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A Cell-Free System for Protein Synthesis from Azotobacter vinelandii

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A CELL-FREE SYSTEM FOR PROTEIN SYNTHESIS FROM *AZOTOBACTER VINELANDII*

by

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Nirenberg and Matthaeii (56) were among the first to demonstrate the requirements for *in vitro* protein synthesis in *Escherichia Coli*; the requirements include ribosomes, which are the structural site of amino acid incorporation and a dialyzed 100,000 x g supernatant fraction which contains the soluble components. Their important contribution provided the molecular basis for the study of the coding problem with their discovery that poly U (an oligonucleotide containing repeating units of 5' uridylic acid) acted as a messenger and that it coded for the synthesis of polyphenylalanine. These relationships were the basis for subsequent studies of the coding and translational problems.

A number of bacterial, mammalian and plant cell-free systems (3-5, 14, 27-29, 37, 40, 44, 53, 60, 71, 78, 86, 87, 88) have since been developed with requirements similar to the *E. coli* system (55, 56, 58, 73, 83, 85). Amino acid incorporation is dependent upon ribosomes, 100,000 x g supernatant or pH 5 fraction, an ATP generating system, GTP, a critical concentration of magnesium, an optimal pH, and optimal concentrations of tRNA, polyamine, and KCl.

Systems from *E. coli* and yeast have been of great use to biology due to the large body of information regarding biosynthetic pathways for cellular constituents as well as the large number of mutants readily available. These resources led to explosive advances in our knowledge of the mechanisms of protein synthesis, the action of antibiotics, the genetic

code, and the structure of protein synthesizing components. The system from Bacillus cereus may be useful for the information it may yield in conjunction with sporulation and germination, two processes which may be simple analogues of differentiation. Systems from plants and animals may yield information peculiar to processes observed in these advanced forms.

A cell-free amino acid incorporating system was developed from Azotobacter for several reasons. First, in order to study protein synthesis directed by natural endogenous message, polyribosomes must be readily available and in reasonable quantities. Azotobacter may be disrupted by osmotic shock (64), an extremely gentle method which permits isolation of large quantities of polyribosomes (62). At least 80 to 90% of the cellular ribosomes are polyribosomes; four-fifths of which are larger than tetramers (62). Furthermore, various size classes of polyribosomes up to the octomer class can readily be separated. Individual size classes of larger polyribosomes are less well resolved and isolated but are readily separated from the smaller size classes (62). Thus we can examine the activities of individual size classes of polyribosomes as well as the kinds of nascent protein (enzymes) synthesized on a given size class of polyribosome.

Second, Azotobacter is an organism in which the study of membrane bound ribosomes may prove significant since Azotobacter has an internal membranous network (62) similar to that found in animal cells to which ribosomes are attached (63) and whose function in protein synthesis is established (34). Thus it is possible to compare the incorporating

activity of "free cytoplasmic" ribosomes and polyribosomes with those associated with membranes. In bacteria, ribosomes attached to cellular membranes have been reported (6, 73) but have not been demonstrated conclusively *in vivo*. An early report of a cell-free protein synthesizing system in *Azotobacter vinelandii* (1959, prior to that reported by Nirenberg) implicated a poorly defined fraction of cell wall and membrane which activly incorporated ^{14}C amino acids (20). However, this system did not exhibit the requirements presently associated with cell-free systems. Therefore, the development of a cell-free system for protein synthesis with clearly defined requirements in *Azotobacter* is then a prerequisite to experiments involving the isolation of membrane bound ribosomes and the characterization of amino acid incorporation associated with these particles.

Finally, *Azotobacter* forms cysts, a dormant, resistant stage, which is similar in some respects to spores. It would be of interest to determine alterations in protein biosynthesis which occur during the formation and germination of these cysts.

MATERIALS AND METHODS

Organism and conditions of culture. Azotobacter vinelandii strain OP, a slime free mutant, obtained from Dr. P. W. Wilson, the University of Wisconsin, Madison, was used in these experiments. The bacteria were grown in a modified Burk's medium containing, in g/1: 0.2 KH_2PO_4; 0.8 K_2HPO_4; 0.2 $MgSO_4 \cdot 7\ H_2O$; 0.09 $CaCl_2 \cdot 2\ H_2O$; 0.0192 $FeCl_3$; 0.00213 Na_2MO_4; 20 sucrose. This modification, a five-fold increase in $FeCl_3 + Na_2M_6O_4$, seems to lengthen the log phase of growth. One liter starter cultures in 2 liter flasks equipped with stainless steel baffles (30) to increase aeration were incubated on a rotary shaker at 32 C well into the log phase. A starter culture was used to inoculate into a five gallon carboy containing 15 liter of medium. The culture was aerated at 20 pounds per square inch pressure through sintered stainless steel spargers (Pall Trinity Micro Corporation) immersed in the culture with air humidified by bubbling through distilled water and sterilized by filtration through sterile cotton. Actively metabolizing cells produced enough heat to maintain the temperature of the culture at about 32 C in spite of the large amount of cool air pumped into the vessel obviating the necessity of using water baths or heating units. Under these conditions, a generation time of 2 hours was observed. Growth was monitored by removing small aliquots aseptically at given time intervals, diluting samples with water, and measuring the absorbancy at 600 mu in a Beckman DB Spectrophotometer. Organisms were grown to an absorbancy of 3.5 - 4.0 in log phase.

Preparation of cell-free extracts. One vol of culture in the log

phase was poured over one vol. crushed ice to halt metabolism rapidly, and all subsequent procedures were performed at or near 0 C. Cells were harvested in a Sharples centrifuge operating at 3,500 x g to yield approximately 45 g of bacterial paste per carboy. Cells were washed once with cold buffer (AVB) 50 mM Tris HCl, pH 7.2; 4 mM Mg acetate; 0.2 mM spermidine; and 100 mM KCl and resuspended in 6 M glycerol-AVB. The cells were equilibrated in glycerol until an internal cell concentration of 3 M glycerol was attained. This suspension was again centrifuged and suspended in a minimal amount of 6 M glycerol-AVB. Two vol. of AVB were injected into one vol. of agitated cell suspension to lyse cells osmotically by a modification of the technique of Robrish and Marr (68). Centrifugation of the mixture at 25,000 x g for 10 min yielded a pellet containing approximately 5% unbroken cells, "hulls" and a viscous, dark, brownish-green supernatant fluid. The supernate was decanted and recentrifuged twice for 1/2 hr. at 30,000 x g in an IEC B20 centrifuge to remove remaining cellular debris. This supernate (S-30) was centrifuged at 140,000 x g in an IEC B35 Preparative ultracentrifuge for 2 hr. to sediment ribosomes. Following centrifugation, 2/3 to 3/4 of this supernate (S-140) was aspirated; the remaining fluid was discarded. The ribosomal pellet was rinsed carefully with AVB and gently suspended in a 10 ml of AVB with a Potter-Elvejhem homogenizer. Ribosomes and S-140 were then centrifuged for 2 hr. at 140,000 x g a second time and the ribosomes were subsequently dissolved in sufficient AVB to give a concentration of 12 mg ribosomal protein per ml as determined by the Lowrey Procedure (10). The S-140 fraction was dialyzed overnight against 100 vol. AVB to remove amino acids and other low molecular weight co-factors

and to adjust the pH and the concentration of spermidine, Mg^{2+}, and K^{+} to the optimal concentrations required for the system. AVB dialysis fluid was changed at least twice during the period of dialysis.

Preincubation of ribosomes. The S-30 fraction was dialyzed against AVB overnight at 4 C and then incubated at 30 C for 1 hr. in a reaction mixture containing: (mM unless otherwise specified) 25 Tris · HCl, pH 7.2; 4 Mg acetate; 100 KCl, 0.2 spermidine; 10 ug/ml pyruvate kinase; 25 each of 20 amino acids; 0.15 2-phospho-enol-pyruvate Na_3 · 5 H_2O (2-PEP); 0.5 ATP; 0.15 each of GTP, CTP, and UTP. The mixture was centrifuged at 140,000 x g for 2 hr. and the ribosomes were washed with AVB.

Amino acid incorporating system. The following components were contained in a 0.4 ml reaction solution (in μmoles unless otherwise specified); 10 Tris · HCl, pH 7.2; 1.6 Mg acetate; 40 KCl; 0.08 spermidine; 4 ug pyruvate kinase; 0.0035 ^{14}C L-phenylalanine (7 mC per mmole); or 0.0035 ^{14}C-L-Lysine, (7 mC per mmole); 10 each of 19 amino acids; 60 2-PEP Na_3 · 5 H_2O; 200 ATP; and 60 each of GTP, CTP, and UTP; 60 - 300 μg ribosomal protein; 120 - 300 μg protein from the S-140 fraction.

Precipitation and washing procedure. The reaction mixtures were incubated for the time indicated. To halt the reaction two solutions were pipetted into each tube in rapid succession; 0.2 ml of solution A (containing 1 mg/ml each of carrier phenylalanine and bovine serum albumin) and 1.0 ml of solution B (containing 1 mg/ml carrier phenylalanine in 10% TCA). Filter membrane pads (0.45 μ pore size) were rinsed with a solution containing 1 mg/ml each of EDTA to remove heavy metal contaminants, 5% TCA titrated

with NaOH to about pH 10, and carrier phenylalanine. Precipitates were then poured onto the Millipore filter pads and washed four times with a solution containing 1 mg/ml carrier phenylalanine in 5% TCA and once with 95% ethanol.

For amino acid incorporation stimulated by poly A, reactions were stopped by the addition of 0.2 ml of a solution containing 1 mg/ml each of protamine sulfate and carrier lysine and 1.0 ml of a solution containing 1 mg/ml carrier lysine and 0.25% Na_2WO_4 in 10% TCA titrated with NaOH to pH 2.

Precipitates were air dried for one hr. and then placed in scintillation vials to which were added 10 ml of scintillation fluor containing toluene, 5 mg/l 2,5-diphenyloxazole (PPO) and 0.1 mg/l dimethyl 2,2-paraphenylene bis 5-phenyloxazole (POPOP). Vials were counted for 10 min. in a Nuclear Chicago scintillation counter at 67% efficiency. Efficiency and quenching were determined by using a barium external standard in conjunction with various quenched standards. All experiments were performed in duplicates or triplicates, and results are expressed in terms of μmoles ^{14}C phenylalanine incorporated per mg ribosomal protein.

Preparation of tRNA. Growth of the organism. Transfer RNA was prepared at the University of Wisconsin, Madison, using the facilities of the Department of Biochemistry.

Azotobacter was inoculated into a 1 l. flask containing an enriched Burk's medium. In addition to components previously listed the medium contained yeast extract and trypticase (B.B.L.) Casitone hydrolysate (Sigma) were each added to a final concentration of 0.5%. The 1 l. starter flask was then inoculated into a stainless steel stir jar containing 15 l. of the

same sterile medium. After growth of Azotobacter, this in turn was inoculated into a 200 l. fermenter containing 150 l. tap water and in g/l: 1.0 KH_2PO_4; 4.0 K_2HPO_4; 1.0 $MgSO_4 \cdot 7\ H_2O$; 0.09 $CaCl_2 \cdot 2\ H_2O$; 0.0192 $FeCl_3$; 0.00213 $NaMoO_4$; 100 sucrose. Growth was followed using a Beckman DB Spectrophotometer according to the procedure previously described. A generation time of 2 hr. was obtained. After growth began to slow down at an OD of 4.0, 2.88 gm $FeCl_3$, 0.3185 gm Na_2MO_4 and 1.4 Kg Trypticase Casitone hydrolysate were added. One generation time later, the cellular growth again slowed. The aeration was increased and 3.5 Kg sucrose were added to the medium. Log phase growth then continued at a slightly slower generation time of 2-1/2 hr. until an OD of 17.0 was reached. The cells were harvested in a Sharples centrifuge to give a yield of approximately 3 Kg of Azotobacter. Cells were washed in 15 l. of a buffer containing .05 M Tris · HCl, pH 7.6.

