

# HYDROSTATIC PRESSURE EFFECTS ON PROTEIN SYNTHESIS

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**ABSTRACT** The effects of high hydrostatic pressure on several phases of cell-free protein synthesis have been examined. The initial rate of polyuridylic acid (poly U)-directed synthesis of polyphenylalanine showed an apparent increase at 100 atm, above which the synthetic rate was reduced sharply with increased pressure up to 640 atm where 95% inhibition was observed. The magnitude of the inhibition of polyphenylalanine synthesis with increased pressure depended strongly on the magnesium salt concentration in the reaction system. Misreading of the poly U message, as measured by insertion of leucine in place of phenylalanine, dropped rapidly with increased pressure from 1 to 350 atm, above which the amount of misreading increased. Enzymatic activation of transfer RNAs (tRNAs) was reduced by increased pressure in the range 100–640 atm, where the rate of tRNA aminoacylation was 80% inhibited. Both nonenzymatic attachment of phenylalanyl-tRNA (phe-tRNA) to the poly U-ribosome complex and stability of the phe-tRNA-poly U-ribosome complex were decreased at high pressures (100–900 atm). The results of the action of pressure on the various phases of cell-free protein synthesis suggest that the major pressure-sensitive element in the protein synthetic machinery is the ribosome.

## INTRODUCTION

Pressure as a distortion agent has been known for a long time to affect cell growth and viability. The effects of high hydrostatic pressures on macromolecular synthetic processes (i.e., protein, DNA, and RNA synthesis) have been studied recently in our laboratory and elsewhere (1–8) with the general conclusion that rates of synthesis are reduced by pressure in both bacterial and mammalian cells. In all cases, protein synthesis was found to be the most pressure sensitive, followed by DNA, and least sensitive RNA synthesis (3, 6, 7). In view of these findings, we thought it worthwhile to study the effect of pressure on synthesis in cell-free systems.

Because of its large sensitivity to high pressures, we have focused on the protein synthetic process. While the work was in progress a report of a study of translation stages by Arnold and Albright appeared (8). Our work confirms and adds to their

findings; an account of our work has been given previously (9). This study has examined the effects of pressure on the processes and components which together produce a polypeptide in response to the information provided in the nucleotide sequence of a messenger RNA (mRNA). In order to perform this investigation, a bacterial cell-free protein-synthesizing system was used so that certain steps in the process could be examined independently. The effects of high pressures on the following processes or states have been established: (a) total cell-free polypeptide synthesis; (b) fidelity of translation of a synthetic mRNA (poly U); (c) enzymatic activation of tRNAs; (d) nonenzymatic attachment of activated tRNAs to ribosomes and mRNA to form an aminoacyl-tRNA-(aa-tRNA)-mRNA-ribosome complex; and (e) stability of this complex at high pressures. The last two were stressed in the study by Arnold and Albright (8).

## EXPERIMENTAL

### *Materials*

The adenosine triphosphate (ATP, sodium salt), guanosine triphosphate (GTP, potassium salt), dithiothreitol, phosphoenolpyruvate, pyruvate kinase, L-amino acids, and the *Escherichia coli* K-12 tRNA (Grade B) were obtained from Calbiochem, Los Angeles, Calif. The poly U was purchased from Schwarz Bio Research, Orangeburg, N.Y. L-Phenylalanine- $^{14}\text{C}$  (4 mCi/mmole), L-leucine- $^{14}\text{C}$  (10 mCi/mmole), and L-amino acid- $^{14}\text{C}$  mix were bought from New England Nuclear Corp., Boston, Mass., and the L-phenylalanine-ring-4- $^3\text{H}$  (21 Ci/mmole) was obtained from Amersham/Searle Corp., Arlington Heights, Ill.

### *Apparatus*

The high pressure apparatus used in this investigation has been described by Yayanos and Pollard (3). The hydraulic fluid consisted of two parts distilled water with one part Xerex antifreeze in order to minimize temperature rise due to compression and to aid in the prevention of rust. A reaction vessel similar to that used in these studies has been described by Koskikallio and Whalley (10) and permits rapid sampling of small-volume pressurized reactions. The temperature of the system was controlled by immersing the entire pressure vessel and, hence, the reaction assembly in a constant temperature water bath.

### *Preparation of Bacterial Extracts*

All extracts used in this study were prepared from *E. coli* W3110 obtained from Dr. Stanley Person of this laboratory. A 10 ml culture of this bacterium was grown from a frozen stock in A-1 minimal medium (2 g  $\text{NH}_4\text{Cl}$ , 6 g  $\text{Na}_2\text{HPO}_4$ , 3 g  $\text{KH}_2\text{PO}_4$ , 5 g  $\text{NaCl}$ , 0.34 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.155 g  $\text{Na}_2\text{SO}_4$ , 4 g glucose, and 1 liter distilled water) with aeration at  $37^\circ\text{C}$ . When the 10 ml culture reached the midlog phase of growth, it was used as an inoculum for a 500 ml vol of nutrient medium (8 g nutrient broth, 5 g glucose, and 1 liter distilled water). This culture was grown with aeration at  $37^\circ\text{C}$  to midlog phase, at which time it was used as an inoculum for an 18 liter nutrient culture. It was grown with vigorous aeration at  $32 \pm 1^\circ\text{C}$  to a titer of  $2-4 \times 10^8$  cells/ml. The 18 liter culture was poured over a surplus of crushed ice, bringing the temperature to  $0-3^\circ\text{C}$  within 5 min. The bacteria were harvested in a continuous flow Sharples centrifuge (Sharples-Stokes Div., Pennwalt Corp., Warminster, Pa.). All of

the following operations were carried out at 3–5°C unless otherwise specified. The bacterial pellet was resuspended in a buffered salt solution (TMK buffer) containing 0.01 M Tris-Cl (pH 7.8), 0.06 M KCl, 0.014 M magnesium acetate, and 0.0001 M dithiothreitol (Cleland's reagent). After a low speed centrifugation, the washed bacterial pellet was weighed and frozen rapidly in dry ice-acetone and stored at –65°C until used.

