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# Cell Free Amino Acid Incorporating Systems from Mesophilic and Thermophilic Bacteria

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# CELL FREE AMINO ACID INCORPORATING SYSTEMS FROM MESOPHILIC AND THERMOPHILIC BACTERIA

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# INTRODUCTION

Amino acid incorporation had first been demonstrated with systems containing naturally occurring messenger RNA (1). Later on it was shown that amino acid incorporation could also occur in the presence of synthetic messenger RNA (2,3). Such synthetic messenger RNA's (synthetic polyribonucleotides) were found to code for specific amino acids. For example, polyuridylic acid (poly U) codes for the incorporation of phenylalanine. In this way it has become possible to assign a codon, or nucleotide triplet, in messenger RNA which codes for a particular amino acid (4). This assignment of codons for amino acids is known as the genetic code. The evidence for these codon assignments comes mainly from work with cell-free extracts of Escherichia coli. However, it is likely that the genetic code of other organisms (5) is similar to the genetic code of E. coli. On the other hand, it is known that the code is degenerate and ambiguous. The former refers to the fact that a given amino acid can be coded by more than one codon. Ambiguity refers to the fact that mistakes can occur in the coding by a particular codon as a result of variations in the experimental conditions of in vitro studies (6).

Cell-free amino acid incorporating systems have also been prepared from other bacteria. These include several strains of Bacillus such as B. brevis (7), B. cereus (8, 9, 10), and B. subtilis (11). Studies involving these various bacterial systems, as well as those from plant and animal sources, have contributed greatly to the development of the currently accepted model for protein synthesis.

In our laboratory we have been interested in a comparison of mesophilic and thermophilic bacteria. Thermophiles are bacteria which grow at relatively high temperatures (about 55-80°C) as compared to mesophiles which grow at moderate temperatures (about 20-40°C). A working hypothesis for the explanation of the phenomenon of thermophily ascribes the latter to physical-chemical differences on the macromolecular level. Both proteins and nucleic acids have been investigated in this connection and a few studies have appeared on the respective protein synthesizing systems of these two groups of organisms. However, so far these studies have been limited to a comparison of protein synthesizing systems from mesophilic strains of E. coli with thermophilic strains of Bacillus (6, 12). Since these organisms represent different genera, the possibility of intergeneric differences cannot be ruled out. For this reason the present investigation was undertaken. The purpose of this research was to isolate and

partially characterize a cell-free amino acid incorporating system from both a mesophilic and a thermophilic strain of one genus, namely Bacillus. The strains chosen for this purpose, were the mesophile B. licheniformis and the thermophile B. stearothermophilus 10.

# MATERIALS AND METHODS

## Organisms and Growth Conditions

A total of four bacterial strains, three mesophiles and one thermophile, were used in this study. The former included B. licheniformis (NRS 243), E. coli, and B. subtilis. The thermophile was B. stearothermophilus 10.

Initially cells of B. licheniformis were grown in slants and large petri dishes on a medium containing 2% Bacto agar (Difco), 1% Trypticase (BBL), and 0.2% yeast extract (Difco). Slants which contained in addition to the above 10 p.p.m. of manganese were used for growing the stock cultures. Slants were grown to the logarithmic phase and the growth was suspended in about 6 ml of sterile water. Two ml of this suspension were used to inoculate one petri dish. The petri dishes were incubated for approximately 6 hr at 37°C and the cells were harvested in the mid-log phase.

Later it became apparent that large amounts of cells would be required for this research. Accordingly, cells of B. licheniformis were grown at 37°C in a liquid medium consisting of 1% trypticase and 0.2% yeast extract. This was carried out by the Upjohn Company in Kalamazoo, Michigan. The cells were harvested in the late-log phase. The yield

was approximately 2,000 g of cells (wet weight) from 500 l of medium. The cells were washed in buffer A, frozen in liquid nitrogen, and stored at -20°C. These were the cells used for the present research.

Cells of B. stearothermophilus 10 were grown in a similar manner in the same liquid but at 55°C. Again this was performed by the Upjohn Company. These cells were harvested in the early-log phase. The yield was approximately 125 g of cells (wet weight) from 500 l of medium. The cells were washed in buffer A, frozen in liquid nitrogen, and stored at -20°C.

Cells of E. coli and B. subtilis, both harvested in the late-log phase, were purchased from General Biochemicals.

## Apparatus

All spectrophotometric measurements were made in a Zeiss model PMQ-II spectrophotometer. A Sorvall model RC-2 refrigerated centrifuge was used for low speed centrifugations (15,500 r.p.m. and below), and a Spinco model L preparative ultracentrifuge was used for high speed centrifugations (above 15,500 r.p.m.). All analytical ultracentrifugation studies were made in a Spinco model E analytical ultracentrifuge, equipped with rotor temperature indicator control, and with both Schlieren and ultraviolet optics. All runs were made between 4° and 10°C. Measure-

ments of the photographic plates were made with a Nikon model 6C microcomparator. Area measurements of ultracentrifuge patterns were made with a Gelman model 39231 planimeter.

Radioactivities of washed protein precipitates were counted in a Nuclear Chicago model 4338 thin-window gas-flow automatic planchette counter.

## Chemicals

Two types of aluminum oxide were used in this study; one was a chromatographic grade (Merck 71707), and the other was a bacteriological grade (Alcoa A-305). Chemicals included the following: magnesium acetate (Matheson Coleman and Bell CB 482), 2-mercaptoethanol (Eastman 4196), potassium chloride (Baker and Adamson 2150), polyvinylsulfuric acid, potassium salt (Eastman 8587), trichloroacetic acid (TCA Mallinckrodt 2928), spermidine trihydrochloride (Nutritional Biochemicals 7319).

Adenosine-5'-triphosphate (ATP, disodium salt), guanosine-5'-triphosphate (GTP, disodium salt), phosphoenolpyruvate kinase (PEP kinase, 10 mg/ml of a suspension in $(NH_4)_2SO_4$), and phosphoenolpyruvate (PEP, tricyclohexylamine salt), were all obtained from Sigma. The phosphoenolpyruvate salt was converted to the free acid form by the use of a washed Dowex (50w-x2; 100-200

mesh) column of approximately 2.5 x 15 cm as described by Nirenberg (13). The solution was stored in small aliquots at -20°C.

Stripped soluble ribonucleic acids (s-RNA) from E. coli, strain B, and B. subtilis were obtained from General Biochemicals. The s-RNA was dissolved in water (20 mg/ml) and stored in small aliquots at -20°C.

Polyuridylic acid (poly U) was purchased from Miles Laboratories as the ammonium salt. The poly U was dissolved in distilled water (5 mg/ml) and stored in small aliquots at -20°C. The distilled water was distilled twice more, the first time from alkaline $KMnO_4$ (96 g NaOH and 5 g $KMnO_4$ eiluted to 1 l; 1 ml was used per 1 of distilled water). $C^{14}$ phenylalanine (phe-$C^{14}$, uniformly labeled, specific activity 10 mc/mM, in 0.01 M HCl) was obtained from International Chemical and Nuclear Corp.

## Reagents

Buffer A consisted of 0.01 M tris(hydroxymethyl) aminomethane, 0.01 M magnesium acetate, and 0.06 M potassium chloride. It was adjusted to pH 7.4 with 1 N hydrochloric acid. Buffer B was identical to buffer A except that it was made 0.006 M in 2-mercaptoethanol just prior to use.