Extraction and isolation of tRNA. The cell paste (1.5 Kg) was mixed with 1.2 l. distilled water and 1.8 l. of a water saturated phenol solution, stirred for 1 hr., and allowed to stand overnight at 0 C. The mixture was centrifuged at 2,000 x g for 30 min. to separate aqueous and phenol phases. With care taken not to disturb the interface, 90% of the aqueous phase was aspirated. In order to inactivate nucleases and to dissociate any ribonucleoprotein complexes, sodium dodecyl sulfate (SDS) was added to the extracted aqueous phase until the solution reached 2% SDS. The solution was stirred for 1 hr. and allowed to stand overnight. Five hundred ml of phenol were added to the solution. It was stirred for 15 min., and allowed to stand for 4 hr. The aqueous layer was again removed as described previously. To

precipitate the RNA one vol. of aqueous phase was added to 2 vol. of 95% ethanol and 0.1 vol. of 20% potassium acetate, pH 5.0. The precipitate was centrifuged, washed with a solution containing 70% ethanol, and 0.05 M potassium acetate prepared by titrating 0.05 M KOH with acetic acid to pH 5.0, and dissolved in 300 ml. of 0.1 M Tris · HCl, pH 7.5. Undissolved material was removed by centrifugation. This solution was then applied to a 3 x 15 cm. DEAE cellulose column equilibrated with 0.1 M Tris · HCl, pH 7.5 at room temperature. The flow rate was adjusted to 100 ml/hr. The column was subsequently washed with 1.3 l. of 0.1 M Tris · HCl, pH 7.5 and eluted with a solution containing 1 M NaCl in 0.1 M Tris · HCl, pH 7.5 to release a low molecular weight RNA fraction composed of 130 nucleotides or less. Two vol. of cold 95% ethanol were added to one vol. of the eluent collected and the mixture was allowed to stand at -20 C overnight for complete precipitation. The precipitate was collected, washed once with 80% ethanol and then twice with 95% ethanol and lyophilyzed. The yield was 800 mg. The transfer RNA was incubated in 0.5 M Tris · HCl, pH 8.0 for 30 min. at 37 C to remove esterified amino acids.

Determination of protein. Protein was determined by a modification of the Lowry method (10). A solution containing 1 ml 2% $CuSO_4 \cdot 5\ H_2O$, 1 ml 4% Na tartarate and 48 ml 3% Na_2CO_3 in 0.1 N NaOH added to 0.5 ml samples containing 10-200 ug of protein. Samples were mixed well and permitted to stand at room temperature for 10 min. The Folin-Ciocalteau reagent diluted 1 part to 1 part water just before use was rapidly pipetted into these tubes and mixed. After 30 min., absorbancies were read at 660 mμ

on a Beckman DB Spectrophotometer. The same procedure was used for protein determinations of crystalline bovine albumin standards. One mg of ribosomal protein is equivalent to an A_{260} of 20. One mg of dialyzed S-100 protein equals on A_{280} of 3.

Reagents. Uniformly labeled ^{14}C-L-phenylalanine was obtained from Nuclear Chicago. ATP, CTP, GTP, and UTP were purchased from the P-L Biochemical Company while pyruvate kinase was obtained from Cal-Biochem. Poly U and poly A with an average sedimentation coefficient of 5.69 S and 9.33 S respectively were purchased from Miles Laboratories. Puromycin was obtained from the Nutritional Biochemicals Corporation. Chloramphenical was a gift from Parke Davis and Company. The Folin-Ciocalteau reagent was purchased from Aloe Scientific. Escherichia coli tRNA was a gift from Dr. Nakamoto at the University of Chicago.

RESULTS

Characteristics of the amino acid incorporating system.

Ribosomes and supernatant fraction requirements. Phenylalanine incorporation into protein does not occur in the absence of ribosomes. The linear relationship between incorporation and ribosomal content is illustrated in Fig. 1. The S-140 fraction is also required for amino acid incorporation (Fig. 2). Low levels of incorporation without added supernatant fraction are probably due to S-140 components adsorbed to ribosomes. Thus both ribosomes and supernatant fraction are required for amino acid incorporation. (Table 1)

Other cofactor requirements. Several other components are necessary for amino acid incorporation (Table 1). ATP and an ATP generating system are also absolute requirements. Both the omission of GTP alone and the omission of GTP, CTP, and UTP decreased incorporation to 24% and 29% respectively; while the presence of GTP, and in the absence of UTP and CTP in the reaction mixture restored incorporation to 70% of the complete system. GTP is required in the rabbit reticulocyte (5, 66) as well as the *E. coli* (21, 48, 55) systems for the peptidization step and release of tRNA from the ribosome. Since incorporating activity decreases 30% in the absence of UTP and CTP, these nucleotide triphosphates are required to some extent. Low levels of incorporation which occur in the absence of GTP or ATP may be the result of interconversions of other nucleoside triphosphates to these triphosphates.

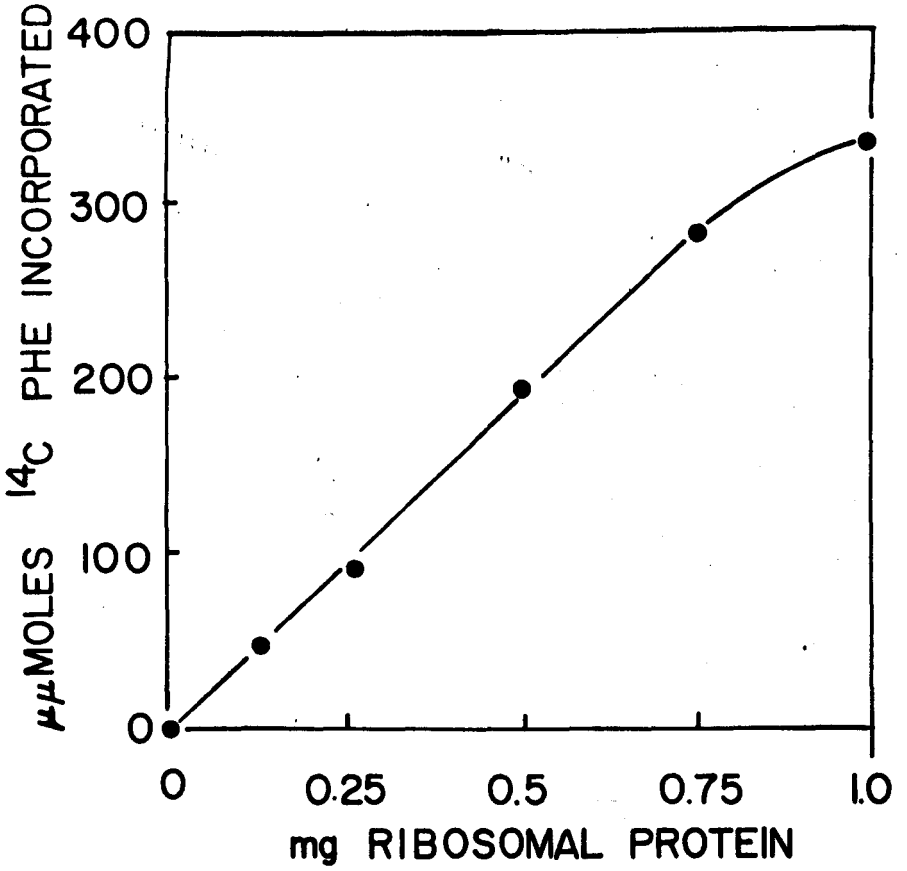

FIG. 1. The effect of ribosomal concentration upon phenylalanine incorporation. The reaction mixture described in Materials and Methods was used. The mixture contained increasing amounts of washed ribosomes and 125 μg S-140 protein.

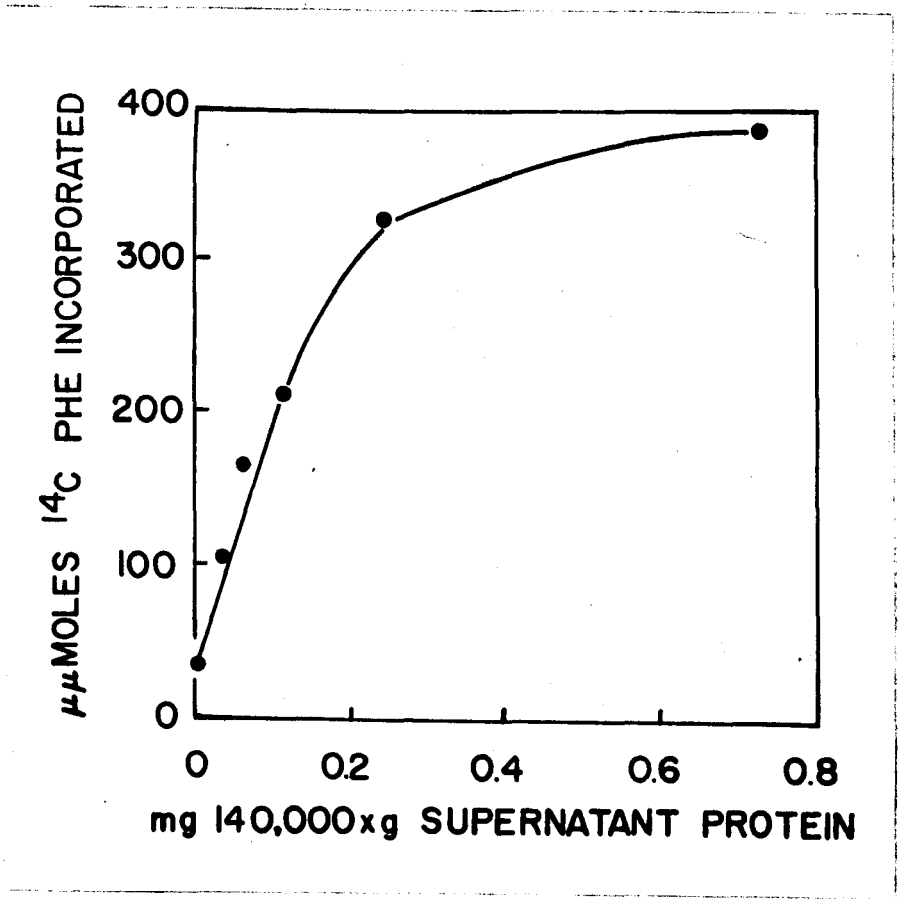

FIG. 2. The effect of S-140 protein fraction upon phenylalanine incorporation. The reaction mixture described in Materials and Methods was used. The mixture consisted of washed ribosomes containing 160 μg protein and increasing quantities of S-140.

Table 1 Requirements for phenylalanine incorporation in the cell-free system.

System	μμmoles ^{14}C phenylalanine incorporated per mg ribosomal protein	Percent incorporation
Complete	228	100
- Ribosomes	0	0
- S-140 fraction	16	7
- ATP, PEP, pyruvate kinase	8	4
- ATP	50	22
- GTP	66	29
- UTP, CTP, GTP	55	24
- UTP, CTP	159	70
+ 9 μg DNAse	228	100
+ 9 μg RNAse	1	0.4
+ 9 μg puromycin	15	7
+ 5 x 10^{-4} streptomycin	22	10
+ 9 μg chloramphenicol	46	20

The reaction mixture described in Materials and Methods was used with deletions or additions of appropriate components. 0.4 ml of the mixture consisted of washed ribosomes containing 120 μg protein and 373 μg S-140 protein.

Antagonists to protein synthesis. DNAse does not affect protein synthesis; however, RNAse, puromycin, streptimycin, and chloramphenicol inhibited incorporation of ^{14}C phenylalanine 99%, 93%, 90%, and 80% respectively.

Inorganic ion requirements. A concentration of 4 mM Mg^{2+} is optimal for amino acid incorporating activity in both the endogenous and poly U-stimulated systems in Azotobacter (Fig. 3). At higher concentrations endogenous incorporation decreases sharply to a minimal level at 10 mM Mg^{2+}. The poly U-stimulated incorporating activity approaches the minimal level at 20 mM Mg^{2+}. Both display little activity at Mg^{2+} concentrations below 1 mM not shown). Since the poly U-stimulated incorporation is decreased by only 40% at 10 mM, it may reflect a higher Mg^{2+} concentration required for initiation of polypeptide synthesis. The skewed curve in Fig. 3 may indicate that higher Mg^{2+} concentrations required to form initiating complexes with poly U than Mg^{2+} concentrations required for translation of natural message, limits the accuracy of polyphenylalanine synthesis.

