12-1973

Binding of Aminoacyl-tRNA to Bacterial Ribosomes

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BINDING OF AMINOACYL-tRNA TO BACTERIAL RIBOSOMES

by

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A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment
of the
Degree of Master of Arts

Western Michigan University
Kalamazoo, Michigan
December 1973

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. Jochanan Stenesh, without whose counsel, wisdom and endless patience this research could not have been completed.

Appreciation is also expressed to the Department of Chemistry and School of Graduate Studies for providing the financial support during this investigation.

Somesh Datt Sharma

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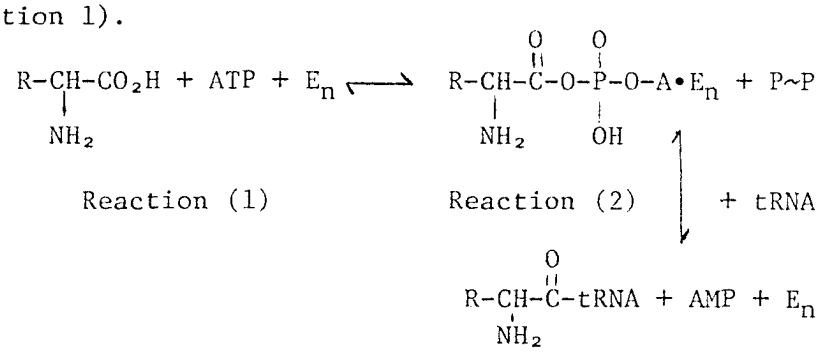
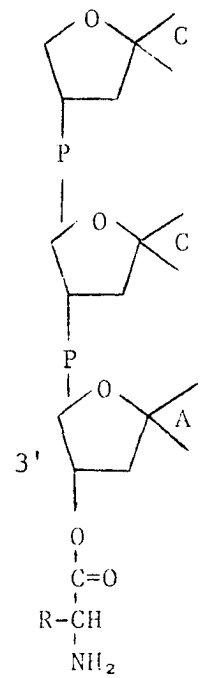
INTRODUCTION

Transfer RNA (tRNA) molecules are low molecular weight molecules (molecular weight 2.2-2.6 x 10^4), which constitute about 10-20% of the total cellular RNA. The tRNA molecule contains about 70-80 nucleotides and most of the tRNA molecules have the unique sequence, C-C-A, at their 3'-terminal. The function of the tRNA molecule is to bind covalently the proper amino acid and then transfer the amino acid to the site of protein synthesis (the ribosomes) where the amino acids are assembled into polypeptides.

The binding of an amino acid to the tRNA molecule (known as amino acid activation) requires adenosine-triphosphate (ATP) and a specific enzyme for each amino acid (amino acyl-tRNA synthetase). For each amino acid there is at least one specific aminoacyl-tRNA synthetase and one specific tRNA. The immediate product of the amino acid activation reaction is an enzyme-bound aminoacyl adenylate (Reaction 1).



The same enzymes have binding sites for specific tRNA's, to which the activated aminoacyl residue is transferred (Reaction 2) resulting in an aminoacyl-tRNA in which the carboxyl group of the amino acid is esterified to the 3'-C of the terminal adenylic acid residue:



This was proved by the fact that the terminal ribosyl group in tRNA can no longer be oxidized by periodic acid (HIO_4) after the tRNA has been charged with the aminoacid.

The tRNA 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). In the next step of protein synthesis a molecule of aminoacyl-tRNA becomes bound to a ribosome-mRNA complex by hydrogen bonding with the appropriate codon and by attaching to a site on the ribosomes.

Thus the synthesis of a polypeptide proceeds via a sequence of reactions which can be conveniently separated into two groups: first the formation of a ribosome-mRNA-tRNA complex, and second the formation of a peptide bond. By simplifying the system, it is possible to eliminate peptide bond formation and to observe only the forma-

tion of the complex (1). This can be done by omitting the enzymes and ATP-regenerating system from the reaction mixture, which then consists merely of washed ribosomes, tRNA, mRNA, and the proper ionic environment.

The binding of tRNA to ribosomes has been observed in many studies (2,3). An exchangeable binding of tRNA to ribosomes was reported by Cannon, Krug, and Gilbert (4). The specific binding of phenylalanyl-tRNA to ribosomes in the presence of polyuridylic acid was demonstrated in the laboratories of Schweet (5-7), Lipmann (1), Kaji and Kaji (8), and Spyrides (9). Binding was reported to be dependent upon GTP and the first transfer enzyme, but not upon peptide bond synthesis. The mechanism of binding, including a possible nonenzymatic binding of aminoacyl-tRNA induced by mRNA, was, however, not clarified until the work of Nirenberg and Leder (10).

Binding is a non-enzymatic reaction (11,12); the essential requirement is merely the proper Mg^{++} concentration, although this can be replaced by Ca^{++} or Mn^{++} ions or by spermidine (13). Depending upon the Mg^{++} concentration, monovalent cations, (K^{+} and especially NH_4^{+}) are stimulatory (13). High concentrations of lithium ions release tRNA from the complex (14). Binding is to the 30S component of the ribosome and it is immaterial whether the tRNA is charged with amino acid or not (11).

The possibility of using polynucleotides of well defined and known sequences as mRNA for protein synthesis was realized from the start, and had been discussed by Matthei (15) in 1963. The first

attempt to put this idea into practice was that of Wahba et. al. (16), who polymerized uridylic acid on to a primer to produce what was thought to be the polynucleotides AUU.....U and CUU.....U. When used as messengers, these appeared to stimulate the incorporation of phenylalanine and small amounts of tyrosine and cysteine, respectively, and the tyrosine appeared to be partially C-terminal. From this it was tentatively concluded that the codons for tyrosine and cysteine are UUA and UUG, and that the translation of mRNA in the 3'⟶ 5' direction corresponds to the synthesis of the protein in the N-terminal⟶ C-terminal direction. Unfortunately, for a number of technical reasons (15), these conclusions proved to be erroneous.

The idea was, however, sound and once the technical difficulties could be overcome, there were two possible ways of using polynucleotides of known sequence. One way represented a straightforward extension of the original method of Nirenberg and Matthei (17) and involved the addition of polynucleotides of known sequence to a cell-free system and a determination of the nature of the polypeptide synthesized. The second way entailed dispensing with the actual synthesis of protein and studying, instead, the specificity with which tRNA was bound to ribosomes in the presence of a given messenger.

In order to determine the minimum chain length of mRNA required for codeword recognition and to test the ability of chemically defined oligonucleotides to induce C^{14}-aminoacyl-tRNA binding to ribosomes, Nirenberg and Leder (10) in 1964 devised a rapid method of detecting this interaction. It is based on the ability of cellulose

nitrate filters to retain ribosomes and any labelled amino acid attached to them. It is this technique that has been used in the present study of the binding of aminoacyl-tRNA to ribosomes from a mesophilic and a thermophilic bacterial strain.

Bacteria are commonly divided into three groups according to the temperature at which they grow. Psychrophiles grow at low temperatures (below 25°), mesophiles grow at physiological temperatures (25-45°) and thermophiles grow at elevated temperatures (about 55-85°).

Thermophilic bacteria have been isolated from numerous sources (18). They have attracted considerable attention because they can grow at temperatures at which normal cellular components break down. Three main theories have been advanced to explain the phenomenon of thermophily. One theory (19) considers thermophily to be a special type of metabolic state, involving high rates of breakdown and synthesis of cellular constituents. A second theory relates thermal stability to the protective action of lipids and attempts to correlate heat stability with the melting points of the cellular lipids (20). A third theory ascribes thermophily to physical and chemical differences of macromolecules from thermophiles as compared to similar molecules from mesophiles.

Most of the evidence accumulated so far supports the latter theory. Such evidence comes mostly from studies of proteins and nucleic acids. Koffler (21) showed that cytoplasmic proteins from thermophilic bacteria are more stable than those from mesophilic bacteria. Stenesh and Koffler (22) demonstrated a striking differ-

ence in heat stability of flagella from these two types of bacteria.

Early studies of nucleic acids yielded ambiguous results. Thus, for example, DNA and transfer RNA (23) from thermophiles showed no unusual heat stability. In most of these studies components from thermophilic strains of Bacillus were compared to similar components of Escherichia coli. Since such comparisons do not exclude intergeneric differences, a systematic study of mesophilic and thermophilic strains of one genus, namely Bacillus, was initiated in our laboratory.

Stenesh and Holazo (24) showed that the ribosomal RNA from thermophilic strains of Bacillus was more heat stable than the ribosomal RNA from mesophilic strains of the same genus. The RNA from thermophiles melted out at higher temperatures and had a higher guanine plus cytosine content than that from the mesophiles. Similar results were obtained in studies of the DNA isolated from the above strains (25). An in vitro protein synthesizing system from a thermophilic strain had a higher optimum temperature than one from a mesophile (26). Some differences were also found in the fatty acid distribution of mesophilic and thermophilic strains of Bacillus (27).

The present work extends the comparative studies of mesophilic and thermophilic strains of the genus Bacillus to the first step in protein synthesis which involves the binding of aminoacyl-tRNA to the ribosomes. Specifically, the study involved the isolation of tRNA, aminoacyl-tRNA synthetase and washed ribosomes from the mesophile B. licheniformis and the thermophile B. stearothermophilus 10; the charging of the tRNA with either ^{3}H-L-Asparagine or ^{3}H-L-Lysine;

and, finally, the binding of the charged tRNA to the ribosomes in the presence of synonym codons (trinucleotides) for the two amino acids (AAU and AAC for asparagine and AAA and AAG for lysine).

MATERIALS AND METHODS

Organisms and Growth Conditions

The bacterial strains used in this experiment were a mesophile Bacillus licheniformis (NRS 243) and a thermophile, Bacillus stearothermophilus 10.

The bacteria were grown initially on slants containing 1% Trypticase (BBL), 0.2% yeast extract (Difco) and 2% Bactoagar (Difco). Stock cultures were grown on the same medium containing in addition 10 ppm manganese. Slants were incubated for 12 hours at 37° for the mesophile and for 10 hours at 55° for the thermophile. At the end of this period, 6 ml of sterile water were added and the contents of 3 slants, a total of 18 ml of inoculum, was transferred to a seed flask containing 2 liters of sterile medium (1% Trypticase and 0.2% Yeast extract). The seed flask was incubated at 37° or 55° for 4 hours with constant aeration.

The contents of the seed flask was inoculated into a 25 liter fermentor (New Brunswick Scientific, Model MF-128S) containing 23 liters of sterile medium and 1 ml of silicone antifoam (Union Carbide Corporation, SAG-471). The bacteria were grown to an absorbance of 1.0 at 540 nm for both the thermophile (55°) and the mesophile (37°). The medium containing the cells was chilled and the cells harvested at 4° using a continuous-flow centrifuge (Sorvall RC-2 with KSB continuous-flow attachment) at 30,000 x g and at a flow rate of 250 ml/min.