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Mix I consisted of 10.0 ml of 2 M tris buffer (pH 7.4), 2 ml of magnesium acetate (variable concentration), 5.0 ml of 2 M potassium chloride, and 3.0 ml of $6.66 \times 10^{-2}$ M ATP. Mix I was stored in small aliquots at -20°C. The magnesium acetate concentration was varied in the mix I depending on the magnesium ion concentration desired in the final incubation mixture (see preparation of incubation mixtures.) The following 5 solutions of magnesium acetate were used in this manner:

| Solution No. | Molarity of magnesium acetate (as added to make mix I) | $Mg^{++}$ mM (in standard incubation mixture of 0.25 ml) |
|---|---|---|
| 1 | $4 \times 10^{-2}$ | 7 |
| 2 | $3.39 \times 10^{-1}$ | 10 |
| 3 | $8.38 \times 10^{-1}$ | 15 |
| 4 | 1.34 | 20 |
| 5 | 1.84 | 25 |

Most experiments were conducted at a magnesium ion concentration of 10, 15 or 20 mM. Any deviations from the above procedure are described in the results and discussion section.

## Isolation of Subcellular Fractions

### A. The S-30 Fraction

The isolation of subcellular fractions followed the

procedure of Nirenberg (13) except for the modifications which are listed at the end of this section. All operations, unless otherwise specified, were performed in a cold room (3-5°C) or in crushed ice. All centrifugations were performed between 3°-7°C.

Approximately 100 g of frozen cells were placed in a large beaker and thawed in approximately 600 ml of cold buffer A (in the cold or for about 1 hr at room temperature). The thawed cells were centrifuged at 11,700 x g for 20 min. The supernatant was decanted and discarded and the pellets were drained. The pellets were resuspended in buffer A and centrifuged as before. After the second centrifugation the discarded supernatant should be clear. The pellets were transferred to prechilled, unglazed porcelain mortars (about 20-30 g of cells per mortar). The wet weight of cells was obtained by weighing the centrifuge tubes before and after transferring the pellets. The cells were ground with twice their weight of alumina (Alcoa, A-305) (ie. 20 g of cells would require 40 g of alumina). Approximately 15 g of alumina were placed in the mortar, and the cells were ground vigorously with a pestle into a thick paste. As the cells broke, the paste became more fluid and additional alumina was slowly added to keep the paste thick. After all the alumina had been added the cells were ground until the paste was homogeneous. After a total

time of about 15 min of grinding the paste was transferred to a 400 ml beaker with 2 volumes, of buffer B (ie. 40 ml for 20 g of cells). The paste and buffer were gently stirred until a homogeneous suspension was obtained. Alumina, intact cells, and cell debris were removed from this suspension by centrifugation at 20,000 x g for 20 min. The supernatant fluid was decanted and collected in a 250 ml erlenmeyer flask which had been placed in crushed ice. The extract was then treated with DNase (1 ug per ml of extract). After 5 min in the crushed ice the extract was centrifuged at 30,000 x g for 30 min. The supernatant solution was removed by aspiration to within 1 cm of the pellet and was again centrifuged at 30,000 x g for 30 min. The upper four-fifths of the supernatant solution was removed by aspiration. This fraction was designated as the S-30 fraction.

B. The W-RIB and S-100 Fractions

In order to obtain the washed ribosome and supernatant fractions, the S-30 fraction was centrifuged at 105,000 x g for 2 hr in a Spinco model L preparative ultracentrifuge. The upper four-fifths of the supernatant were removed by aspiration. The lower one-fifth was decanted and discarded. The ribosomal pellets were resuspended in the initial volume of buffer B (40 ml for 20 g of cells) by gentle homogenization ( 7 to 8 passes) in a Potter-Elvejehm homogenizer. This suspen-

was again centrifuged at 105,000 x g for 2 hr and the supernatant solution was decanted and discarded. The ribosomal pellets were suspended in one-fourth the original volume of buffer B as described previously. The suspension was again centrifuged at 10,000 x g for 5 min to remove aggregates. The washed ribosomal fraction was designated as W-RIB. The original 105,000 g supernatant was dialyzed against four liters of buffer B for 8 hr. The dialyzing medium was changed at the end of 4 hr. This fraction was designated as S-100.

All fractions (S-30, W-RIB, and S-100) were transferred to 1 ml ampules, frozen in the vapor of liquid nitrogen and then stored in a liquid nitrogen tank (Linde). All the fractions were analyzed for protein by the method of Lowry (14), using bovine serum albumin as a standard.

The cell-free extracts from E. coli were prepared according to the above procedure. However, for the preparation of the cell-free extracts from the various strains of Bacillus, the procedure was somewhat modified. The entire volumes of the supernatants were decanted and no fractional aspiration was used. The ribosomes were collected after one centrifugation at 105,000 x g and suspended in buffer B. Any further modifications of the isolation procedure are given in the results and discussion.

## Incubation Mixture

A. Preparation of mix II - Mix II was prepared prior to each experiment by mixing the following: 250 ul of the appropriate mix I; 1 ul of 2-mercaptoethanol; 250 ul of PEP; 4-5 ul of PEP kinase suspension; 250 ul of water; and 50 ul of phe-$C^{14}$ solution.

B. Preparation of incubation mixture - The components of the incubation mixture were added in the following sequence. 80 ul of mix II; poly U solution (5 mg/ml); s-RNA solution (20 mg/ml); and 0.160 ml of subcellular fractions. These consisted of either the S-30 fraction, or varying amounts of W-RIB and S-100 fractions. The mix II was introduced into a 15 ml conical centrifuge tube with a 100 ul Hamilton syringe having a Chaney adapter. The poly U and s-RNA solutions were introduced with a regular 10 ul Hamilton syringe. Mohr pipettes were used for the subcellular fractions. After all the components were added to each reaction mixture the tube was held at an angle of approximately 45° and rolled gently between two fingers. Violent stirring or shaking was avoided. The reaction mixture was then incubated. For most experiments the incubation was at 37°C for 45 min. Other conditions will be described in the results and discussion section. All the reaction mixtures were run in duplicate with appropriate controls. The variation between duplicates was not more than 10%.

## Measurement of Phenylalanine Incorporation

Protein was precipitated at the end of the incubation by the addition of 3.0 ml of 10% trichloroacetic acid at 3°C. The reaction mixtures were stirred on a Vortex mixer and placed in a water bath at 90°-95°C for 20 min to hydrolyze RNA. Tubes were then chilled in ice for at least 60 min. Protein precipitates were dispersed by stirring with a Vortex mixer, and each suspension was filtered under suction through a Millipore filter (HA Millipore filter, 25 mm in diameter, 0.45 u pore size, Millipore Filter Corp.). Each precipitate was then 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 min under an infrared lamp and counted. 1000 counts per minute were equivalent to 166 uu-moles of phenylalanine incorporated.