Monovalent cations such as K^+ or NH_4^+ which have similar physical properties are required in both bacterial and mammalian systems (28, 37, 55, 74, 78) and have been implicated in the binding of tRNA to ribosomes for initiation of protein synthesis (55). The Azotobacter system also requires K^+ for protein synthesis. Both the endogenous and poly U-stimulated systems are enhanced maximally 3-fold and 20-fold respectively at a concentration of 0.1 M K^+ (Fig. 4). Incorporation of ^{14}C phenylalanine into protein was stimulated in the endogenous and poly U systems 3-fold and 7-fold res-

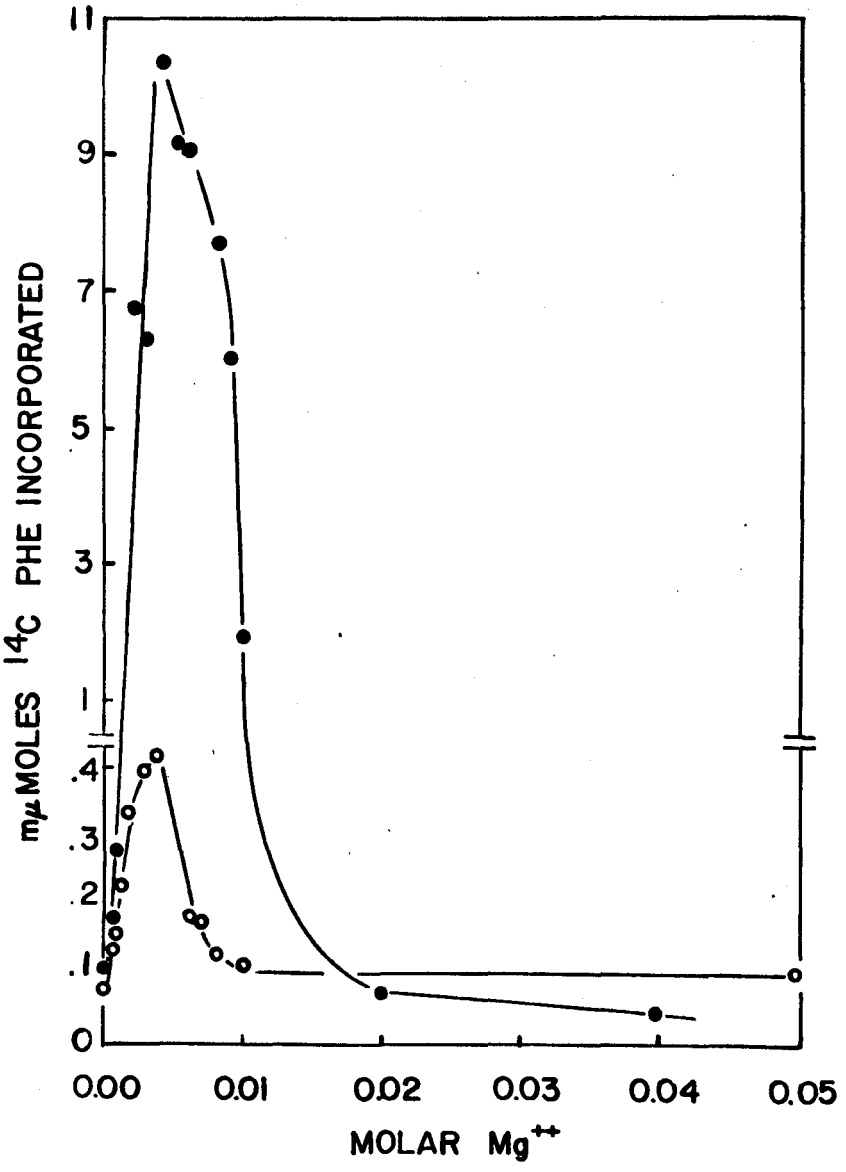

FIG. 3. The effect of Mg^{2+} concentration upon phenylalanine incorporation. The reaction mixture described in Materials and Methods was used. Washed ribosomes containing 250 μg protein and 520 μg S-100 for the poly U-stimulated system (●—●) and 186 μg S-140 protein for the endogenous system (○—○). Poly U-stimulated systems contained 100 μg poly U and 180 μg tRNA. Zero magnesium represents 0.75 mM Mg^{2+} since ribosomes were extracted and washed in AVB and S-140 was dialyzed against AVB.

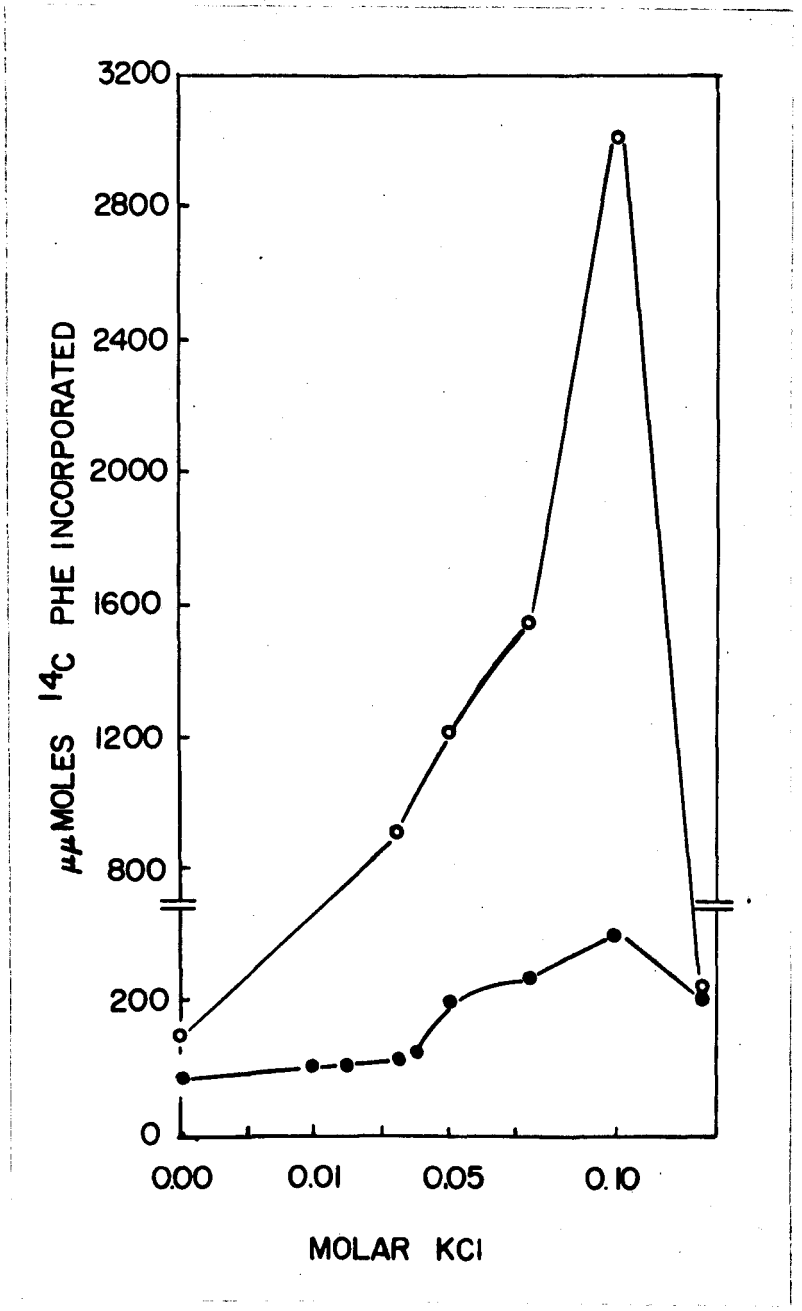

FIG. 4. The effect of K^+ upon phenylalanine incorporation. The reaction mixture described in Materials and Methods was used. The K^+ concentration was varied. Washed ribosomes containing 255 μg protein and 200 μg S-140 protein prepared with K^+ free AVB and were added to the system. Endogenous incorporation (●—●); poly U-stimulated incorporation (o—o) using 100 μg poly U and 180 μg tRNA.

pectively within a broad range from 2 mM to 80 mM NH_4^+.

Polyamine requirements. Spermidine shows maximal enhancement of endogenous incorporation at a concentration of 0.2 mM in the system (Fig. 5).

ATP concentration. ATP is an absolute requirement for incorporation of ^{14}C phenylalanine into hot TCA-insoluble material

This cofactor has been associated with charging tRNA with amino acids (2, 23, 24, 35, 36) as well as with repairing the 3' terminal end of deacylated tRNA species (64). Optimal concentrations of 25 mM and 50 mM have been demonstrated for the poly U and endogenous-stimulated systems respectively (Fig. 6). Concentrations above this level are inhibitory, probably due to effective removal of Mg^{2+} from ribosomal binding sites through the neutralization of phosphate residues in ATP (89). The lower optimal ATP concentration in the poly U compared to the endogenous system may reflect the same mechanism.

pH dependence. The optimal pH of this system is 7.2. This optimum may reflect the conformation and biological activity of ribosomal proteins and/or enzymatic activity of other protein fractions associated with the system.

Temperature dependence. Incorporation in the poly U-stimulated system is highest at 40 C. However, basal incorporation is highest within the range between 30 C and 40 C (Fig. 7). Protein synthesis in the endogenous and poly U-stimulated system decrease to 10% and 6% respectively at 0 C.

Kinetics of endogenous incorporation. Endogenous incorporation at 30 C increases linearly for the first ten minutes after which there is little

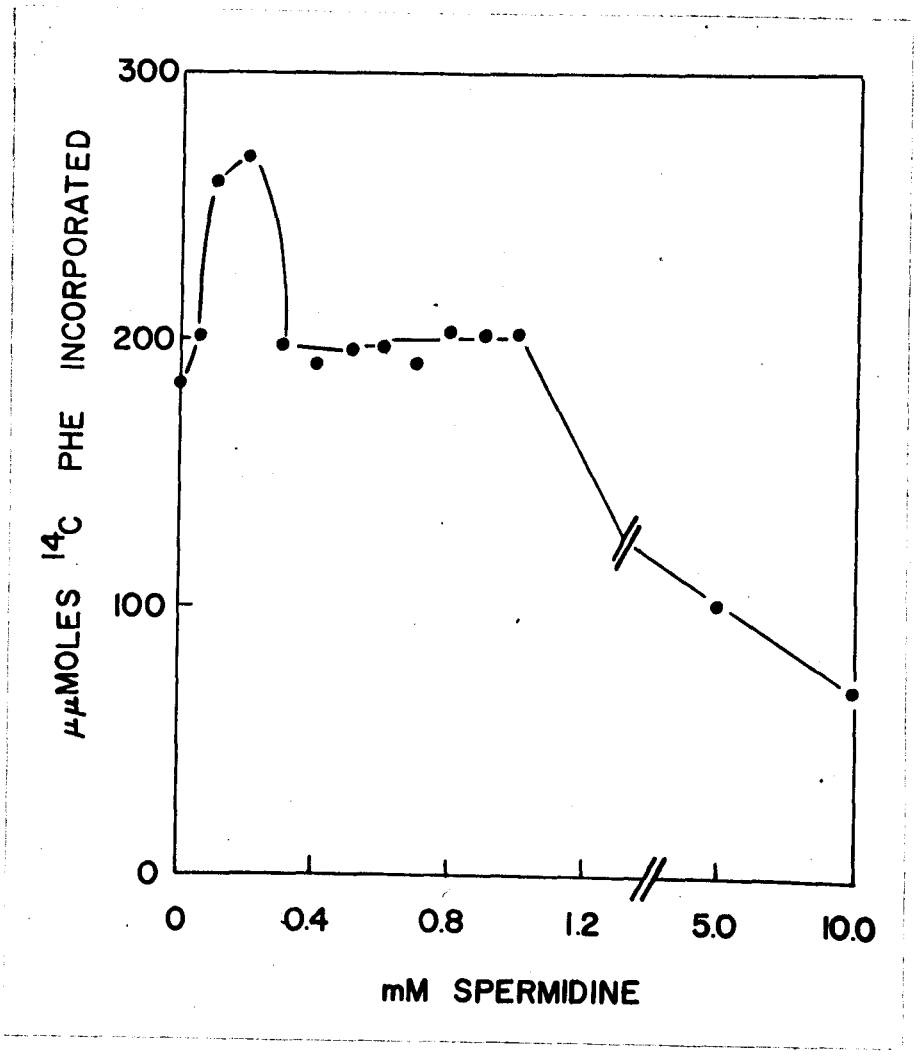

FIG. 5. The effect of spermidine upon endogenous phenylalanine incorporation. The reaction mixture described in Materials and Methods was used. Washed ribosomes containing 260 μg protein and 520 μg S-140 protein were added to all tubes. Zero molarity represents 0.06 mM spermidine since ribosomes were extracted and washed in AVB and S-140 was dialyzed against AVB.