The procedures for disruption of the bacteria and preparation of the extracts have been described by Nirenberg (11). In all cases, dithiothreitol was substituted for mercaptoethanol. Disruption of the bacterial cells was performed by two passages of the concentrated cell suspension through a French press at 16,000–24,000 psi. Additional dithiothreitol (0.1  $\mu$ mole/ml) was added to the lysate, and the cellular debris was removed by a low speed centrifugation. After DNase treatment and a 37°C incubation with GTP, ATP, pyruvate, pyruvate kinase, and 20 amino acids (at the same concentrations as used in cell-free synthesis) to remove ribosomes from endogenous mRNA, the bacterial extract was fractionated by centrifugation at 30,000 *g* for 30 min. The top four-fifths of the supernatant of each tube was removed and designated the S-30 fraction, containing enzymes, tRNAs, ribosomes, and small molecules. A portion of the S-30 was further fractionated by a 2 hr 100,000 *g* centrifugation, and the top four-fifths of the supernatant containing enzymes, tRNAs, and small molecules was decanted. The ribosomal pellet was resuspended by stirring at slow speed for 2 hr over a magnetic stirrer, washed 3 times by centrifugation, resuspended in TMK buffer, and designated W-Rib. The S-30 and S-100 preparations were dialyzed against 100 vol of TMK buffer for 15–18 hr with one change of the buffer at 8 hr. The S-30, S-100, and W-Rib preparations were rapidly frozen in dry ice-acetone and stored at –65°C until used. The protein content of the S-30, S-100, and W-Rib preparations was determined by a modification (12) of the method of Lowry et al. (13).

#### *Cell-Free Synthesis of Polyphenylalanine*

The synthesis of polyphenylalanine was initiated by adding poly U to the rest of the reaction mixture described in the legend of Fig. 1. The reaction mixture was divided into two portions, one of which was used to fill the high pressure reaction syringe and placed under pressure, and the other of which was held at 1 atm and 24°C as a control. The pressure reaction apparatus was assembled as rapidly as possible (2–3 min) at room temperature (22–24°C), and pressure was applied over a 1 min period.

#### *Assay for Misreading of Poly U*

The most frequently occurring misreading event in the cell-free protein-synthesizing system described above is the insertion of leucine in place of phenylalanine in the poly U-directed polypeptide (14). The misreading of the poly U as a function of both  $Mg^{++}$  concentration and pressure was examined by two methods. In one method, two parallel poly U-directed cell-free reactions were run as described in the legend of Fig. 1, one containing phenylalanine- $^{14}C$  at the specific activity and concentration noted above and the other containing unlabeled phenylalanine at 0.2  $\mu$ mole/ml in place of phenylalanine- $^{14}C$  and leucine- $^{14}C$  at 0.2  $\mu$ mole/ml and 10 mCi/mM in place of the unlabeled leucine. These reactions were sampled and assayed for the amounts of phenylalanine- $^{14}C$  and leucine- $^{14}C$  incorporated into the polypeptide products. In the other method, the incorporation of phenylalanine- $^3H$  and leucine- $^{14}C$  was followed in a single reaction. The phenylalanine- $^3H$  (40 mCi/mM) and leucine- $^{14}C$  (10 mCi/mM) were both supplied at 0.2  $\mu$ mole/ml in place of the phenylalanine- $^{14}C$  and unlabeled leucine in the reaction mixture described above. Samples of 200  $\mu$ l were taken as described in Fig. 1. All samples were filtered on Whatman GF/A glass fiber filters which had been soaked

in 95% ethanol. Each filter sample was washed with two 3 ml portions of ice-cold 5% TCA and then with 3 ml of 95% ethanol. The filters were placed in 3-ml glass vials and allowed to dry overnight. Toluene-PPO-POPOP<sup>1</sup> scintillation fluid (2.5 ml) was added to each vial, and the samples were counted in a Nuclear-Chicago liquid scintillation spectrometer (Nuclear-Chicago Corp., Des Plaines, Ill.). Corrections were made for <sup>3</sup>H/<sup>14</sup>C crossover, and the <sup>14</sup>C/<sup>3</sup>H ratio was determined for each sample.

#### *Reaction System for Studying the Aminoacylation of E. coli tRNA*

The components were mixed in the order listed. The system used for studying the effects of high pressure on the aminoacylation of *E. coli* tRNA contained the following ingredients in micromoles per milliliter unless otherwise specified: 100 Tris-Cl buffer (pH 7.8); 50 KCl; 1.0 ATP; 0.03 GTP; 10 magnesium acetate; 0.4 dithiothreitol; 75 phosphoenolpyruvate; 1.6  $\mu$ g/ml pyruvate kinase; 0.51 (1  $\mu$ Ci) amino acid-<sup>14</sup>C mix containing L-amino acids-<sup>14</sup>C alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tyrosine, and valine; 0.12 mg/ml bovine serum albumin; 4  $\mu$ g/ml S-100 protein; and 2 mg/ml *E. coli* tRNA. All components except the tRNA were mixed at 0°C and then warmed to 24°C. The reaction was initiated by adding the tRNA, also at 24°C, to the reaction mixture. Samples (50–200  $\mu$ l) were taken into 3.0 ml of ice-cold 10% TCA, allowed to stand at 0°C for at least 30 min but not more than 2 hr, and filtered and washed with 2 vol of ice-cold 5% TCA on Millipore membrane filters (Millipore Corporation, Bedford, Mass.). The filters were glued to planchets, allowed to dry, and counted as described above.