The cells were resuspended in about 300 ml of buffer I (0.01 M Tris-HCl (pH 7.4), 0.01 M magnesium acetate and 0.06 M NH_4Cl), and recentrifuged at 11,700 x g for 20 minutes. The cells were then frozen at -20°. The yield of cells (wet weight) was about 50 g for B. stearothermophilus and 40 g for B. licheniformis.

Isolation of Ribosomes

The cells were thawed, washed with buffer I and suspended in 2 volumes (w/v) of buffer II (Buffer I plus 0.006 M 2-mercaptoethanol and 0.006 M spermidine); the suspension was disrupted in a French press (Aminco, Model 5-596) at 18,000 lb/in^2. Deoxyribonuclease (200μg/25 g of cells) was added and the cell debris was removed by two 30 minute centrifugations at 30,000 x g (Sorvall, Model RC-2). The first supernatant fluid was collected in its entirety, and the second supernatant fluid was withdrawn to within about 1 cm above the pellet.

The ribosomes were collected by a two hour centrifugation at 105,000 x g (Spinco, Model L) and suspended in 25% the original volume of buffer II. These crude ribosomes were then purified by cycles of low and high speed centrifugations (5 min. at 10,000 x g followed by two hours at 105,000 x g and resuspension of the pellet). After two such cycles, the ribosomes were suspended in 10% of the volume of buffer II using buffer III (0.5 M Tris-HCl (pH 7.2), 0.25 M NH_4Cl, 0.05 M magnesium chloride) and centrifuged for 5 minutes at 10,000 x g to give the final preparation of washed ribosomes. The ribosome were transferred to 2 ml ampules (0.1 ml in each

ampule), frozen in the vapor of liquid nitrogen and stored in liquid nitrogen.

Preparation of Aminoacyl-tRNA Synthetase

Crude aminoacyl-tRNA synthetase was prepared according to the procedure of Kelmers et. al. (28). All the steps were carried out at 4° unless otherwise specified. Bacterial cells (30 g) were thawed, dispersed in 60 ml buffer IV (0.01 M Tris-HCl, pH 7.4, containing 0.01 M magnesium acetate and 0.01 M reduced glutathione), and broken in a French pressure cell at 10,000-12,000 lb/in^2. Four volumes of buffer were then added and the mixture was centrifuged at 35,000 x g for 40 minutes and then at 78,000 x g for three hours to remove particulate matter. The supernatant solution was dialyzed overnight against two liters of buffer IV. Streptomycin sulfate (0.1 volume of a 10% solution) was then added to the dialyzed solution to precipitate out the nucleic acids. After stirring for three hours, the precipitate was removed by centrifugation at 16,000 x g for ten minutes. The pH of the supernatant was then adjusted to 7.5 with 0.1 M KOH and maintained at that pH during the addition of solid ammonium sulfate to 65% saturation. The mixture was stirred for 30 minutes and the precipitate recovered by centrifugation at 16,000 x g for 10 minutes. The precipitate was dissolved in 100 ml of buffer IV and dialyzed overnight against 6 liters of buffer. Glycerol (0.25 volume) was added to the enzyme solution which was then stored at -20°. Crude aminoacyl-tRNA synthetase prepared in this manner was stable for at least one year.

Isolation of tRNA

Crude tRNA was prepared by a slight modification of the method described by Gutcho (29).

Phenol (Coleman, Matheson and Bell; reagent grade without preservative) was distilled, and the distillate between 178°-181° was collected in a round bottom flask and stored at -20° in a stoppered vessel. Just prior to use the phenol was melted at 65° and adjusted to contain 12% by volume of water; the resulting 88% phenol solution was chilled to 4°.

To 45 g of frozen cells were added 85 ml of 88% phenol. After 1.5 to 2.0 hours of stirring at room temperature, the uniform, well dispersed slurry was treated with 198 ml of deionized, doubly distilled, water. After one hour of additional stirring, the slurry was left to settle overnight at room temperature. On the following day, 95 ml of 88% phenol were added and the mixture was stirred for 30 minutes. Deionized, doubly distilled, water (220 ml) was added. Stirring was continued for one hour and the suspension was again left to settle overnight at room temperature. The top aqueous layer was removed by syphoning and was then centrifuged at 16,000 x g for one hour. To the aqueous layer were added successively 0.1 volume of 20% potassium acetate (pH 5.2) and two volumes of 95% ethanol. The precipitate which contained the transfer RNA was removed the following day by syphoning off the supernatant and centrifuging the remaining slurry at low speed (2500 rpm). The precipitate was washed once with cold 75% ethanol and once with cold isopropanol,

and dried in a vacuo at room temperature. The tRNA was then dissolved in a small amount of water, lyophillized and stored over silica at -20°.

Assay of (amino acid acceptor) tRNA activity

The tRNA was assayed by ''charging'' it with ^{3}H-labelled amino acids. The standard assay for this ''amino acid acceptor activity'' followed the procedure of Kelmers et. al. (28) with some modifications.

The reaction mixture, in a final volume of 0.2 ml, contained the following, in order of addition: sufficient homologous crude aminoacyl-tRNA synthecase (about 0.05 mg) to obtain maximal formation of aminoacyl-tRNA; tris-HCl buffer (pH 7.4), 20 micromoles; magnesium acetate, 2 micromoles; ATP, 0.4 micromoles; KCl, 1 micromole; ^{3}H-labelled L-Asparagine or L-Lysine, 0.1 micromoles (specific activity 50-200 μC/μmole); tRNA, 0.02 to 4.0 absorbance units at 260 nm. The reaction mixture was incubated at 37° for 10 minutes (the optimum time for incubation for the two amino acids was determined in a separate experiment). The reaction was stopped by the addition of 3 ml of cold 10% trichloroacetic acid. The mixture was left to stand in the cold room for at least one hour before a 0.1 ml portion of it was poured on a Millipore filter (HA Millipore filter, 25 mm diameter, 0.45μ pore size) and washed 10 times with 5 ml portions of cold 5% trichloroacetic acid. The filter was dried under an infrared lamp and then suspended in 10 ml of scintillation fluid (100 g of naphthalene, 4 g PPO, and 50 mg POPOP per liter of 1,4-di-

oxane), and counted in a liquid scintillation counter (Nuclear Chicago Model Mark II).

Preparation of charged tRNA

In order to remove (strip) any attached amino acids, the tRNA was dissolved in 0.5 M Tris-HCl buffer, pH 8.8 at a concentration of 20 mg/ml, and was incubated for sixty minutes at 37°. The mixture was then directly dialyzed for 24 hours at 0-4° against several changes of a large volume of distilled water.

For the charging with a labelled amino acid, the reaction mixture contained the following in a final volume of 1 ml, in order of addition: 0.05 micromole of ^{3}H-L-Asparagine or ^{3}H-L-Lysine (specific activity 50-200 μC/mole), 100 micromoles of Tris-HCl buffer, pH 7.4, 2.5 micromoles of magnesium acetate, 4 micromoles of ATP, 10 micromoles of reduced glutathione, 1 mg of stripped tRNA and 0.1 to 0.2 ml of homologous aminoacyl-tRNA synthetase. The mixture was incubated for 30 minutes at 37°. At the end of the incubation period, the sample was chilled in ice, an equal volume of redistilled phenol (88%) was added, and the mixture was shaken vigorously at 4° for 10 minutes using a vortex mixer. The mixture was centrifuged at 32,000 x g for 30 minutes, the water (upper) layer was removed, and the phenol layer was washed once with 1 ml of buffer V (0.001 M Tris-HCl buffer, pH 7.4, containing 0.01 M magnesium chloride). To the combined water layers, 0.1 volume of 20% potassium acetate solution was added, and the charged tRNA was precipitated with 2 volumes of ethanol at -20°, and allowed to stand

at -20° for at least two hours. The precipitate was collected by centrifugation at 32,000 x g for 10 minutes, washed once with 67% ethanol, and then dissolved in 1 M NaCl solution and stirred for two hours. Insoluble material was discarded after centrifugation at 32,000 x g for 20 minutes. The supernatant was dialyzed for at least 24 hours at 0-4° against several changes of distilled water. The charged tRNA thus obtained was lyophillized and stored over silica gel at -20°.

Assay of Aminoacyl-tRNA Binding

The binding of ^{3}H-aminoacyl-tRNA to ribosomes in presence of appropriate codons (''binding assay'') was assayed by the procedure of Nirenberg and Leder (10).

Each 100 µl of reaction mixture contained the following which were added in the order indicated:

5.0 micromoles of Tris-HCl, pH 7.2

0.5 micromoles of Magnesium chloride

4.50 Absorbance units at 260 nm of ribosomes (dispersed in buffer III, approximately 0.040 mg/ml and 0.033 mg/ml ribosomal protein of B. lichenifor-mis and of B. 10

0.2 Absorbance units at 260 nm of codons (trinucleotides), approximately 10 A_{260} units per ml.

^{3}H-aminoacyl-tRNA, approximately 900-1600 counts per minute and 1 to 3 A_{260} units.

Timing and the order of addition are of utmost importance. Tubes were kept at 0°C prior to incubation. ^{3}H-aminoacyl-tRNA was added last to initiate the reaction (less binding was obtained if

the trinucleotide was added last), and the tubes were immediately incubated in a water bath at 24° for 20 to 30 minutes.

After incubation, the tubes were placed in ice and each reaction mixture was immediately diluted with 5.0 ml of buffer III at 0 to 3°. A cellulose nitrate filter (HA Millipore filter, 25 mm diameter, 0.45 μ pore size) in a glass holder was washed under gentle suction with 5 ml of buffer III at 0 to 3°. The diluted reaction mixture was immediately poured on the filter under suction and washed three times with 5 ml portions of buffer III at 0 to 3° to remove unbound ^{3}H-aminoacyl-tRNA. The ribosomes and the aminoacyl-tRNA bound to them are retained on the filter. Since the reaction mixtures were not deproteinized, it was important to dilute and wash the ribosomes immediately after incubation, to use cold buffer, and to allow relatively little air to be pulled through the filter during the washing procedure. The filter was removed from the holder and dried under an infra-red lamp for 5 to 10 minutes. The dried filters were placed in vials containing 10 ml of scintillation fluid and counted in a Nuclear Chicago Model Mark II liquid scintillation counter.

Protein Determination

Protein was determined by the method of Lowry (30) using Bovine serum albumin as a standard.

One hundred ml of 2% Na_2CO_3 in 0.1 N NaOH were mixed with 1 ml of 1% $CuSO_4 \cdot 5H_2O$ and 1 ml of 2% sodium potassium tartrate. To 1 ml of sample were added 5 ml of this reagent. After ten minutes or more, 0.5 ml of 1 N Folin-Ciocalteau reagent were added with

immediate mixing on a vortex stirrer. After at least 30 minutes at room temperature, the absorbance was measured at 750 nm.