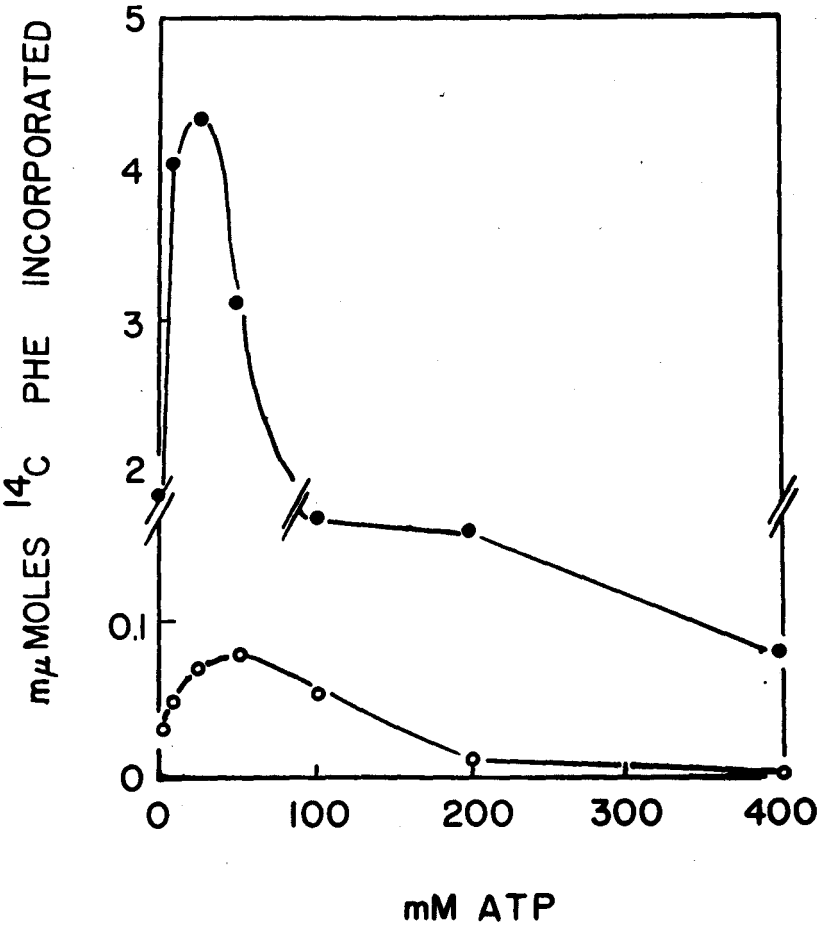

FIG. 6. The effect of ATP concentration upon phenylalanine incorporation. The reaction mixture described in Materials and Methods was used. The ATP concentration was varied as indicated. Washed ribosomes containing 135 μg protein and 187 μg S-140 protein were added. The endogenous system (o——o); the poly U-stimulated system (●—●) included 100 μg poly U and 180 μg tRNA.

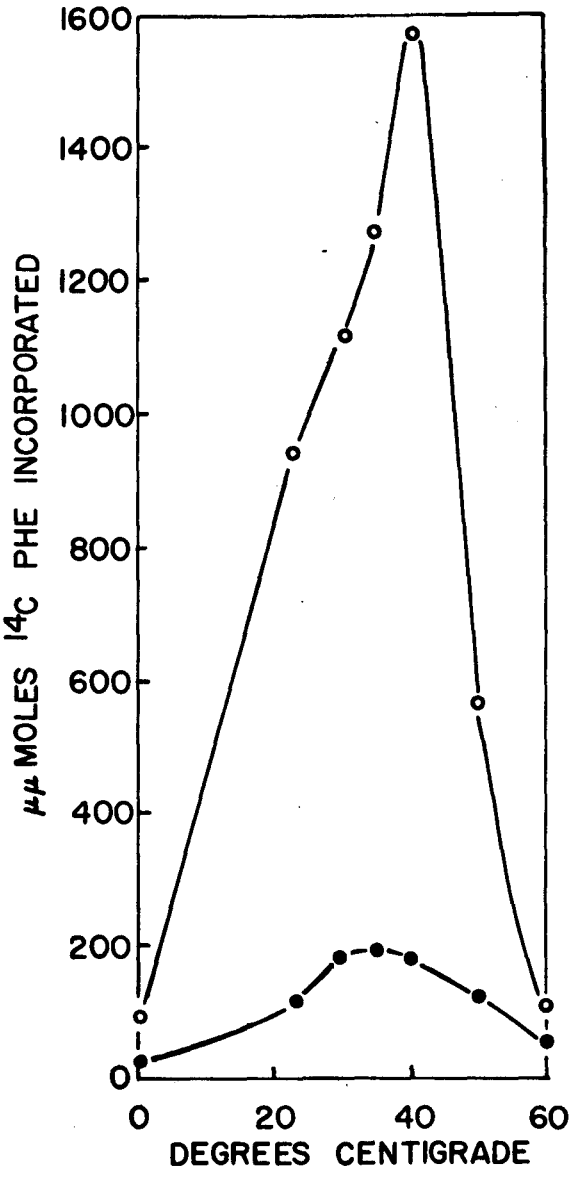

FIG. 7. The effect of temperature on phenylalanine incorporation. The reaction mixture described in Materials and Methods was used. Tubes were incubated at indicated temperatures. Washed ribosomes containing 300 μg protein and 250 μg S-140 protein were added to all tubes. Endogenous system (●—●); poly U system (o—o) contained 100 μg poly U and 180 μg tRNA.

additional increase. At the end of twenty minutes incorporation is virtually complete (Fig. 8).

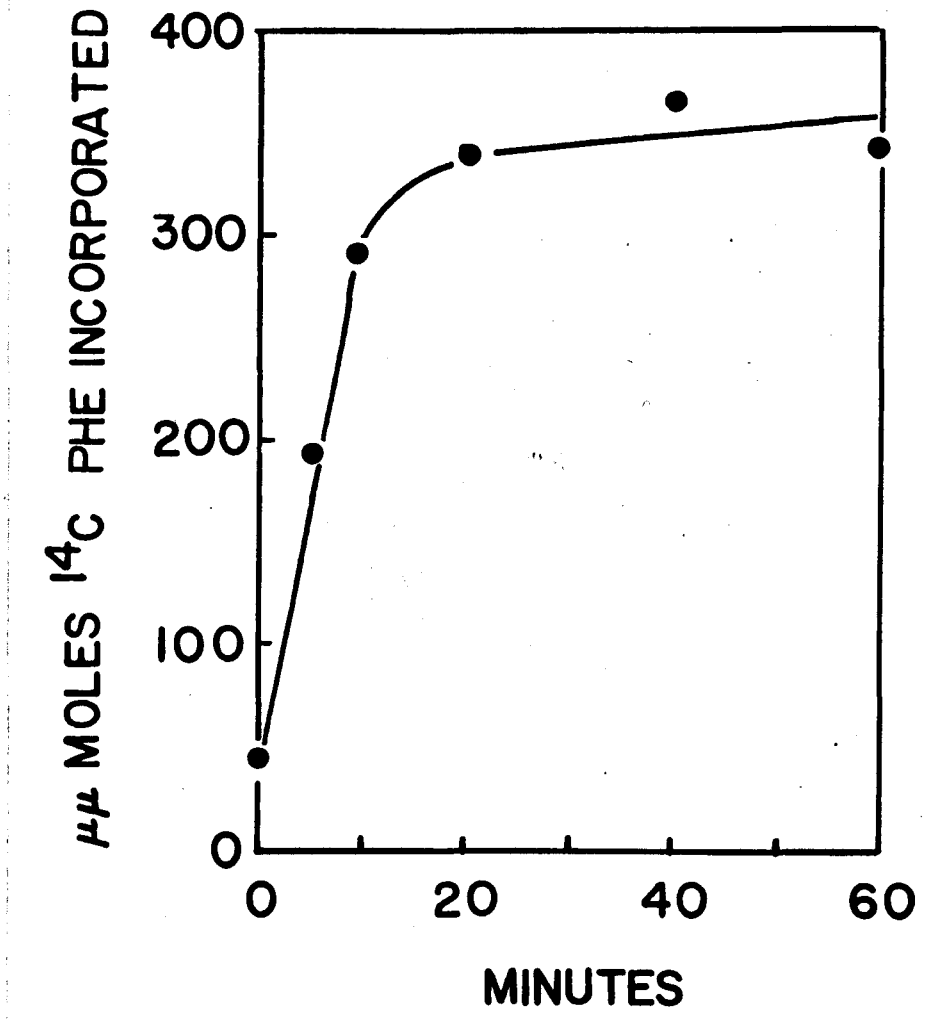

FIG. 8. Kinetics of endogenous ^{14}C phenylalanine incorporation. The reaction mixture described in Materials and Methods was used. Washed ribosomes containing 260 μg protein and 520 μg S-140 protein were added to reaction mixtures.

Characteristics of the poly U-stimulated incorporation of phenylalanine.

Poly U participation. Poly U stimulates incorporation of ^{14}C phenylalanine into polyphenylalanine. With the system described in Materials and Methods, 0.1 mg poly U/255 ug ribosomal protein saturates the system in the presence of excess tRNA (Fig. 9).

Participation of tRNA. Radioactive phenylalanine incorporation in the basal system may be enhanced as much as 5-fold by the addition of tRNA. When tRNA is added to a reaction mixture containing poly U, stimulation may be as high as 20 to 25 fold (Table 2). The relationship between tRNA and ^{14}C phenylalanine incorporation is shown in Fig. 10. Under the conditions of the experiment illustrated 180 ug tRNA/0.4 ml reaction mixtures gives maximal incorporation. Higher concentrations of tRNA inhibit ^{14}C phenylalanine incorporation possibly due to competition for Mg^{2+} by ribosomes, poly U and tRNA.

To determine the role of tRNA in protein synthesis in this system, the kinetics of amino acid incorporation into charged tRNA and polypeptides were examined. The kinetics of in vitro incorporation of ^{14}C phenylalanine into hot TCA (nascient protein) and cold TCA (nascient protein and aminoacyl tRNA) precipitable fraction is shown in Fig. 11. Incorporation into cold TCA precipitable material proceeded at 150% the rate of hot TCA precipitable material and reached a final level 52% greater than the hot TCA precipitable fraction. The rate of incorporation into hot TCA soluble components (difference between the curves of cold and hot TCA precipitable fractions, respectively amino acyl tRNA) is equal to that into protein. Experiments in which the S-140 fraction alone was incubated in the incorporating system

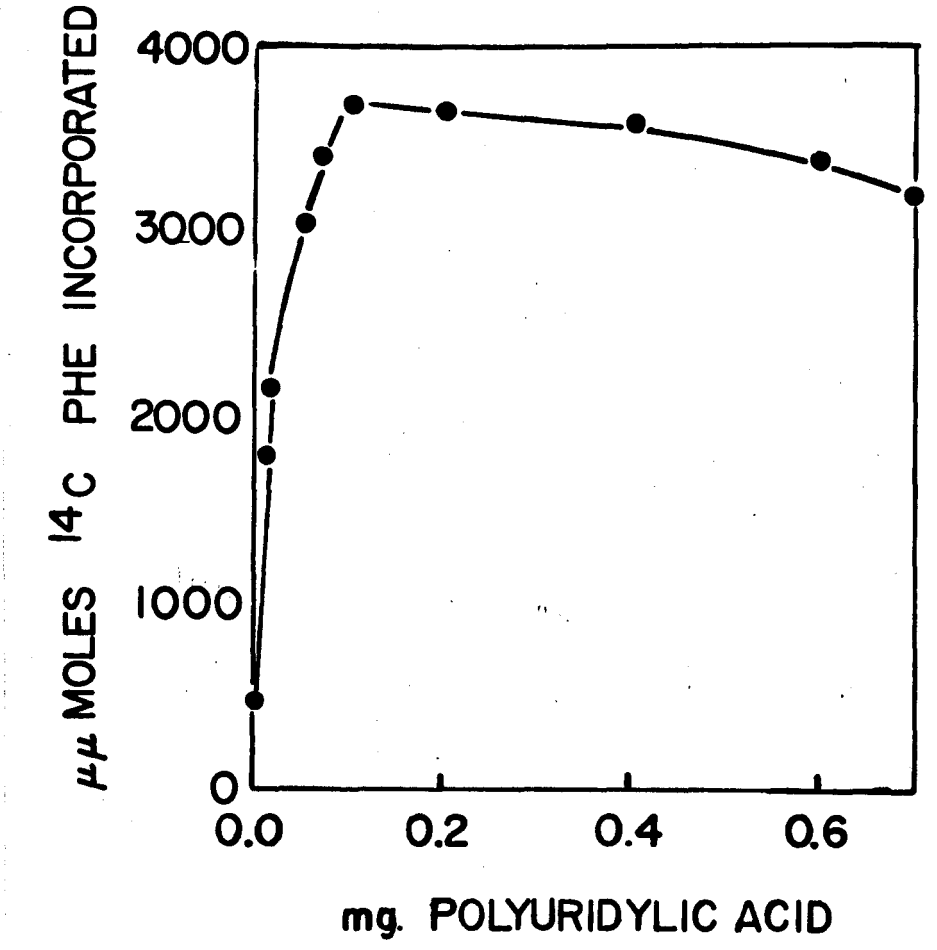

FIG. 9. The effect of poly U upon the stimulation of phenylalanine incorporation. The reaction mixtures described in Materials and Methods was used. Washed ribosomes containing 255 μg protein, 400 μg S-140 protein and 180 μg tRNA were added to the reaction mixtures.

Table 2 Requirements for the stimulation of polyphenylalanine synthesis.

System	μμMoles ^{14}C phenylalanine incorporated per mg ribosomal protein	Percent
Basal	172	100
+ tRNA	897	520
+ pU	472	274
+ pU + tRNA	3152	1890

Reaction mixtures were prepared as described in Materials and Methods. Washed ribosomal containing 250 μg protein and 350 μg S-140 protein were added to reaction mixtures. 180 μg tRNA and 100 μg poly U were added to appropriate tubes.