#### *Preparation of Phenylalanyl-<sup>14</sup>C-tRNA for Use in Binding Studies*

The method used for preparing phenylalanyl-<sup>14</sup>C-tRNA (phe-<sup>14</sup>C-tRNA) has been adapted from the procedures of von Ehrenstein and Lipmann (15) and Moldave (16). The composition of the reaction system was as follows (in micromoles per milliliter unless otherwise specified): 100 Tris-maleate buffer (pH 7.0); 10 magnesium acetate; 0.8 dithiothreitol; 3 ATP; 20 phosphoenol pyruvate; 40 mg/ml pyruvate kinase; amino acid mix (minus phenylalanine); 0.1 each amino acid (see composition of cell-free reaction system for list of these amino acids); 0.013 phenylalanine-<sup>14</sup>C (SA 384 mCi/mM, New England Nuclear Corp.); 0.88 mg/ml S-100 protein; and 9.5 mg/ml *E. coli* K-12 tRNA. The above components were mixed at 0°C in the order listed and incubated at 24°C for 1 hr. The following procedures were carried out at 4°C. The 10 ml reaction was treated with 10 ml of water-saturated redistilled phenol, and the mixture was shaken vigorously for 1 hr. The phenol and aqueous phases were separated by centrifugation at 15,000 g for 20 min. The top aqueous layer containing the phe-<sup>14</sup>C-tRNA and other unlabeled aa-tRNA was removed and saved. The phenol layer was washed with 1 vol of distilled water by shaking for 1 hr. The water-phenol phases were separated and the aqueous phases combined. 0.1 vol of a 20% (w/w) solution of potassium acetate was added to the aqueous phase. The aa-tRNAs were precipitated by addition of 2 vol of 95% ethanol at –20°C; precipitation was allowed to proceed at 0°C for 30 min. The precipitate was collected by centrifugation at 15,000 g for 10 min. The precipitate was then dissolved in 10 ml of distilled water and dialyzed at 4°C for 8 hr against 10 vol of distilled water with three changes. Phe-<sup>14</sup>C-tRNA was recovered at approximately 5 mg/ml and  $8.1 \times 10^{-5}$  mCi/ml. The phe-<sup>14</sup>C-tRNA preparation was divided into 0.5 ml portions, quickly frozen in dry ice-acetone, and stored at –65°C.

<sup>1</sup> PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]-benzene.

### *Binding of Phe-<sup>14</sup>C-tRNA to Form the Phe-<sup>14</sup>C-tRNA-Poly U-Ribosome Complex*

The system used to study the effects of pressure on the formation and stability of the aa-tRNA-mRNA-ribosome complex and the phe-<sup>14</sup>C-tRNA-poly U-ribosome complex specifically has been described by Nirenberg and Leder (17). The samples were filtered immediately on Millipore membrane filters (0.45  $\mu$  pore size, 25 mm diameter) and washed with two 3 ml portions of the same solution. The filters were glued to aluminum planchets, allowed to dry, and counted as described above. The amount of radioactivity retained by the filter was a measure of the extent of phe-<sup>14</sup>C-tRNA binding in the complex.

## RESULTS

### *Characteristics of the Cell-Free Protein-Synthesizing System*

The kinetics of polyphenylalanine synthesis in a cell-free reaction was shown in Fig. 1. Synthesis is dependent on the presence of poly U. Factors to consider are, first,

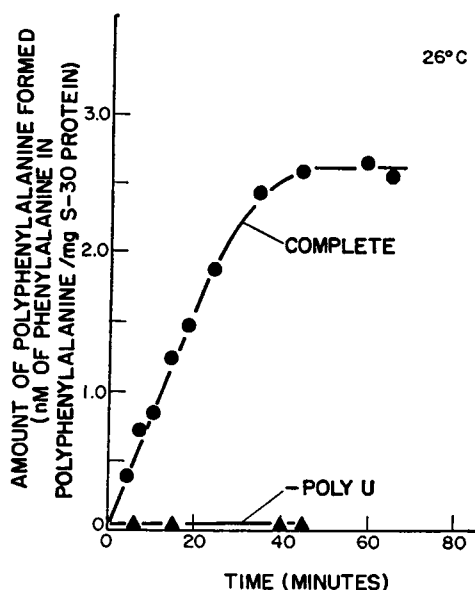


FIGURE 1 Time-course of a typical poly U-directed synthesis of polyphenylalanine. The cell-free protein-synthesizing system contained the following components in micromoles per milliliter unless specified: 100 Tris-Cl buffer (pH 7.8); 50 KCl; 16.8 magnesium acetate; 0.1 dithiothreitol; 1 ATP; 0.03 GTP; 7.5 phosphoenolpyruvate (sodium salt); 1.6 mg phosphoenolpyruvate kinase; 0.2 each of 19 L-amino acids (glycine, alanine, serine, aspartic acid, asparagine, glutamic acid, glutamine, isoleucine, leucine, cysteine, histidine, tyrosine, tryptophan, proline, threonine, methionine, arginine, lysine, and valine); 0.2 phenylalanine-<sup>14</sup>C (4 mCi/mmol,  $8 \times 10^{-4}$  mCi); 4 mg S-30 protein; and 160 mg poly U. Samples (200  $\mu$ l) were taken immediately into ice-cold 10 % trichloroacetic acid (TCA) and held for at least 2 hr. The precipitates were then placed in suspension by rapid mixing on a Vortex mixer (Scientific Industries, Inc., Lake Worth, Fla.) and placed in an 85°C water bath for 30 min in order to solubilize the phenylalanine-<sup>14</sup>C residues attached to cold acid-precipitable tRNAs. The samples were placed at 0°C for 1–2 hr. The remaining precipitable material was collected and washed with ice-cold 5 % TCA on Millipore membrane filters (0.45  $\mu$  pore size, 25 mm diameter). The filters were glued to aluminum planchets and counted under a gas-flow counter operated in the Geiger-Müller region (Nuclear-Chicago Corp., model D-47 with Q-gas) with an efficiency of approximately 25 %.

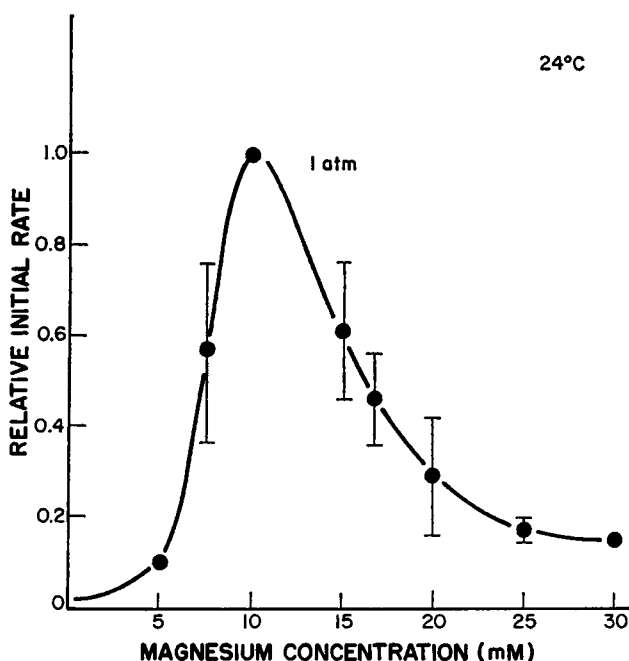


FIGURE 2 Dependence of initial rate of polyphenylalanine synthesis on magnesium concentration in the reaction system. The relative initial rate is the ratio of the initial rate measured at the given magnesium concentration to the initial rate measured at 10 mM magnesium concentration. The composition of the reaction system employed was described in the legend to Fig. 1.