# RESULTS AND DISCUSSION

## Preliminary Studies with Bacillus licheniformis

The first experiments with B. licheniformis were unsuccessful, that is, no poly U-directed phenylalanine incorporation was observed. For these experiments, the cells were ground with a chromatographic grade of alumina (Merck), using a 3:1 ratio of alumina to cells. This mixture was extracted with 8 volumes of buffer B yielding a very dilute S-30 fraction having a protein concentration of only 2.3 mg/ml. According to the literature (13), the S-30 fraction should have a protein concentration of about 3-12 mg/ml. In order to obtain a more concentrated extract the cells were ground with the same alumina but the ratio of alumina to cells was changed from 3:1 to 2:1 and the mixture was extracted with only 2 volumes of buffer B. Using this method an extremely thick paste was obtained that yielded only a few ml of S-30 fraction upon centrifugation. It was obvious that the protein was not adequately extracted from the alumina when this method was used. To overcome these difficulties the cells were ground with a fine, bacteriological grade alumina (Alcoa A-305), using a 2:1 ratio of alumina to cells, and the mixture extracted with 2 volumes of buffer B. The S-30 fraction from this

procedure had a protein concentration of 10.6 mg/ml which was of satisfactory magnitude. Even so, no phenylalanine incorporation was observed by using either this S-30 fraction or the W-RIB and S-100 fractions prepared from it.

Since it is known that nucleases are associated with subcellular preparations of Bacillus strains (15, 16), the possibility existed that these nucleases degraded some of the added messenger RNA (poly U), or the endogenous soluble RNA, or even the ribosomal RNA during these in vitro experiments. This would, of course, prevent amino acid incorporation. In order to check for this possibility, cells were broken using the standard procedure except that buffer B contained a common nuclease inhibitor. Two such experiments were performed and in the one case the buffer contained spermidine trihydrochloride (0.8 u-mole/ml) and in the other case the buffer contained potassium polyvinyl-sulfate (4 mg/ml). In neither experiment was any incorporation obtained. This was true for both S-30 fractions and the combination of S-100 and W-RIB fractions.

Another possible difficulty relates to the wash procedure of the protein ppt. Conceivably, a more thorough wash procedure might decrease the counts of the controls to such low levels that sample counts would be

detected even if the sample counts were not very much higher than those of the controls. Accordingly, the following wash procedures were used.

A. Five 5 ml aliquots of cold 5% TCA (standard procedure)

B. Ten 5 ml aliquots of cold 5% TCA

C. Instead of filtering the protein ppt directly on the Millipore filter, it was first collected by centrifugation in a clinical centrifuge. The ppt was then resuspended in 3 ml of an ether-ethanol solution (1:1, V/V), centrifuged and resuspended again in 3 ml of the ether-ethanol solution. The ppt was then centrifuged and resuspended in 5 ml of 5% TCA and filtered on a Millipore filter.

The various incubation mixtures which were analyzed in this study were incubated at 37°C for 30 min (Experiment I) or else treated with 3 ml of 10% TCA and not incubated (Experiment II). The results are given in Table I.

This study showed that with the standard wash procedure (procedure A, No. 1-6), no incorporation was obtained with or without added poly U (No. 1-2, 5-6). Furthermore, the zero time controls (No. 3-4) had the same counts as the incubation mixtures (No. 5-6) wash

Table 1. Effect of wash procedure on radioactivity determinations.

| No. | Poly U ug | Experiment | Wash Procedure | CPM |
|---|---|---|---|---|
| 1 | 0 | I | A | 250 |
| 2 | 0 | I | A | 264 |
| 3 | 25 | II | A | 294 |
| 4 | 25 | II | A | 241 |
| 5 | 25 | I | A | 209 |
| 6 | 25 | I | A | 322 |
| 7 | 25 | I | B | 238 |
| 8 | 25 | I | B | 179 |
| 9 | 0 | I | C | 77 |
| 10 | 0 | I | C | 86 |
| 11 | 25 | II | C | 76 |
| 12 | 25 | II | C | 79 |
| 13 | 25 | I | C | 73 |
| 14 | 25 | I | C | 88 |

procedure B, although more rigorous than A, gave essentially the same results (No. 7-8). Wash procedure C was the most rigorous and indeed the total counts were less in all cases (No. 9-14). However, even here, there was no detectable incorporation either in the absence or in the presence of added poly U. Since

the background count with this instrument is about 12 cpm it is clear that even with this rigorous wash procedure some radioactivity must have adhered to the protein ppt. Because in such amino acid incorporation studies relative differences rather than the absolute and precise CPM are important, the simpler but less efficient wash procedure A was used for all subsequent experiments.

Another factor which could mean the difference between good, poor and no incorporation is the molecular weight of the poly U. This is the case even if the cell fractions are fully active. If the poly U has a smaller molecular weight than about 30,000 (about 100 nucleotides), no incorporation will take place (13).

In order to check the quality of the purchased poly U, two samples of poly U were examined in the analytical ultracentrifuge. One sample had been stored in distilled water (5 mg/ml) for 3 months and the other sample was freshly prepared. The stored sample was diluted 1:3 with 0.05 M phosphate buffer (pH 7.5) containing 0.1 M NaCl. The freshly prepared sample was dissolved in the same buffer, to a concentration of 3 mg/ml. The centrifuge runs were made at 59,780 RPM and the temperature was held between 7-8°C. The sedimentation coefficients were corrected to a medium of water at 20°C ($s^{W}_{20}$). The $s^{W}_{20}$ value of the stored sample

was 5.2 and that of the freshly prepared one was 4.0.

Thus there had been no obvious degradation in the poly U because of prolonged storage. The stored sample had actually a slightly higher $s^{w}_{20}$ and this can be attributed to the fact that the concentration of the stored poly U was less than that of the freshly prepared one. Poly U which has a sedimentation coefficient of about 4.0-5.2 would have an average molecular weight between 50,000-100,000 according to the gel filtration data supplied by the Miles Chemical Company. It is thus certainly of sufficiently large size to direct amino acid incorporation.

A final check on the various components in the reaction mixture was performed by checking the ATP regenerating system. This system was tested for activity by the addition of lactic acid dehydrogenase, ADP and DPNH (17). A rapid decrease in absorbance at 340 mu was observed due to the disappearance of DPNH, and thus, both the PEP and the PEP kinase were acceptable.

Since no incorporation could be obtained with B. licheniformis in spite of the careful examination of all the above variables and possible sources of difficulties, it was decided to proceed first with a well characterized model system, namely that of E. coli.

## Studies with Escherichia coli

Cells of E. coli were broken according to the standard procedure. Buffer B for these experiments consisted of 0.01 M tris, 0.014 M magnesium acetate, 0.06 M potassium chloride, and 0.06 M 2-mercaptoethanol. It was adjusted to pH 7.8 with 1 N HCl. For the preparation of Mix I a 1.4 M solution of magnesium acetate was used and the final incubation mixture was 23 mM in magnesium. The S-30, W-RIB, and S-100 fractions were obtained as described above. Standard incubation mixtures (see Materials and Methods) were incubated at 37°C for 15 min. The protein concentrations of the various fractions were as follows.

| Fraction | Protein mg/ml |
|---|---|
| S-30 | 15.5 |
| W-RIB | 22.4 |
| S-100 | 12.5 |

Table 2. Phe-$C^{14}$ incorporation by the S-30 fraction

| No. | Poly U ug | s-RNA (E. coli) ug | CPM* | uu-moles phe-$C^{14}$ / mg protein |
|---|---|---|---|---|
| 1 | 0 | 0 | — | — |
| 2 | 25 | 0 | 355 | 23 |
| 3 | 50 | 0 | 398 | 26 |
| 4 | 25 | 200 | 703 | 46 |

* Corrected for the control (No. 1) which was 511 CPM

It can be seen from Table 2 that the addition of poly U led to an increase in the incorporation of phenylalanine. There was also a small amount of incorporation directed by endogenous messenger RNA as can be seen by comparing the controls (No. 1) with similar controls in Table 1 (No. 1-2). It is further evident from Table 2 that a pronounced increase in incorporation resulted upon the addition of s-RNA to the incubation mixture. Table 3 summarizes the result of a similar experiment.