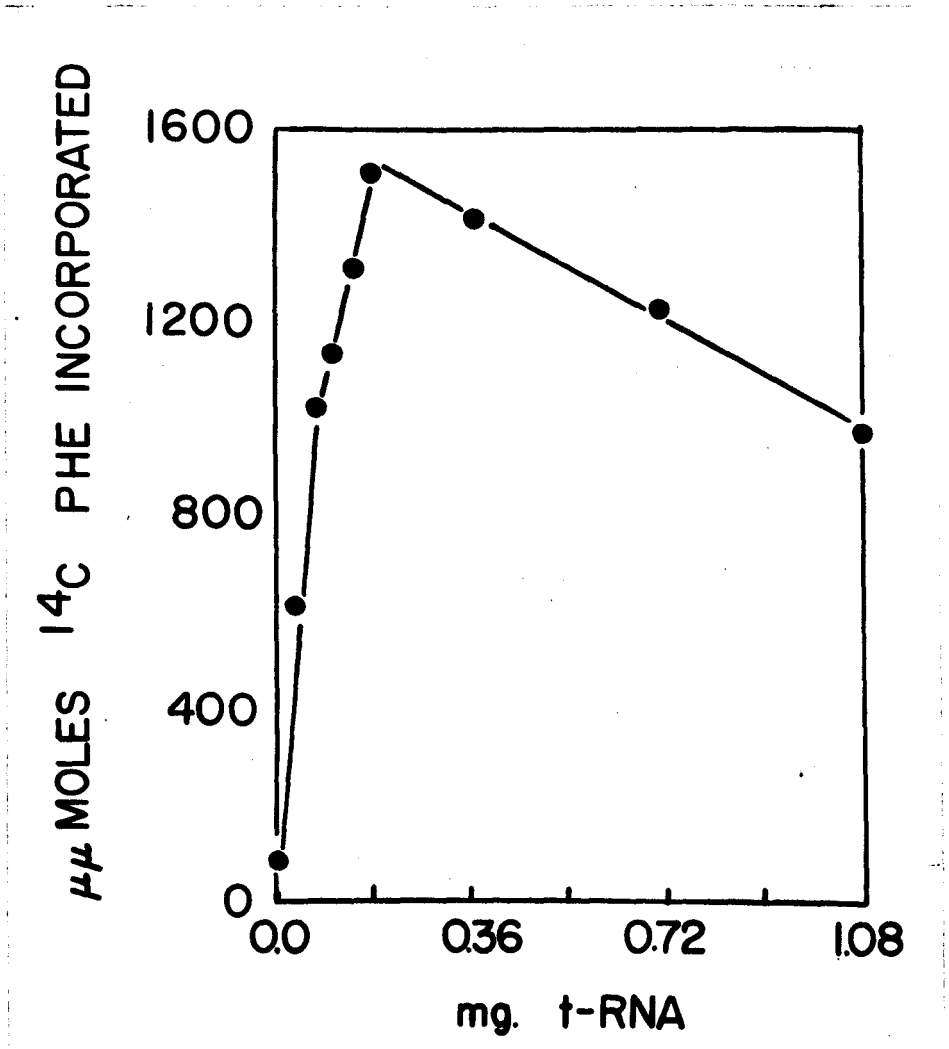

FIG. 10. The effect of tRNA upon the stimulation of polyphenylalanine synthesis. Reaction mixtures were prepared according to Materials and Methods washed ribosomes containing 270 μg protein, 112 μg S-140 protein, and 100 μg poly U were added to each tube.

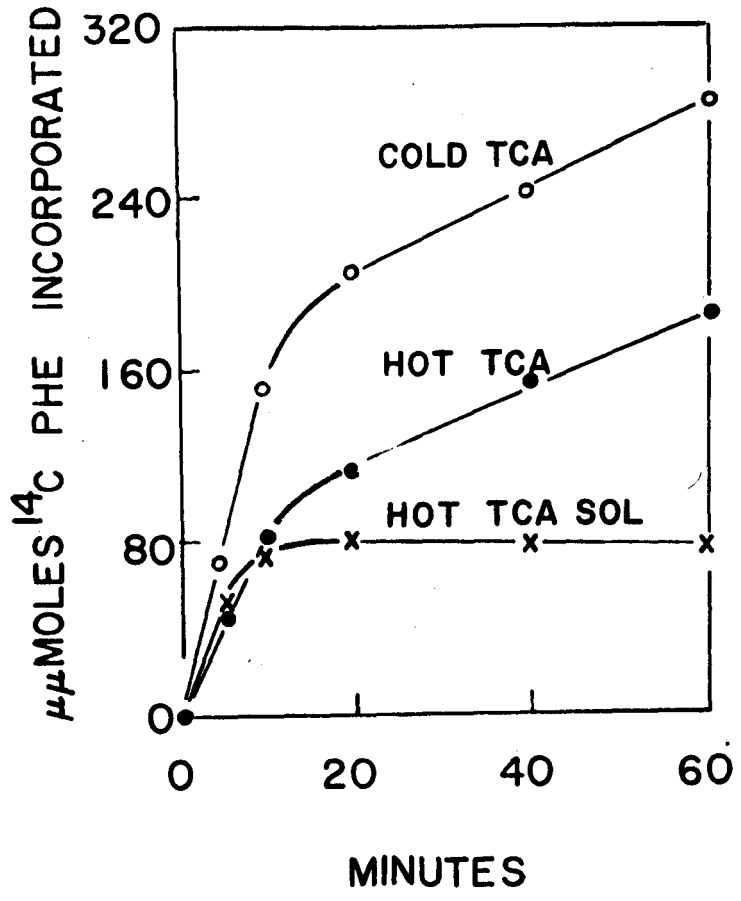

FIG. 11. The time course of incorporation of ^{14}C phenylalanine into hot and cold TCA precipitable material. Duplicate aliquots of 0.4 ml were withdrawn at times indicated and ice cold TCA added. One sample of each time interval was heated at 90 C for 15 min. All samples were washed as described under Materials and Methods with ice cold 5% TCA within 2 hours after precipitation. (o—o) Cold TCA precipitable counts; (●—●) hot TCA precipitable counts; X-X hot TCA soluble counts (difference between cold and hot TCA precipitable counts). Washed ribosomes containing 200 μg protein and 320 μg S-140 proteins were added to reaction mixtures.

revealed that almost all the differences in final incorporation observed in Fig. 2 were due to components found in the cold TCA precipitable fraction. We conclude from this that the amino acyl tRNA is an intermediate in the synthesis of polypeptides.

E. coli tRNA was examined as a substitute for Azotobacter tRNA in the system. The RNA from either source stimulated incorporation in the presence of poly U or poly A to levels characteristic of poly U and poly A system. (Table 3) This finding reemphasizes the similarities between biological systems of microorganisms.

Table 3 Substitutions of Azotobacter tRNA with E. coli tRNA and the requirements for poly U and poly A-stimulation.

System	μmoles ^{14}C phe incorporated /mg ribosomal protein	Fold Stimulation	μmoles ^{14}C Lys incorporated /mg ribosomal protein	Fold Stimulation
Basal	202	1.0	294	1.0
+ Azot. tRNA	420	2.0	460	1.5
+ poly U	450	2.2	480	1.0
+ Azot. tRNA + poly U	5537	30	—	—
+ Azot tRNA + poly A	—	—	2192	7.3
+ E. coli tRNA	430	2.1	450	1.5
+ E. coli tRNA + poly U	4442	22.0	—	—
+ E. coli tRNA + poly A	—	—	1987	6.6

Reaction mixtures were prepared according to Materials and Methods. 100 μg each poly U and poly A, 180 μg Azotobacter (Azot.) tRNA and E. coli tRNA were added to appropriate tubes. Reaction mixtures consisted of washed ribosomes containing 300 μg protein and 287 μg S-140 protein in addition to other factors described in Materials and Methods.

Variables associated with ribosomal activity.

The extent of amino acid incorporation in the endogenous and poly U-stimulated experiments depended upon the phase of growth in which the cells were harvested, how rapid cellular metabolism was halted before harvest, the mode of cell lysis, pretreatment of the concentrated ribosomes and the mode of storage of frozen ribosomes and S-140 fractions.

Phase of growth. Ribosomes isolated from cells growing in mid-log phase demonstrated a greater endogenous activity than those isolated from late in the log phase (Table 4). Similarly, poly U directed ^{14}C polyphenylalanine synthesis was greater using ribosomes from the mid log cells than from the late log phase ribosomes. Stationary phase cells yielded ribosomes whose endogenous activity was below the sensitivity of measurement; poly U-directed incorporation with these ribosomes was poor also. Ribosomes extracted from mid-log cultures demonstrated the greatest efficiency in polypeptide formation and apparently contained higher quantities of "active ribosomes" necessary for the initiation of polypeptide synthesis. As the culture approached and entered into the stationary phase probably less endogenous message and "active ribosomes" were present.

Rapidity of cessation of cell metabolism. Prior to harvest, variation in the time taken to cool log phase cells to 0 C influences the activity of the ribosomes (Table 5). Pouring cells onto crushed ice and thus cooling to 0 C within 15 seconds yielded a ribosomal preparation with the highest endogenous activity and high poly U-stimulated activity. This is due to the high level of polyribosomes in the ribosomal preparation as shown by sucrose

Table 4 Ribosomal activity of extracts from Azotobacter obtained by harvesting at several phases of growth.

Phase of growth from which ribosomes were obtained	μμMoles ^{14}C phe. incorporated/mg ribosomal protein	μμMoles ^{14}C phe. incorporated/mg ribosomal protein	Fold Stimulated
	Basal	poly U, tRNA	
Mid-log	180	8291	52
Late-Log	150	5737	40
24 hour-stationary	0	352	--

Reaction mixtures were prepared according to Materials and Methods. Mid-log phase washed ribosomal containing 69 μg protein, washed late-log phase ribosomes containing 174 μg protein washed stationary-phase containing 45 μg protein, 287 μg S-140 protein, 100 μg poly U and 180 μg tRNA were added to appropriate tubes. All results were normalized to uumoles ^{14}C phenylalanine incorporated per mg ribosomal protein.

Table 5 The effect of rapidly halting cellular metabolism upon ribosomal activity.

Ribosomes extracted from cultures which were cooled to 0 C within the following period of time	μμmoles ^{14}C phe incorporated/mg ribosomal protein	μμmoles ^{14}C phe incorporated/mg ribosomal protein	Fold Stimulation
	Basal	+ poly U tRNA	
15 seconds	428	9805	23
15 minutes	203	4647	23
2 hours	50	7447	149
12 hours	52	1011	20

Reaction mixtures were prepared as described in Materials and Methods. Washed ribosomes were added containing the following amounts of protein: 15 seconds cooled, 120 μg; 15 minutes cooled, 135 μg; 2 hours cooled, 324 μg; 12 hours cooled, 130 μg. To all reaction mixtures 253 μg S-140 protein was added. 100 μg poly U and 180 μg tRNA were added to appropriate tubes. Incorporation was normalized in terms of uumoles ^{14}C phenylalanine incorporated/mg ribosomal protein.

density centrifugation. Polyribosomes comprised more than 40-50% of those ribosomes found in these preparations. When cells were cooled to 0 C within 15 minutes by placing culture flasks in an ice bath, they yielded ribosomal preparations with one-half the endogenous activity and less poly U-stimulated polyphenylalanine synthesis. Ribosomes extracted from cultures requiring 2 hours to cool contained little endogenous activity but high poly U-stimulated activity. Radioactive phenylalanine incorporation was not as high as with ribosomes extracted from rapidly cooled cells. Ribosomes extracted from cultures cooled to 0 C during a period of 12 hours yielded very low endogenous activity and low poly U-stimulated activity.

Preincubation. The endogenous activity of ribosomes from log phase cells can be reduced greatly by preincubation. In this case, poly U-stimulated incorporation usually attains 6 mumoles, but has reached as high as 18 mumoles. During preincubation polyribosomes are converted to monosomes as demonstrated by sucrose density centrifugation (not shown). The non-preincubated control contains the normal complement of polyribosomes in addition to monosomes. The preincubated preparation showed complete loss of polyribosomes with the concomittant increase in monosomes probably due to the completion of protein synthesis as a result of message run-off. (Table 6)

Mode of cell rupture. We have previously shown the importance of the mode of cell lysis in obtaining active ribosomal preparations (62). Ribosomes prepared from cells disrupted by sonic oscillation demonstrate lower endogenous and poly U-stimulated incorporation than do ribosomes obtained from osmotically shocked cells (Table 7). Since the poly U-stimulation of these

Table 6 The effect of storage and preincubation of ribosomes upon incorporation.