the amount of phenylalanine which is available for enzymatic attachment to tRNA decreases as the reaction proceeds. Second, there is no mechanism for chain termination. This may prevent ribosome release and reutilization. Third, the bacterial RNases produce shorter and shorter pieces of poly U with time. All of these factors affect the final amount of polyphenylalanine synthesized but not the initial rate of synthesis. For this reason, the initial rate of synthesis between 3 and 20 min was used to measure the effect of pressure on the cell-free synthesis of polyphenylalanine. The effects of temperature, magnesium ion concentration, potassium ion concentration, and pH on polyphenylalanine synthesis were studied. The temperature dependence showed two components on an Arrhenius plot. The activation energy calculated was 35 kcal/mole from 11 to 26°C compared with 13 kcal/mole from 26 to 37°C.

The magnesium ion concentration dependence proved to be important in the interpretation of our work and is shown in Fig. 2. The initial rate at the various magnesium ion concentrations reached a maximum at approximately 10 mM  $Mg^{++}$  and varied sharply on both sides of the maximum. Potassium ion concentration had less effect. There was a shallow maximum between 60 and 80 mM  $K^+$ . The optimum pH was 7.8, and less than 10% change occurred for 0.4 pH unit on each side.

### Effect of Pressure on Initial Rate of Polyphenylalanine Synthesis

The pressure dependence of the initial rate of polyphenylalanine synthesis was examined by comparing the initial rate of synthesis in a pressurized reaction with that in a 1 atm control reaction run simultaneously, as shown in Fig. 3. To check that the process of sample removal did not vitiate the data, a test was made of a reaction at 1 atm which was pressurized just before sampling. Thus, sample removal under pressure was tested. No effect was observed. The temperature increase due to compression was less than 2°C, and tests of temperature dependence showed that this is negligibly important. The pressure-induced pH change was also too small to produce an effect.

The possibility of secondary effects produced by a pressure-induced pH change can be excluded on the basis that (a) the buffering system, Tris-Cl (pH 7.8), is an organic buffer with a small volume change upon ionization and, therefore, is only slightly affected by pressure (18), and (b) the pH dependence of the initial reaction is negligible over a broad pH range. The sharpness of the magnesium ion concentration dependence prompted an investigation of the effects of pressure at two magnesium ion concentrations: one at the optimum of Fig. 2 and the other well above

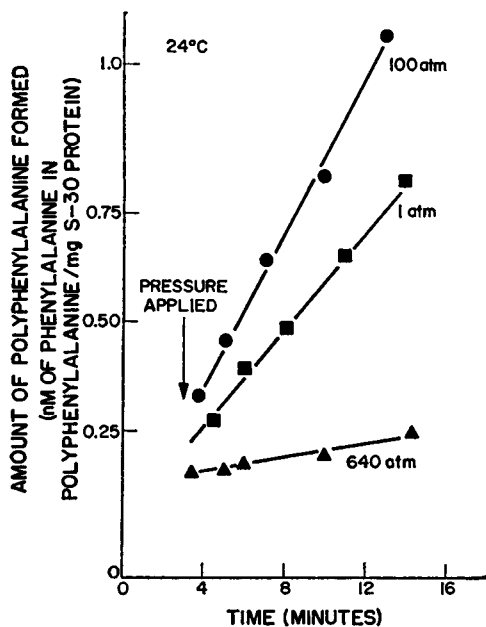


FIGURE 3

FIGURE 3 Initial kinetics of polyphenylalanine synthesis at two high pressures. The composition of the reaction systems was described in Fig. 1.

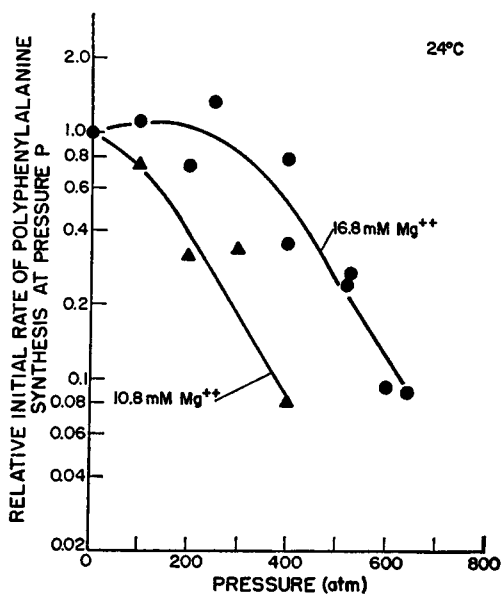


FIGURE 4

FIGURE 4 Pressure dependence of the initial rate of polyphenylalanine synthesis at two magnesium concentrations. The relative initial rate is defined as the ratio of the initial rate at pressure to the initial rate at 1 atm.

the optimum. The results of many kinetic studies of the pressure effect at the two magnesium ion concentrations are shown in Fig. 4. These results indicate a significantly greater pressure sensitivity at the lower magnesium ion concentration.

The capacity of the protein synthetic machinery to recover from an inhibitory pressure was studied. Fig. 5 shows the synthetic ability of the polyphenylalanine synthetic system after application of 920 atm, a totally inhibitory pressure, for 12 min after the reaction has been initiated. While the pressure-treated reaction does not synthesize as much polyphenylalanine before exhausting itself, the rate of synthesis immediately after pressure treatment approximately parallels the initial rate of the 1 atm control 11 min earlier.