Table 3. Phe-$C^{14}$ incorporation by the S-30 fraction

| NO. | Poly U ug | s-RNA (E. coli) ug | CPM* | uu-moles phe-$C^{14}$ / mg protein |
|---|---|---|---|---|
| 1 | 0 | 0 | — | — |
| 2 | 25 | 0 | 431 | 28 |
| 3 | 25 | 0 | 62 | 4 |
| 4 | 25 | 0 | 485 | 32 |

* Corrected for the control (No. 1) which was 407 CPM

Here, however, incubation mixture No. 3 was kept in ice 1 hr before incubation. No. 4, on the other hand, was prepared by keeping the combination of Mix II and poly U in ice for 1 hr before the addition of the S-30 fraction

and subsequent incubation. It is apparent that once the S-30 fraction has been added to the Mix II and poly U, the entire mixture must be incubated immediately. However, as shown by No. 4, it is possible to set up a large number of incubation mixtures provided that these contain initially only Mix II and poly U and that the protein is introduced into the tubes just prior to the incubation.

After these successful experiments with the S-30 fraction of *E. coli*, experiments were designed using the W-RIB and S-100 fractions prepared from the S-30 fraction. Typical results are summarized in Table 4.

Table 4. Phe-$C^{14}$ incorporation by the W-RIB and S-100 fractions.

| Poly U ug | s-RNA (*E. coli*) ug | W-RIB ml | S-100 ml | CPM* | uu-moles phe-$C^{14}$ / mg rib. prot. |
|---|---|---|---|---|---|
| 0 | 0 | 0.08 | 0.08 | — | — |
| 25 | 0 | 0.12 | 0.04 | 439 | 27 |
| 25 | 0 | 0.08 | 0.08 | 435 | 40 |
| 25 | 0 | 0.04 | 0.12 | 562 | 104 |
| 25 | 200 | 0.12 | 0.04 | 1876 | 116 |

* Corrected for the control (no poly U) which was 374 CPM

In this experiment the ratio of W-RIB to S-100 was varied. It appears that changes in the amount of the S-100 fraction (the enzymes) are more crucial than changes in the W-RIB fraction. Furthermore, it appears that in general the W-RIB and S-100 system is more active than the S-30 system. In agreement with results shown in Table 3, the S-100 and W-RIB system is also greatly stimulated by the addition of s-RNA. This is in line with recent papers on amino acid incorporation with E. coli systems where s-RNA has been added (18) in contrast to the early work which was conducted in the absence of added s-RNA (13).

In a second experiment (Table 5) the poly U concentration was varied using the best conditions of Table 4, ie. 0.04 ml W-RIB, 0.120 ml S-100, and 200 ug of s-RNA.

Table 5. Phe-$C^{14}$ incorporation by the W-RIB and S-100 fractions.

| Poly U ug | s-RNA (E. coli) ug | W-RIB ml | S-100 ml | CPM* | uu-moles phe-$C^{14}$ / mg rib. prot. |
|---|---|---|---|---|---|
| 0 | 0 | 0.04 | 0.120 | — | — |
| 5 | 200 | 0.04 | 0.120 | 1247 | 231 |
| 15 | 200 | 0.04 | 0.120 | 1685 | 312 |
| 35 | 200 | 0.04 | 0.120 | 1765 | 326 |
| 45 | 200 | 0.04 | 0.120 | 1770 | 327 |

* Corrected for the control (no poly U) which was 376 CPM

It can be seen from Table 5 that an increase in the concentration of poly U leads to an increase in phe-$C^{14}$ incorporation and that a level of about 25 ug saturates this system as pointed out by Nirenberg (13). The phe-$C^{14}$ incorporation was, therefore, poly U dependent and the level of incorporation was of comparable magnitude to that found by other workers (19).

These successful experiments with the E. coli system constituted a good check on the various components in the incubation mixture and the general experimental techniques. Because of these results it was decided to return to a species of the Bacillus system but, to use first a species (B. subtilis) from which a cell-free amino acid incorporating system had already been prepared by other workers.

## Studies with Bacillus subtilis

Cells of B. subtilis were broken according to the standard procedure. The conditions for preparing the extracts and the incubation mixtures were the same as for E. coli in the previous experiments. No incorporation was obtained in experiments using either S-30 or W-RIB and S-100 fractions, supplemented in all cases with B. subtilis s-RNA. A protein analysis of the various fractions indicated that cell breakage had been insufficient. The protein concentrations for the various fractions were as follows:

| Fraction | Protein mg/ml |
|---|---|
| S-30 | 2.3 |
| W-RIB | 28.0 |
| S-100 | 1.6 |

Since it appeared that B. subtilis cells were harder to break than those of E. coli, another batch of B. subtilis cells was broken using the coarser alumina (Merck). This resulted in a very thick paste so that an additional 1.5 volumes of buffer were added to the extract in the mortar. This procedure resulted in much more concentrated preparations of subcellular fractions (Table 6). Even these preparations, however, gave no incorporation of phe-$C^{14}$. In order to pinpoint the difficulty it was decided to prepare the subcellular fractions once more from E. coli and cross them with the appropriate fractions from the B. subtilis preparation. The E. coli cells were broken according to the standard procedure using buffer B. The protein concentrations of the subcellular fractions are given in Table 6.

Table 6. Protein concentration of subcellular fractions.

| Fraction | B. subtilis mg/ml | E. coli mg/ml |
|---|---|---|
| S-30 | 26.2 | 18.3 |
| W-RIB | 22.0 | 19.8 |
| S-100 | 24.3 | 11.8 |

The results of the experiment involving both E. coli and B. subtilis cells are given in Table 7. In all cases the molarity of the magnesium acetate used in the preparation of Mix I was $8.38 \times 10^{-1}$ M so that the final concentration of magnesium in the incubation mixture was 15 mM. The incubations were carried out for 15 min at 37°C.