Chemicals and Reagents

Ammonium chloride - Baker Chemical

Ammonium sulfate - Merck

Adenosine-5'-triphosphate (ATP, disodium salt) - Sigma

^{3}H-L-Asparagine - Schwarz/Mann

Bactoagar - Difco

Bovine serum albumin - Sigma

Copper sulfate - Merck

Deoxyribonuclease (DNase) - Worthington Biochemicals

1,4-Dioxane - Mallinckrodt

Ethanol - Commercial Solvents Corporation

Folin - Ciocalteau reagent - Fischer Scientific

Glutathione - Nutritional Biochemicals

Glycerol - Mallinckrodt

Isopropanol - Mallinckrodt

^{3}H-L-Lysine - Schwarz/Mann

Magnesium acetate - Baker Chemical

Magnesium Chloride - Baker Chemical

Mercaptoethanol - Eastman Organic Chemicals

Naphthalene - Baker Chemical

Potassium acetate - Baker Chemical

Potassium Chloride - Baker Chemical

Potassium Hydroxide - Mallinckrodt

Scintillation fluid - Nuclear Equipment Chemical Corporation

Silicone antifoam - Union Carbide SAG-471

Sodium chloride - Merck

Sodium carbonate - Merck

Sodium potassium tartrate - Baker Chemical

Spermidine trihydrochloride - Nutritional Biochemicals

Streptomycin sulfate - Nutritional Biochemicals

Trichloroacetic acid (TCA) - Matheson Coleman and Bell

Trinucleotides:	AAC - P-L Biochemicals AAU - Miles	Codons for asparagine
	AAA - Sigma AAG - Miles	Codons for lysine

Tris(hydroxymethyl)aminomethane (Tris) - Sigma

Trypticase - Baltimore Biological Laboratory

Yeast extract - Difco

RESULTS AND DISCUSSION

Yield and Activity of tRNA

The yield and the amino acid acceptor activity of the tRNA are summarized in Table I.

It can be seen that the yield of tRNA was essentially the same for both organisms but that both the specific activity and the total activity were higher (approximately double) for B. stearothermophilus than for B. licheniformis.

Table II shows the specific activity of the isolated ^{3}H-L-Asparaginyl-tRNA, and ^{3}H-L-Lysyl-tRNA. The concentration of charged tRNA was estimated by spectrophotometry, assuming that 24 absorbance units at 260 nm are equivalent to 1 mg/ml tRNA. The specific activity in terms of $\mu\mu$moles of amino acid per mg tRNA was calculated with the help of the information that 0.0053 μmole of ^{3}H-L-Asparagine gave 4×10^{5} counts per minute, and 0.002 μmoles of ^{3}H-L-Lysine gave 2.76×10^{5} counts per minute.

Binding of Asparaginyl-tRNA

Requirements for the binding assay

The requirements for the binding of ^{3}H-Asn-tRNA (^{3}H-Asparaginyl-tRNA) to ribosomes by the binding assay are shown in Table III. The complete system contained the components described under methods. The incubation was at 24° for 20 minutes. It is clear that little ^{3}H-Asn-tRNA was retained on the filters after incubation in the

Table I

Yield and Amino Acid Acceptor Activity of tRNA

Organism	Yield*	Amino Acid	Specific Activity	Total Activity
	g		cpm/mg	cpm
B. licheniformis	0.66	^{3}H-Asparagine	1.80×10^{4}	1.20×10^{7}
		^{3}H-Lysine	1.02×10^{4}	0.67×10^{7}
B. stearothermophilus	0.58	^{3}H-Asparagine	5.05×10^{4}	3.33×10^{7}
		^{3}H-Lysine	1.85×10^{4}	1.22×10^{7}

* gram tRNA per 45 g of cells

Table II

Specific Activity of Isolated Aminoacyl-tRNA

Organism	Amino Acid	Specific Activity *	Specific Activity **
B. licheniformis	^{3}H-Asparagine	16,650	215
	^{3}H-Lysine	9,720	70
B. stearothermophilus	^{3}H-Asparagine	43,200	580
	^{3}H-Lysine	16,050	116

* cpm/mg

** $\mu\mu$moles of amino acid/mg tRNA

Table III

Requirements for Binding of Aminoacyl-tRNA to Ribosomes*

Modifications	Binding of Aminoacyl-tRNA ($\mu\mu$moles of ^{3}H-Asparaginyl-tRNA bound/mg ribosomal protein) B. lich.**	B. 10***
Complete system	29	66
- AAC	2.5	5.0
- Ribosomes	0	0
- Mg^{++}	0.85	2.45
Complete system	128	63
- AAU	6.5	4.5
- Ribosomes	0	0
- Mg^{++}	2.0	1.6

*Conditions for this binding assay are described under effect of magnesium concentration on page 23

**B. lich. (B. licheniformis)

***B. 10 (B. stearothermophilus)

absence of either an asparagine codon (AAU or AAC), ribosomes or magnesium ions. All three of these components were required for binding and therefore for retention of ^{3}H-Asn-tRNA on millipore filters.

The counts per minute were converted to $\mu\mu$moles of ^{3}H-Asn-tRNA bound to 4.5 absorbance units at 260 nm of ribosomes. These data were subsequently changed to $\mu\mu$moles of ^{3}H-Asn-tRNA bound per mg ribosomal protein using the information that 4.5 A_{260} units of B. licheniformis and B. stearothermophilus ribosomes are equal to 0.040 mg and 0.033 mg ribosomal protein respectively. The values have been corrected for blanks, that is binding in the absence of magnesium ions which accounted to at most 5% of the total counts (Table III).

Effect of magnesium concentration

The codons used for asparagine were AAC, AAU and the effect of Mg^{++} concentration on the binding of ^{3}H-Asn-tRNA in the presence of these codons is shown in Figures 1 and 2. Each point represents a 100 μl reaction mixture containing 18.3 $\mu\mu$moles of B. stearothermophilus ^{3}H-Asn-tRNA (1372 counts per minute, 0.73 A_{260} units) and 13.4 $\mu\mu$moles of B. licheniformis ^{3}H-Asn-tRNA (1035 counts per minute, 1.43 A_{260} units). The other components of the reaction mixture were the same as described under methods.

The binding in response to codon AAC is shown in Figure 1. Maximum binding was observed at 0.02 M Mg^{++} both in the case of B. licheniformis and B. stearothermophilus. An increase in the

Figure 1

Binding of Asparaginyl-tRNA as a Function of Magnesium Concentration

Codon AAC

Incubation temperature 24°

Incubation time 20 min.

