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Inhibition of Cell-Free Protein Synthesis by Hydrostatic Pressure[1]

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Pressure inhibition of cell-free polypeptide synthesis is manifested in the same manner as that observed in the intact cell: (i) starting at approximately 200 atm, there is a progressive inhibition with increasing pressures; (ii) there is complete inhibition at 680 atm; (iii) incorporation into polypeptide is instantaneously reversible after pressure release and proceeds at a rate parallel to an atmospheric control; and (iv) the volume change of activation (ΔV^*) is 100 cm^3/mole. Peptide bond formation per se can occur at a pressure level which is totally inhibitory to polypeptide synthesis. The one investigated step in translation that is inhibited in an identical manner is the binding of aminoacyl-transfer ribonucleic acid (AA-tRNA) to the ribosome-messenger RNA (mRNA) complex. The volume change of activation (ΔV^*) calculated for the binding reaction is also 100 cm^3/mole. Thus, the inability of AA-tRNA to bind to ribosomes and mRNA under pressure, possibly in conjunction with translocation, appears to be responsible for the observed inhibition of the translational mechanism.

Increasing hydrostatic pressures progressively inhibit whole-cell protein biosynthesis in *Escherichia coli* at the level of translation (1, 7, 14). The volume change of activation (ΔV^*) calculated by Landau for this inhibition was 100 cm^3/mole (7). We have previously shown that cellular amino acid permeability, amino acid activation, aminoacyl-transfer ribonucleic acid (AA-tRNA) formation, and polysomal integrity do not appear to be affected by pressure in the intact cell (14). Recently, Arnold and Albright (1) have reported a pressure inhibition of cell-free AA-tRNA binding to ribosomes and an even greater susceptibility to pressure of dipeptide formation.

We now wish to report the results of pressure experiments on cell-free synthesizing systems. Since pressure effects do not always manifest themselves in cell-free preparations in the same manner as in whole-cell experiments (1, 8, 11, 17), the validity of results from the cell-free preparations had to be established. These investigations have yielded quantitative data on pressure inhibition of such systems programmed by natural and synthetic messenger RNA species (mRNA), inhibition of peptide bond formation per se, and the inhibition of AA-tRNA binding to the ribosome-mRNA complex.

[1] This paper is taken in part from a thesis that was submitted by J. R. S. in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology, Rensselaer Polytechnic Institute.

MATERIALS AND METHODS

Materials. Uniformly ^{14}C-labeled L-phenylalanine (355 μCi/μmole), uniformly ^{14}C-labeled L-amino acid mixture (232 μCi/μmole), puromycin-methoxy-^3H (N) dihydrochloride (935 μCi/μmole), and ^{14}C-phenylalanyl tRNA (0.16 μCi/mg, 0.416 μmoles/mg) were purchased from New England Nuclear Corp. Adenosine triphosphate (ATP), guanosine triphosphate (GTP), phosphoenolpyruvate (PEP), pyruvate kinase, polyuridylic acid (poly U), and alumina 305 were purchased from Sigma Chemical Co. Unlabeled amino acids and bovine serum albumin (BSA) were obtained from Nutritional Biochemical Corp., and deoxyribonuclease (E.C. 3.1.4.5.) was from Worthington Biochemical Corp. MS_2 viral RNA was purchased from Miles Laboratories.

Organism and preparation of cell-free extracts. *Escherichia coli* strain K-12, ATCC 10798, mid-log harvest, was obtained as a frozen cell paste from Miles Laboratories, Inc. The procedure for the preparation of cell-free extracts was that of Nirenberg (12). Preincubation of the S-30 fraction was necessary and was carried out at 37 C for 80 min. Dialysis against 120 volumes of standard buffer was carried out in the cold (4 C) for 10 hr, with the buffer changed at 4 hr. The S-30 fraction protein content was estimated by the method of Lowry (9) to be 15 mg/ml. The S-30 fraction was quickly frozen and stored in small samples at −20 C.

Extraction of ribosomes and transfer factor preparation. Ribosomes were removed from the S-30 fraction by centrifugation at 105,000 × g for 2 hr and were subsequently washed five times by the method of Ravel (13). Ribosomes were diluted to 25 mg of protein per ml (9) and were stored at −20 C for no more than 2 weeks. The S-100 supernatant fraction was removed by aspiration from the initial pelleting of ribosomes and was utilized as a source of transfer factors. The transfer factors were extracted by ammonium sulfate precipitation by the method of Ravel (13). The resulting 40 to 65% $(NH_4)_2SO_4$ fraction contained about 20 mg of protein per ml, as determined by the method of Lowry (9). The transfer factors were frozen quickly and stored in small samples at −20 C for no more than 2 weeks.