It can be seen from Table 7 that there was no incorporation with any of the B. subtilis systems (No. 5-8). However, when E. coli ribosomes were added to either the S-30 or the S-100 fraction of B. subtilis, significant incorporation was obtained (No. 9-11). On the other hand when E. coli S-100 fraction was added to either the S-30 or the W-RIB fraction of B. subtilis, no incorporation was obtained (No. 12-14). This proves that the inactivity of the B. subtilis system was due to the W-RIB fraction. One would conclude, therefore, that the B. subtilis W-RIB fraction is either devoid of the required 70S ribosomes or else that these ribosomes, if present, are degraded or altered during the incubation and thus lose their activity. In order to decide which of these possibilities was applicable here, the W-RIB fraction and the incubation mixtures containing ribosomes were analyzed by means of the analytical ultracentrifuge. These experiments showed that while the initial W-RIB fraction did contain the 70S ribosomes, these were completely

Table 7. Phe-$C^{14}$ incorporation by *E. coli* and *B. subtilis* systems

| No. | Poly U ug | s-RNA 200 ug | S-30 0.08 ml | S-30 0.160 ml | S-100 0.08 ml | W-RIB 0.08 ml | CPM | Corrected CPM | uu-moles phe-$C^{14}$ mg rib. prot. |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | | *E. coli* | | | 559 | | |
| 2 | 25 | *E. coli* | | *E. coli* | | | 4297 | 3738 | 215* |
| 3 | 0 | 0 | *E. coli* | | | *E. coli* | 527 | | |
| 4 | 25 | *E. coli* | *E. coli* | | | *E. coli* | 3736 | 3209 | 334 |
| 5 | 0 | 0 | | *B. sub.* | | | 234 | | |
| 6 | 25 | *B. sub.* | | *B. sub.* | | | 238 | | |
| 7 | 0 | 0 | | | *B. sub.* | *B. sub.* | 206 | | |
| 8 | 25 | *B. sub.* | | | *B. sub.* | *B. sub.* | 222 | | |
| 9 | 25 | *B. sub.* | *B. sub.* | | | *E. coli* | 1194 | 960 | 100 |
| 10 | 0 | 0 | | | *B. sub.* | *E. coli* | 207 | | |
| 11 | 25 | *B. sub.* | | | *B. sub.* | *E. coli* | 1325 | 1118 | 116 |
| 12 | 25 | *B. sub.* | | | *E. coli* | *B. sub.* | 229 | | |
| 13 | 25 | *B. sub.* | *B. sub.* | | *E. coli* | | 221 | | |
| 14 | 0 | 0 | | | *E. coli* | *B. sub.* | 188 | | |

*uu-moles phe-$C^{14}$ / mg total protein

degraded when suspended in the incubation mixture. Further details of this experiment are given under the section on ultracentrifugation.

### Studies with Bacillus licheniformis

Cells of B. licheniformis were broken according to the standard procedure using buffer B. S-30, S-100, and W-RIB fractions were obtained and stored in liquid nitrogen. Incubations were for 15 min at 37°C, and the magnesium concentration in the final incubation mixtures was 15 mM. The fractions were as follows.

| Fraction | Protein mg/ml |
|---|---|
| S-30 | 10.6 |
| W-RIB | 10.8 |
| S-100 | 9.0 |

When 200 ug of B. subtilis s-RNA were added to incubation mixtures containing the S-30 fraction of B. licheniformis and 50 ug of poly U, 54 uu-moles of phe-$C^{14}$ were incorporated per mg of total protein. However, when the magnesium ion concentration in the incubation mixture was reduced to 10 mM or when the poly U concentration was lowered to 25 ug no incorporation was observed. Similarly, incorporation was obtained in experiments using the S-100 and

W-RIB of B. licheniformis together with 50 ug of poly U and 200 ug of B. subtilis s-RNA and a final magnesium concentration of 15 or 20 mM. Again, no incorporation was observed when the poly U concentration was lowered to 25 ug or when the final magnesium concentration was lowered to 10 mM.

In the light of these preliminary experiments several other variables were investigated using incubation mixtures containing 50 ug of poly U and having a final magnesium ion concentration of 20 mM. Incubation was at 37°C for 15 min. Some of these results are given in Table 8.

It can be seen from Table 8 that more phe-$C^{14}$ is incorporated when the W-RIB:S-100 ratio is increased (No. 1-4). This is the opposite of the finding with E. coli (Table 4) and indicates that ribosome stability may also be a factor with B. licheniformis systems (Table 7). This is in accord with the demonstration of nuclease activity in subcellular fractions from Bacillus (15). When the S-100 fraction is omitted from the incubation mixture (No. 5) or when it is denatured (No. 6-7) no incorporation is obtained. In this respect the B. licheniformis system differs from that of B. cereus (8) where evidently enough enzymes are absorbed to the ribosomes so that incorporation is obtained without the addition of an S-100 fraction.

Table 8. Phe-$C^{14}$ incorporation by B. licheniformis fractions.

| | Poly U ug | s-RNA (B. sub.) ug | W-RIB ml | S-100 ml | CPM | Corrected CPM | uu-moles phe-$C^{14}$ / mg rib. prot. |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0.08 | 0.08 | 239 | — | — |
| 2 | 50 | 200 | 0.04 | 0.120 | 320 | 81 | 31 |
| 3 | 50 | 200 | 0.08 | 0.08 | 502 | 263 | 51 |
| 4 | 50 | 200 | 0.120 | 0.04 | 731 | 492 | 63 |
| 5 | 50 | 200 | 0.160 | 0 | 126 | — | — |
| 6 | 50 | 200 | 0.08 | 0.08* | 136 | — | — |
| 7 | 50 | 200 | 0.120 | 0.04* | 203 | — | — |

* The S-100 was denatured at 70°C for 10 min.

In another experiment the effect of ribosome washing and dialysis of the S-100 fraction was investigated. The cells were broken according to the standard procedure. Once and twice centrifuged W-RIB fractions and dialyzed and undialyzed S-100 fractions were obtained and frozen in liquid nitrogen. Experiments were performed with these four fractions in order to ascertain their effect on the incorporating system. Incubation mixtures were incubated for 15 min at 37°C at a final magnesium ion concentration of 20 mM. The protein concentrations of these fractions were as follows:

| Fraction | Protein mg/ml |
|---|---|
| W-RIB (once centrifuged) | 14.5 |
| W-RIB (twice centrifuged) | 11.2 |
| S-100 (dialyzed) | 9.9 |
| S-100 (undialyzed) | 11.1 |

The results of this experiment are given in Table 9.

It is evident from Table 9 that an increase in the incorporation of phe-$C^{14}$ is observed when a dialyzed S-100 fraction is used. Approximately the same increase in incorporation is observed when the ribosomal fraction is twice centrifuged. A combination of both dialysis of the S-100 fraction and washing of the ribosomes by double centrifugation leads to an even higher incorporation. However, in view of the loss of ribosomes upon

Table 9. Phe-$C^{14}$ incorporation by B. licheniformis fractions.

| No. | Poly U ug | s-RNA (B. sub.) ug | W-RIB 0.120 ml | S-100 0.04 ml | CPM | Corrected CPM | uu-moles phe-$C^{14}$ / mg rib. prot. |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | Once Cent. | undial. | 229 | — | — |
| 2 | 50 | 200 | Once Cent. | undial. | 651 | 422 | 41 |
| 3 | 0 | 0 | Once Cent. | dial. | 248 | — | — |
| 4 | 50 | 200 | Once Cent. | dial. | 814 | 566 | 55 |
| 5 | 0 | 0 | Twice Cent. | undial. | 232 | — | — |
| 6 | 50 | 200 | Twice Cent. | undial. | 642 | 410 | 52 |
| 7 | 0 | 0 | Twice Cent. | dial. | 272 | — | — |
| 8 | 50 | 200 | Twice Cent. | dial. | 818 | 546 | 70 |

repeated centrifugation it was decided to perform all the remaining experiments with ribosomes prepared in the usual manner (once washed).