Treatment of Ribosomes	μmoles ^{14}C phe incorporated/mg ribosomal protein	μmoles ^{14}C phe incorporated/mg ribosomal protein	Fold stimulation
	Basal	+ poly U tRNA	
Fresh ribosomes	234	2880	12
Frozen ribosomes & frozen S-100	185	3700	20
Storage of ribosomes at 0 C for 48 hours	161	6687	42
Preincubation of ribosomes	0	18,112	—

Fresh ribosomes were obtained by procedures described in Materials and Methods. Frozen ribosomes were quick frozen by submerging samples in liquid nitrogen and stored therein. Preincubated ribosomes were prepared as described in Materials and Methods. Reaction mixtures were prepared with components outlined in Materials and Methods. Washed ribosomes containing 330 μg protein and 250 μg S-140 protein and washed preincubated ribosomal containing 45 μg protein and 287 μg S-140 protein are added to appropriate tubes.

Table 7 The effect of the mode of cell lysis upon the incorporation of phenylalanine.

Mode of cell lysis prior to ribosomal isolation	μμmoles ^{14}C phe incorporated/mg ribosomal protein	μμmoles ^{14}C phe incorporated/mg ribosomal protein	Fold Stimulation
	Basal	+ poly U, tRNA	
Osmotic, shock	150	6000	40
Sonication	105	2895	27

Ribosomes extracted from osmotically lysed cells were prepared as described in Materials and Methods. 0.25 g cell/ml AVB were sonicated using a Bronsen sonifier set at 4 amps for 3 five-second intervals. Subsequent to lysis ribosomal preparations followed the procedure outlined in Materials and Methods. Reaction mixtures were prepared according to Materials and Methods. Washed ribosomal containing 120 μg protein from sonicated cells, 174 μg protein from osmotically lysed cells and 286 μg S-140 protein were added to appropriate tubes.

ribosomes is minimal, they are somehow damaged by the cavitation process.

Stability of the cell-free extracts. Ribosomes and the S-140 fraction when quick frozen and stored in liquid nitrogen remain active over a period of at least 2 weeks (Table 6). Longer periods of time were not tested. After 2 weeks of storage there is an 18% decrease in the basal level of amino acid incorporation. On the other hand, there is a 16-fold stimulation in ^{14}C phenylalanine incorporation by poly U compared to a 12-fold stimulation with the fresh ribosomal preparation. This may be due to the stripping of message from ribosomes during the process of freezing or thawing.

Ribosomes and S-140 may be stored at 0 C for at least 48 hours and still retain activity (Table 6). Endogenous incorporation is decreased on the second day compared to that of the first day. At the same time, not only does poly U-stimulation increase by the second day as compared to the first day, but additional levels of ^{14}C polyphenylalanine are synthesized. This is presumably due to the translation of nascent message at 0 C and subsequent release of ribosomes capable of initiating protein synthesis.

Kinetics of poly U stimulation.

Poly U stimulated incorporation of ^{14}C phenylalanine proceeds linearly. Most striking is an initial lag of 3 minutes (Fig. 12). Preincubation of the ribosomes increases the lag period to eleven minutes (Fig. 13). To determine whether the ribosomes undergo a change during the preparation of ribosomes we compared their kinetics of incorporation with the original S-30 fraction. An initial lag of three minutes was also observed with the S-30 fraction (Fig. 14). However, when ribosomes were preincubated for 15 minutes with poly U and tRNA at 0 C prior to the addition of the S-140 fraction the lag disappeared (Fig. 15). Similarly, the initial lag disappeared when preincubated ribosomes prepared as described in Materials and Methods were mixed with tRNA, poly U and the rest of the system excluding the S-140 fraction. Reaction mixtures were incubated for 15 minutes before the addition of S-140 (Fig. 16). The highest levels of ^{14}C phenylalanine incorporation was observed under these latter conditions (18 mumoles ^{14}C phenylalanine incorporated/mg ribosomal protein).

Poly A-stimulated ^{14}C lysine incorporation. Poly A and tRNA stimulate ^{14}C lysine incorporation 10-fold under the same conditions in which poly U stimulated ^{14}C phenylalanine incorporation was 24-fold (Table 3). Lesser stimulation of ^{14}C lysine incorporation in the Azotobacter system may be due to the fact that poly A itself precipitates at concentrations above 10 ug/ml in the presence of 3 mM Mg^{2+} (26). Precipitated polymer probably does not bind to the ribosome and also removes Mg^{2+} from solutions necessary for optimal polypeptide synthesis.

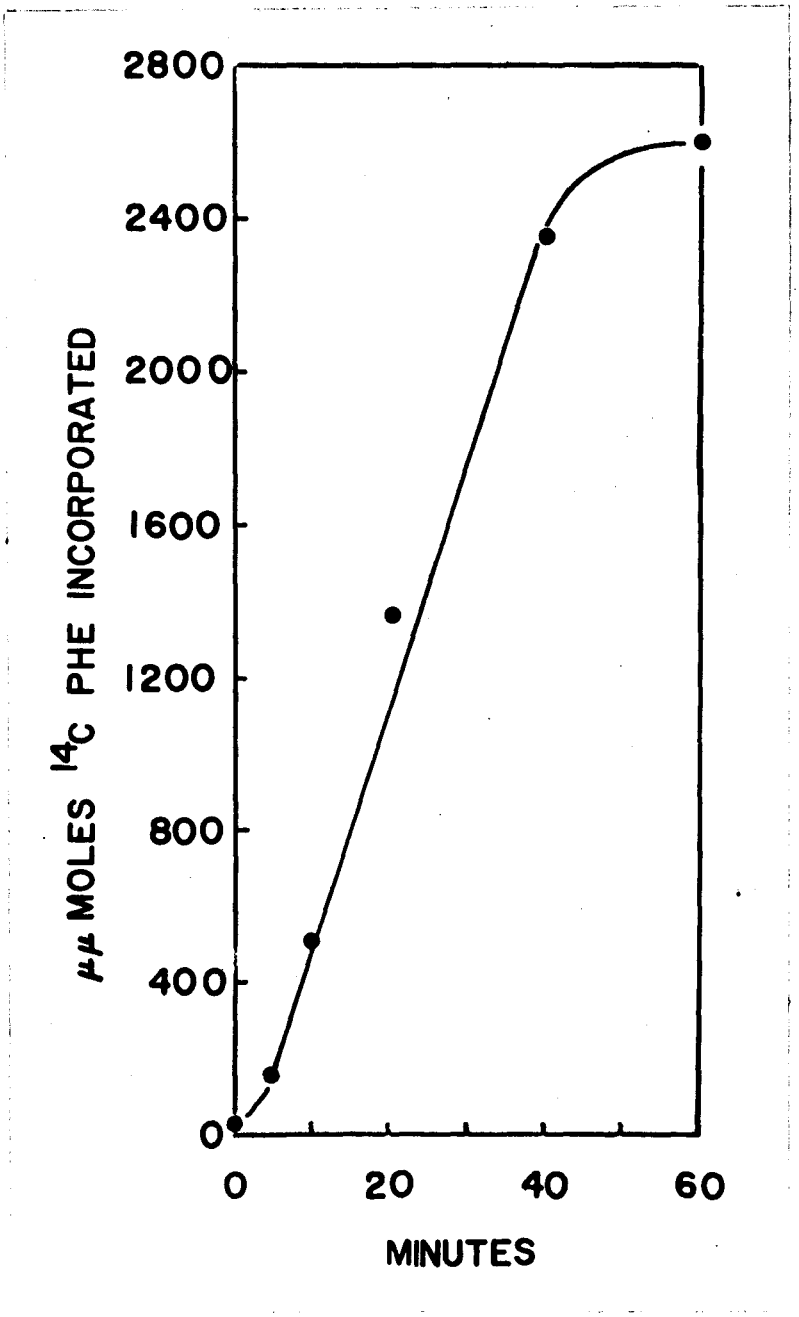

FIG. 12. Kinetics of poly U-stimulated phenylalanine incorporation. Reaction mixtures were prepared as described in Materials and Methods. Washed ribosomes containing 270 μg protein, 200 μg S-140 protein, 180 μg tRNA and 100 μg poly U were added to the reaction mixtures.

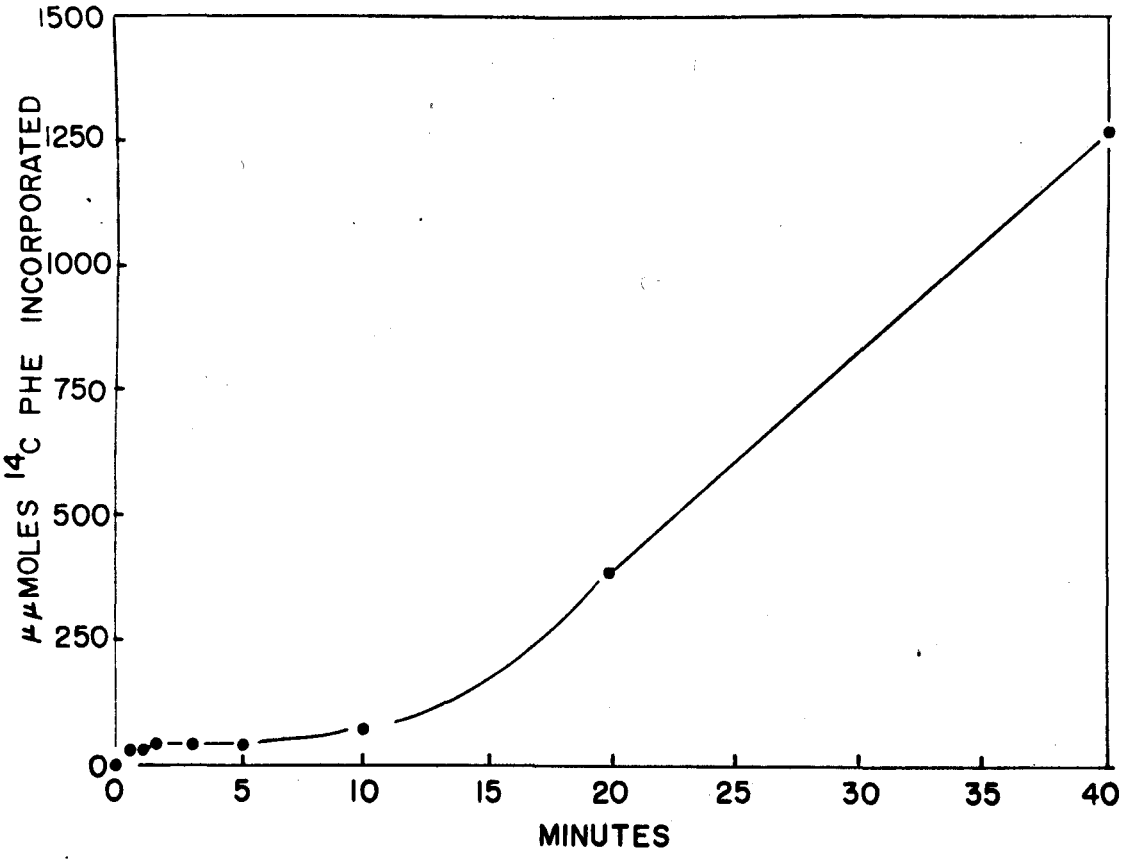

FIG. 13. Kinetics of phenylalanine incorporation using preincubated ribosomes prepared according to the procedure described in Materials and Methods. Reaction mixtures contained per 0.4 ml 100 μg poly U, 180 μg tRNA. Washed ribosomes containing 200 μg protein and 75 μg S-140 protein. Aliquots were removed and protein precipitated as described in Materials and Methods at given time intervals.

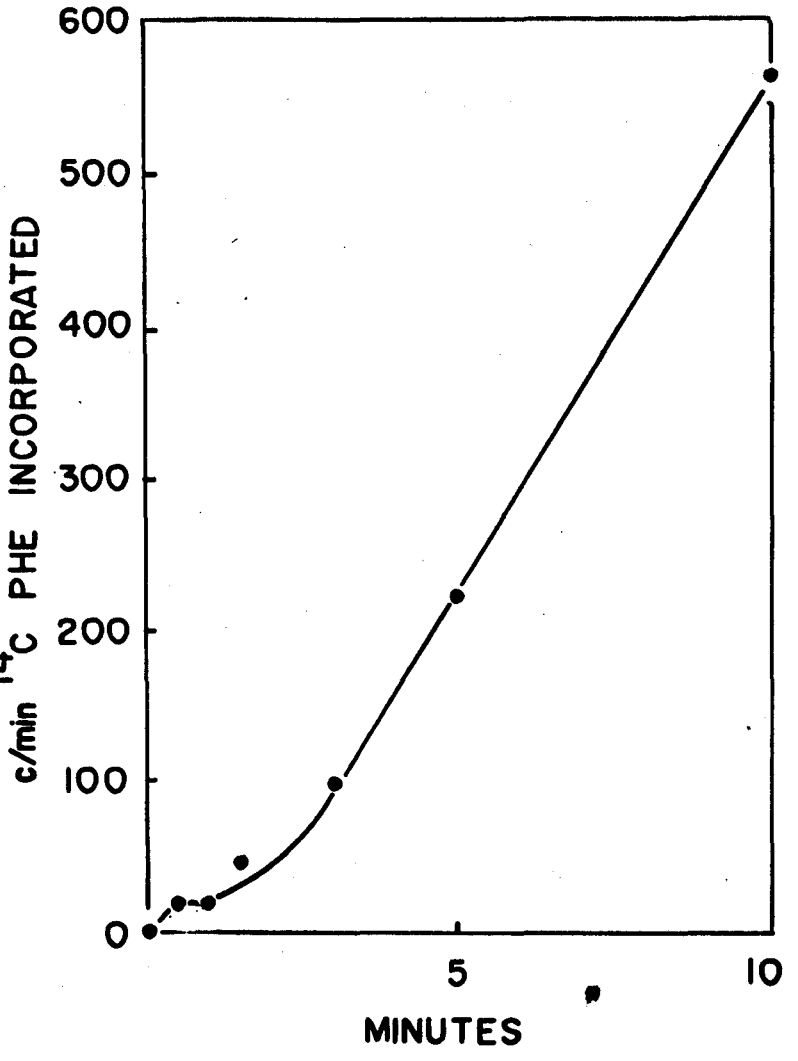

FIG. 14. A time course of phenylalanine incorporation in S-30 fractions stimulated by poly U and tRNA. The S-30 fraction was dialyzed against AVB overnight and then added directly to reaction mixtures prepared as described in Materials and Methods.