Conditions for in vitro polyphenylalanine formation. All components were warmed to 37 C and were present in the concentrations described in each figure. The reaction was initiated by the addition of S-30 extract. When all components were mixed, 1.05-ml fractions were added to small glass tubes that were stoppered on each end with rubber, serum tube caps (Fig. 1a, oil and S. S. pellet omitted). Three samples, each in duplicate, were set up. One sample was placed in the pressure reactor described by Landau (8), and the reactor was filled with 37 C water. The appropriate experimental pressure was applied, and the reactor was incubated for 5 min at 37 C after pressure application. The other two samples were the atmospheric controls. The first of these was sampled at the moment of pressure application. The second control was taken at the point of pressure release. Termination of polyphenylalanine synthesis was accomplished by pouring the contents of each sample into 8 ml of ice-cold 10% trichloroacetic acid containing 340 mg of BSA. The extraction procedure followed that reported by Changchien and Aronson (2), except that five 5-ml samples of ice-cold 5% trichloroacetic acid were used to wash the membrane filter (Millipore Corp.), and 15 ml of Aquasol was used as the scintillation fluid. All samples were assayed for radioactivity by counting in an Intertechnique SL-30 liquid scintillation spectrometer.

Conditions for MS_2 RNA-directed polypeptide synthesis. The procedure was the same as that listed for the poly U-directed system, except that MS_2 viral RNA was used in place of poly U in the reaction mixture. The sampling and extraction procedures were identical to those used for the poly U-directed synthesis.

In vitro puromycin-polyphenylalanine formation. A poly U-directed synthesizing system was initiated and placed in the bottom portion of a small glass tube (Fig. 1a, fluid B). Approximately 0.15 ml of immersion oil was layered on top of this, and 0.05 ml of tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.5, containing μCi of ^{3}H-puromycin was gently layered on top of the oil (Fig. 1a, Fluid A). The Tygon collar was used because it afforded a greater attractive surface for the oil than did the glass walls of the tube and prevented premature mixing of the two solutions. When assembled, this miniature mixing vessel was placed in a pressure reactor, the remaining volume of the reactor was filled with 37 C water, and a pressure of 680 atm was applied to the reactor. The onset of pressure occurred 3 min after the poly U-directed synthesizing system was initiated. Ten minutes after pressure application, the contents of the reaction vessel were mixed by continually inverting the reactor and allowing the stainless-steel pellet to slide back and forth in the glass vessel. The pressure reactor was then chilled to 1 to 2 C 2 min after mixing of the contents of the vessel. Pressure was released, and the contents were sampled in the same manner as for polyphenylalanine formation (2). A corresponding control sample was treated identically, but in the absence of any pressure. A third sample, to correct for any puromycin-polyphenylalanine formation that occurred at 1 to 2 C in the time interval between pressure release and sampling, was also taken. In this sample, maintained at atmospheric pressure, the ^{3}H-puromycin was not mixed with the poly U-directed synthesizing system until the contents of the glass reaction vessel were at 1 to 2 C. This base-line value was then subtracted from both pressure and control valves.