#### *Effect of Pressure on the Fidelity of Translation of Poly U*

The fidelity of translation of synthetic mRNAs has been shown to depend upon ionic concentrations (namely, magnesium ions), pH, and temperature (14, 19). We examined the effect of pressure on the precision of translation of the poly U message by observing the frequency of incorporation of leucine into the poly U-directed polypeptide. First, we examined the effect of  $Mg^{++}$  concentration. Our find-

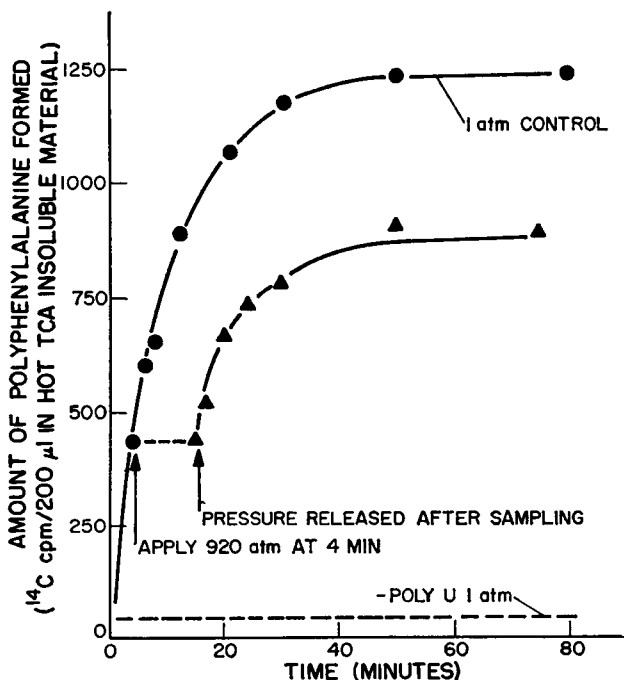


FIGURE 5 Reversibility of the pressure inhibition of the cell-free reaction. Concentrations of components in the reaction system were given in Fig. 1 (1000 cpm/200  $\mu$ l hot TCA-insoluble material = 625 pmoles phenylalanine- $^{14}C$  in polyphenylalanine/mg S-30 protein).



ings indicated a steady increase in misreading from 5 to 20 mM  $Mg^{++}$ . These agreed with work by Szer and Ochoa (14) which showed that misreading increases with increasing magnesium concentration from approximately 10 to 20 mM. Studies of the effect of pressure on fidelity of translation of poly U shown in Fig. 6 indicate that the amount of misreading in the pressurized system decreases with increasing pressure from 1 atm to approximately 350 atm, where an apparent minimum in this effect occurs. Above 350 atm the misreading again increases up to 470 atm, where the levels of synthesis are too low for accurate estimates of ratios.

### *Effect of Pressure on Aminoacylation of tRNAs*

Because supply of aa-tRNAs must be available for selection by the translation complex, a decrease in this supply would result in a decrease in rate of polypeptide chain

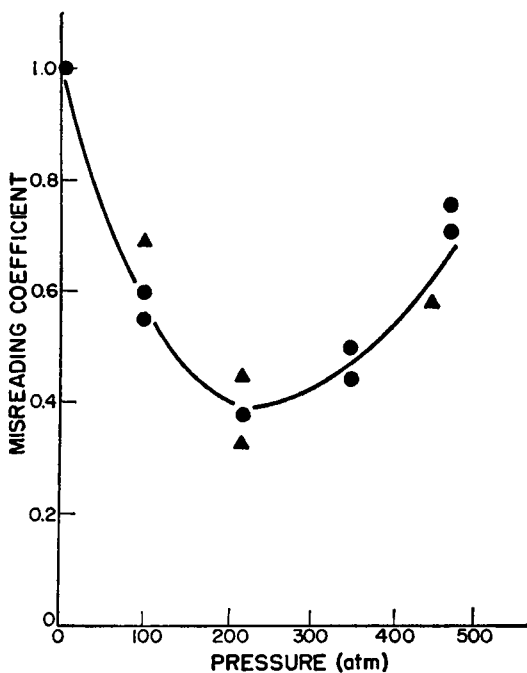


FIGURE 6

FIGURE 6 Pressure dependence of misreading of the poly U message. The misreading coefficient is defined as the ratio of the leucine/phenylalanine (leu/phe) content in the poly U-directed polypeptide product at pressure to the leu/phe content in the 1-atm product. The concentrations of components in the reaction system were listed in the legend of Fig. 1. Each point represents the average ratio obtained from triplicate samples in a single experiment: (—●—) double label experiment; (—▲—) single label experiments. Detail is given in the text. A misreading coefficient of 1.0 corresponds to an incorporation of one leucine residue to every seven phenylalanine residues, on the average.

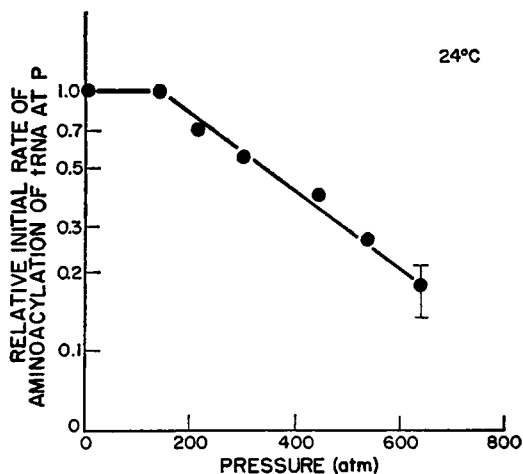


FIGURE 7

FIGURE 7 Pressure dependence of the initial rate of aminoacylation of *E. coli* tRNA. Details of the reaction system and sampling techniques are given in the text.

elongation. Accordingly, the effect of pressure on the rate of aa-tRNA formation was studied. The initial rate of formation (first 10 min) of aa-tRNAs in a pressurized reaction was compared with that in a 1 atm control reaction with results as shown in Fig. 7. In these experiments, the preparation (S-100) containing aminoacyl-activating enzymes had to be diluted a factor of 3000 in order to make the reaction kinetics slow enough to be followed by available methods. Even then the reaction kinetics were linear only for 15 min with a rapid leveling taking place between 15 and 20 min. These reaction mixtures contained 10 mM magnesium acetate, a concentration at which the synthesis of polyphenylalanine was approximately 50% inhibited at 150 atm, while at the same pressure the aminoacylation reaction was not affected. It should be noted that the aminoacylation reaction system measured the attachment of 15 different amino acids to their specific tRNAs. Hence, only the average pressure effect on the 15 different aminoacylating reactions is indicated in Fig. 7.