△—△ B. stearothermophilus

○—○ B. licheniformis

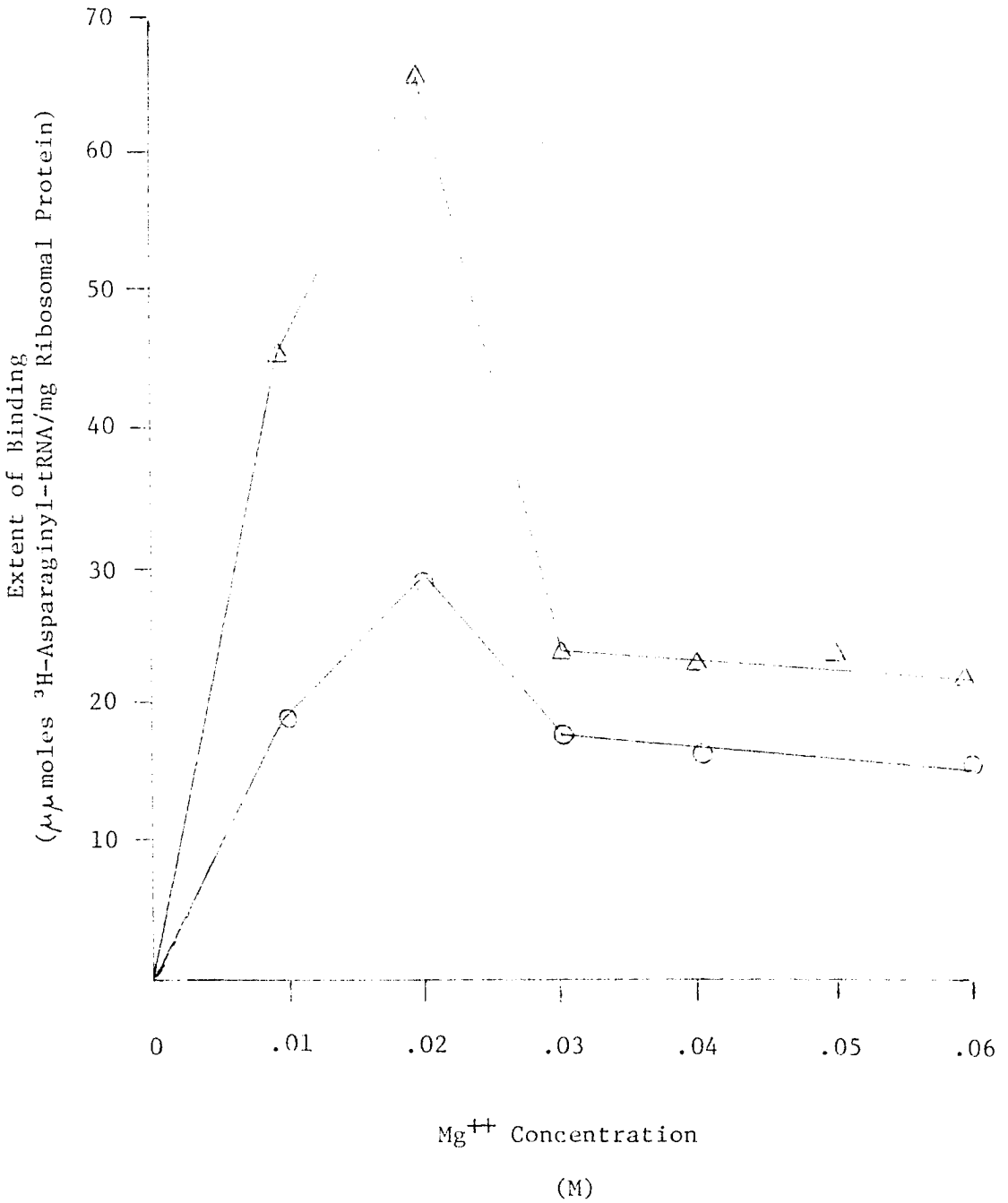


Figure 2

Binding of Asparaginyl-tRNA as a Function of Magnesium Concentration

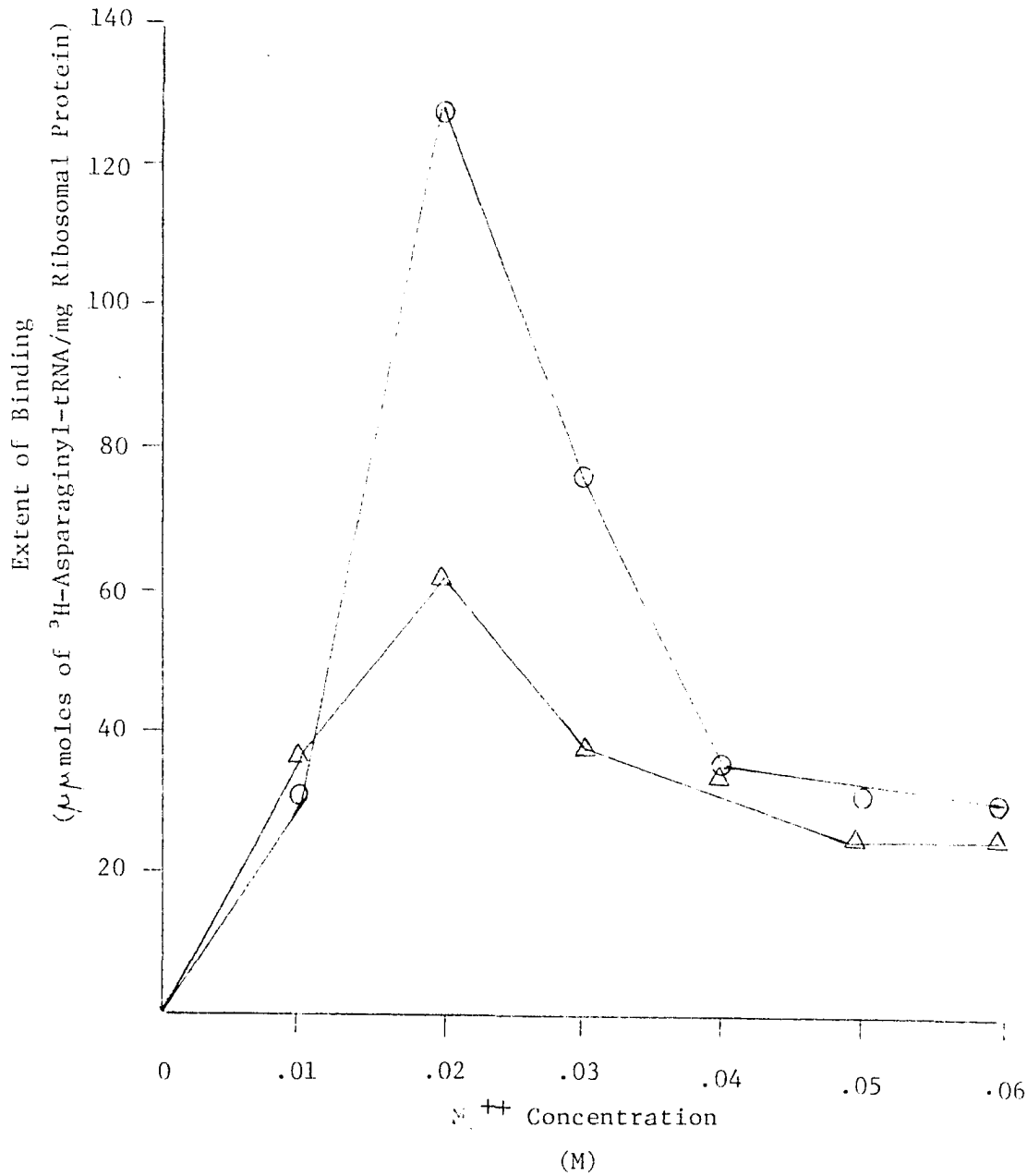
Codon AAU

Incubation temperature 24°

Incubation time 20 min.

△—△ B. stereothermophilus

○—○ B. licheniformis



Mg^{++} concentration from 0.02 M to 0.03 M resulted in a sharp decline in the binding of ^{3}H-Asn-tRNA to ribosomes of B. stearothermophilus and to a somewhat smaller decline in B. licheniformis. At Mg^{++} concentrations above 0.02 M, the binding was essentially constant for both B. stearothermophilus and B. licheniformis. The decline is probably due to changes in the state of aggregation of the ribosomes as a function of (Mg^{++}).

Figure 2 shows the dependence on Mg^{++} concentration of the binding of ^{3}H-Asn-tRNA to ribosomes in the presence of the codon AAU.

The curves are similar to those in Figure 1 with respect to the optimal magnesium concentration (0.02 M). The curves levelled off at a somewhat higher magnesium concentration (0.04 M), but the major difference between Figures 1 and 2 is the fact that the binding in response to codon AAU was about twice as great for B. licheniformis as it was for B. stearothermophilus (i.e. the reverse of that in Figure 1). However, the amount of ^{3}H-Asn-tRNA used with B. licheniformis ribosomes was double that used with B. stearothermophilus ribosomes (1.43 and 0.73 A_{260} units respectively). If we divide the amount of ^{3}H-Asn-tRNA bound to B. licheniformis ribosomes at 0.02 M by two, the activity of Asn-tRNA in binding to B. licheniformis ribosomes is approximately equal to that of its binding to B. stearothermophilus ribosomes in response to codon AAU and one fourth in response to codon AAC. Thus, considering the two synonym codons for asparagine, the mesophilic system preferentially uses AAU over AAC. It is tempting to speculate that this may be a reflection of the

base composition of the DNA which has been shown to have a higher A-T content in the mesophiles than in the thermophiles (25).

Effect of time

Figure 3 shows the effect of time on the binding of ^{3}H-Asn-tRNA to ribosomes in response to the AAC codon. The reaction mixture used was that described under methods. The reaction was completed within 40 minutes for B. licheniformis and 50 minutes for B. stearothermophilus, but maximum binding occurred after 20 minutes in both systems.

When the codon AAU was used (Figure 4), maximum binding was again obtained after 20 minutes and the reaction levelled off after 30 minutes in the case of B. stearothermophilus and 40 minutes in the case of B. licheniformis. It is interesting to note that essentially equal amounts were bound at 10 and 30 minutes in both the B. licheniformis and the B. stearothermophilus system.

Effect of temperature

The dependence of the binding on the incubation temperature in response to codon AAC is shown in Figure 5. The optimal binding for the mesophile was at 24° while that for the thermophile was at 55°. These results are in agreement with earlier studies in this laboratory which showed that thermophiles carry out in vitro protein synthesis at higher temperatures than mesophiles. While the stability of the ^{3}H-Asn-tRNA-ribosome-mRNA complex for B. stearothermophilus decreased sharply above 55°, the actual number of mole of