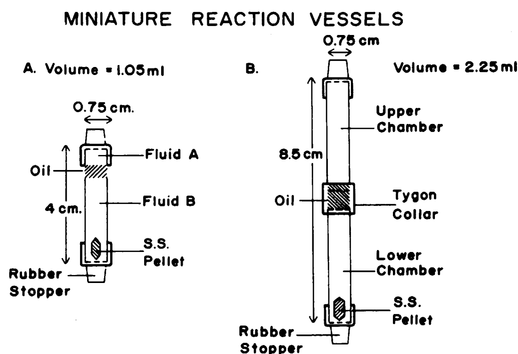

FIG. 1. *Miniature reaction vessels.*

Conditions for in vitro binding of phenylalanyl-tRNA. All components were warmed to 37 C, and ^{14}C-phenylalanyl-tRNA was added last to initiate the binding reaction. The reaction vessel contained 1.05 ml of reaction mixture (minus tRNA) separated from 0.25 ml of buffer containing the ^{14}C-phenylalanyl-tRNA by 0.15 ml of immersion oil (similar to Fig. 1b). A stainless-steel pellet was also present in the bottom chamber and was used to mix the two solutions after the desired pressure level had been applied. After the application of pressure and the mixing of the two solutions, the pressure reactor was placed in a 37 C incubator until time for release and sampling. Corresponding atmospheric control experiments were also performed. To terminate the binding reaction, pressure was released, and 0.2-ml samples were removed and diluted 40-fold with ice-cold buffer (0.5 M Tris-hydrochloride, pH 7.7, containing 0.16 M ammonium chloride and 0.012 M $MgCl_2$). The diluted reaction mixture was immediately poured over a cold membrane filter (Millipore Corp.; 0.45-μm pore size) and washed with three 8-ml samples of this same buffer. The time interval between release of pressure and dilution of the sample was 35 sec; filtering and

washing were complete within 1 min after dilution. The filters were then dried at 80 C for 10 min, placed in vials to which 15 ml of Aquasol was added, and assayed for radioactivity in a liquid scintillation counter.

RESULTS

Cell-free poly U-directed synthesis. The conclusion that the probable action of pressure upon the whole cell occurred during translation (1, 7, 14) necessitated the employment of cell-free synthetic systems to investigate translational events.

The results obtained with such an in vitro synthesizing system are presented in Fig. 2. Incorporation of ^{14}C-phenylalanine into trichloroacetic acid-insoluble polyphenylalanine at 37 C and 1 atm was linear for about 30 min and leveled off by 45 min. Application of 136 atm to this system had no effect, but pressure levels of 204 atm and above resulted in a progressive inhibition of polyphenylalanine synthesis (Fig. 2). At 680 atm, there was a complete inhibition of all incorporation, the same level at which whole-cell incorporation ceased. When samples were taken after release of all pressure levels used, incorporation of ^{14}C-phenylalanine instantaneously resumed at a rate which paralleled the atmospheric control. A logarithmic plot of incorporation per 5 min versus pressure exhibits a straight line between 204 and 544 atm (Fig. 3, top). The slope of this line yields a ΔV^* of 100 cm^3/mole, equal to that obtained from the whole cell. Thus, whole-cell and poly U-directed cell-free systems overtly appear to respond to pressure in the same manner.

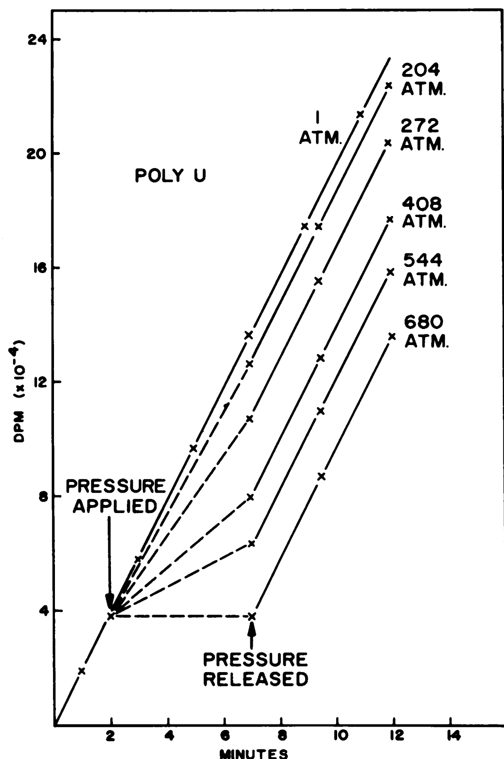

FIG. 2. *Effect of pressure on cell-free polyphenylalanine synthesis at 37 C. The complete reaction mixture (1.05 ml) contained the following per milliliter: 100 µmoles of Tris-hydrochloride (pH 7.6), 58 µmoles of KCl, 12 µmoles of magnesium acetate, 6 µmoles of 2-mercaptoethanol, 2.4 µmoles of ATP, 0.28 µmoles of GTP, 8.75 µmoles of PEP, 58 µg of pyruvate kinase, 95 µg of poly U, 0.5 µCi of* ^{14}*C-phenylalanine, 0.25 µmoles of each of the other 19 amino acids, and 6 mg (protein) of S-30 fraction. The S-30 extract was added last to initiate the reaction. The reaction mixture was treated as described in Materials and Methods.*