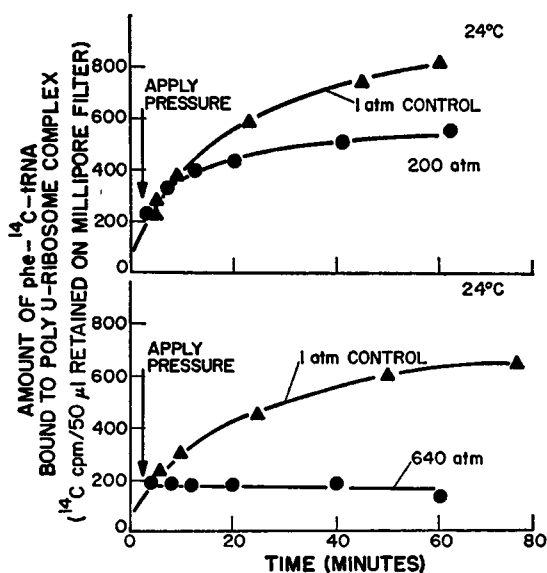


FIGURE 8 Effect of pressure on formation of the phe-tRNA-poly U-ribosome complex. The reaction system contained the following ingredients (in micromoles per milliliter unless specified): 100 Tris-acetate buffer (pH 7.0); 50 KCl; 5–50 magnesium acetate (specified for a given experiment); 0.2 mg/ml poly U; 0.52 mg ribosomal protein/ml W-Rib; and 1 mg/ml phe- $^{14}\text{C}$ -tRNA ( $1.6 \times 10^{-6}$  mCi/ml). All components except the phe- $^{14}\text{C}$ -tRNA were mixed at 0°C and warmed to 24°C. The phe- $^{14}\text{C}$ -tRNA also at 24°C was added to initiate the binding reaction at zero time. To assay for the amount of phe- $^{14}\text{C}$ -tRNA bound in the complex, each 50  $\mu\text{l}$  sample was taken into 3 ml of an ice-cold solution of 0.1 M Tris-acetate (pH 7.0), 0.05 M KCl, and 0.02 M magnesium acetate. The samples were filtered immediately on Millipore membrane filters (0.45  $\mu$  pore size, 25 mm diameter) and washed with two 3 ml portions of the same solution. The filters were glued to aluminum planchets, allowed to dry, and counted as described above. The amount of radioactivity retained by the filter was a measure of the extent of phe- $^{14}\text{C}$ -tRNA binding in the complex.

### *Attachment of Phe-tRNA to the Poly U-Ribosome Complex*

The recognition of the correct amino acid in the sequence is achieved by specific attachment of the aa-tRNA to the messenger-ribosome complex. A technique used to study this interaction was developed by Nirenberg and Leder (17). Pressure effects on this stage were studied by Arnold and Albright (8); our data, described below, agree with theirs. Kinetic studies of the formation of the phe- $^{14}\text{C}$ -tRNA-poly U-ribosome complex indicated that the rate of complex formation was proportional to the difference between the maximum amount of complex formed at large times (e.g., 120 min) and the amount of complex formed at any previous time. An analysis of kinetic data showed that the proportionality constant for this relationship was a measure of the dissociation rate constant for the complex. Hence, there was no simple measure of the effect of pressure on rate of formation of the complex. The kinetics of formation of the phe- $^{14}\text{C}$ -tRNA-poly U-ribosome complex are shown for two pressures in Fig. 8. In both cases, pressure reduces the ability of the phe-tRNA to attach and remain attached to the complex. The effect is also greater at the higher pressure.

It is suggested that kinetic measurements shown in Fig. 8 can be described by two processes: one process for the attachment of phe-tRNA to the complex and the other process for the detachment of the phe-tRNA. In order to test this idea, the phe- $^{14}\text{C}$ -

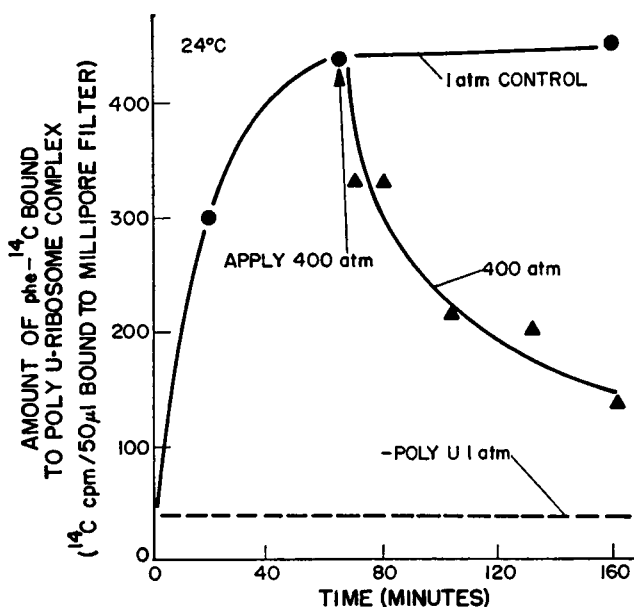


FIGURE 9 Effect of pressure on the stability of the phe-tRNA-poly U-ribosome complex formed at 1 atm and 24°C. The reaction was allowed to proceed to the maximum level of phe-tRNA binding. Pressure was applied to one portion while the other portion was held at 1 atm and 24°C. The reaction system composition and assay procedures were described in Fig. 8.

