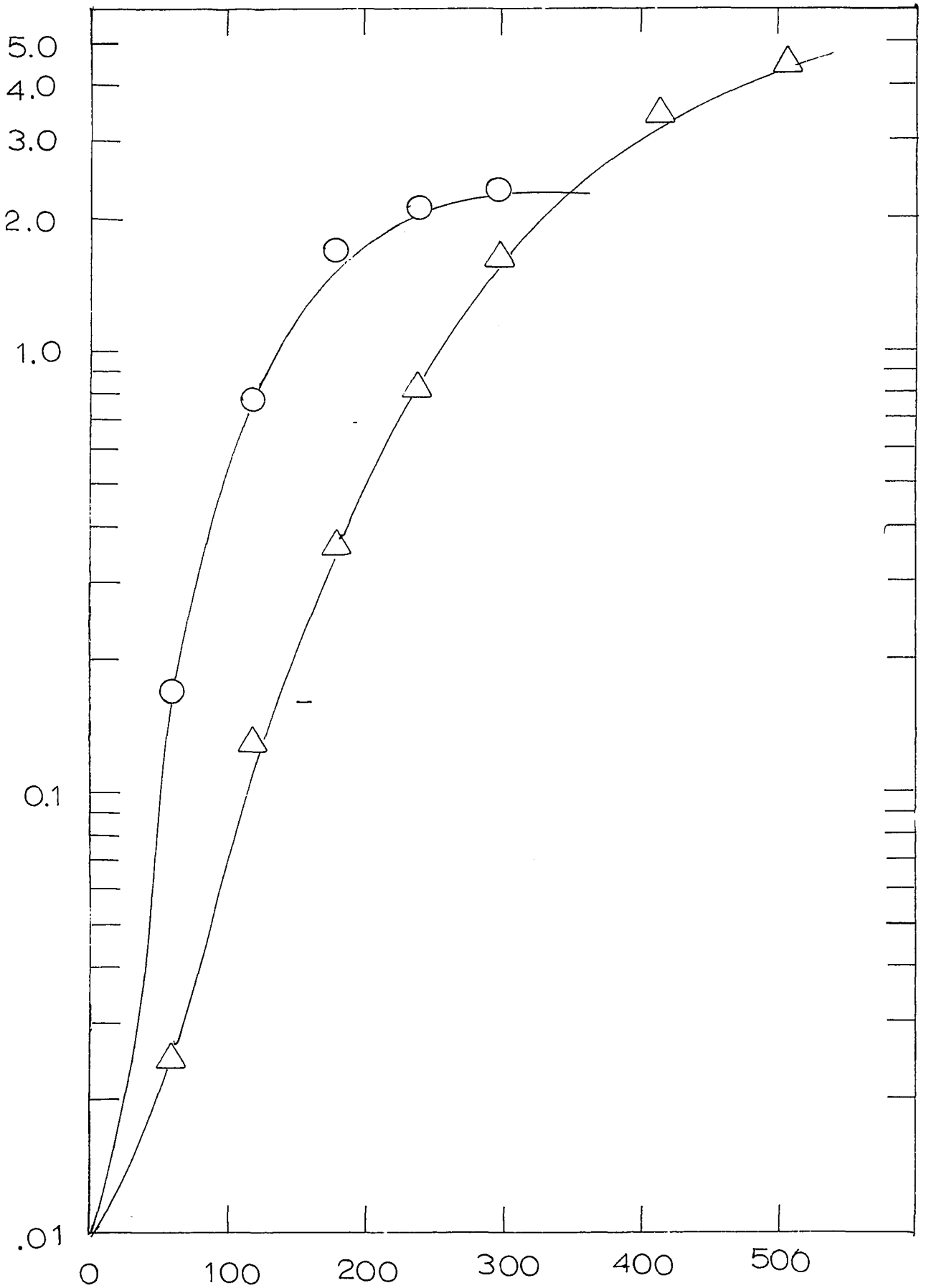
1% trypticase

0.2% yeast extract

1 ml silicone antifoam (per 22 l of medium)

Fermentation conditions :

Standard



Isolation of Subcellular Fractions

An identical isolation procedure was used for both the mesophile and the thermophile. It was a modified version of the one used by Nirenberg for E. coli (22). All operations, including centrifugations, were performed at 4 C or in crushed ice.

Approximately 80 gm of frozen cells were placed in a large beaker and thawed, while being gently stirred, in approximately 600 ml of cold buffer A. The thawed cells were centrifuged at 11,700 x g for 20 minutes. The supernatant was discarded and the cells were weighed and suspended in 1½ volumes of buffer C (W/V) with gentle stirring. When the cells were evenly suspended in the buffer, the solution was poured into a prechilled French pressure cell. Pressure was raised to 18,000 psi in approximately one minute, and it was kept at that level for about another minute. The outlet valve was then opened slightly to release the subcellular mixture at a rate of approximately 10 ml per minute into an erlenmeyer surrounded by crushed ice. Meanwhile the pressure in the cell was maintained at 18,000 psi. The broken cells were then treated with DNase (approximately 1 ug per ml of broken cells). After 5 minutes, the mixture was centrifuged at 30,000 x g for 30 minutes. The supernatant was removed by a pipette to within 1 cm above the pellet and was again centrifuged at 30,000 x g for 30 minutes. The supernatant was again removed to within 1 cm above the pellet. This supernatant fraction was designated as the S-30 fraction. The S-30 fraction was then

centrifuged at 105,000 x g for 2 hours in a Spinco preparative centrifuge. The upper four-fifths of the supernatant were removed and dialyzed against two liters of buffer B for approximately 17 hours. The dialyzing medium was changed at the end of 4 hours. This supernatant fraction was designated as S-100. The lower one-fifth was discarded. The pellet was resuspended in one-fourth of the original volume of buffer C, by gentle homogenization in a Potter-Elvejehm homogenizer (about 10 passes). This subcellular fraction was designated as the ribosomal fraction.

Both the S-100 and the ribosomal fraction were transferred to 2 ml ampules, frozen in the vapor of liquid nitrogen and stored in a liquid nitrogen tank. Both fractions were analyzed for protein concentration by the method of Lowry (23), using bovine serum albumin as a standard.

The activity of the fractions of both bacteria was very high. About 45% of the activity was lost by the end of two months. Therefore, the subcellular fractions of both strains were obtained on the same day and all experiments were performed with both strains simultaneously.

Incubation Mixture

The components of the incubation mixture were delivered into specially shortened 15-ml conical centrifuge tubes using Eppendorf pipettes with disposable polycarbonate tips, in the following sequence: 10 ul ammonium chloride solution, 10 ul magnesium acetate solution, 100 ul of mix II, 10 ul of poly U, 10 ul sRNA,

100 ul of premixed ribosomal and S-100 fractions (in the ratio of 30:70), and 10 ul of puromycin if used. The ribosome fraction contained approximately 23 mg/ml in both strains (i.e. 0.7 mg per incubation mixture). The S-100 fraction contained approximately 16 mg/ml and 26 mg/ml of protein for B. licheniformis and B. stearothermophilus, respectively (i.e. 1.1 and 1.8 mg per incubation mixture). The volume of the final incubation mixture was held constant at 250 ul. In some cases, appropriate amount of water was added to replace certain deleted components.

Unless otherwise specified, a standard incubation mixture contained: tris (100 mM), ATP (9.53×10^{-4} M), GTP (2.86×10^{-4} M), 2-mercaptoethanol (8.4 mM), PEP (7.4 mM), PEP kinase (4 ug protein), phe-C^{14} (25 mu-moles), ammonium chloride (100 mM), magnesium acetate (4 mM), spermidine trihydrochloride (4 mM), poly U (300 ug), B. subtilis sRNA (200 ug), premixed ribosomal and S-100 fractions, and PM (10^{-3} M). Incubation time was 30 minutes.