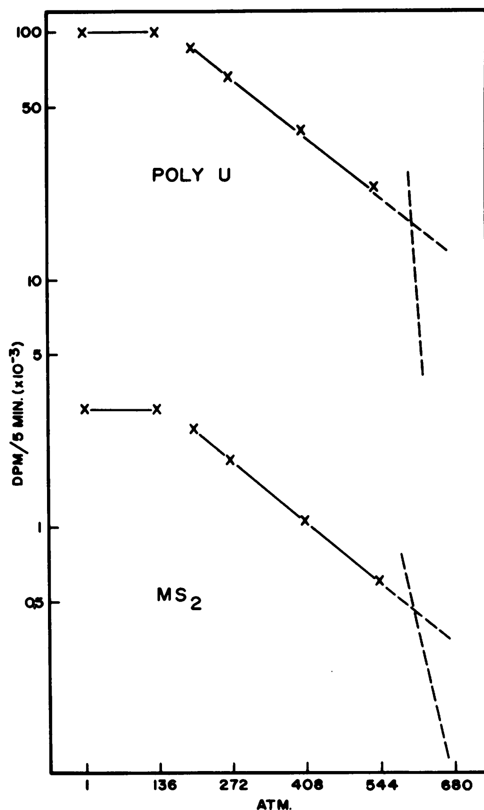

FIG. 3. *Logarithmic plot of* ^{14}*C-phenylalanine incorporation into polypeptide per 5 min at 37 C versus pressure level in a cell-free synthesizing system. Top, Polyuridylic acid-directed synthesis; bottom,* MS_2 *viral RNA-directed synthesis.*

MS_2 viral RNA-directed cell-free synthesis. An in vitro system programmed with natural mRNA should approximate, to a greater degree, the processes that occur in the intact cell. The results from such a system, directed by MS_2 viral RNA, are presented in Fig. 4. Pressure up to 136 atm did not inhibit incorporation at 37 C, but pressures of 204 atm and above progressively inhibited incorporation into polypeptide. At 680 atm, a complete inhibition of synthesis occurred. Sampling of reaction mixtures after release of pressure resulted in a resumption of incorporation, the rate of which paralleled the atmospheric control rate (Fig. 4). The ΔV^* calculated for this system was 100 cm^3/mole (Fig. 3, bottom), in agreement with both whole-cell and poly U-directed synthesizing systems. In a previous report (14), it was pointed out that, since all the amino acids were not tested for their ability to permeate into the intact cell under 680 atm, it was possible that the entrance of one or more of them could be limited by this pressure. However, when using a natural mRNA to direct a cell-free system, no permeability barrier is present and all amino acids are available. The fact that the ΔV^* of 100 cm^3/mole is the same as in the whole cell would seem to rule out amino acid permeability as a factor in pressure inhibition of protein synthesis.

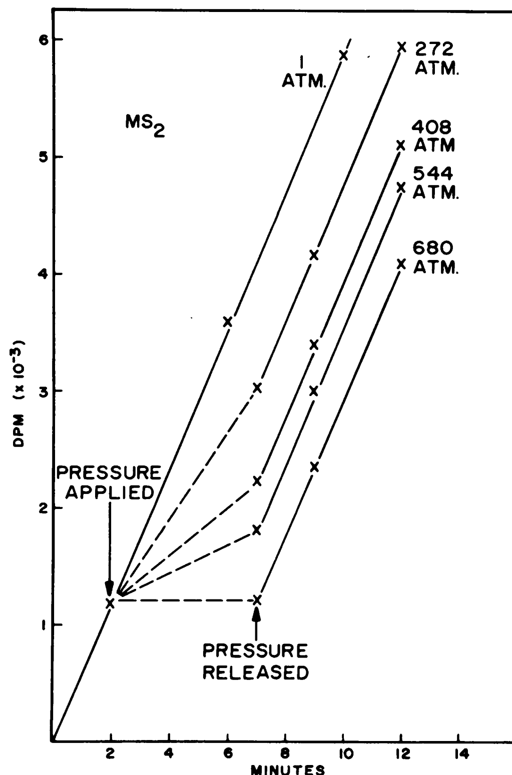

FIG. 4. *Effect of pressure on MS_2 viral RNA-directed cell-free protein synthesis at 37 C. The complete reaction mixture (1.05 ml) contained the following per milliliter: 50 μmoles of Tris-hydrochloride (pH 7.5), 7 μmoles of magnesium acetate, 30 μmoles of ammonium chloride, 5 μmoles of 2-mercaptoethanol, 2.3 μmoles of ATP, 0.3 μmoles of GTP, 8.5 μmoles of PEP, 50 μg of pyruvate kinase, 150 μg of stripped E. coli K-12 tRNA, 0.75 μCi of ^{14}C-phenylalanine, 0.25 μmoles of each of the other 19 amino acids, 150 μg of MS_2 viral RNA, and S-30 fraction (6 mg of protein). The S-30 was added last to initiate the reaction.*