tRNA-poly U-ribosome complex was formed at 1 atm. When the amount of the complex reached a maximum, the reaction mixture was divided into two portions. One part was held at 1 atm at 24°C, while the other portion was subjected to 400 atm at 24°C. The results of this experiment are given in Fig. 9. Clearly, the increased pressure destabilizes the complex and phe-tRNA is either released or hydrolyzed so that the phenylalanyl- $^{14}\text{C}$  group is released from the complex. In order to determine whether the decrease in amount of phenylalanine- $^{14}\text{C}$  (i.e., phe- $^{14}\text{C}$ -tRNA) indicated by Fig. 9 was due to an increase in rate of hydrolysis of phe-tRNA at high pressure or to an actual release of phe- $^{14}\text{C}$ -tRNA from the complex, the amount of phe- $^{14}\text{C}$ -tRNA remaining in the reaction was measured as a function of time (Fig. 10). It was found that the total amount of phe- $^{14}\text{C}$ -tRNA in both the control and pressurized reactions decreased as a function of time. This decrease, however, was apparently due to the deacylation or hydrolysis of unbound phe- $^{14}\text{C}$ -tRNA, since in the control the amount of bound phe- $^{14}\text{C}$ -tRNA remained constant from 70 to 160

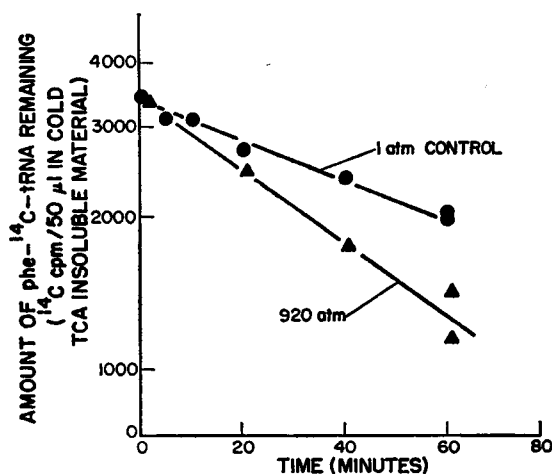


FIGURE 10

FIGURE 10 Effect of pressure on the stability of phe- $^{14}\text{C}$ -tRNA. Phe- $^{14}\text{C}$ -tRNA was incubated in Tris-maleate buffer (pH 7.0) with 0.05 M KCl and 0.02 M magnesium acetate at 1 atm and at 920 atm at 24°C. Sampling and assay procedures were detailed in Fig. 7.

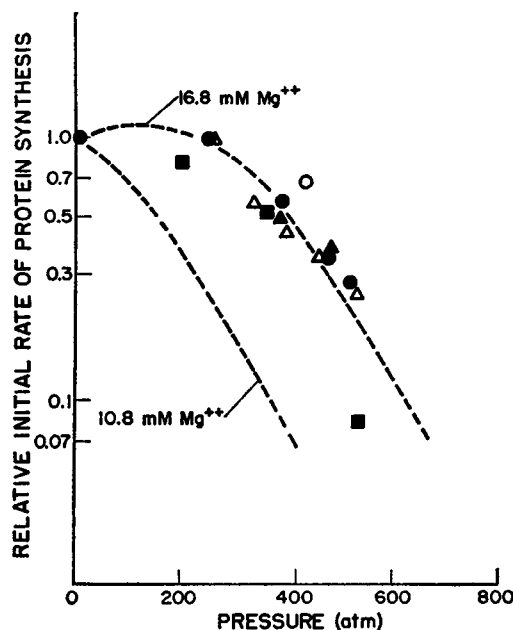


FIGURE 11

FIGURE 11 Effect of high pressure on the rates of protein synthesis in various organisms. The relative initial rate is the ratio of the initial rate at pressure to the initial rate at 1 atm. These data were obtained from the following sources: *E. coli* —, (—○—) Pollard and Weller (1); (—●—) Landau (4, 5); (—■—) Yayanos and Pollard (3); HeLa cells —, (—△—) Landau (6); *Vibrio marinus* —, (—▲—) Albright and Morita (7). All studies except those of Albright and Morita were done at 37°C, and the investigation of Albright and Morita was performed at 15°C. The dotted lines indicate the results of the cell-free study shown in Fig. 4.

min, while the free phe-<sup>14</sup>C-tRNA was deacylated. In addition, the decrease in amount of phe-<sup>14</sup>C-tRNA in the pressurized reaction was only slightly greater (about 30 %) than that found for the control. It should be noted that, while these measurements do not preclude the possibility that cleavage of the phenylalanyl residue of the bound phe-tRNA is facilitated by pressure resulting in an observed apparent decrease in bound phe-tRNA, the measurements shown in Fig. 9 were made at 900 atm where there is a 30 % decrease in amount of phe-<sup>14</sup>C-tRNA relative to the 1 atm control, but an 85 % decrease in amount of bound phe-<sup>14</sup>C-tRNA. In addition to the above results, analysis of the kinetic data indicated that both the rate constant for complex formation and rate constant for complex dissociation increased with increasing pressure. The predominance of the dissociation process masks the effect of pressure on association.

## DISCUSSION

The relevance of results obtained with a cell-free system to the functioning of an intact living cell has been approached by comparing the effects of pressure on protein synthesis in various kinds of cells (1, 3, 5-7) with the results of this study. The comparison is shown in Fig. 11. The effects of pressure on rate of protein synthesis in living organisms are remarkably similar to the pressure effect on initial rate of polyphenylalanine synthesis in the cell-free system at the higher magnesium salt concentration. This comparison suggests that the magnitude of pressure required to produce a change in the rate of protein (or polypeptide) synthesis is approximately the same in both the cell-free and the *in vivo* studies. The similarity of the pressure effect on cell-free protein synthesis to the effect on protein synthesis in such a broad spectrum of organisms with greatly differing degrees of complexity suggests that the pressure-sensitive elements are very likely the same in all of these systems.

In each of the processes studied (i.e., polyphenylalanine synthesis, misreading of the poly U, tRNA aminoacylation, and attachment of phe-tRNA to the ribosome-poly U complex), the action of high pressure is such that an increase in pressure above 1 atm at a given magnesium concentration produces an effect which correlates with the effect observed at a lower magnesium concentration at 1 atm. For example, the effect of pressure on total polyphenylalanine synthesis (cf., Fig. 4) at 16.8 mM magnesium acetate concentration shows a slight increase in synthetic rate for pressures from 1 to approximately 300 atm, above which there is a rapid decrease in rate with increasing pressure. Comparing the pressure dependence of the rate with the magnesium concentration dependence (Fig. 2), it is found that 16.8 mM magnesium concentration lies on the high magnesium side of the optimum concentration. As the magnesium ion concentration is decreased from 16.8 mM, the rate of synthesis increases approximately a factor of 1.7 at the 10 mM optimum concentration and decreases rapidly as the concentration falls below 11 mM. This result suggests a correlation between the effect of increased pressure at a given magnesium concentration and decreased magnesium concentration at a given pressure (namely, 1 atm).