Figure 3

Binding of Asparaginyl-tRNA as a Function of Incubation Time

Codon AAC

Incubation temperature 24°

Mg^{++} concentration 0.02 M

△—△ B. stearothermophilus

○—○ B. licheniformis

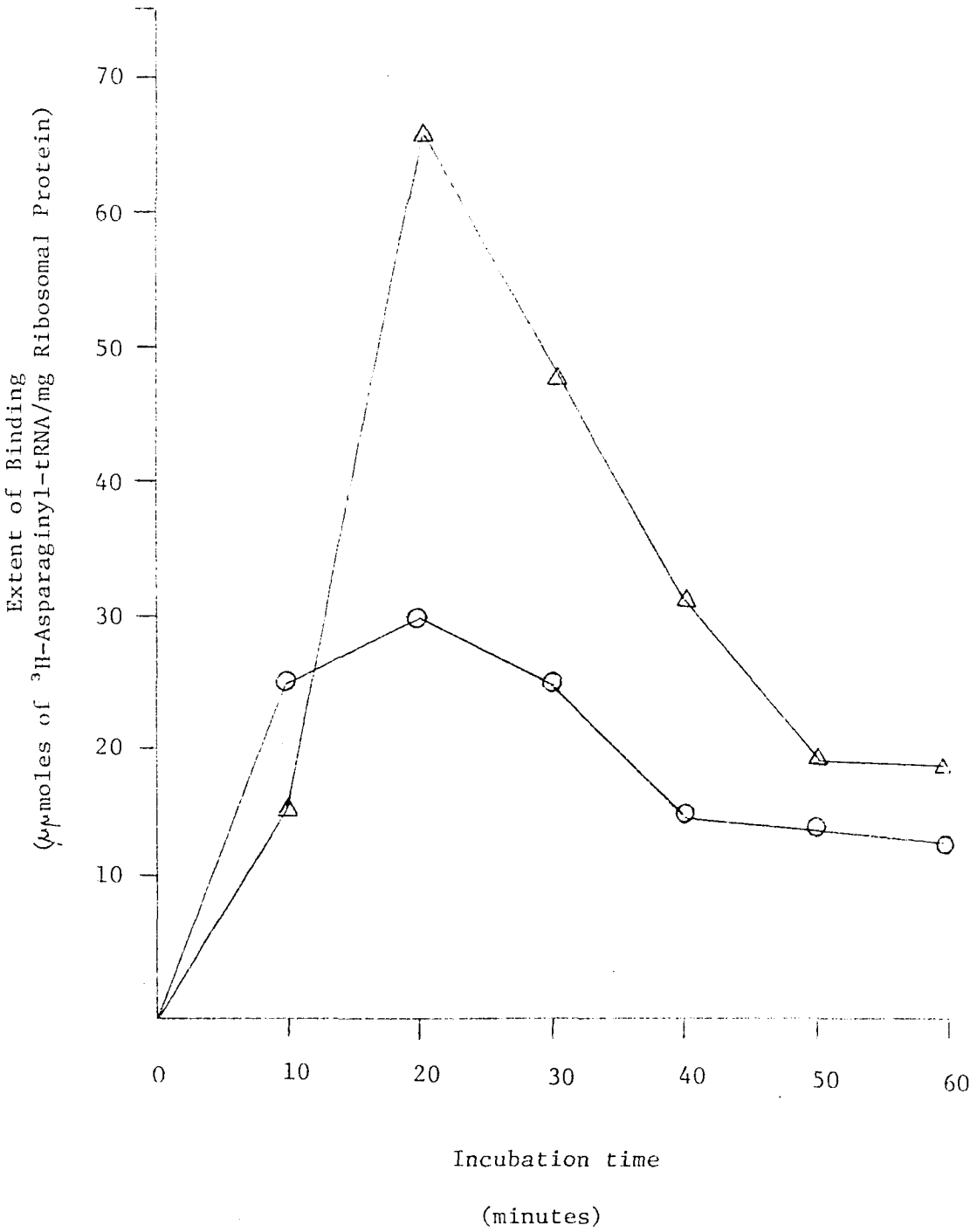


Figure 4

Binding of Asparaginyl-tRNA as a Function of Incubation Time

Codon AAU

Incubation temperature 24°

Mg^{++} concentration 0.02 M

△—△ B. stearothermophilus

○—○ B. licheniformis

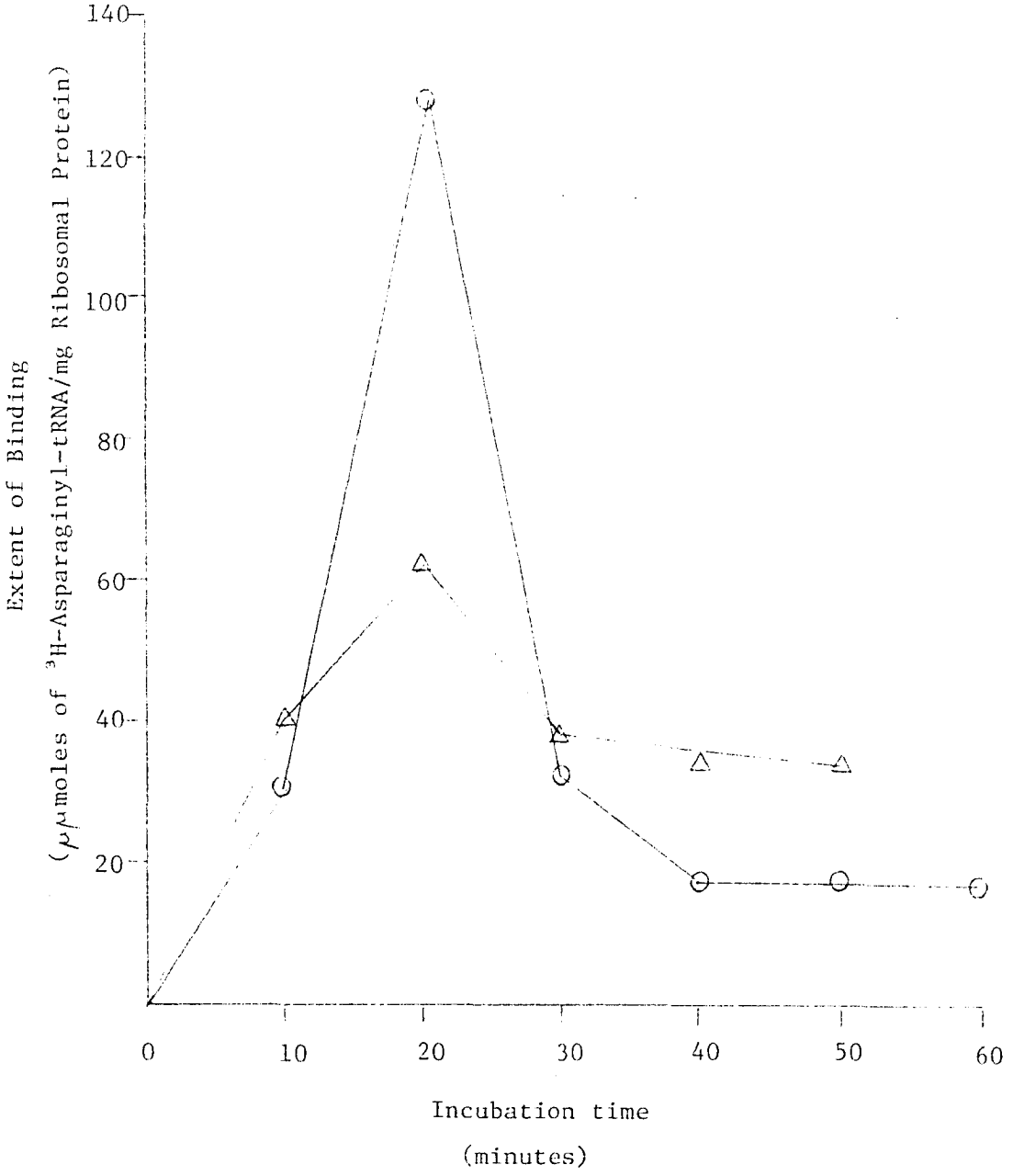


Figure 5

Binding of Asparaginyl-tRNA as a Function of Incubation Temperature

Codon AAC

Incubation time 20 min.

Mg^{++} concentration 0.02 M

△—△ B. stearothermophilus

○--○ B. licheniformis

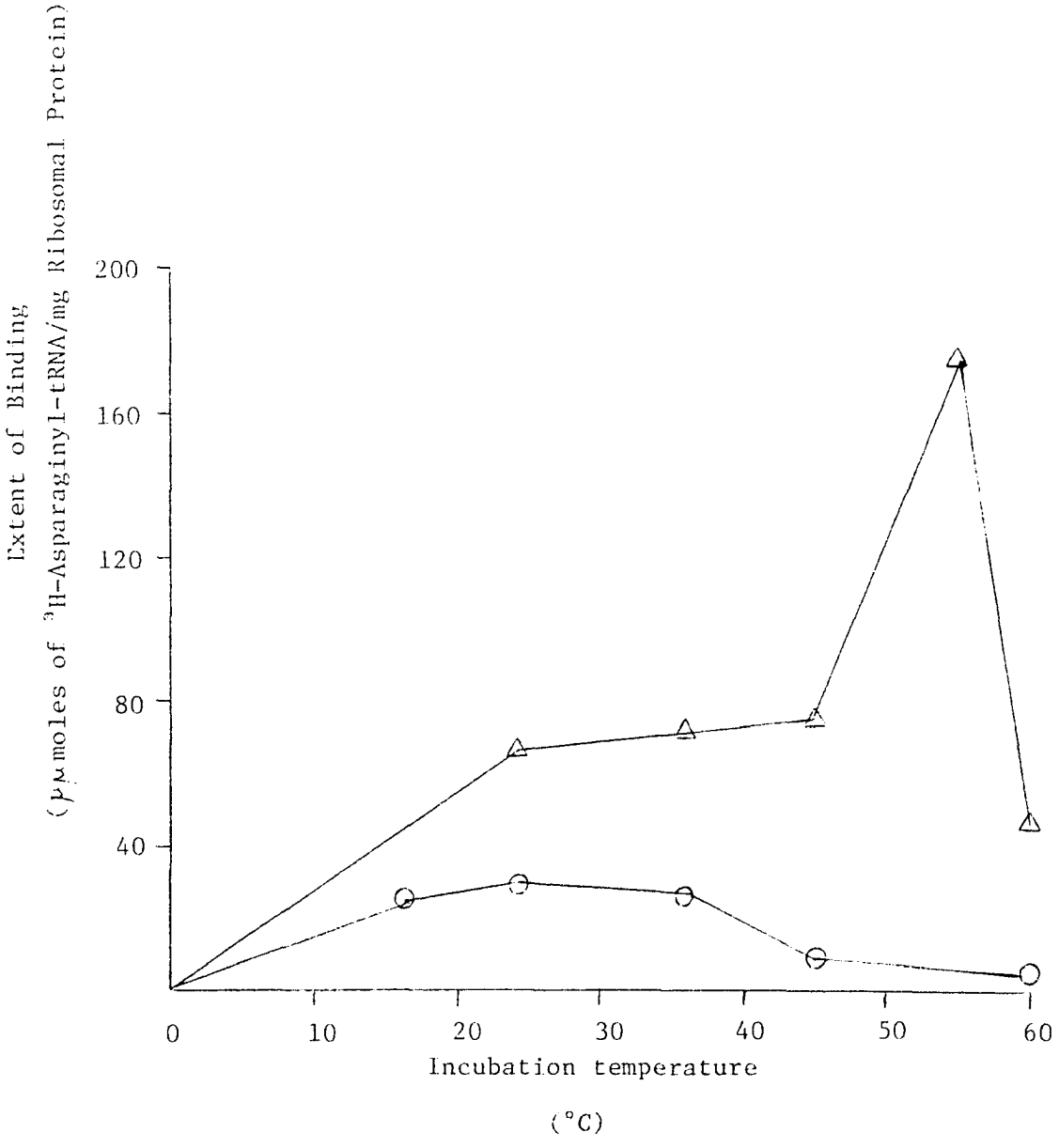


Figure 6

Binding of Asparaginyl-tRNA as a Function of Incubation Temperature

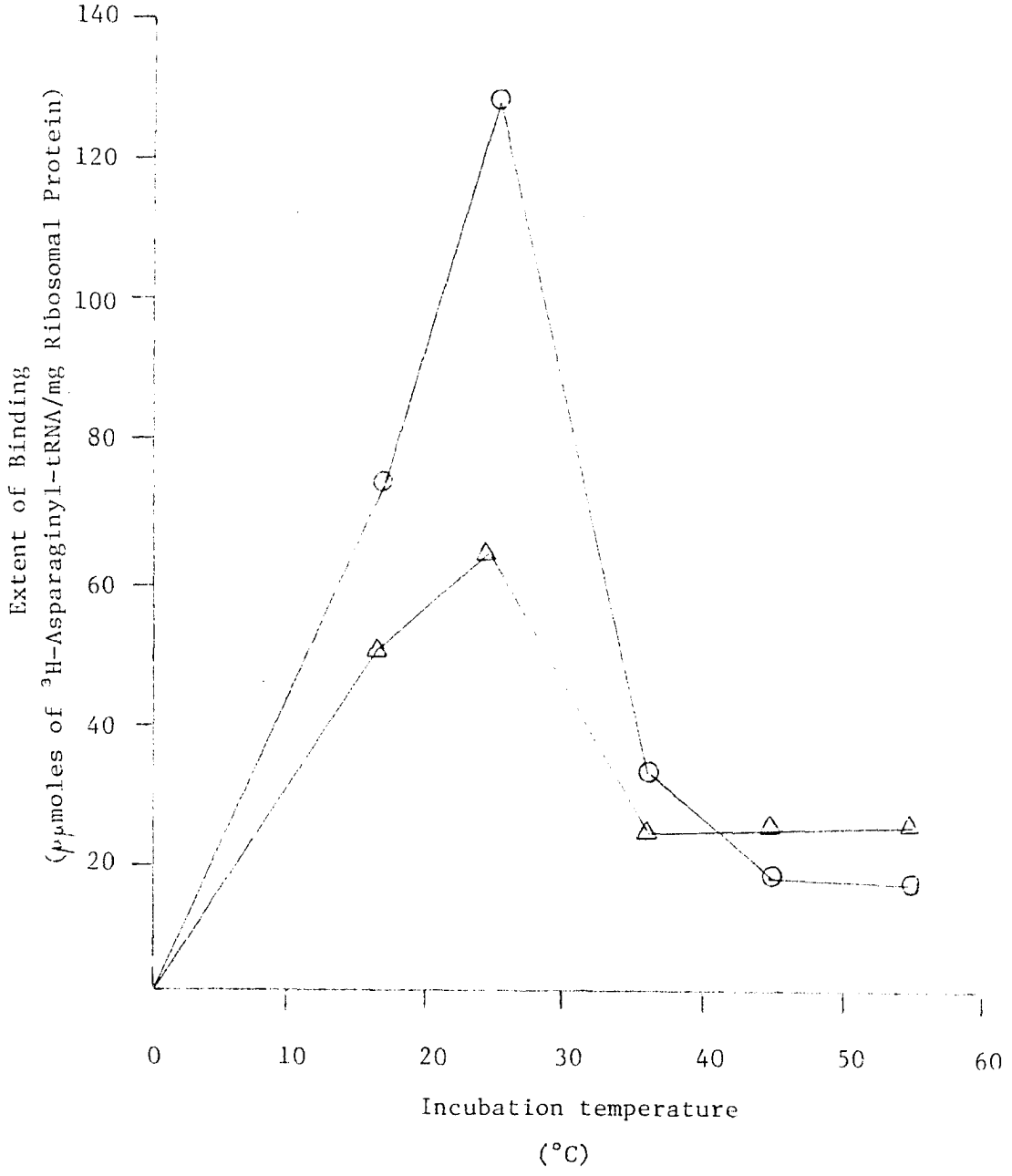
Codon AAU

Incubation time 20 min.