Studies with B. stearothermophilus 10

Cells of B. stearothermophilus 10 were broken according to the standard procedure using buffer B. S-100 and W-RIB fractions were obtained and stored in liquid nitrogen. Incubation mixtures had a magnesium ion concentration of 10 mM and were incubated for 45 min at 37°C. The protein concentrations of the fractions were as follows.

| Fraction | Protein mg/ml |
|---|---|
| W-RIB (once centrifuged) | 25.0 |
| S-100 (dialyzed) | 7.6 |

The results of the experiment are given in Table 10.

By comparing these data with those in Tables 8 and 9 it can be seen that under the conditions of these experiments the incorporation of phe-$C^{14}$ by B. stearothermophilus 10 systems was approximately 8 to 10 times that given by B. licheniformis systems. This can be accounted for by a number of reasons. The B. stearothermophilus 10 cells were harvested in the early-log phase whereas the B. licheniformis cells were harvested in the late-log phase. Early-log phase cells are apparently more active in protein synthesis (13) and the

Table 10. Phe-$C^{14}$ incorporation by B. stearothermophilus 10

| No. | Poly U ug | s-RNA (B. sub.) ug | W-RIB ml | S-100 ml | CPM | Corrected CPM | uu-moles phe-$C^{14}$ mg rib. prot. |
|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0.08 | 0.08 | 740 | — | — |
| 2 | 50 | 0 | 0.08 | 0.08 | 7539 | 6799 | 567 |
| 3 | 0 | 200 | 0.08 | 0.08 | 826 | — | — |
| 4 | 50 | 200 | 0.04 | 0.120 | 3096 | 2356 | 393 |
| 5 | 50 | 200 | 0.08 | 0.08 | 10189 | 9449 | 787 |
| 6 | 50 | 200 | 0.120 | 0.04 | 12048 | 11308 | 628 |
| 7 | 50 | 200 | 0.08 | 0.08 | 6187 | 5447 | 454* |
| 8 | 50 | 200 | 0.160 | 0 | 4024 | 3284 | 137 |
| 9 | 50 | 200 | 0 | 0.160 | 651 | — | — |

* The S-100 was denatured at 70°C for 10 min.

systems are probably less damaged because of ribonuclease activity than is the case for the late-log phase cells. Moreover, it has been shown (15) that the ribosomes of thermophilic species of Bacillus are more stable to nuclease degradation than is the case for the mesophilic strains. Further evidence for the decreased effect of nuclease in the thermophilic preparations comes from the fact that these systems actively incorporate even in the absence of added s-RNA presumably because endogenous s-RNA has not been degraded by nucleases. Under the same conditions, systems of B. licheniformis are inactive. These findings as well as other results with B. subtilis and those of studies on ribosome stability (15, 20) indicate that the problem of nucleases may be a serious one for mesophilic strains of Bacillus in general.

The protein concentration in the S-100 fraction was much lower than in any other of our active preparations, yet the incorporation was much higher. This indicates that the level of enzymes and or enzyme activity is higher in S-100 extracts from early-log phase cells. Some incorporation also occurred in systems containing only ribosomes (No. 8). This is probably due to the fact that the ribosomes were only centrifuged once and therefore may still have had various enzymes associated with them. When the S-100 fraction of B. stearothermophilus 10 was denatured

(Table 10, No. 7) a large amount of incorporation still occurred whereas under the same conditions the B. licheniformis system was inactive (Table 8). This is in excellent agreement with the theory that macromolecules of thermophiles are more stable than similar components of mesophiles.

## Comparative Studies with B. licheniformis and B. stearothermophilus 10

Four comparative studies were performed on B. licheniformis and B. stearothermophilus 10. The parameters studied were time, poly U concentration, magnesium, and temperature. All the incubation mixtures (except the controls) contained 200 ug of B. subtilis s-RNA. The experiments were performed with W-RIB and S-100 fractions. The experimental conditions for these studies are given in Table 11.

Results of the temperature study are given in Table 12. These data show the relative stability of the preparations from B. stearothermophilus 10. at 37 and 55°C. Unfortunately the preparations from B. licheniformis which came from cells that had been stored for 9 months were so inactive even at 37°C that no conclusions can be drawn as to their relative stability at 37 and 55°C.

Results of the time course study are given in Fig. 1. It is seen that for both organisms the incorporation is

Table 11. Experimental conditions.

| Experiment | Organism | Temp. °C | Poly U ug | $Mg^{++}$ mM | Incubation time min |
|---|---|---|---|---|---|
| Time course study | B. lich. | 37 | 50 | 20 | — |
| | B. stear. | 37 | 50 | 20 | — |
| Poly U concentration study | B. lich. | 37 | — | 20 | 45 |
| | B. stear. | 37 | — | 20 | 45 |
| Magnesium ion concentration study | B. lich. | 37 | 75 | — | 45 |
| | B. stear. | 37 | 75 | — | 45 |
| Temperature study | B. lich. | — | 75 | 15 | 45 |
| | B. stear. | — | 75 | 10 | 45 |

Table 12. Temperature study.

| Organism | Temp. | Corrected CPM | uu-moles phe-$C^{14}$ / mg rib. prot. |
|---|---|---|---|
| B. stearothermophilus 10 | 37 | 7768 | 432 |
| | 55 | 2143 | 119 |
| B. licheniformis | 37 | 100 | 7 |
| | 55 | 40 | 3 |

essentially linear during the first 20-25 min and levels off farily abruptly at that time.

Results of the poly U concentration study are given in Fig. 2. There were two definite differences in the response to poly U between the two systems. For B. stearothermophilus 10 incorporation was pronounced even with very low concentrations of poly U and it leveled off at about 35 ug of poly U per incubation mixture. For B. licheniformis, however, no incorporation was obtained below about 15 ug of poly U per incubation mixture and the incorporation leveled off only at about 75 ug of poly U. Both these differences can be understood in terms of the increased nuclease activity of the preparations from B. licheniformis. It should be noted that the absolute amount of incorporation in this, and all other experiments, was always less for B. licheniformis. This is undoubtedly a reflection of the fact that the B. licheniformis fractions were prepared from late-log phase cells while those of B. stearothermophilus 10 were prepared from early-log phase cells.

Fig. 3 shows the effect of varying the magnesium ion concentration on the phe-$C^{14}$ incorporation. In all experiments the volume of the incubation mixture was increased from the standard 0.25 ml to 0.30 ml by the addition of water and 1 M magnesium acetate in order to achieve the various magnesium ion concentrations.