Cell-free puromycin-polyphenylalanine formation at 680 atm. The incorporation of puromycin into the carboxy terminal end of nascent peptides involves the formation of a peptide bond (3). Puromycin competes with the incoming AA-tRNA for the carboxy, terminal end of peptidyl-tRNA bound to the 50*S* subunit. Thus, by allowing the synthesis of polyphenylalanine to occur before the application of 680 atm, a portion of the polysomes will be in a puromycin-reactive state. The addition of ^3H-puromycin to this system under 680 atm and the subsequent analysis for formation of ^3H-puromycin-polyphenylalanine will indicate the capability of this system to form a peptide bond (Table 1). The average percentage of radioactive label extracted as puromycin-polyphenylalanine from the pressure sample was 84% of that obtained from the atmospheric control (range 71–93%), an amount sufficient to permit polypeptide synthesis to continue if the system were not affected by pressure elsewhere.

Additional GTP was not added in a control

TABLE 1. *Reactivity of ribosome-bound polyphenylalanyl-tRNA with ^3H-puromycin*[a]

Additions to reaction components	Counts/min at 1 atm[b]	Counts/min at 680 atm[b]	Counts per min at 680 atm/counts per min at 1 atm
+GTP, +^3H-puromycin ...	561	472	84%
−GTP, +^3H-puromycin ...	129	120	93%

[a] Reaction mixture components were the same as described for poly U-directed polypeptide synthesis, except for the omission of ^{14}C-phenylalanine and the addition of 35 μCi of ^3H-puromycin and ^{12}C-phenylalanine (0.25 μmoles/ml.).

[b] Base-line value of 55 counts/min already substracted.

experiment that was performed to determine whether we were actually observing the puromycin reaction. Here, the levels of both control and pressure samples dropped as expected, but they still retained the same relative proportions. All values are corrected for any puromycin-polyphenylalanine formation occurring between release of pressure and addition of trichloroacetic acid.

Cell-free ^{14}C-phenylalanyl-tRNA binding at 37 C. The binding of phenylalanyl-tRNA in the presence of poly U, washed ribosomes, GTP, transfer factors, and ions is complete within 10 min at 37 C. The rate, however, is linear within the first 8 min (Fig. 5). AA-tRNA binding was initiated after the experimental pressure level was attained. The application of increasing pressures above 204 atm progressively inhibited the binding reaction; no binding is observed at 680 atm. When pressure is released, AA-tRNA binding resumes at a rate parallel with the atmospheric control (Fig. 5). Since we used ribosomes free of any bound AA-tRNA and initiated the reaction under pressure, we could determine that the inhibition observed was a consequence of an inability of the AA-tRNA to bind at a free site rather than an inability to bind because the site had been occupied, a point not clarified by the work of Arnold and Albright (1).

When a logarithmic plot of rate versus pressure level was made, the slope of the straight-line portion yielded ΔV^* of 100 cm^3/mole (Fig. 6). Thus, the inability of AA-tRNA to bind to ribosomes and mRNA under pressure is quantitatively identical to the inhibition of protein synthesis in the whole cell.