After all components were added to each incubation mixture, the tube was held at an angle of approximately 20 degrees and rolled gently between two fingers to provide mixing. The tubes were covered with marbles and incubated at appropriate temperatures, and for appropriate lengths of time. All the determinations were performed in duplicate and with appropriate controls. The variation between duplicates was less than 10%.

Measurement of Phenylalanine Incorporation

Protein was precipitated at the end of the incubation by the addition of 3 ml of 10% trichloroacetic acid at 4 C. The preicpitate was dispersed by stirring on a Vortex mixer and the reaction tube was placed in a water bath at 85-90 C for 20 minutes to hydrolyze RNA. The tubes were then kept at 4 C for at least one hour. Then the protein precipitate was again dispersed by stirring, and each suspension was filtered under suction through a Millipore filter. Each precipitate was washed with five 5 ml aliquots of cold 5% trichloroacetic acid. The millipore filters were glued with rubber cement to disposable planchettes, dried for 5-10 minutes under an infrared lamp and the radioactivity was counted with an automatic gas-flow planchette counter. Each planchette was counted for ten minutes. 160,000 counts per minute were equivalent to 25 mu-moles of phenylalanine incorporated.

RESULTS AND DISCUSSIONS

Preliminary Studies

Activity of the B. licheniformis system

Previous work from this laboratory had resulted in the preparation of cell free systems from B. licheniformis and B. stearothermophilus 10 which exhibited poly U dependent phenylalanine incorporation (11). It was found that the thermophilic system was comparatively more active and more heat stable. However, under those original conditions, the extent of phenylalanine incorporation in the mesophilic system was only about 10% that of the thermophilic system. In order to have a valid comparison of the effect of drugs on the two systems, it was deemed necessary to raise the activity of the mesophilic system to a level comparable to that of the thermophilic system. Several approaches were tried in order to achieve this.

The original incubation mixture contained, among other components, potassium chloride (80 mM), magnesium acetate (15 mM) and ribosomal protein (about 2.5 mg). The concentrations of these components were varied in the hope of improving the activity of the mesophilic system. Preincubation methods such as those of Nirenberg (22) and Hirashima (24) were tried. Subcellular fractions were prepared from cells harvested at different stages of growth. No improvement resulted from any of these approaches. Finally we arrived at a system in which we replaced the potassium chloride (80 mM) with ammonium chloride

(100 mM), and added spermidine (4 mM) as an inhibitor for nucleases which have previously been shown to be present in the preparation (7). The magnesium concentration was decreased from 15 mM to 4 mM, because of the co-operative effect of spermidine and magnesium. The amount of ribosomal protein used in each incubation mixture was also decreased to about 0.7 mg. With a 400% increase of poly U and a 30% increase of sRNA, we increased the extent of incorporation in the thermophile by approximately a factor of 9 and in the mesophile by a factor of 30. At this point, the incorporation of the mesophile was about 85% that of the thermophile. We then proceeded with the comparative studies of the two systems.

Incorporation temperature of the B. stearothermophilus 10 system

Preliminary experiments indicated that the B. stearothermophilus 10 system incorporated more at 37 than at 55 C. Since the intact organism grows better at 55 than at 37 C, it would have been desirable to have similar conditions for the cell free phenylalanine incorporation. Three attempts were made in the hope of shifting the optimal incorporation temperature of B. stearothermophilus 10 to a higher level. One of these involved omission of the heterologous B. subtilis sRNA from the incubation mixture. The reasoning was that, conceivably, the thermal lability of the sRNA from this mesophile might be obscuring the thermal stability of the other components. As can be seen from Figure 6, the incorporation for both systems was still higher at 37 than at

Figure 6. Phenylalanine Incorporation in the Absence of B. Subtilis sRNA

(No PM was present in this experiment)

Ordinate : mu-moles phe-C^{14} per mg ribosomal protein

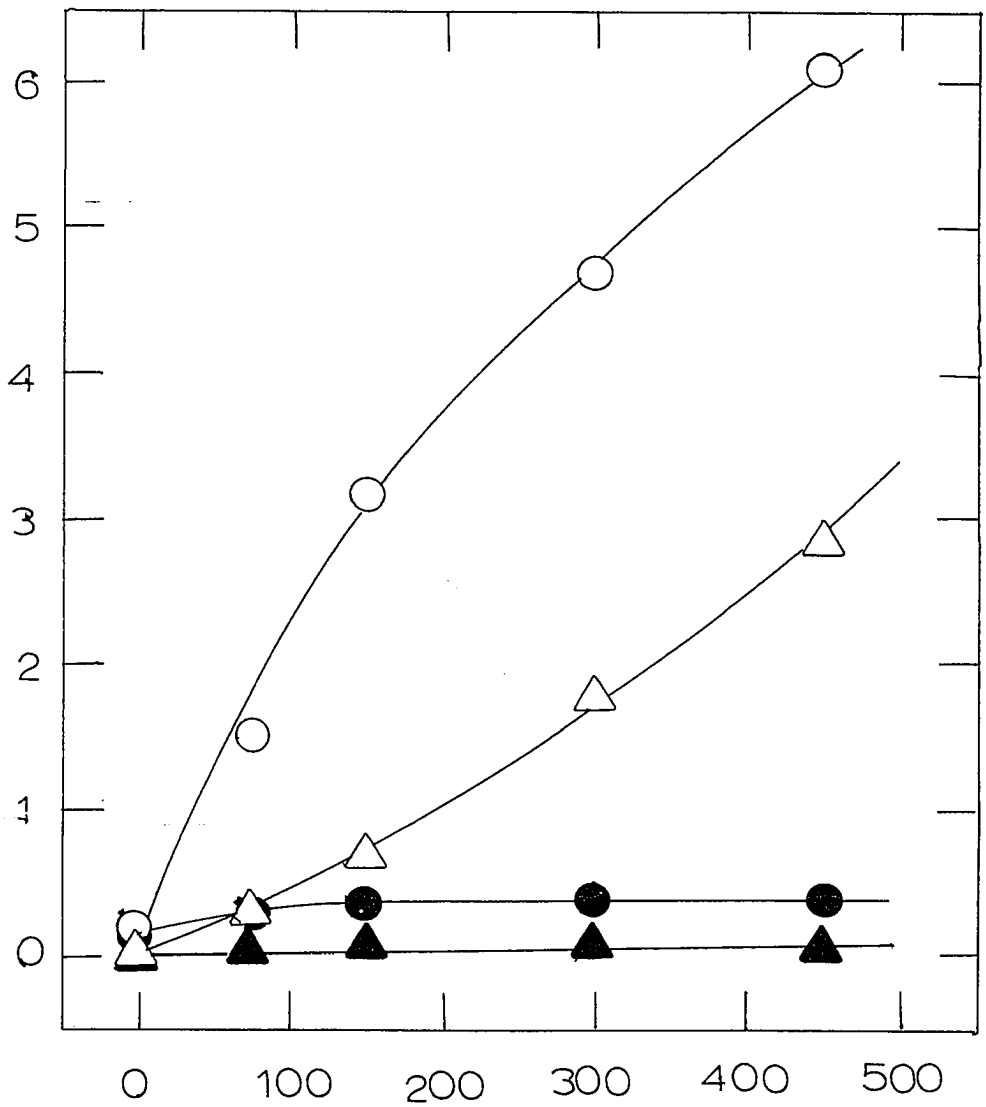
Abscissa : poly U (ug/incubation mixture)

(△) B. licheniformis at 37 C

(▲) B. licheniformis at 55 C

(○) B. stearothermophilus 10 at 37 C

(●) B. stearothermophilus 10 at 55 C



55 C and, as before, the thermophile system incorporated more at 37 than the mesophile system. The incorporation at 37 was a normal, poly U dependent, reaction except that the incorporation was only about 50% of that in the presence of 200 ug B. subtilis sRNA.