Fig. 1. Time course study of phe-$C^{14}$ incorporation

( o ) B. licheniformis (right ordinate)

( ● ) B. stearothermophilus 10 (left ordinate)

ordinate: $\frac{\text{uu-moles phe-}C^{14}}{\text{mg rib. prot.}}$

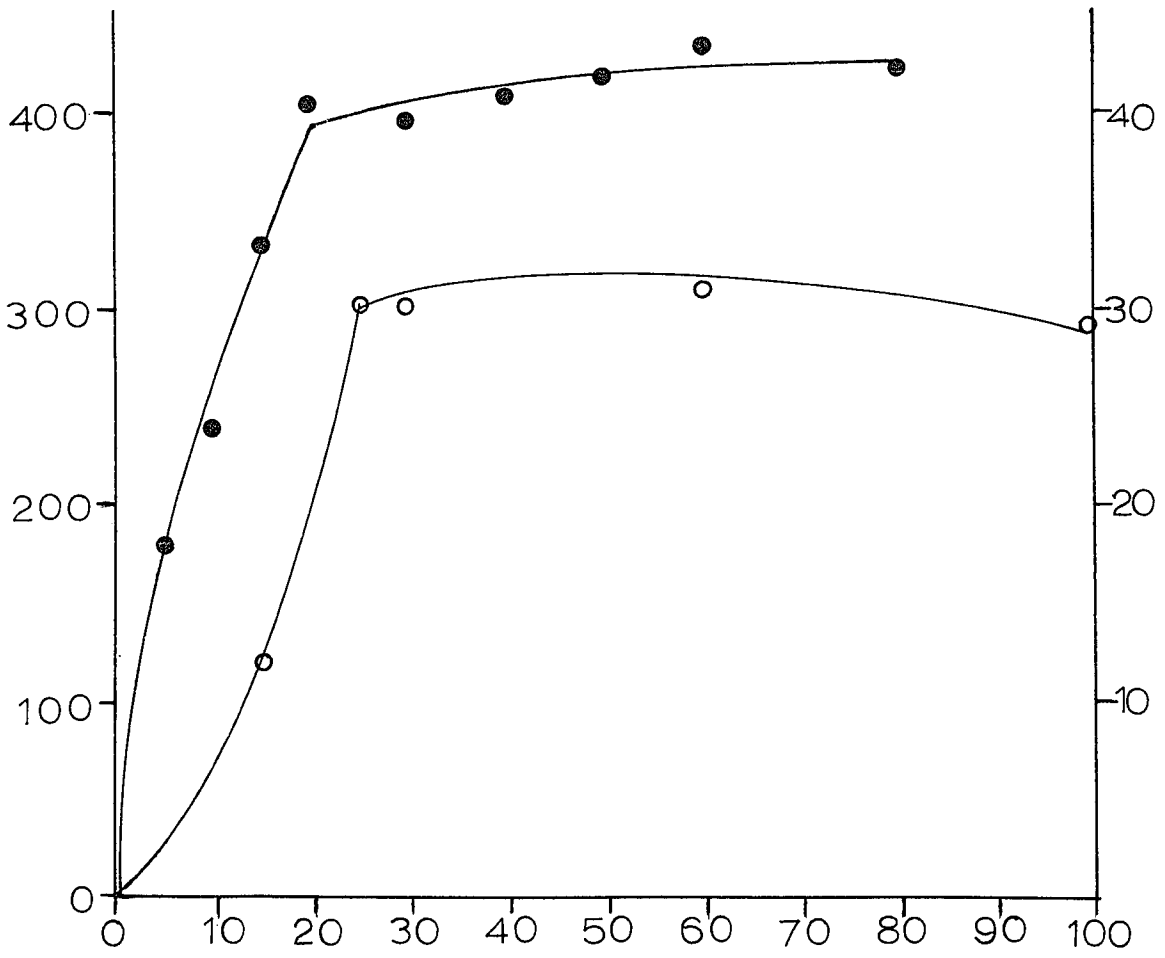


Fig. 2. Poly U concentration study.

(○) B. licheniformis (right ordinate)

(●) B. stearothermophilus 10 (left ordinate)

ordinate: $\frac{\text{uu-moles phe-C}^{14}}{\text{mg rib. prot.}}$

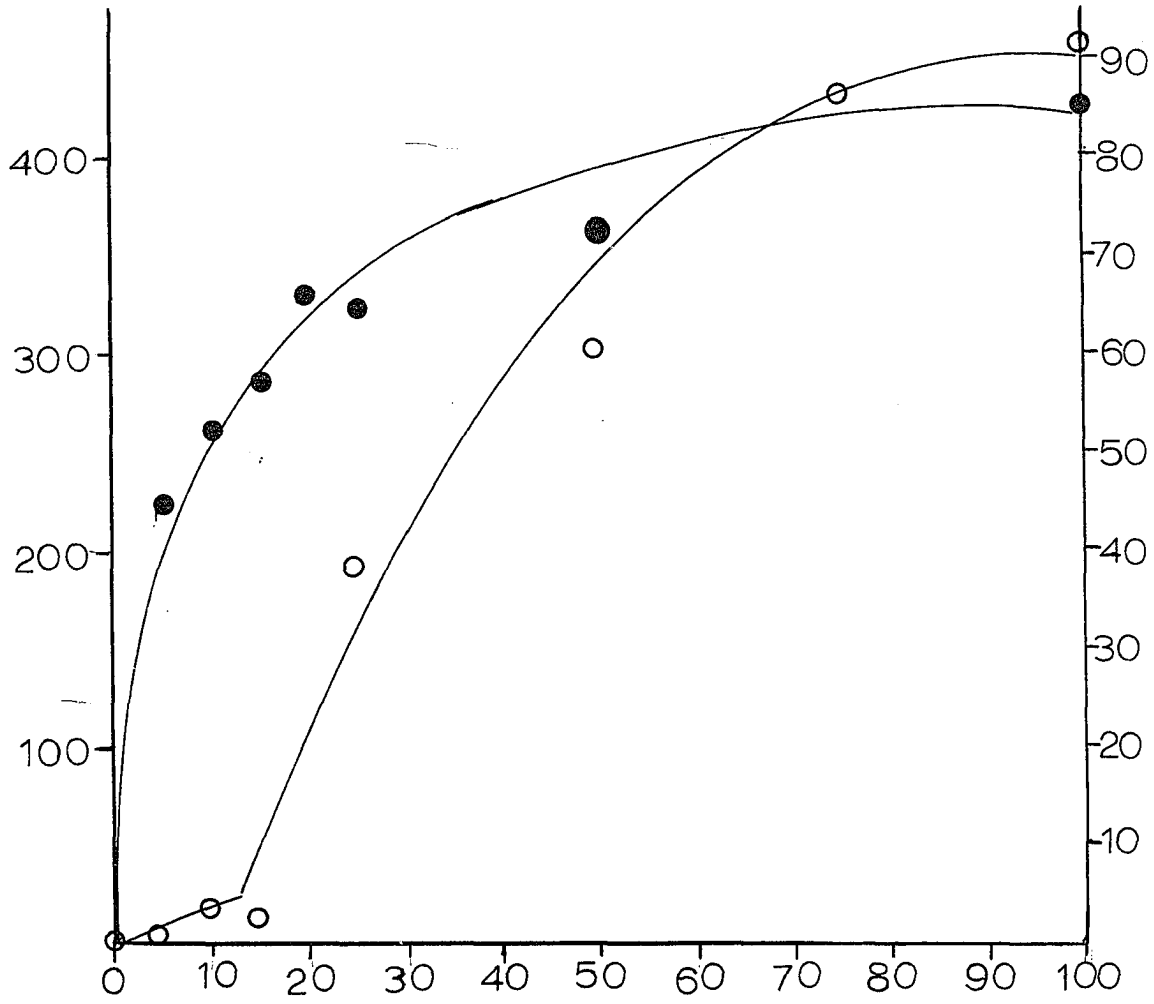
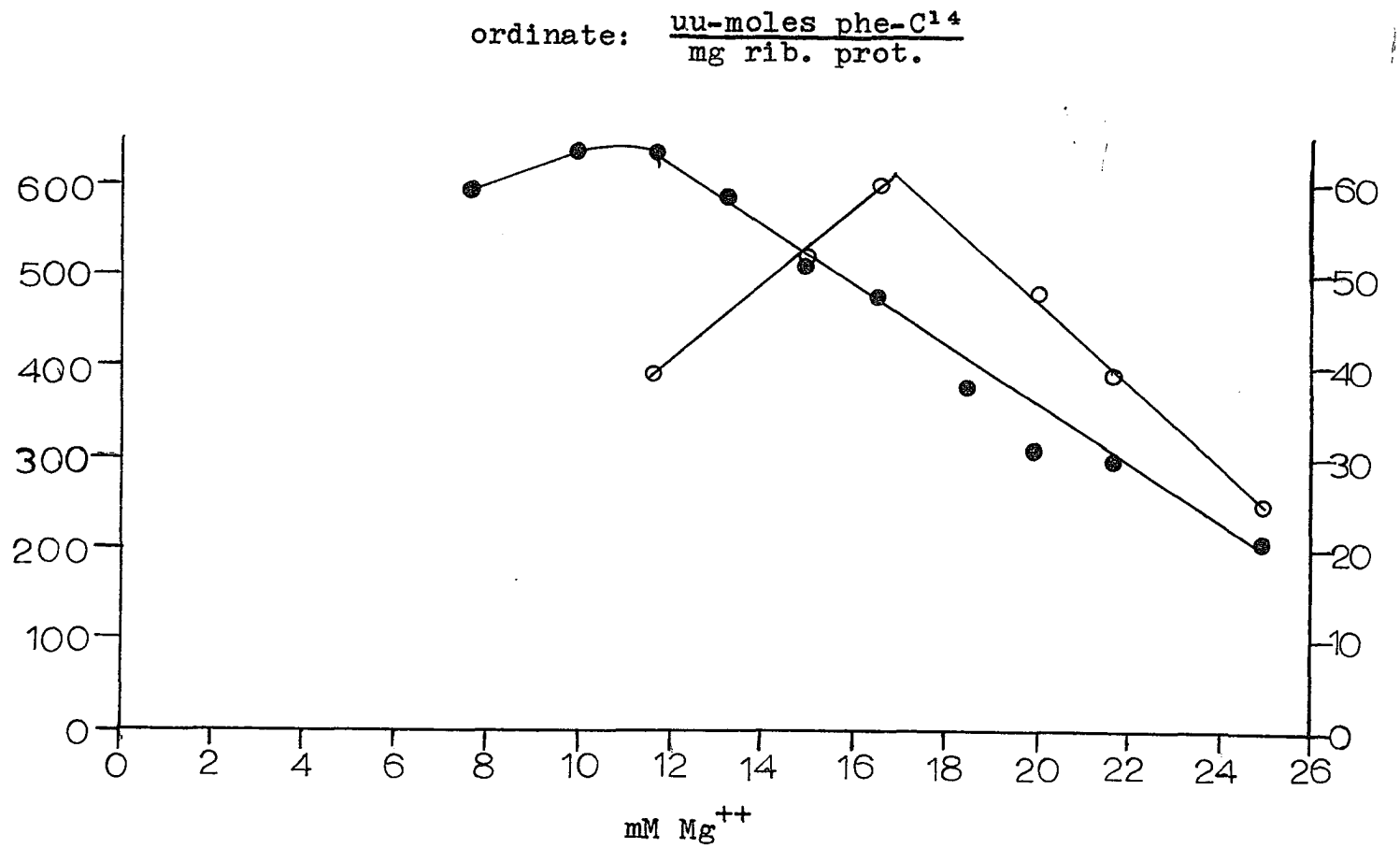