Mg^{++} concentration 0.02 M

△—△ B. stearothermophilus

○—○ B. licheniformis



^{3}H-Asn-tRNA bound/mg ribosomal protein at 60° was approximately equal to that bound at 18°. There was no binding of B. licheniformis ^{3}H-Asn-tRNA to ribosomes above 37°, to any significant extent.

The dependence of the binding on the incubation temperature in response to codon AAU is shown in Figure 6. The binding for B. licheniformis was again optimal at 24° and very limited above 37°. The results for B. stearothermophilus were, however, strikingly different from those shown in Figure 5. The optimum temperature for binding was clearly at 24°, rather than at 55° and the increased binding at 45° and 55° over that of B. licheniformis system was insignificant.

Thus the results for binding of asparaginyl-tRNA show that not only does the mesophilic system preferentially uses codon AAU over AAC but also that if codon AAU is used, even the thermophilic system uses it best at lower temperatures. In other words AAU, as compared to AAC, is clearly a mesophilic (low temperature) codon. Since the AAU codon would base pair with a UUA anticodon in tRNA by means of 6 hydrogen bonds while an AAC codon would pair with GUU anticodon via 7 hydrogen bonds, the former would indeed be expected to be less stable at higher temperatures.

The binding data for asparaginyl-tRNA is summarized in Table IV.

Binding of Lysyl-tRNA

Requirements for the binding assay

The second aminoacid used in this study, lysine can be coded by the two triplets AAA and AAG. The requirements for the binding

Table IV

Binding of ^{3}H-Asparaginyl-tRNA to Ribosomes

Codon	Variable	Optimum		Binding*	
		M	T	M	T
AAC	Mg^{++} (M)	0.02	0.02	8.7	12.0
	Time (min.)	20	20	8.7	12.0
	Temperature (°)	24	55	8.7	32.4
AAU	Mg^{++} (M)	0.02	0.02	38.2	11.4
	Time (min.)	20	20	38.2	11.4
	Temperature (°)	24	24	38.2	11.4

* Percent of added ^{3}H-asparaginyl-tRNA bound per mg of ribosomal protein; M refers to the mesophile (B. licheniformis) and T to the thermophile (B. stearothermophilus).

assay using ^{3}H-Lys-tRNA (^{3}H-Lysyl-tRNA) and ribosomes from B. licheniformis and B. stearothermophilus are shown in Table V. The complete reaction mixture contained 6.96 μμmoles of B. stearothermophilus ^{3}H-Lys-tRNA (963 counts per minute, 1.4 A_{260} units) or 7.00 μμmoles of B. licheniformis ^{3}H-Lys-tRNA (972 counts per minute, 2.4 A_{260} units) and the components described under methods. The data of Table V show that little ^{3}H-Lys-tRNA was bound to ribosomes in the absence of codons or magnesium ions as was the case when ^{3}H-asparaginyl-tRNA was used as described in the previous section. In contrast to the data in Table III the data in Table V indicate that with respect to binding of lysyl-tRNA both codons led to essentially the same binding by the mesophilic and the thermophilic system.

Effect of magnesium concentration

The variation of binding of ^{3}H-lysyl-tRNA to ribosomes as a function of Mg^{++} concentration in the presence of codon AAA is shown in Figure 7. Each point represents a 100 μl reaction mixture as described above. The same procedure as described in the case of ^{3}H-asparaginyl-tRNA was used to convert counts per minute to μμmoles of ^{3}H-lysyl-tRNA bound per mg of ribosomal protein.

As in the case of asparaginyl-tRNA essentially no binding (5% at most) was observed in the absence of Mg^{++} ions as shown in Table V. The maximum amount of ^{3}H-lysyl-tRNA bound to ribosomes in both organisms was observed at a Mg^{++} concentration of 0.02 M. Increasing the Mg^{++} concentration from 0.02 M to 0.03 M resulted in a large decrease of binding in both systems and the binding levelled

Table V

Requirements for Binding of Aminoacyl-tRNA to Ribosomes*

Modifications	Binding of Aminoacyl-tRNA ($\mu\mu$moles of ^{3}H-Lysyl-tRNA bound/mg ribosomal protein) B. lich.**	B. 10***
Complete system	44.00	58.50
- AAA	2.00	2.25
- Ribosomes	0	0
- Mg^{++}	1.50	1.20
Complete system	17.20	22.20
- AAG	1.35	2.50
- Ribosomes	0	0
- Mg^{++}	0.92	1.12

*Conditions for this binding assay are described under requirements for binding assay on page 40

**B. lich. (B. licheniformis)

***B. 10 (B. stearothermophilus)

Figure 7

Binding of Lysyl-tRNA as a Function of Magnesium Concentration

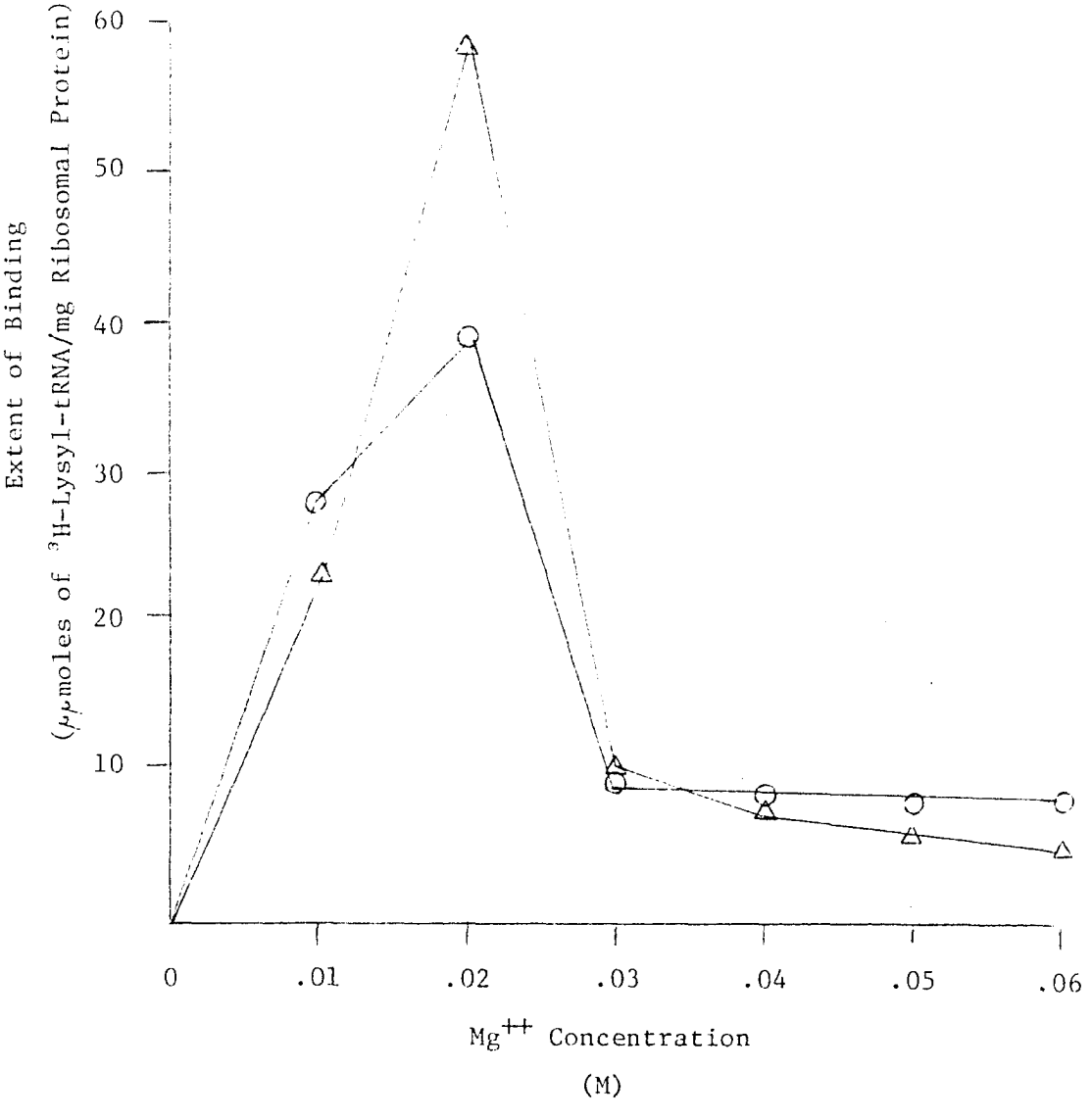
Codon AAA

Incubation temperature 24°

Incubation time 20 min.

△— △ B. stearothermophilus

○— ○ B. licheniformis



off at a magnesium concentration of 0.03 M. These results are similar to those obtained in the binding of ^{3}H-asparaginyl-tRNA to ribosomes in the two systems.

Figure 8 shows the effect of Mg^{++} concentration on binding in response to the codon AAG. Maximum binding was again at a molarity of 0.02 in both organisms. As before the binding decreases when the Mg^{++} concentration is raised to 0.03 M and levels off at that point.

It can be seen from Figures 7 and 8 that both AAA and AAG lead to binding of B. stearothermophilus ^{3}H-lysyl-tRNA, which is greater than that of B. licheniformis ^{3}H-lysyl-tRNA. No inversion of the relative extent of binding was noted here as was observed with the codons AAC and AAU for asparaginyl-tRNA. The reason that AAA does not appear to be a mesophilic codon as compared to AAG (as AAU was compared to AAC) might be due to unusually tight binding to ribosomes of the homotrinucleotide AAA as compared to AAG. This is supported by the finding (Thesis, Schechter) that poly . is a more effective synthetic messenger in in vitro amino acid incorporating systems than copolymers.

Effect of time

The binding of ^{3}H-lysyl-tRNA to ribosomes as a function of time in the presence of codons AAA and AAG is shown in Figures 9 and 10. As in the case of asparaginyl-tRNA, maximum binding was observed at 20 minutes and the reaction levelled off after about 30-40 minutes. This was true for both B. licheniformis and B. stearothermophilus.

Figure 8

Binding of Lysyl-tRNA as a Function of Magnesium Concentration

Codon AAG

Incubation temperature 24°

Incubation time 20 min.