The second attempt at raising the optimal incorporation temperature for B. stearothermophilus 10 was based on the findings of Friedman and Weinstein for B. stearothermophilus 2184 (9). They reported that cell free systems from this organism incorporated more phenylalanine at 37 than 65 C at low magnesium concentrations, but the reverse was true when the magnesium concentration was raised to 18 mM. Accordingly, we investigated the incorporation of our B. stearothermophilus 10 system at varying magnesium concentrations at both 30 and 55 C. The results are shown in Figure 7.

This experiment was performed at a time when we were still using potassium as the cation, in the absence of spermidine. Potassium was at 80 mM and sRNA was at 150 ug per incubation mixture. Incubation time was 45 minutes.

It was found that at 30 C and at high poly U concentration, the optimal magnesium ion concentration was 10 mM; whereas at 55 C and at the same poly U concentration, the optimal magnesium concentration was at 15 mM. At the lower end of the poly U concentrations, magnesium was optimal at 15 mM at 30 C and at 20 mM at 55 C. This showed, in agreement with Friedman and Weinstein, that at higher temperatures, a higher magnesium concentration is required. However, under our conditions, the

Figure 7. The effect of Magnesium Ions on Phenylalanine Incorporation

(This experiment was performed with B. stearothermophilus 10 in the absence of spermidine and ammonium. Potassium was at 80 mM and sRNA was at 150 ug per incubation mixture. Incubation time was 45 minutes).

Ordinate : mu-moles phe-C^{14} per mg ribosomal protein

Abscissa : poly U (ug/incubation mixture)