Fig. 3. Magnesium ion concentration study.

(o) B. licheniformis (right ordinate)

(●) B. stearothermophilus 10 (left ordinate)



Both preparations showed a dependance on the magnesium concentration. The exact concentration was more critical for the fractions from B. licheniformis because the curve exhibited a sharper optimum than was the case for the fractions from B. stearothermophilus 10. For B. licheniformis the optimum magnesium concentration was about 16-18 mM while it was about 10-12 mM for B. stearothermophilus 10. Thus, with respect to magnesium much as was the case with respect to poly U, there were some definite differences between the system from the mesophile and that from the thermophile.

## Ultracentrifugation Studies

The W-RIB preparations from each of the organisms studied were examined in the analytical ultracentrifuge under two conditions. Under the first condition the W-RIB preparation was used directly at a magnesium ion concentration of 10 mM. Under the second condition an incubation mixture was prepared containing W-RIB, S-100, Mix II, and a final magnesium ion concentration of 15 mM (ie. exactly as in the incorporation studies except that poly U was omitted in order to avoid the formation of polyribosomes). The results of these experiments are given in Table 13.

It could be seen from the ultracentrifugation patterns that those systems that were active in incorporating phe-$C^{14}$ did indeed have a large percentage of the

Table 13. Ultracentrifugation data.

| Organism | Conditions: W-RIB 10 mM $Mg^{++}$ | Conditions: Incubation Mixture 15 mM $Mg^{++}$ | Sedimentation Coefficient* $s^{W}_{20}$ | | | | Percent of 70S ribosomes** |
|---|---|---|---|---|---|---|---|
| E. coli | X | | 32.5 | 49.4 | 66.1 | 92.7 | 42 |
| | | X | 31.2 | 45.6 | 58.9 | 78.3 | 57 |
| B. subtilis | X | | 29.1 | 49.4 | 75.5 | 95.3 | 6 |
| | | X | 25.6 | 39.7 | | | 0 |
| B. licheniformis | X | | 29.4 | 50.4 | 67.1 | 94.3 | 42 |
| | | X | 28.5 | 45.7 | 61.0 | 85.4 | 42 |
| B. stearothermophilus 10 | X | | | 52.0 | 69.8 | 101.6 | 64 |
| | | X | 32.1 | 46.4 | 61.1 | 81.8 | 69 |

* The underlined component represents the 70S ribosomes

** Obtained from area measurements of the various components

ribosomal material present as 70S ribosomes (about 40-60% in all cases). Preparations of B. subtilis on the other hand which were not active in these experiments showed a completely degraded profile and no 70S ribosomes could be detected. B. stearothermophilus 10 which had the most activity also had the highest percentage of 70S ribosomes. An amino acid incorporating system from cell-free extracts must have both 70S ribosomes and active enzyme systems. These conditions are apparently fulfilled best by extracts from early-log phase cells.

## SUMMARY

An amino acid incorporating system was obtained from cell free extracts of E. coli and two strains of Bacillus, a mesophile B. licheniformis, and a thermophile B. stearothermophilus 10. The mesophile was obtained from late-log phase cells and the thermophile from early-log phase cells. Both systems were polyuridylic acid dependent and the mesophile required added soluble RNA. The thermophile was more active with or without added soluble RNA.

The enzyme preparation of the thermophile was more heat stable than that of the mesophile. Both cell free systems were examined with respect to magnesium ion concentration, polyuridylic acid concentration, and length of time for incorporation. Analytical ultracentrifugation studies showed the requirement of the 70S ribosomal particle for amino acid incorporation.

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