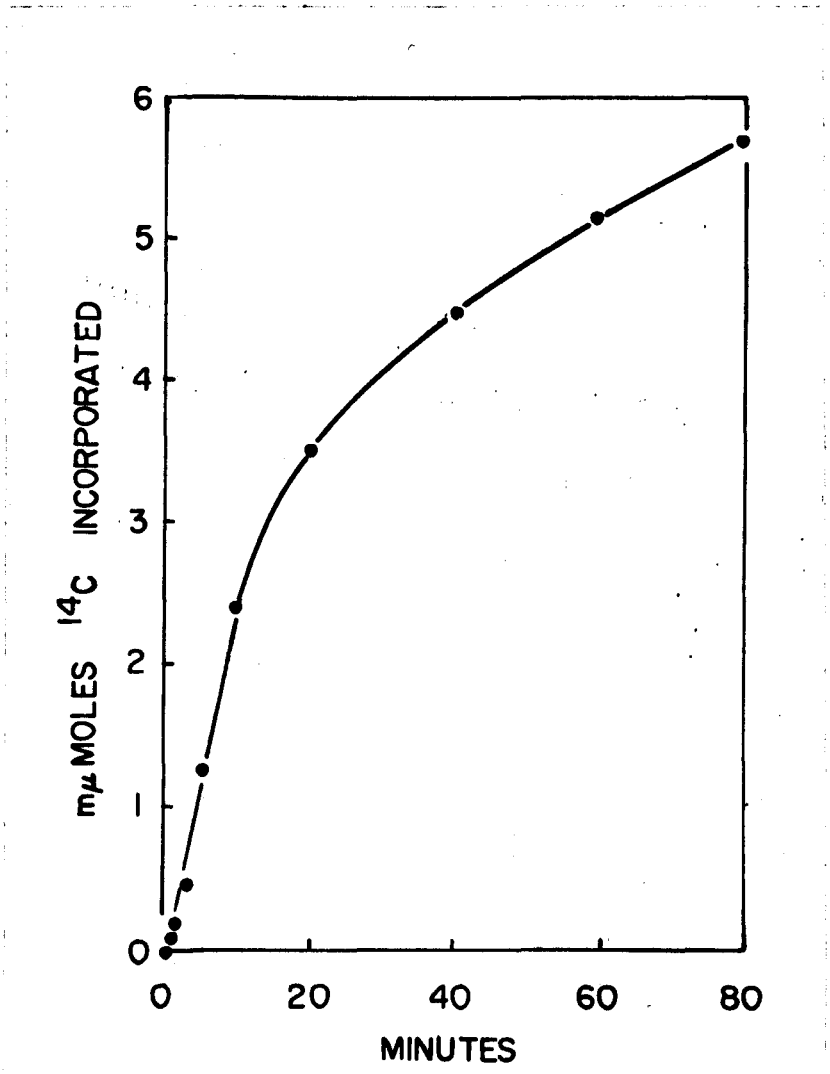

FIG. 15. A time course of phenylalanine incorporation following preincubation of ribosomes. Preincubated washed ribosomes containing 300 μg protein, 180 μg tRNA, 100 μg poly U are added to the system and incubated for 15 minutes at 0 C. Reactions were initiated by adding 372 μg S-140 protein.

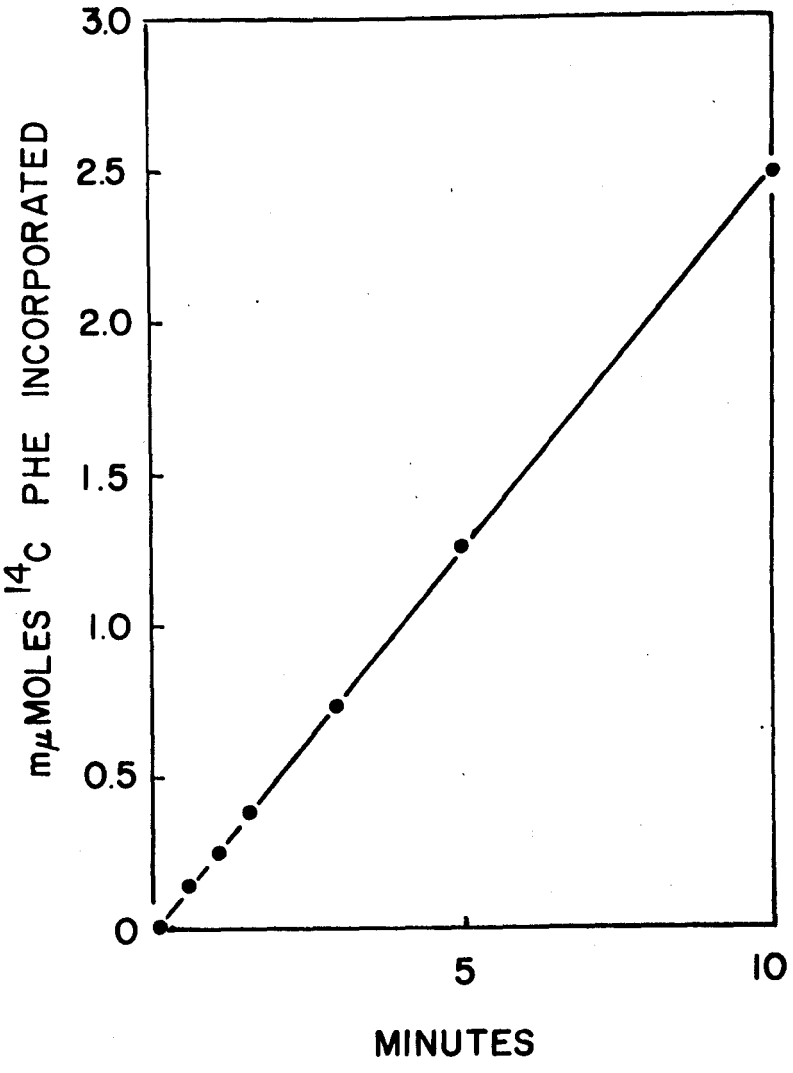

FIG. 16. A time course of phenylalanine incorporation using pre-incubated ribosomes. Reaction mixtures are prepared according to procedures outlined in Materials and Methods. Washed ribosomes containing 45 μg were preincubated with 180 μg tRNA, 100 μg poly U and the system for 15 minutes at 0 C. S-140 protein (283 ug) was added to commence protein synthesis.

Membrane bound ribosomes.

Since Azotobacter has an internal membranous network to which ribosomes are attached (62), we performed the following experiment to characterize amino acid incorporation with ribosomes bound to membranes compared with ribosomes found free in cytoplasmic extracts. Log phase cells were disrupted by osmotic shock. Hulls were washed seven times with AVB until there was no absorbance at 260 mμ in the supernatant fluid to extract all remaining free ribosomes. The hulls were separated from unbroken cells by centrifugation through a 20% sucrose-AVB solution, washed twice and treated with 0.5% sodium deoxycholate to release ribosomes contained within the hulls. Ribosomes released by the deoxycholate treatment were tested in the cell-free system for endogenous and poly U-stimulated activity. Amino acid incorporation directed by endogenous message is at most five times greater than incorporation directed by endogenous message associated with cytoplasmic ribosomes. Poly U-directed incorporation of polyphenylalanine was 34% that of polyphenylalanine synthesis observed with "free ribosomes" and stimulation was 3.6-fold greater than endogenous using membrane bound ribosomes (Table 8).

Table 8 The comparison of ribosomal activity of free ribosomes to activity of ribosomes extracted from hulls.

Origin of ribosomal	μμmoles ^{14}C phe incorporated/mg ribosomal protein	μμmoles ^{14}C phe incorporated/mg ribosomal protein	Fold stimulated
	Basal	+ poly U, tRNA	
Cytoplasm	150	6000	40.0
Hulls	742	3380	4.6

Log phase cultures were harvested and lysed by osmotic shock. Hulls were washed 7 times to extract free ribosomes, separated from intact cells by low speed centrifugation through a 20% sucrose-AVB solution, and were treated with 0.5% deoxycholate-AVB. Ribosomes were isolated as described in Materials and Methods. Reaction mixtures were prepared as stated in Materials and Methods. Washed free ribosomes containing 174 μg protein, washed bound ribosomes containing 75 μg protein and 286 μg S-140 protein were added to appropriate tubes results were normalized in terms of μμmoles ^{14}C phenylalanine incorporated per mg ribosomal protein.

DISCUSSION

The requirements and characteristics for protein synthesis in the Azotobacter cell-free system are similar in many respects to those of other cell-free systems reported. This implies that the basic mechanisms for protein synthesis have not been altered drastically in the course of phylogenetic evolution. However, subtle differences have been observed between systems which suggest that the basic protein-synthesizing machinery, ribosomes, tRNA, and enzymes have indeed varied.

Antagonists to protein synthesis. Ribosomes are required for cell-free systems, yet not all antagonists to protein synthesis whose site of action is associated with these particles are equally effective for each system. RNAse, puromycin, streptomycin and chloramphenicol are all effective inhibitors of phenylalanine incorporation in the Azotobacter system as well as in other bacterial systems (40, 48). But the Azotobacter differ in its sensitivity compared to yeast (14), rabbit reticulocyte (86), and wheat embryo systems (3).

Magnesium requirement. Incorporation in Azotobacter has been shown to be dependent upon the Mg^{2+} concentration. Polysome studies in Azotobacter (62) indicate polyribosomes disaggregate completely to subunits below 10^{-4} M Mg^{2+}. The cation Mg^{2+} plays the role of neutralizing negative changes present in the backbone of RNA in the E. coli ribosome (19, 32, 47, 82) in order for the subunits to assume configurations which allow association through hydrogen bonding. In this regard Mg^{2+} plays a similar role in Azotobacter as well as in mammalian systems (59, 83) in stabilizing the subunits,

the binding of mRNA to subunits (7, 61) and the binding of tRNA with the 30 S (55) and 50 S (16) subunits. Optimal Mg^{2+} concentrations for the translation of endogenous directed message are different for the various cell-free protein synthesizing systems developed (14, 32, 40, 60), but they all fall within the same order of magnitude. These differences may reflect variations in the RNA content of ribosomes from different systems, or perhaps quantities of divalent cations already adsorbed to the ribosome prior to extraction. The observed relationship between Mg^{2+} and phenylalanine incorporation shown in Fig. 3 for poly U directed polyphenylalanine synthesis possibly indicates that a higher Mg^{2+} concentration is required to initiate protein synthesis than is required to translate endogenous message. Other systems (32, 60, 67) require a Mg^{2+} concentration that is twice or nearly twice the Mg^{2+} concentration necessary for endogenous directed protein synthesis. Higher Mg^{2+} concentrations than that of optimal conditions cause misreading of the message. Poly U stimulates the incorporation of leucine (UUA, UUG, CUU, CUA, CUC, CUG) at these high Mg^{2+} concentrations by distorting the poly U-ribosome complex which also might allow initiation of protein synthesis with N-formyl methionyl tRNA (UUG, AUG, CUG and to a lesser extent GUG) in E. coli (1, 18, 39, 76, 81, 84). Since the codons for leucine and N-formyl methionine are degenerate and share common codons, CUG and UUG, it is possible that an error in the reading of the first codon may be responsible for the binding of N-formyl methionyl tRNA instead of leucyl tRNA to the ribosome. It is also possible that an initiation codon attached to the 5' end of polyuridylic acid would initiate

protein synthesis more efficiently and permit incorporation of phenylalanine more accurately at Mg^{2+} concentrations optimal for endogenous protein synthesis than in the absence of such a codon. In the Azotobacter system, experiments with poly U showed maximal phenylalanine incorporation at 4 mM. Ten mM Mg^{2+} may have permitted initiation efficiently but miscoding due to this high Mg^{2+} concentration possibly reduced phenylalanine incorporation.