a. Incorporation at 30 C

b. Incorporation at 55 C

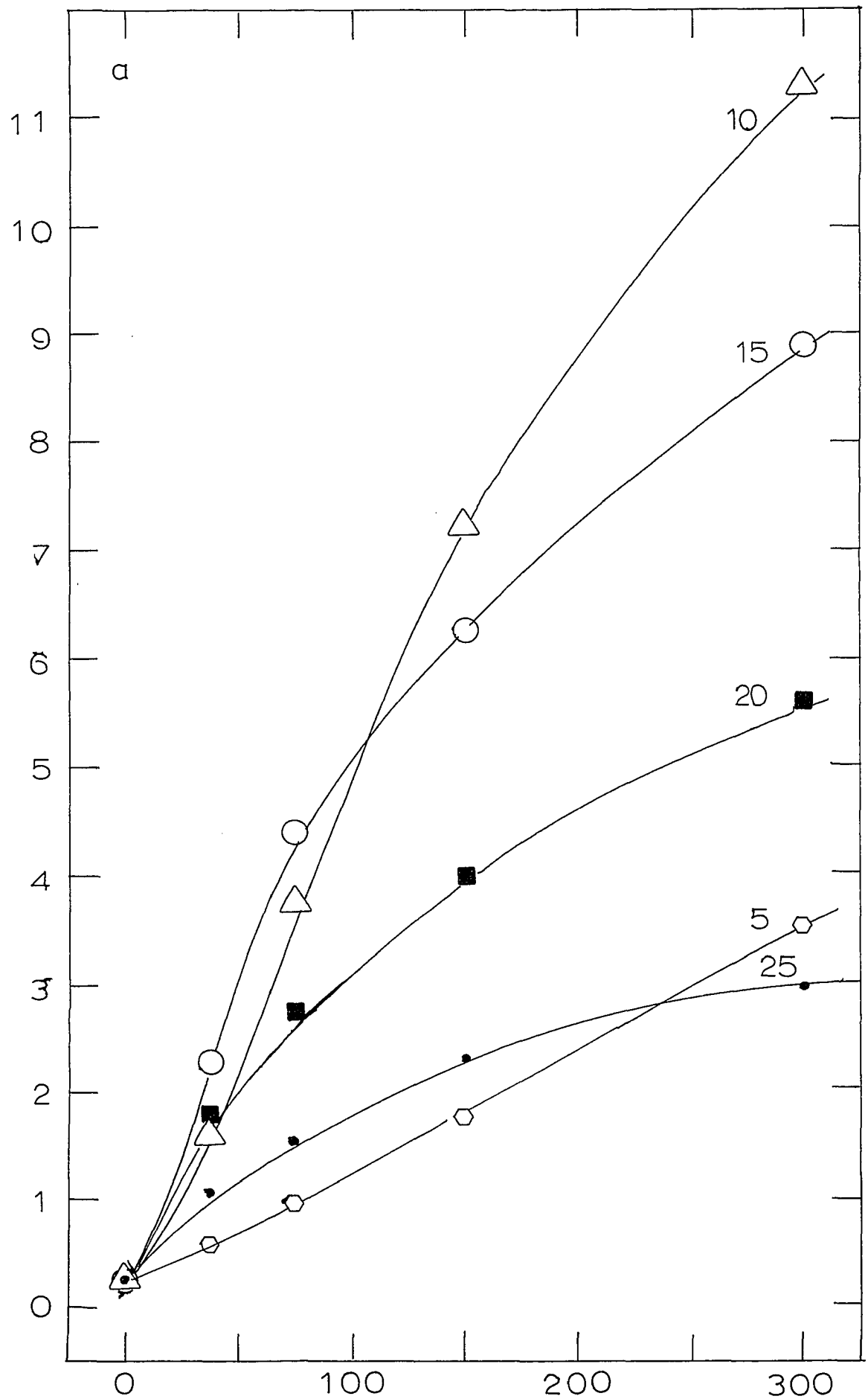
(⬡) magnesium at 5 mM

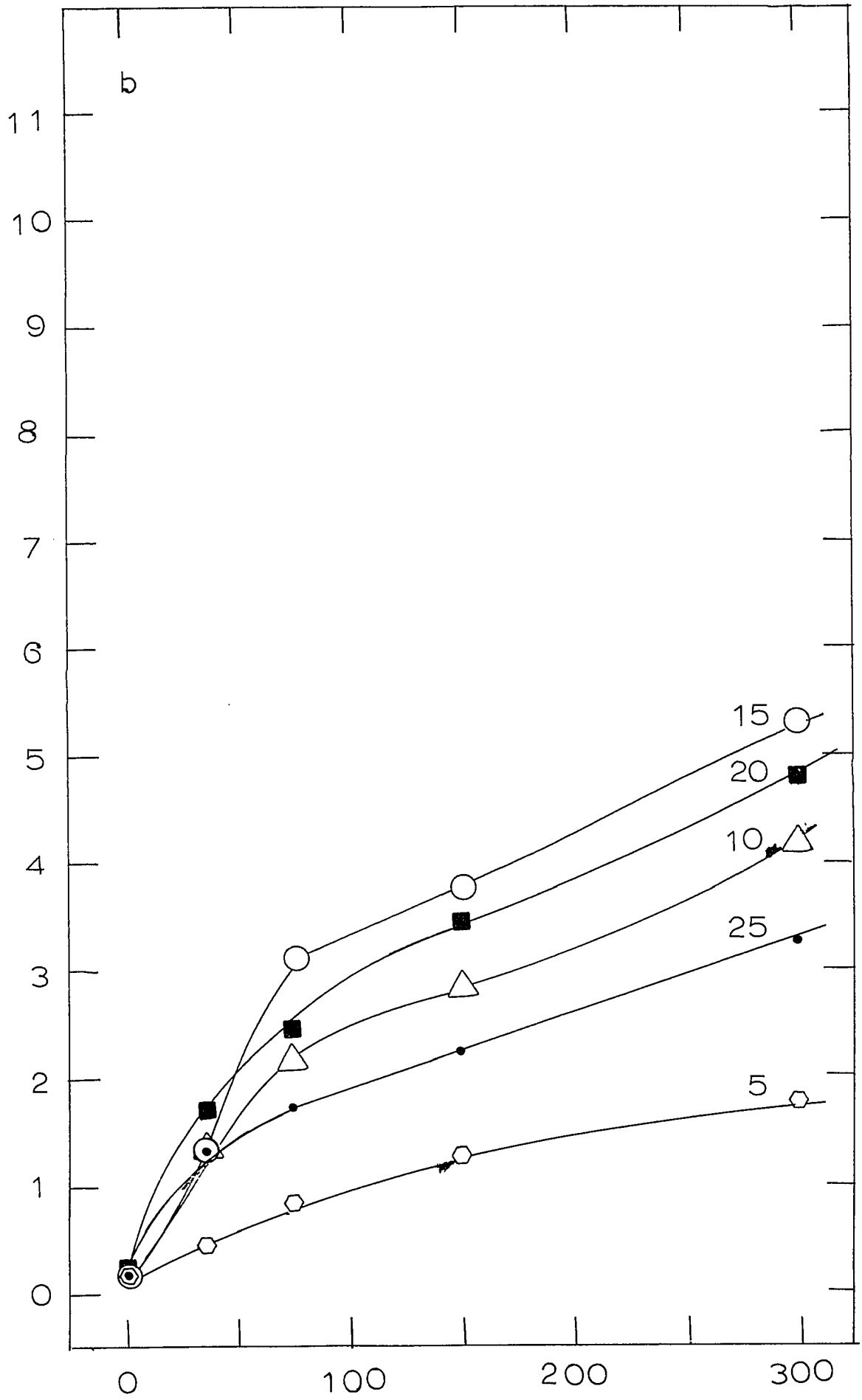
(△) magnesium at 10 mM

(◯) magnesium at 15 mM

(■) magnesium at 20 mM

(•) magnesium at 25 mM





incorporation at 30 C was still higher than at 55 C.

The third attempt at raising the optimal incorporation temperature for B. stearothermophilus 10 was based on the findings of Algranati and Lengyel for another thermophile (10). They used an incubation mixture which was similar to ours but differed in some of the ionic concentrations.

Previous work in our laboratory had shown that at an ammonium concentration of 100 mM, the optimal magnesium concentration was 4 mM. In the present experiment, we varied both the ammonium and the magnesium concentrations hoping to find conditions so that the incorporation of the B. stearothermophilus 10 system would be greater at 55 than at 37 C (Table 1, incubation for 30 minutes).

At 37 C, it was found that in both B. licheniformis and B. stearothermophilus 10, the incorporation decreased with increasing magnesium concentration regardless of the ammonium ion concentration. However, at 55 C, the incorporation increased with increasing magnesium concentration, regardless of the ammonium concentration. This again showed that more magnesium was needed at the higher temperature.

The optimal ammonium concentration for B. licheniformis at 37 and 55 C was at 60 mM; that for B. stearothermophilus 10 was between 60 and 30 mM.

At each set of ammonium and magnesium concentrations, the % activity of the incorporation at 55 C compared to that of 37 C was always higher for B. stearothermophilus 10 compared to B.

Table 1. Effect of Varying Magnesium and Ammonium Ion Concentrations

NH_4^+ (mM)	Mg^{++} (mM)	uu-moles phe-C^{14} per mg ribosomal protein					
		B. licheniformis			B. stearothermophilus		
		37 C	55 C	% Activity (55/37)	37 C	55 C	% Activity (55/37)
100	4	3186	374	12	3740	593	16
100	8	2509	920	37	3369	1627	48
100	10	2113	1114	53	3047	1628	54
60	4	3557	518	15	3878	899	23
60	8	2570	1034	40	3434	1677	49
60	10	2107	1149	55	2893	1702	59
30	4	3221	694	22	4790	1685	35
30	8	2529	1103	44	2944	2055	69
30	10	1824	897	49	2373	1716	72

licheniformis. This showed again, that the thermophile was more heat stable. Where the heat stability (% activity - 55/37) was greatest for B. stearothermophilus, was at an ammonium concentration of 30 mM and magnesium 10 mM; in other words, low ammonium and high magnesium. It would be of interest to pursue this investigation using a still lower ammonium and high magnesium concentration, and perhaps using potassium in addition.

Thus, while the above represent good evidence for the thermophilic character of the cell free system for B. stearothermophilus 10, we were still unable to increase the incorporation for this strain at 55 C over that at 37 C. Consequently, the puromycin reaction was investigated using the standard incubation mixture (see Materials and Methods).

Assessment of various drugs

We had available six anticancer drugs of which three were antibiotics (puromycin, actinomycin D, mitomycin C) and the other three were antimetabolites (6-mercaptopurine, 5-fluorouracil, 8-azaquanine). All six were tested for inhibition of the two incorporating systems at various concentrations, but all showed little effect except for puromycin where a substantial effect was observed. We, therefore, chose puromycin for this investigation.

Studies of the Puromycin Reaction

Effect of puromycin concentration

Various organisms are known to be sensitive to puromycin at different concentrations. For example, puromycin inhibits protein synthesis completely, in the intact organisms, at a concentration of 4×10^{-4} M in E. coli and at a concentration of 1.5×10^{-5} M in B. subtilis (12). It was also reported by Kammen (25) that B. subtilis was killed by low (5.5×10^{-5} M), but not by high (1.8×10^{-4} M) concentrations of puromycin. This paradoxical observation was attributed to the possible lethality of accumulated long, non-functional peptides expected at low concentrations, but not at high concentrations of the antibiotic.

It was therefore important to find out what effect puromycin concentration might have in the two cell free systems under study and whether the effect would be identical, or different, in the two systems.

Puromycin, its final concentration varying from 10^{-7} M to 10^{-2} M, was added to the incubation mixtures. The incubation mixtures which did not contain puromycin were used as controls. The first half of the experiment was done in the absence of poly U, which was the exogenous messenger-RNA (mRNA) used in our experiments (Figure 8a). The second half of the experiment was performed in the presence of poly U (Figure 8b). In doing so, it was possible to see the effect of puromycin on endogenous and exogenous amino acid incorporation. (The amount of radioactive polyphenylalanine incorporated in the first half of the experiment was subtracted from that incorporated in the second

Figure 8. Effect of Puromycin Concentration

(This experiment was performed at 37 C)

a. Endogenous Polyphenylalanine Synthesis

Ordinate : uu-moles phe-C^{14} per ug ribosomal protein

Abscissa : PM concentration (M)