One prediction based on this correlation is that, if pressure is applied to a reaction at the optimum 10 mM magnesium concentration, then a sharp decrease in rate of synthesis with increasing pressure should be observed. This prediction is verified by the results shown in Fig. 4. It should be noted that the apparent increase in polyphenylalanine synthetic rate between 1 and 300 atm at 16.8 mM magnesium concentration can be attributed partly to the decreased misreading in this pressure range (Fig. 6), where the greater fidelity of phenylalanine insertion at close to the 1 atm rate would produce an apparent increase in rate of polyphenylalanine synthesis. A similar explanation can partly account for the increase in synthetic rate as the magnesium concentration is reduced from 16.8 to 10 mM. Results of the studies of magnesium concentration dependence of misreading of poly U show that a significant decrease in misreading occurs as the magnesium acetate concentration is decreased from 20 to 10 mM. In this case, the predicted effect of increased pressure at 16.8 mM magnesium would be a rapid decrease in misreading over the pressure range extending from 1 to approximately 300 atm. This prediction is confirmed by the results shown in Fig. 6. The action of pressure on the recognition mechanisms in the translation complex is clearly not simple. There appear to be two separate effects of pressure on the fidelity of the reading process. First, there is the initial decrease in misreading (greater fidelity) as pressure is increased from 1 to approximately 300 atm. Second, above 300 atm the misreading appears to increase with increasing pressure. The first process can be implicated in the correlation between pressure effect and magnesium concentration described above. The second process might be attributed to pressure-induced distortions in the translation complex such that the precise interactions between the poly U codons and the anticodons of the aa-tRNAs and among the aa-tRNAs, the peptidyl-tRNAs, and the ribosome cannot occur and insertion of miscoded amino acids (in this case, leucine) takes place.

In the studies of the effect of pressure on initial rate of tRNA aminoacylation, it was found that the rate at 5 mM magnesium acetate concentration and 1 atm was approximately 30% less than the rate at 10 mM. The effect of high pressure on the rate of aminoacylation (Fig. 7) indicated that, as pressure is increased above 1 atm at 10 mM magnesium concentration, there is no effect up to approximately 100 atm. Above 100 atm, there is an exponential decrease of aminoacylation rate with increasing pressure. These findings substantiate the correlation between the effect of increased pressure at a given magnesium concentration and decreased magnesium concentration at 1 atm.

The attachment of phe-tRNA to the poly U-ribosome complex showed a sharp dependence on magnesium concentration. The amount of phe-tRNA which could be bound in an excess of poly U and ribosomes increased from very little (less than 5%) at 5 mM magnesium concentration to maximum (100%) at 22 mM and was constant up to 42 mM magnesium concentration. The effect of pressure applied to this reaction at 20 mM magnesium concentration would be predicted to decrease the

amount of binding. The results of pressure studies (Fig. 8) confirm this prediction. It should be noted that the binding of phe-tRNA to the poly U-ribosome complex in this system is mediated by noncovalent interactions. The amount of phe-tRNA bound to the complex at a given pressure reached the same level relative to the 1 atm control whether pressure was applied just after initiation of the complexing reaction or after the maximum level of complex formation had been at 1 atm. These results suggest that the phe-tRNA-poly U-ribosome complex dissociated until the equilibrium level had been reached.

The above considerations indicate that one very significant element involved in the action of pressure is the interaction of magnesium ions with anionic groups in the macromolecular components of the protein-synthesizing apparatus. This suggestion is supported by the fact that both magnesium ions and phosphate groups show large volume decreases on passage from the associated state to the dissociated state (20–22). Hence, the interaction between magnesium ions and phosphate groups is strongly affected by pressure.

The studies of magnesium concentration dependence of the processes described above suggest that the ionic equilibria involving magnesium ions may be critical. By application of pressure, the magnesium ion-phosphate interaction might be dissolved. While the magnesium ion is mobile and can leave the immediate environment, the phosphate groups are immobile since they form the backbone of the nucleic acid molecules (namely, rRNA or tRNA). The electric fields of the neighboring unshielded negatively charged phosphate groups would produce large forces which would stress the RNA molecule and which could produce a conformation change not only in the RNA molecule but, more significantly, in the ribosome. The sharp dependence of translational processes on magnesium concentration and the large effect of pressure on these processes, together with the correlations noted above in comparing pressure effects with magnesium-dependence, make the proposed action of pressure on protein synthesis an attractive mechanism which can be tested further.

These studies and those of Arnold and Albright (8) exclude the possibility that high pressure acts on only one of the processes involved in the translation of mRNA. The data from the cell-free studies, however, can be examined to determine the relative sensitivities of the various processes which are required for translation of mRNA. Our experiments indicate that the supply of phe-tRNAs to the translation complexes is not the primary element, and we suggest that the most sensitive element to pressure action on polyphenylalanine synthesis must then be among the macromolecular components which are involved in (a) the formation of the translation complex; (b) the events which occur during the elongation cycle (i.e., peptidyl transfer and/or translocation); and/or (c) the checking and selection of aa-tRNAs by the translation complex. The ribosome is very likely that element, and its pressure sensitivity would be reflected in the pressure effect on each of the three functions described above.

Recent studies have demonstrated the cooperative nature of ribosomal function (23-25). Hence, any perturbation introduced on one part of the ribosome could have many manifestations, including changes in fidelity of translation of mRNA, changes in rates of synthesis, and changes in structural integrity. The findings of this study have demonstrated that high pressure has large effects on rate of synthesis, fidelity of translation, and stability of the translation complex. These results strongly implicate the ribosome or an interaction requiring participation of the ribosome as the major sensitive element for the action of high pressure on protein synthesis.

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