△—△ B. stearothermophilus

○—○ B. licheniformis

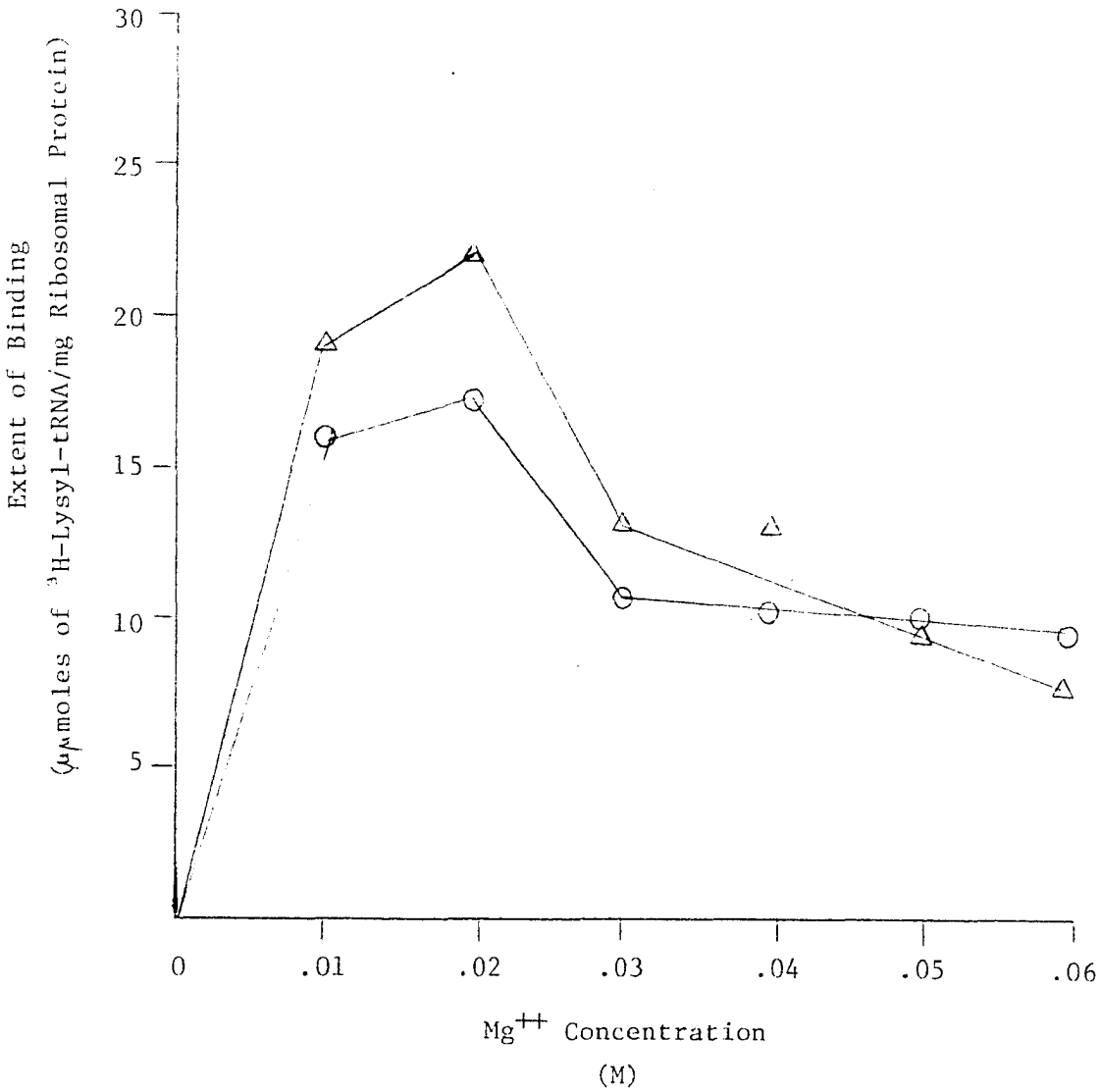


Figure 9

Binding of Lysyl-tRNA as a Function of Incubation Time

Codon AAA

Incubation temperature 24°

Mg^{++} concentration 0.02 M

△—△ B. stearothermophilus

○—○ B. licheniformis

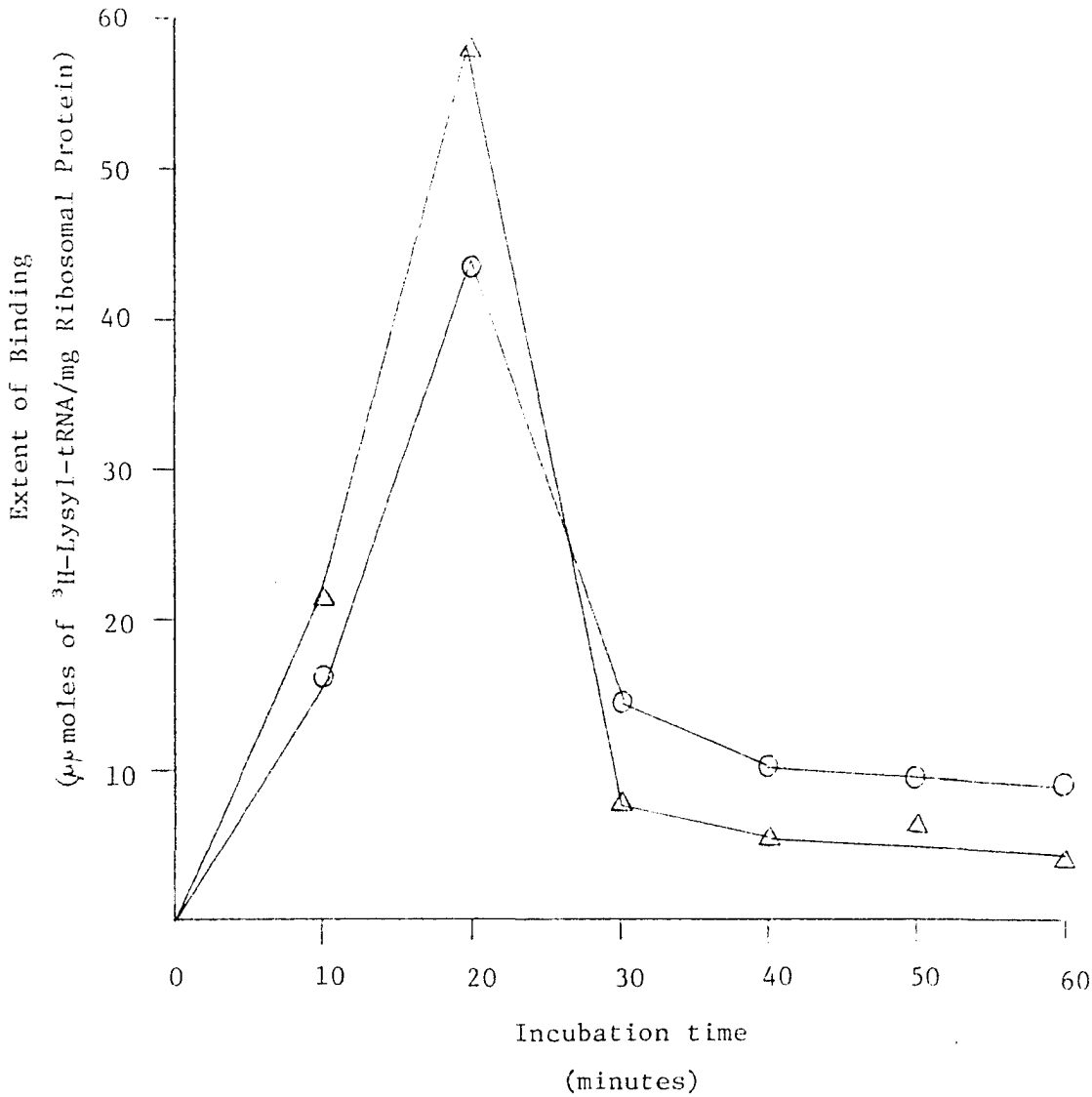


Figure 10

Binding of Lysyl-tRNA as a Function of Incubation Time

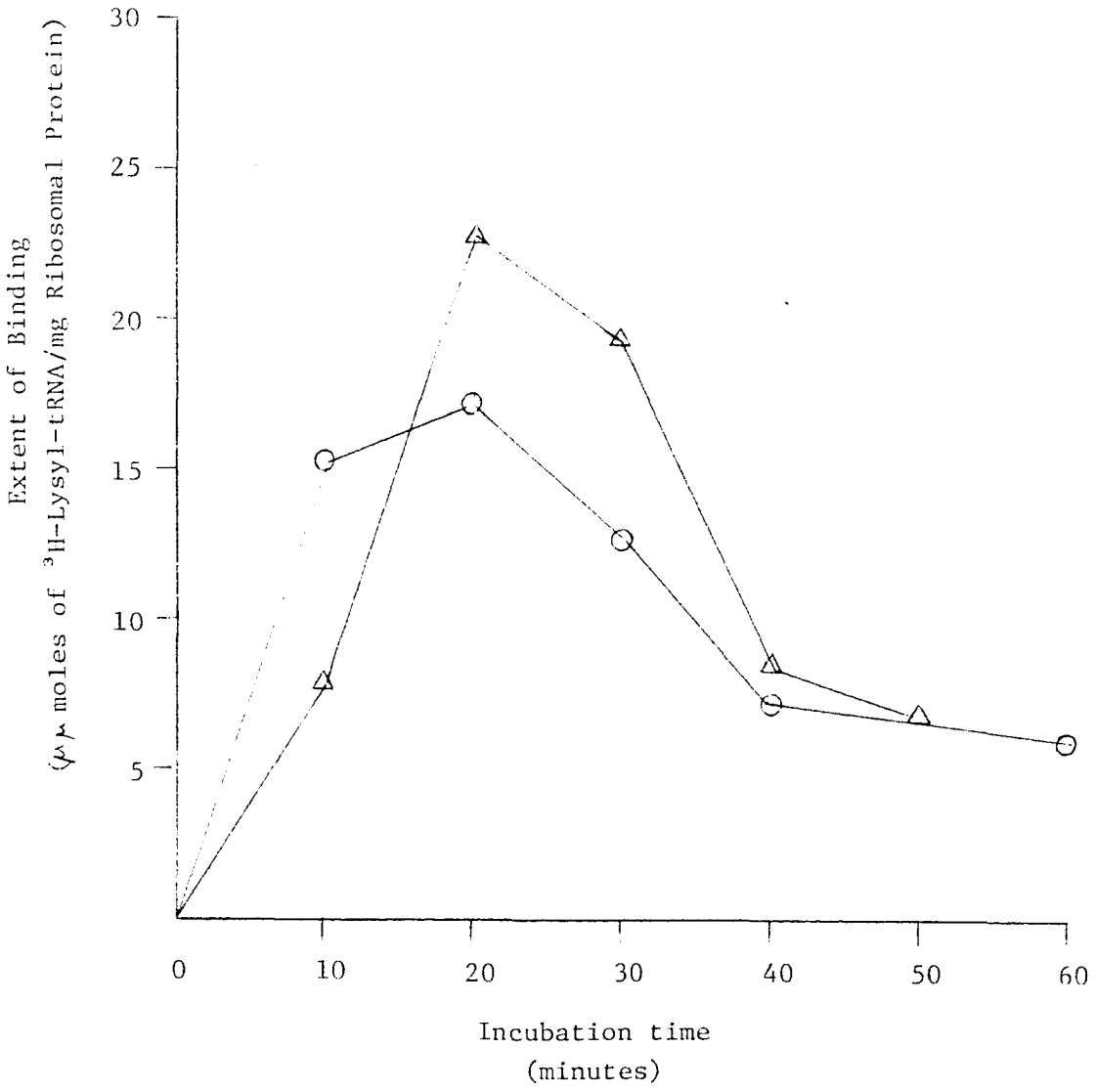
Codon AAG

Incubation temperature 24°

Mg^{++} concentration 0.02 M

△—△ B. stearothermophilus

○—○ B. licheniformis



Effect of temperature

The dependence of the binding on the incubation temperature in response to codons AAA and AAG is shown in Figures 11 and 12, respectively. Each point represents a 100 µl reaction mixture as described before, at a magnesium concentration of 0.02 M and using an incubation time of 20 minutes.