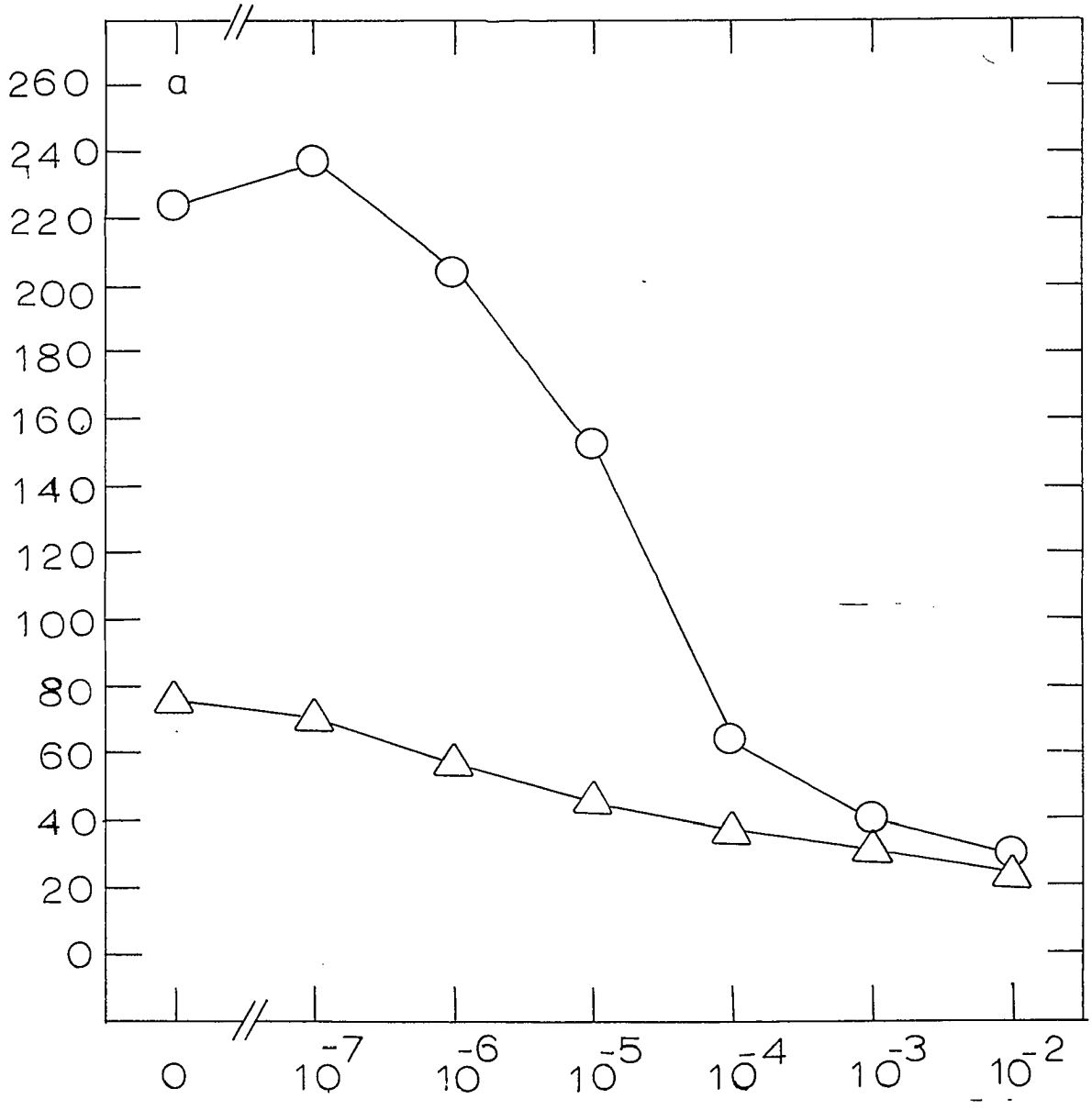
b. Exogenous Polyphenylalanine Synthesis

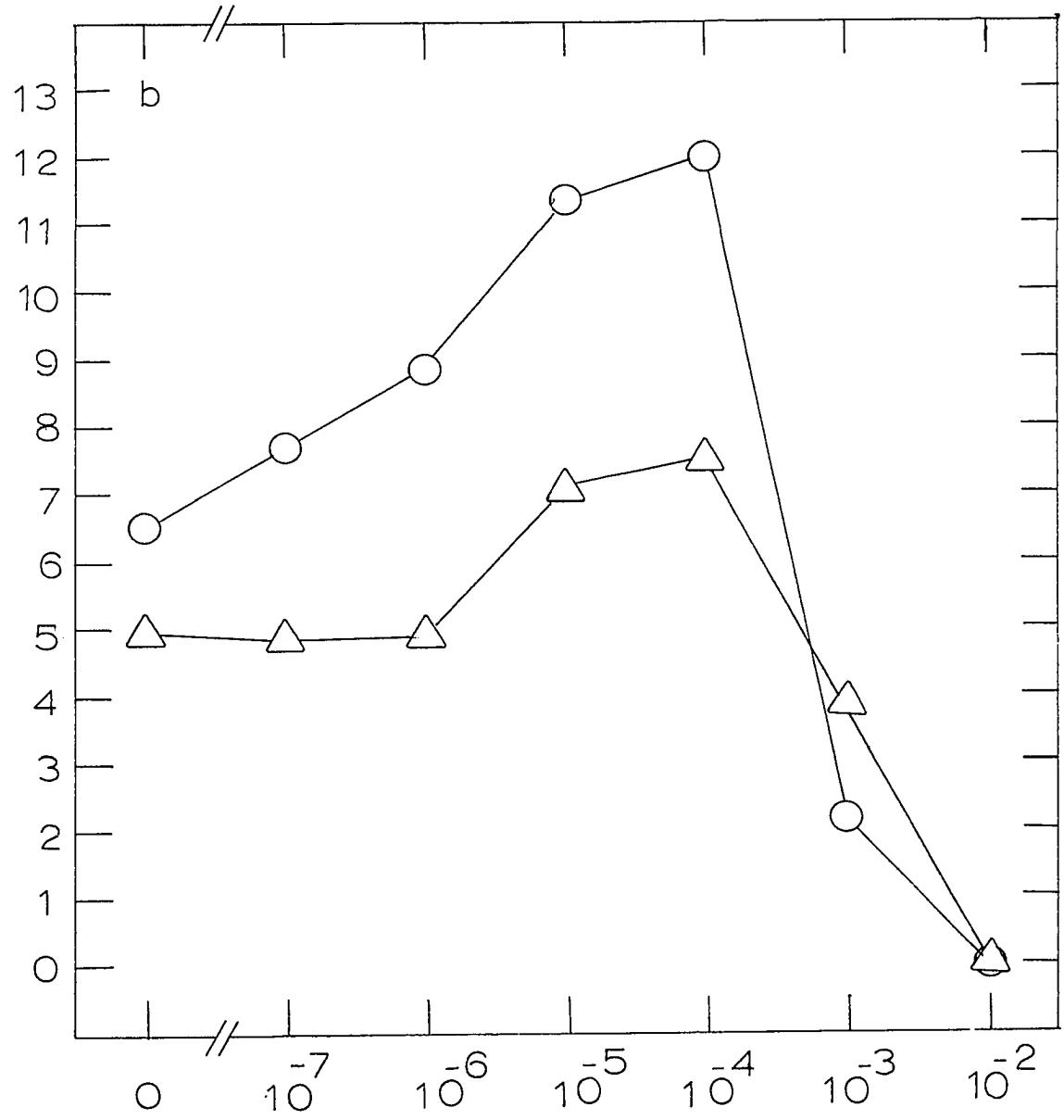
Ordinate : mu-moles phe-C^{14} per ug ribosomal protein

Abscissa : PM concentration (M)

(△) B. licheniformis

(○) B. stearothermophilus 10





half to give the net exogenous incorporation values).

The results showed that, in the control incubation mixtures, the endogenous and exogenous incorporations in B. stearothermophilus 10 were about 300% and 130% respectively, of those in B. licheniformis. This suggested two possibilities: (1) that the endogenous mRNA is more heat stable in the thermophilic system; (2) that the nuclease activity is higher in the mesophilic system and, therefore, the endogenous mRNA is more readily degraded.

The results also showed that endogenous incorporation in B. stearothermophilus 10 constituted about 3% of the exogenous incorporation, while in B. licheniformis it was only 1%.

The effect of puromycin concentration was evident in both the endogenous and the exogenous systems. The endogenous incorporation in both bacteria was more strongly inhibited with increasing concentrations of puromycin, except that a slight stimulation was observed in B. stearothermophilus 10 at a puromycin concentration of 10^{-7} M (Table 2). No stimulation was observed in the endogenous incorporation in B. licheniformis.

In the exogenous system, both stimulation and inhibition due to puromycin were detected in the two bacteria. Stimulation of incorporation in B. licheniformis occurred between the puromycin concentrations of 10^{-6} and about 5×10^{-3} M; that of B. stearothermophilus 10 between the puromycin concentration of at least 10^{-7} and about 5×10^{-3} M. Maximum stimulation in both bacteria occurred at 10^{-4} M. Maximum inhibition was at 10^{-2} M, which was essentially 100% inhibition.