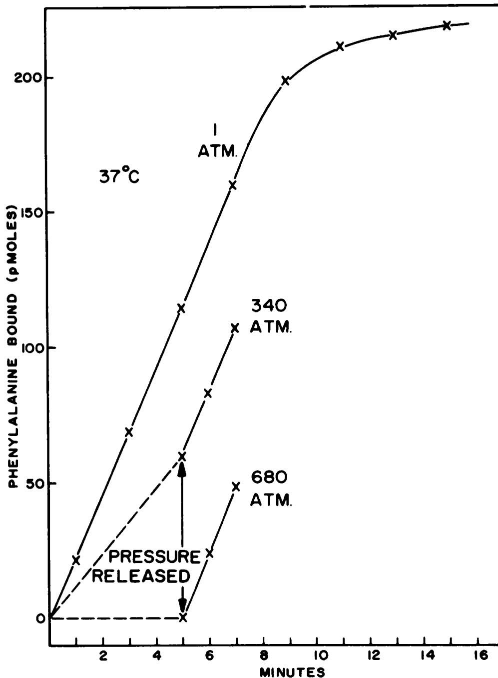

FIG. 5. *Effect of pressure on cell-free binding of ^{14}C-phenylalanyl-tRNA at 37 C. The final concentrations of the reaction components were per milliliter: 0.05 M Tris-hydrochloride (pH 7.7), 0.012 M 2-mercaptoethanol, 0.08 M ammonium chloride, 0.08 M KCl, 0.012 M magnesium chloride, 12 µg of poly U, 0.4 mg of washed ribosomes, 0.5 mg of tRNA charged with 302.5 pmoles of ^{14}C-phenylalanine, 0.2 mM GTP, and 200 µg of transfer factors. The components were added as described in Materials and Methods. Expressed as picomoles of phenylalanine bound.*

DISCUSSION

Pressure inhibition of cell-free polypeptide synthesis is manifested in the same manner as that observed in the intact cell; (i) starting at approximately 200 atm, there is a progressive inhibition with increasing pressures; (ii) there is complete inhibition at 680 atm; (iii) incorporation into polypeptide is instaneously reversible after pressure release and proceeds at a rate parallel to the atmospheric control;

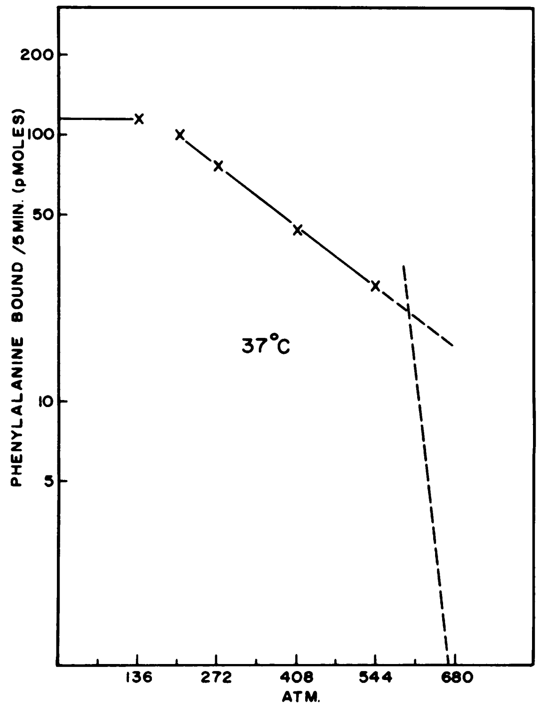

FIG. 6. *Logarithmic plot of phenylalanyl-tRNA bound per 5 min at 37 C versus pressure. Expressed as picomoles of phenylalanine bound per 5 min.*

and (iv) the calculated ΔV^* is 100 cm^3/mole. The one investigated step in translation that is inhibited in an identical manner is the binding of AA-tRNA to the ribosome-mRNA complex. The volume change calculated for the binding reaction is also 100 cm^3/mole. The fact that peptide bond can be formed under pressure conditions which completely inhibit protein synthesis and the observation of Arnold and Albright (1) that dipeptide formation is more sensitive than AA-tRNA binding indicates that translocation of the AA-tRNA may also be involved in the pressure inhibition of the translational mechanism. It is evident that the binding of AA-tRNA to the ribosome involves a volume increase, i.e., the volume of the AA-tRNA-mRNA-ribosome complex is greater than that of the individual molecules. This most likely reflects a configurational change in the ribosome itself.

Arnold and Albright have also reported what seems to be a reversible disruption of a poly U-ribosome complex under pressure (1). Our previous data (14) indicated no such disruption in experiments with whole-cells. Several workers (4-6, 10, 15, 16) have investigated the effect of hydrostatic pressures generated during centrifugation on the components of cell-free synthesizing systems. They conclude, in general, that configurational changes may occur in the ribosome which would prevent the formation of complete ribosomes and inhibit polysome formation. However, in all cases, AA-tRNA-mRNA-ribosome complexes which were already formed were not disrupted by pressure. It must be noted that the experiments of Arnold and Albright (1) did not utilize AA-tRNA.

We have approached this particular problem by utilizing the pressure techniques and apparatus described in this paper, and the detailed analysis will be presented in a forthcoming report from this laboratory.

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