The results in Figure 11 are similar to those in Figure 5 and show that the mesophilic system bound the lysyl-tRNA optimally at 24° while the thermophilic system bound optimally at 55°. This pattern is in agreement with the previously indicated finding that AAA did not act as a ''mesophilic'' codon and hence would indeed not be expected to lead to maximal binding at low temperatures in thermophilic systems (in contrast to AAU, see Figure 6).

The results in Figure 12 are generally similar to those in Figure 11 except that the thermophilic system did not exhibit a well defined optimal temperature (conceivably this lies above 55°) but rather a broad peak between 24-55°. Likewise, the binding in the mesophilic system did not drop off as rapidly and sharply beyond 24°.

The binding data for lysyl-tRNA are summarized in Table VI.

Figure 11

Binding of Lysyl-tRNA as a Function of Incubation Temperature

Codon AAA

Incubation time 20 min.

Mg^{++} concentration 0.02 M

△—△ B. stearothermophilus

○—○ B. licheniformis

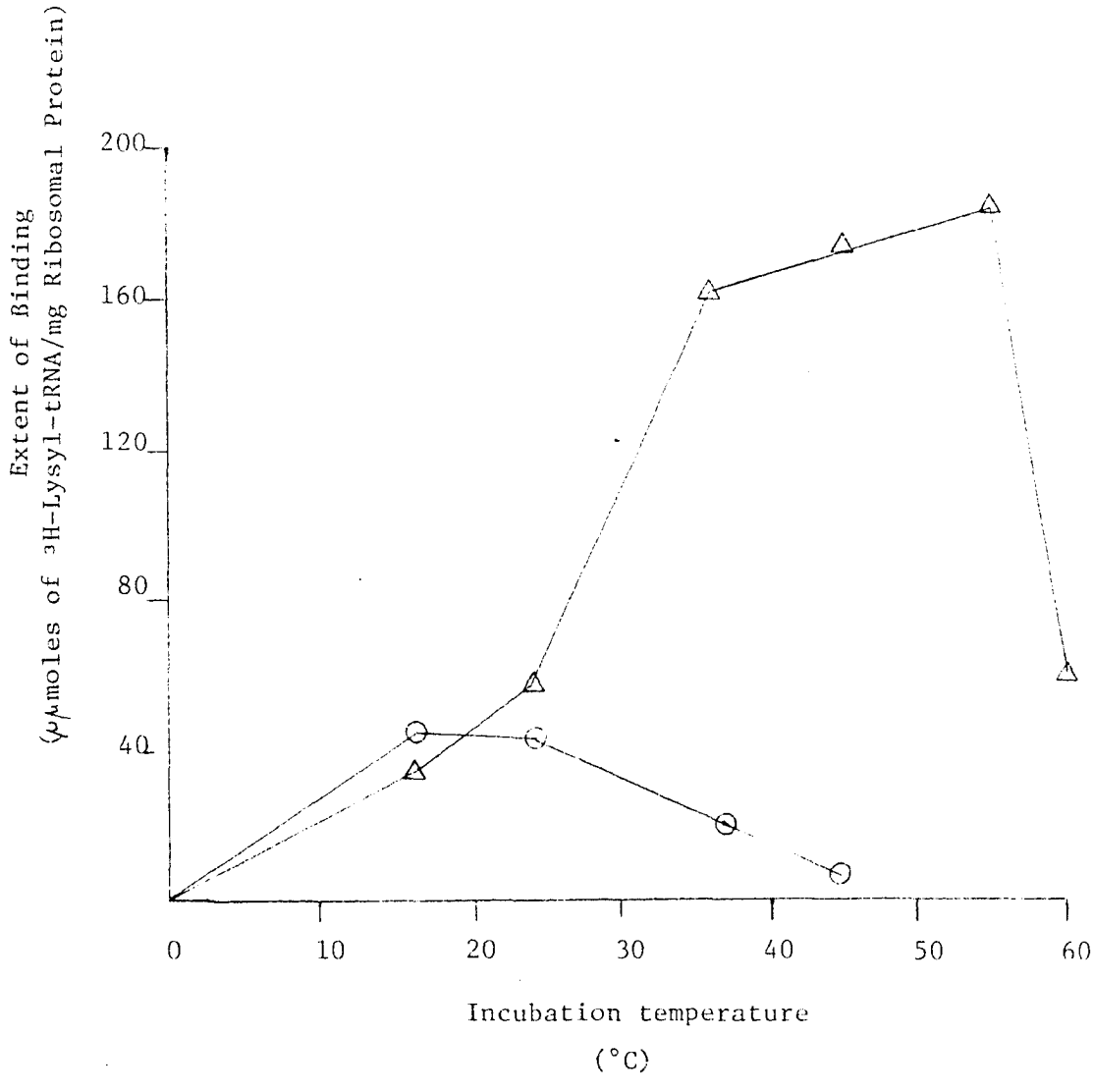


Figure 12

Binding of Lysyl-tRNA as a Function of Incubation Temperature

Codon AAG

Incubation time 20 min.

Mg^{++} concentration 0.02 M

△—△ B. stearothermophilus

○—○ B. licheniformis

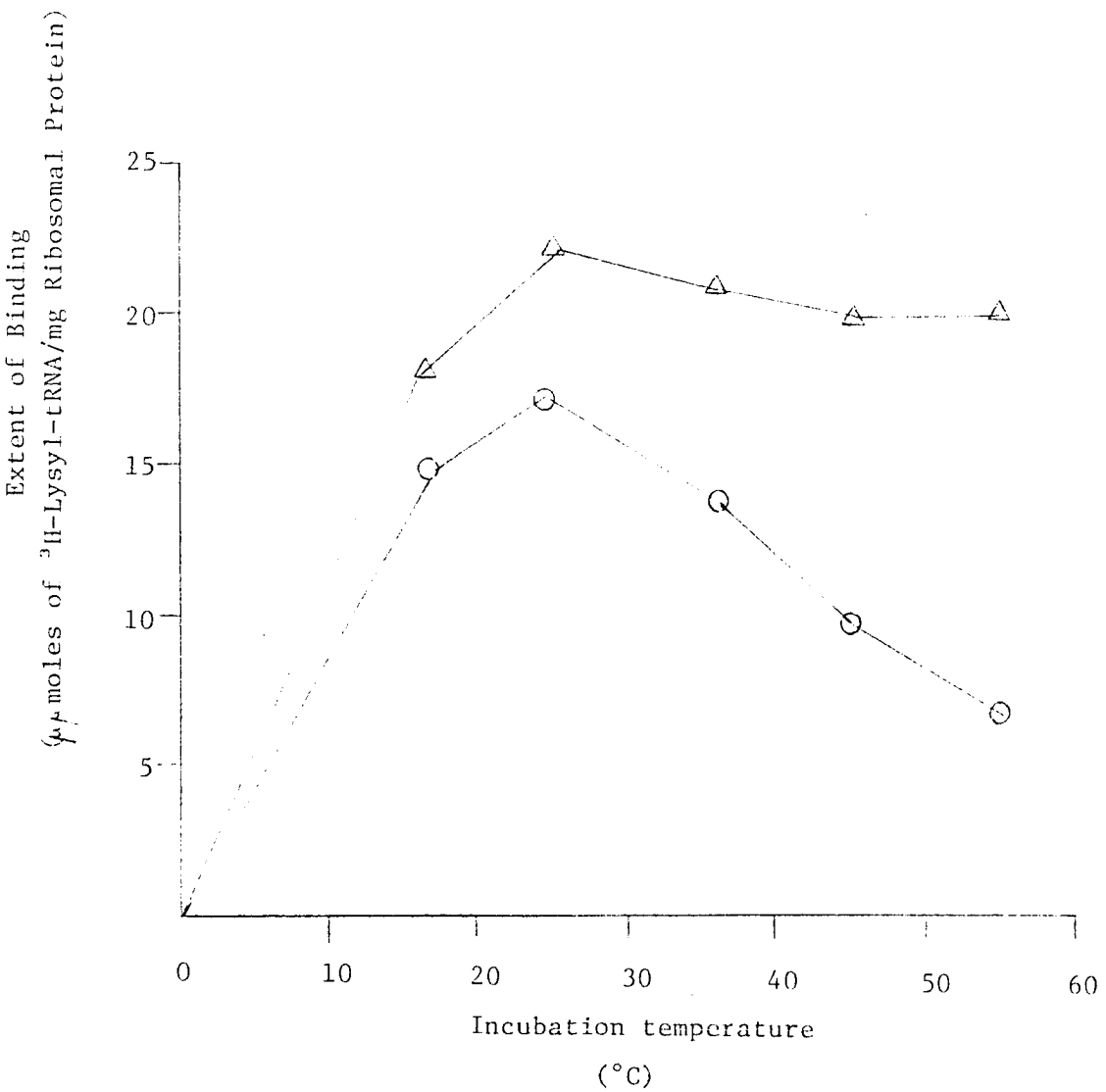


Table VI

Binding of ^{3}H-Lysyl-tRNA to Ribosomes

Codon	Variable	Optimum		Binding*	
		M	T	M	T
AAA	Mg^{++} (M)	0.02	0.02	22.6	28.1
	Time (min.)	20	20	22.6	28.1
	Temperature (°)	24	55	22.6	88.5
AAG	Mg^{++} (M)	0.02	0.02	9.8	10.5
	Time (min.)	20	20	9.8	10.5
	Temperature (°)	24	24	9.8	10.5

* Percent of added ^{3}H-asparaginyl-tRNA bound per mg of ribosomal protein; M refers to the mesophile (B. licheniformis) and T to the thermophile (B. stearothermophilus).

SUMMARY

Transfer RNA, aminoacyl-tRNA synthetase and washed ribosomes were isolated from the mesophile B. licheniformis and the thermophile B. stearothermophilus. The tRNA was charged with ^{3}H-Asparagine and ^{3}H-Lysine to give labelled aminoacyl-tRNA.

The binding of both ^{3}H-asparaginyl-tRNA and ^{3}H-lysyl-tRNA to ribosomes required a codon (trinucleotide) as well as an appropriate Mg^{++} concentration in both organisms. In the presence of synonym codons for the two amino acids, maximum binding was observed at a Mg^{++} concentration of 0.02 M after a 20 minute incubation.

The mesophilic system preferentially used the codon AAU over AAC in the binding of asparaginyl-tRNA whereas with respect to the binding of lysyl-tRNA both codons (AAA and AAG) led to essentially the same binding by the two systems. Both AAA and AAG led to binding of B. stearothermophilus ^{3}H-lysyl-tRNA, which was greater than that of B. licheniformis ^{3}H-lysyl-tRNA. Binding was also found to be temperature dependent.

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VITA

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