Table 2. Effect of Puromycin Concentration

PM conc. (M)	Percent of Original Polyphenylalanine Synthesis			
	Endogenous		Exogenous	
	licheniformis	stearothermophilus	licheniformis	stearothermophilus
0	100	100	100	100
10^{-7}	95	106	98	118
10^{-6}	75	91	99	136
10^{-5}	60	68	142	173
10^{-4}	49	28	154	184
10^{-3}	34	18	78	33
10^{-2}	31	13	0	1

In both the endogenous and the exogenous systems, B. stearothermophilus 10 showed a higher sensitivity towards puromycin than B. licheniformis.

These results are very fascinating indeed. The stimulation due to puromycin indicated by an increase in the radioactive polyphenylalanine synthesized could be interpreted as a stimulation in the rate of polypeptide synthesis. Therefore, puromycin, being an analogue of sRNA, competes for the polymerizing phenylalanine, and in so doing must have by-passed a rate-limiting-step in the extension of the peptide chains.

Hardesty (26) in 1963 and Burka (27) in 1964, observed that when intact reticulocytes were incubated with puromycin and the size distribution of isolated polysomes measured, there was a marked fall in the number of large polysomes and an accompanying increase in the number of 80S monosomes. Similar observations have been made on liver polysomes of rats treated with puromycin (28) and in cell free preparations of reticulocytes and rat liver (29). Puromycin-induced polysome breakdown is, however, dissociable from puromycin-induced release of polypeptide chains; for example, in the absence of an energy source or in the presence of another inhibitor of protein synthesis, cyclohexamide, puromycin causes the release of ribosome-bound polypeptides, but fails to cause appreciable release of ribosomes from the polysomes (30, 31). Therefore, polysomal breakdown is not due to instability caused by removal of the peptide chains per se. Rather, the release of ribosomes appears to be due to at least

in part, the resumption of protein synthesis from the point of peptidyl-sRNA cleavage, leading to continued movement of ribosomes along the mRNA. If the rate-limiting-step in the extension of peptide chains (and hence in ribosome movement) is the binding of specific aminoacyl-sRNA's to the ribosome-messenger complex, puromycin might accelerate the progression of ribosomes by by-passing this binding step. As a consequence the number of polysomes would decrease and the number of monosomes would increase.

Our data agreed perfectly with, and supported, the above hypothesis. If the action of puromycin was limited to releasing short peptide chains, a stimulation would not have been observed; we would only see an increased inhibition with increased puromycin concentration due to the formation of small acid-soluble peptides and therefore less radioactive polyphenylalanine which is precipitable. Hence there must be an increase in the rate of polypeptide synthesis (see experiment on incorporation rate); an increase which must be great enough to offset the formation of small acid-soluble peptides (at a puromycin concentration of 10^{-4} M, for example). At higher concentrations of puromycin (10^{-2} M, for example), the increased synthesis rate was offset by the formation of small acid-soluble peptides. It would be of interest to investigate the possible polysome breakdown in the two cell free systems in the presence of puromycin; this could easily be done by incubating the cell free systems with the drug and analyzing the sedimentation patterns of the polysomes.

If the above argument is valid, one would then ask why the

thermophilic system was more sensitive to puromycin. It might be that the above possible rate-limiting-step (binding of amino-acyl-sRNA) is slower in the thermophile than in the mesophile and hence the effect of PM would be more pronounced in the former. Or else it is possible that PM binds more readily to the ribosomes of the thermophile. This possibility might be investigated by performing some binding studies of PM to ribosomes.

Puromycin effect on incorporation temperature

The extent of phenylalanine incorporation at different temperatures, with and without puromycin, was investigated in this experiment. The temperature range covered was from 20 to 70 C (Figure 9).

From the data, it was observed that both cell free systems were very temperature sensitive. The optimal temperature of incorporation for B. licheniformis, was at 32 C; whereas for B. stearothermophilus 10 it was at 27 C. This was a strange phenomenon since one would expect that the optimal temperature for incorporation for the thermophilic system would be higher than that for the mesophilic one in spite of the fact that they were cell free systems. However, it was evident from the graphs that the thermophilic system was more active and heat stable throughout the temperature range. The incorporation of the mesophilic system at 20, 30, and 70 C represented 47, 65, and 13%, respectively, of incorporation of the thermophilic systems at these same temperatures (both systems without PM).

Figure 9. Puromycin Effect on Incorporation Temperature

Ordinate : mu-moles phe-C^{14} per mg ribosomal protein

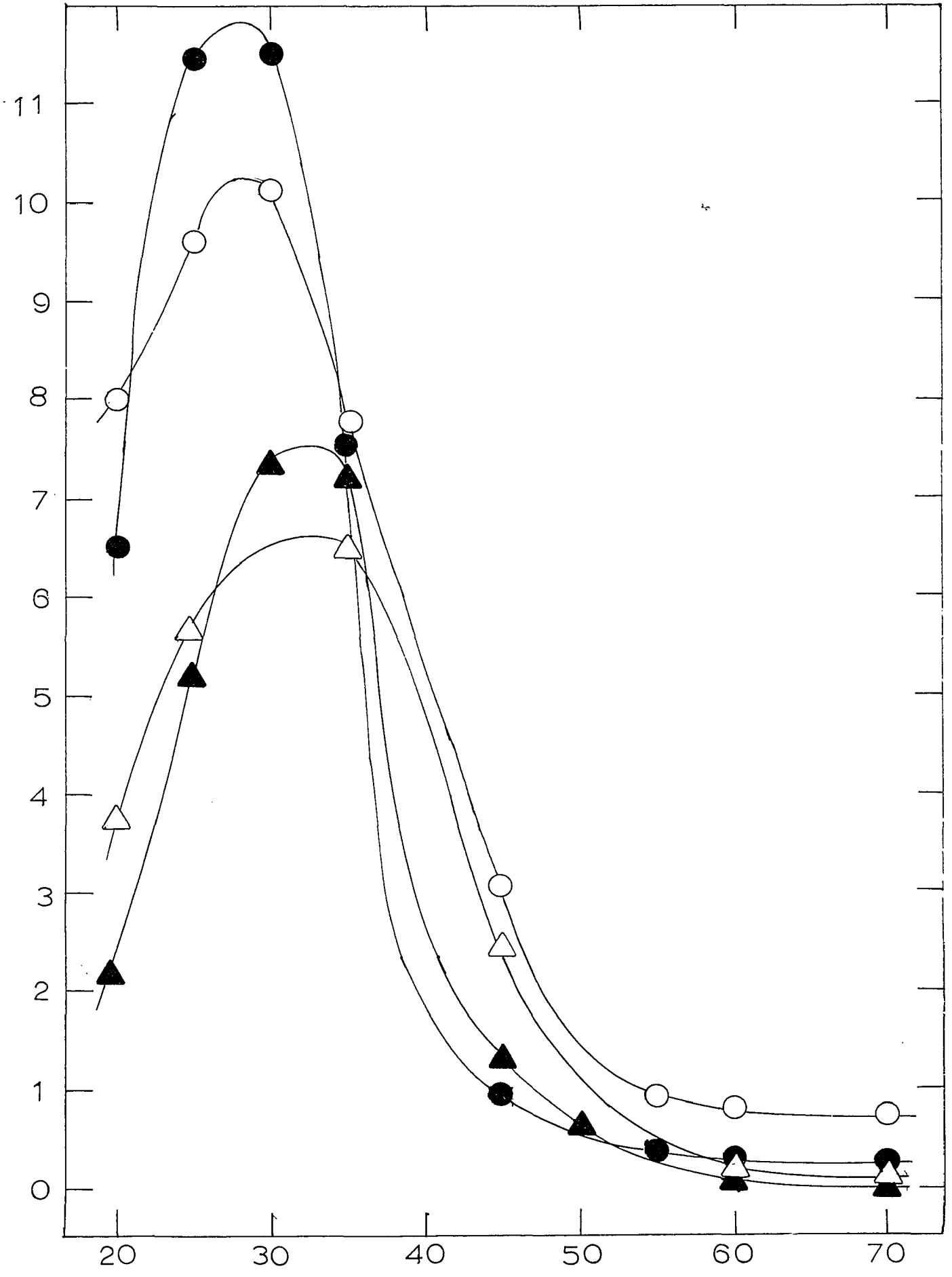
Abscissa : temperature (^{o}C)