Potassium requirement. The high K^{+} (0.1 M) required for optimal incorporation is similar to values reported for several other cell-free systems (28, 37, 44, 55, 78). The inhibitory effect observed with concentrations greater than 0.1 M in Azotobacter (Fig. 4) as well as other systems (28, 37, 78) may be due to competition between K^{+} and Mg^{2+} for binding sites on the ribosome as shown with E. coli ribosomes. High K^{+}/Mg^{2+} ratios, in the order of 100/1, lead to disaggregation of ribosomes (13, 32). Since the divalent cation binds two phosphate residues (13, 32) this implies that Mg^{2+} influences the tertiary structure of the ribosomes thereby rendering it functional. However, K^{+} is only able to bind one phosphate residue and has little influence on ribosomal tertiary structure. This alters conditions necessary for aggregation of polysomes and subunits. Since high concentrations of K^{+} have deleterious effects upon the protein synthesizing machinery, investigators believed that this concentration requirement was an artifact and that this concentration probably was not significant in vivo. However, Schultz and Solomon (75) demonstrated that growing E. coli cells accumulate K^{+} and that the intracellular concentration is at least 0.1 M. Lubin and Ennis (43) then observed that protein synthesis, but not DNA or RNA

synthesis ceases in an E. coli mutant which is unable to concentrate large amounts of K^+. This attached biological significance to the 0.1 M K^+ requirement which already had been deemed significant for the binding of tRNA to ribosomes (55).

Polyamine requirement. Polyamines such as spermidine act as divalent inorganic cations and are found associated with ribosomes in vivo. It has been reported that putrescine and spermidine represent 4% of the total cellular nitrogen and 22% of the cellular nonprotein nitrogen contained in Azotobacter (80); therefore the requirement for polyamines in the Azotobacter cell-free protein synthesizing system is biologically significant. These cations play roles similar to Mg^{2+} in neutralizing negative charges: they stabilize as well as aid in the formation of polysomes (47) and vitiate the disaggregating action of high concentrations of K^+ upon the ribosome. This additional stability afforded the ribosome is presumably due to the length of the polyamine molecule and its binding to phosphate residues which are spatially remote in contrast to residues to which Mg^{2+} may bind.

ATP requirement. ATP is a requirement for protein synthesis (Table 1) in the Azotobacter as well as other systems. Low levels of incorporation observed in its absence are probably due to interconversions of nucleotide triphosphates added to the system. Ommission of ATP and the ATP generating system almost eliminates protein synthesis entirely. The small amount of incorporation probably reflects the low levels of other triphosphates which are converted to ATP. Once these have been converted to ATP, a further source of ATP does not exist. Optimal concentrations for ATP (Fig. 6) for

the endogenous directed and poly U-directed systems differ. This is probably due to removal of Mg^{2+} from ribosomal binding sites through absorption of Mg^{2+} to the backbone of tRNA. This relationship between amino acid incorporation and ATP concentration has been observed in other systems (28, 40, 60) as well as in the Azotobacter system.

Poly U and poly A-stimulation. The Azotobacter cell-free system like other systems synthesizes polyphenylalanine or polylysine upon the addition of poly U or poly A respectively. Poly U stimulation of ^{14}C phenylalanine incorporation is as high as forty-fold when the procedures described in Materials and Methods are used. Poly A-stimulation is less, by comparison, than poly U-stimulation for possibly three reasons. First, poly A precipitates at concentrations above 10 µg/ml in the presence of 3 mM Mg^{2+} (26) and 250 µg/ml of poly A in the presence of 4 mM Mg^{2+} was tested for the stimulation of polylysine incorporation in the Azotobacter system. Second, polylysine molecules are difficult to precipitate due to their structure and charge (31). Thus, it is likely that additional amounts of ^{14}C lysine are incorporated butthese are not detected (31). Third, the conditions which allow for maximal protein synthesis when poly U is used may not be optimal for the initiation of amino acid incorporation and to subsequent polypeptide synthesis when poly A is used.

Transfer RNA requirement: substitution of E. coli tRNA for Azotobacter tRNA. The requirement for tRNA (Fig. 10, 11) as an intermediate for protein synthesis is well documented for other systems (11, 40, 55). In this study it was found that E. coli tRNA could substitute for Azotobacter tRNA

efficiently in the Azotobacter cell-free system as a requirement for the synthesis of polyphenylalanine and polylysine. However, when tRNA molecules such as tyrosine were examined from two phylogenetically distinct organisms, E. coli and yeast, the primary structures of tyrosine tRNA differed greatly for each respective organism (25). Enzyme fractions from one organism are unable to esterify amino acids utilizing tRNA from the original organism. However, once amino acylated, the tRNA molecules are active in the incorporation of amino acids in either system (9, 25). (The biological activity of the added intermediate may be due to both the similar anticodon and the identical pentanucleotide sequence found in all E. coli and yeast tRNA studied (88).) These sequences are probably related to the binding of the tRNA to the ribosome. Thus, the amino acid activating enzyme systems of both E. coli and Azotobacter are compatible while those of E. coli and yeast are not. Therefore, apparent compatibility between the E. coli and the Azotobacter amino acid activating enzyme systems may have a phylogenetic basis.

Variables effecting ribosomal activity. The stimulation of the incorporation of a specific amino acid by a synthetic polymer such as poly U serves as a means of detecting to what degree ribosomes isolated from extracts are associated with endogenous message and to what degree ribosomes from these same extracts are capable of initiating protein synthesis by the introduction of new message. The extent of the stimulation observed is dependent upon the phase of growth in which cells are harvested, the rapidity of halting cellular metabolism before harvest, the pretreatment of ribosomes, and the mode of storage of ribosomes and S-140 fractions.

Dependence upon phase of growth. As cells proceed from the log

phase of growth into the stationary phase, less endogenous incorporation is detected. This presumably reflects a slowing of cellular metabolism and the synthesis of lower levels of mRNA than during the log phase of growth. Schultz and Solomon (75) have shown that as the culture ages, the intracellular K^+ concentration falls. The lowered intracellular potassium concentration observed in an aging culture may regulate protein synthesis by inhibiting the binding of tRNA to the ribosome. A possible explanation for the appearance of only single ribosomes and subunits as shown by sucrose density gradients (62) and low endogenous and poly U-directed phenylalanine incorporation (Table 4) might be the following. As cells approach stationary phase, translation of endogenous message decreases due to the falling concentration of potassium. As a result, message attached to polyribosomes is not read and may be subjected to nucleases responsible for disaggregating polysomes to monosomes. Since most monosomes may be associated with message, poly U-directed synthesis of polyphenylalanine decreases as the culture approaches the stationary phase. Although some cell-free protein synthesis occurs during the late log phase (Table 4, line 2), due to the presence of RNAse in the S-140 fraction, only small amounts of poly U may be available to bind to ribosomes which have terminated endogenous protein synthesis during the period of cell-free reactions.

Dependence upon the rapidity of halting cell metabolism. Ribosomes incorporated less ^{14}C phenylalanine directed by endogenous message as the rapidity of halting cell metabolism was decreased. Polysome profiles of these same extracts which were not subjected to the concentrating and washing

procedures described in Materials and Methods showed increasing disaggregation with increasing periods of cooling so that at the end of a slow cooling period of 2 hours polyribosomes completely disaggregated to monosomes (62). Interestingly enough, poly U stimulation increased by the end of the 2 hour period of cooling.

Stability of ribosomes. Ribosomes are stable when stored at 0 C for 2 days. Endogenous incorporation decreases (Table 4) which relates to the fact that protein synthesis occurs to a small extent at 0 C (Fig. 7). With the decrease in endogenous incorporation there is a concomittant increase in stimulation to poly U and polyphenylalanine synthesis. This indicates that as protein synthesis terminates, ribosomes or subunits are available to initiate protein synthesis. Closely related to this is the fact that endogenous incorporation falls as poly U-stimulation and poly U-stimulated polyphenylalanine levels increase in using a procedure in which ribosomes are preincubated with mRNA and tRNA. (Table 5) Initiation of polyphenylalanine synthesis in the E. coli system is dependent upon the presence of the above factors and has been shown to be temperature dependent (55). The preincubation time required in the Azotobacter system is 50% longer than the required for the E. coli system (55) which may also indicate a temperature dependence. Kinetic studies of polyphenylalanine incorporation stimulated by poly U indicated an initial lag in the Azotobacter system comparable to the lag observed in the reticulocyte system but longer than the lag in the E. coli system. The preincubation procedure described above abolished the lag indicating that the requirements for initiation had

probably been met.

Kinetics of endogenous and poly U-stimulated phenylalanine incorporation. The Azotobacter system is saturated by 0.100 mg poly U/255 ug ribosomal protein. Mammalian systems require similar quantities of poly U for maximal stimulation; yet the E. coli system requires only one-tenth this amount (79). Barandes and Nirenberg have shown that more than 80% of ^{3}H poly U incubated with the E. coli system is degraded before significant polyphenylalanine synthesis has occurred (9). Tritiated degradation products appear at a rate not directly related to the rate of protein synthesis observed (9). The degredation is probably due to RNAse and polynucleotide phosphorylase which are also present in Azotobacter (38). However, the Azotobacter system can incorporate levels of ^{14}C phenylalanine comparable to the E. coli system using the conditions outlined in Fig. 16.

Endogenous-directed ^{14}C pheylalanine incorporation proceeds for 20 minutes before leveling off. This period is shorter than in the E. coli system (48) and the L1220 mouse ascites system (60) but is similar to other bacterial (40), yeast (14), and mammalian systems (53, 78). Forty percent of the ribosomes in this incorporating system are distributed among dimers, trimers, tetramers and pentamers. The remaining amounts are found in the monosome and subunit regions. However, when S-30 extracts are layered on a sucrose density gradient, more than 65% of the ribosomal population are polyribosomes larger than pentamers. Electron microscopic studies of various fractions from these gradients indicate the presence of polyribosomes consisting of as many as 40 ribosomes (62). This indicates that a population

of ribosomes are on a polycistronic message. Amino acid incorporation associated with these aggregates should proceed for longer periods of time than actually observed using preparations described in Materials and Methods. This is due to the fact that more time is required to read a lengthy message than a short message. Since the polyribosome distribution appears to represent an accurate picture of protein synthesis in vivo due to the fact that Azotobacter is lysed gently by osmotic shock obviating harsher methods of lysis which cause shear of the fragile polyribosomal structure (62), sucrose density centrifugation of Azotobacter extracts is an ideal technique for the isolation of polyribosomes to study enzyme synthesis using intact mRNA and to study coding regulatory mechanisms. The fact that poly U directed protein synthesis proceeds for periods up to one hour indicates that the ribosomes and S-140 fraction are stable and that endogenous levels of protein synthesis reflect the size of bound message.

Membrane bound ribosomes. Ribosomes isolated from within the hulls of Azotobacter which are released by 0.5% sodium deoxycholate have higher endogenous levels of ^{14}C phenylalanine incorporation in spite of the procedures used suggesting that these ribosomes are either more stable to fragmentation or are associated with message of enormous proportions. Apparently most ribosomes in this preparation are associated with message, ergo few ribosomes are available to initiate protein synthesis upon the addition of poly U. Therefore, this may indicate that a major proporation of message translation occurs upon membrane bound ribosomes.

SUMMARY

Cell-free extracts were prepared from logarithmically growing cells in which metabolism was halted rapidly. The cells were disrupted by osmotic shock. Amino acid incorporation was dependent upon ribosomes, 140,000 x g supernatant fraction (S-140) ATP, an energy generating system, a critical concentration of Mg^{2+} (4 mM), an optimal pH (7.2), tRNA and optimal concentrations of spermidine (0.2 mM) and K^{+} (0.1 M). Poly U and poly A directed the synthesis of polyphenylalanine and polylysine respectively. E. coli tRNA could substitute for Azotobacter tRNA in the synthesis of polyphenylalanine or polylysine directed by poly U or poly A respectively. Ribonuclease, streptomycin, puromycin and chloramphemical inhibited protein synthesis. Endogenous amino acid incorporation proceeded linearly for ten minutes before it leveled off after 20 minutes. Poly U-directed protein synthesis proceeded linearly for periods up to one hour. Frozen ribosomes and S-140 were stable if frozen quickly and stored in liquid nitrogen. Endogenous incorporation of phenylalanine occurs at 0 C to a limited extent but is highest within the range of 30 C to 40 C. Ribosomal activity reflected the growth phase of the organism, the rapidity of cessation cell metabolism, the preincubation of ribosomes, and the mode of cell rupture. Since Azotobacter has an extensive internal membranous network to which ribosomes are attached it was possible to compare activity of free ribosomes to those attached to membranes.

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