(△) B. licheniformis without PM

(▲) B. licheniformis with PM

(○) B. stearothermophilus 10 without PM

(●) B. stearothermophilus 10 with PM



In the presence of puromycin, we witnessed both inhibition and stimulation. There was no shift in the optimal temperature of incorporation for a given strain. In the mesophilic B. licheniformis system, inhibition due to puromycin was observed throughout the temperature range except within the temperature range of 27-36 C where stimulation was observed. A similar effect was observed in the thermophilic B. stearothermophilus 10 system, except that the stimulation range was 21-34 C. The wider stimulation temperature range showed, as in the previous experiment, that B. stearothermophilus 10 was more sensitive to puromycin.

The stimulation by puromycin over a narrow, and mesophilic, temperature range may well tie in with its stimulatory effect discussed previously. It would indicate that whatever is involved in the PM stimulation may either involve an enzyme or else have a non-enzymatic temperature dependence.

Puromycin effect on incorporation rate

It was shown in the last experiment that at the end of 30 minutes of incubation, the optimal incorporation of B. licheniformis was at a higher temperature than that of B. stearothermophilus 10 (at 32 and 27 C, respectively). Since, in the intact cells, B. licheniformis grows optimally at a lower temperature than B. stearothermophilus 10 (at around 37 and 55 C, respectively), it is expected that similar temperatures would hold for the cell free systems. This we did not find. However, so far

we had only investigated the extent of incorporation at the end of thirty minutes at various temperatures. Conceivably, the rate of polyphenylalanine synthesis by the thermophile at 55 C might be faster than at 37 C and vice versa for the mesophile. Accordingly, we decided to compare the rates of incorporation of polyphenylalanine synthesis in the cell free systems of the thermophile and the mesophile at 37 and 55 C (Figure 10).

The results showed that, in the absence of puromycin, the rate of polyphenylalanine synthesis was faster in the thermophile than in the mesophile at either 37 or 55 C. However, in both systems, the rate of synthesis was faster at 37 than at 55 C.

In the presence of puromycin, the initial rate of synthesis (0-10 minutes) was slower and the latter rate (after 10 minutes) was faster for both systems at 37 compared to that at 55 C. (The initial portions of the curves, i.e. 0-10 minutes, were verified in another experiment not shown here). Moreover, there was an unexpected stimulation due to puromycin in both the mesophilic and the thermophilic systems. At the puromycin concentration of this experiment (10^{-3} M), inhibition should normally occur as was illustrated in the puromycin concentration experiment. The results were rechecked and verified; the possibility of error was unlikely. However, when the activity of the ribosomal/S-100 fraction was analyzed, it was found that at the time of this experiment (approximately three weeks after isolation of the fractions), the activity loss for B. licheniformis was 35%, whereas the activity loss for B. stearothermo-

Figure 10. Puromycin Effect on Incorporation Rate

Ordinate : mu-moles phe-C^{14} per mg ribosomal protein

Abscissa : time (minutes)


( ) B. licheniformis at 37 C without PM


( ) B. licheniformis at 37 C with PM


( ) B. stearothermophilus 10 at 37 C without PM

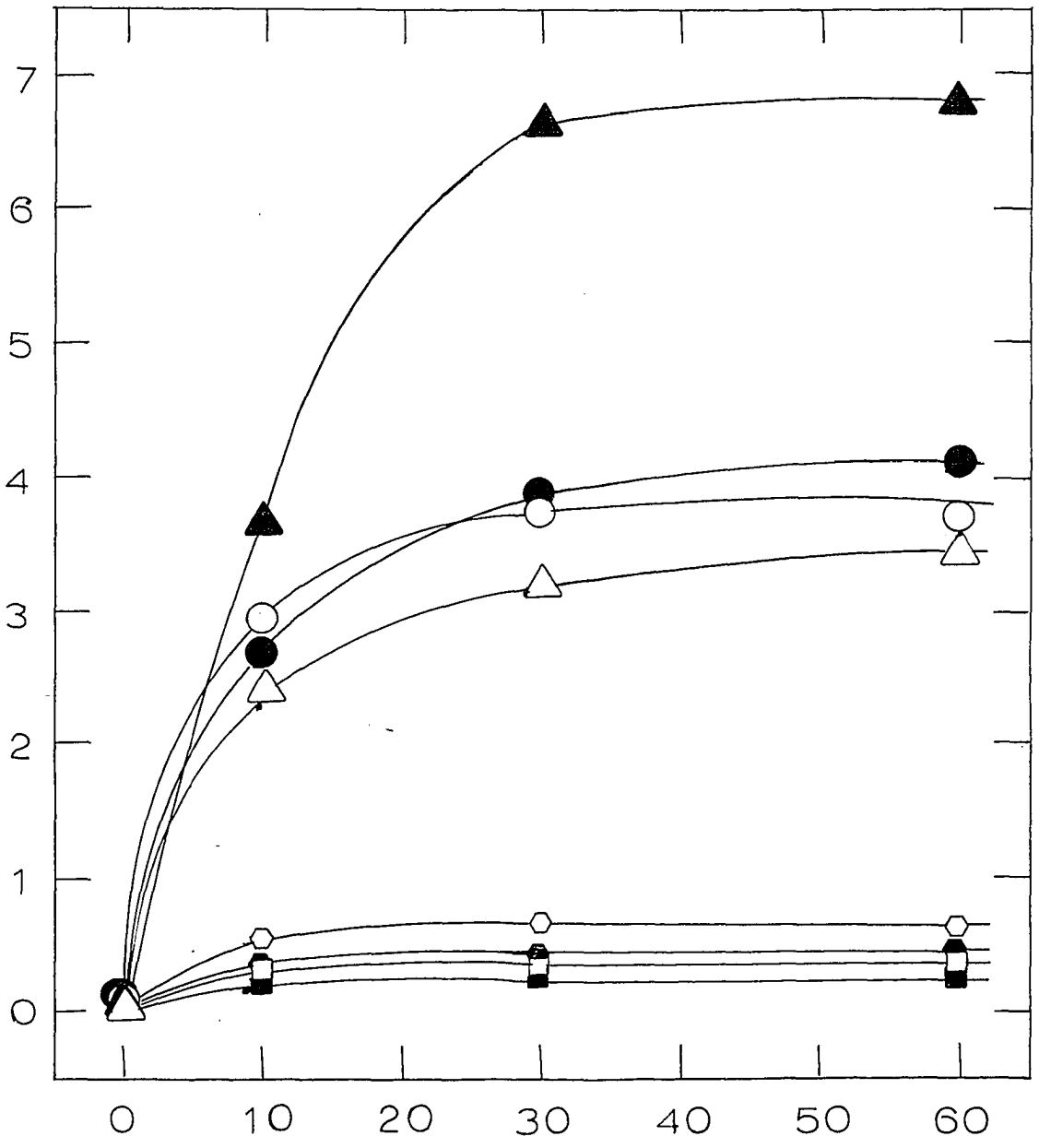

( ) B. stearothermophilus 10 at 37 C with PM


( ) B. licheniformis at 55 C without PM


( ) B. licheniformis at 55 C with PM


( ) B. stearothermophilus 10 at 55 C without PM


( ) B. stearothermophilus 10 at 55 C with PM



philus 10 was 42%.

Okamoto (32) and Mostafa (33) showed the presence of inhibitory factors of protein synthesis in the soluble fraction (S-100) of bacterial extracts. Recent research in our laboratory indicated the presence of similar factors in B. stearothermophilus 10. Therefore, the possibility exists that such factors might have been responsible for the observed loss of activity, and which might be related to the unexpected stimulation due to puromycin.

Competition between puromycin and sRNA

Since the action of PM is thought to be due to its similarity in structure to aminoacyl-sRNA, one would expect a competitive effect between PM and sRNA for the reaction with the growing polypeptide chain. Figure 11 shows the results of an experiment investigating this competition.

In the absence of PM, the B. licheniformis system showed dependence on exogenous B. subtilis sRNA while the B. stearothermophilus 10 system was essentially independent of added sRNA. Saturation points were at 100 ug and 400 ug of sRNA for B. licheniformis and B. stearothermophilus 10, respectively. These findings are inexcellent agreement with previous findings from our laboratory which demonstrated increased nuclease activity in the mesophilic strains. Preparations from these mesophiles would, therefore, be expected to depend on exogenous sRNA.

In the presence of puromycin, however, a stimulation due to sRNA was observed in both systems. At zero sRNA concentration,

Figure 11. Competition between Puromycin and sRNA

(The experiment was performed at 37 C and with sRNA obtained from B. subtilis).

Ordinate : mu-moles phe-C^{14} per mg ribosomal protein

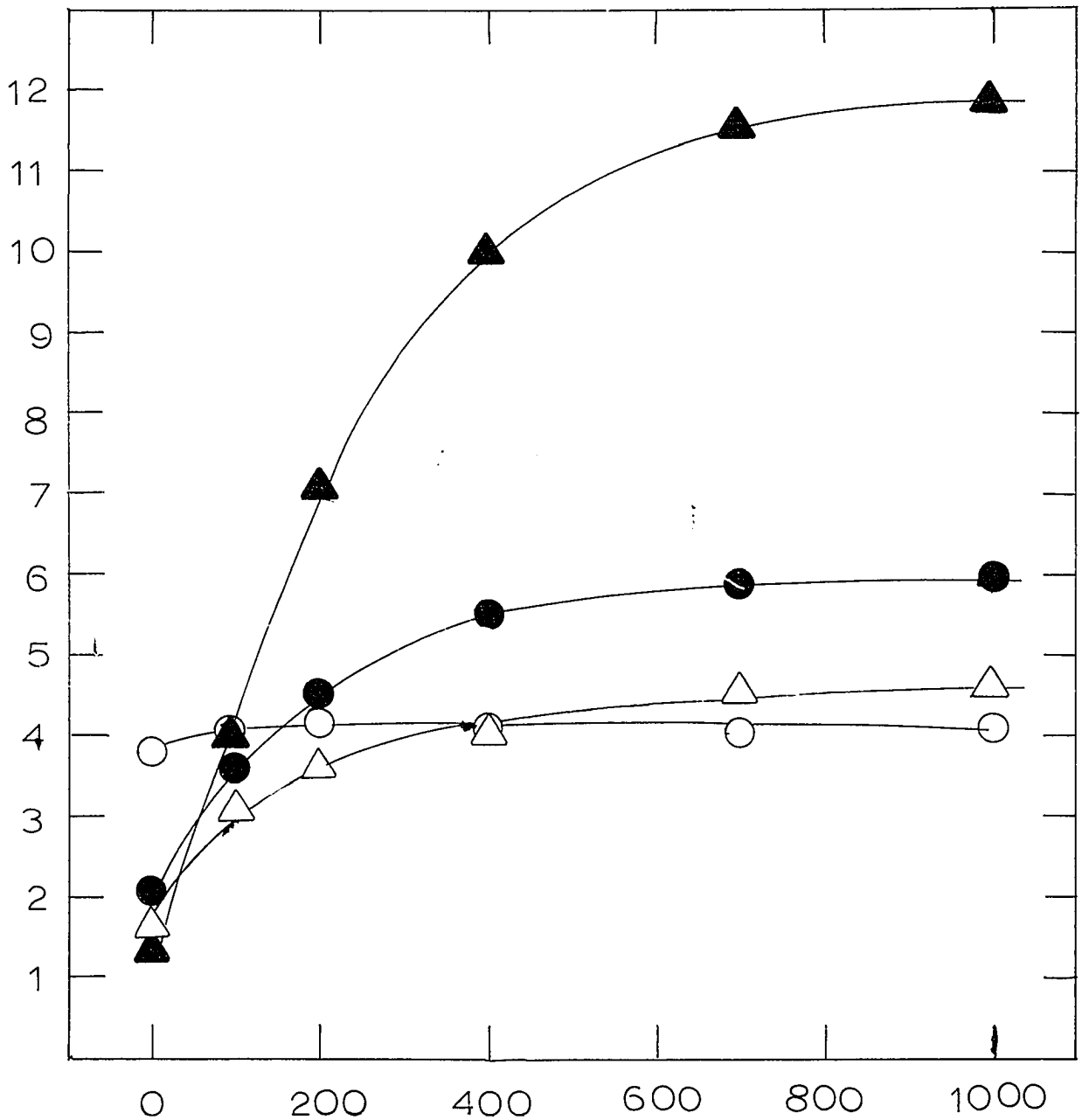
Abscissa : sRNA (ug/incubation mixture)

(△) B. licheniformis without PM

(▲) B. licheniformis with PM

(○) B. stearothermophilus 10 without PM

(●) B. stearothermophilus 10 with PM



addition of puromycin caused an inhibition which was more extensive for B. stearothermophilus 10. (This is in agreement with the previous statement that the B. stearothermophilus 10 system is more sensitive to puromycin. Stimulation in B. licheniformis occurred at an sRNA concentration of 25 ug; that in B. stearothermophilus at an sRNA concentration of 175 ug. At an sRNA concentration of 1,000 ug, the stimulation in B. licheniformis was almost 3 fold; whereas in B. stearothermophilus 10, it was only 1.5 fold. Hence it was again evident that the B. licheniformis system was more sensitive to sRNA than that of B. stearothermophilus 10.

If the only effect of additional sRNA was to compete and dilute out the puromycin present, the initial inhibition due to puromycin should be brought up gradually to the control level with increasing sRNA concentration; stimulation would not have occurred. On possible explanation was that there was a cooperative effect between sRNA and puromycin. Since PM apparently accelerates the reactions of protein synthesis, such a system can utilize larger amounts of sRNA for polyphenylalanine synthesis. It should also be pointed out here that the extent of polyphenylalanine synthesis at an sRNA concentration of 1,000 ug, was by far the greatest in this whole investigation.

SUMMARY

Puromycin was used in a comparative study of cell free protein synthesizing systems from the mesophilic B. licheniformis and the thermophilic B. stearothermophilus 10. Both inhibition and stimulation due to the antibiotic were observed. Inhibition occurred around either high (10^{-2} M) or low (10^{-7} M) concentrations of the drug. Stimulation occurred between 10^{-6} to 10^{-4} M. The thermophile was found to be more sensitive to puromycin.

Optimal temperatures of incorporation for B. licheniformis and B. stearothermophilus were at 32 and 27 C. Addition of puromycin did not shift the temperature optima of the two systems, although puromycin stimulation was observed in the neighborhood of both optima.

The rate of protein synthesis was faster in B. stearothermophilus 10 than in B. licheniformis throughout the temperature range of 20-70 C. In the presence of puromycin, an initial inhibition followed by stimulation were observed in both systems at 37 C; only inhibition was observed at 55 C.

The addition of exogenous B. subtilis sRNA, in the presence of puromycin, led to great stimulation of phenylalanine incorporation.

The stimulatory effect of puromycin was thought to be due to the bypassing of the aminoacyl sRNA binding which may have been the rate-limiting-step.

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VITA

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