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# Role of Bacterial Ribosomes in Barotolerance

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The effects of high hydrostatic pressures on protein synthesis by whole cells and cell free preparations of *Escherichia coli*, *Pseudomonas fluorescens*, and *Pseudomonas bathycetes* were determined. Actively growing cells of *P. bathycetes* and *P. fluorescens* were less sensitive than were *E. coli* cells. Protein synthesis by cell free preparations of *E. coli* and *P. fluorescens* showed the same extent of inhibition as their respective whole cell preparations, whereas cell free preparations of *P. bathycetes* showed a marked increase in pressure sensitivity over whole cells. Protein synthesis by hybrid protein synthesizing cell free preparations (the ribosomes from one organism and the S-100 supernatant fraction from another) demonstrated that response to high pressure is dependent on the source of the ribosome employed. A hybrid system containing *E. coli* ribosomes and *P. fluorescens* S-100 shows the same sensitivity to pressure as a homologous *E. coli* system, whereas a hybrid containing *P. fluorescens* ribosomes and *E. coli* S-100 shows the greater pressure tolerance characteristic of the *P. fluorescens* homologous system.

The effect of high hydrostatic pressure on the biochemical process of microbial organisms has been the subject of numerous reports (1-4, 6-8, 10, 11, 13, 14). In *Escherichia coli*, the translation phase of protein synthesis is most sensitive to high pressure and may well represent the primary biochemical process limiting the growth of this organism at high pressures. Pope and Berger (12), suggested that protein synthesis may in fact be the primary limiting factor for growth in most, if not all, prokaryotes at high pressures. There are, however, differences of opinion regarding the mechanism whereby protein synthesis in *E. coli* is inhibited by high pressure. Landau (6, 7) showed that where inhibition of protein synthesis occurs, it is immediate upon application of pressure and that synthesis resumes at the 1-atmosphere (atm) rate upon release of pressure. It was calculated that protein synthesis in *E. coli* is accompanied by an increase in a volume change of activation ($\Delta V^*$) of approximately 100 $cm^3$/mol at 37 C. Schwarz and Landau (13, 14) showed that protein synthesis by cell free preparations of *E. coli* also have a $\Delta V^*$ of 100 $cm^3$/mole at 37 C. These authors reported that a pressure of 670 atm completely inhibited protein synthesis yet had no immediate effect on amino acyl transfer ribonucleic acid (aa-tRNA) formation, amino acid permeability, polysomal integrity, or peptide bond formation. The reaction found to be inhibited by high pressure in a manner quantitatively indentical to that shown by whole cells occurred between the steps of aa-tRNA formation and peptide bond formation, and it was postulated to directly involve the ribosome by possibly altering the binding capacity of the aa-tRNA to the ribosome-messenger RNA complex or interfering with translocation. Arnold and co-workers (1, 2, 4) and Hildebrand and Pollard (5) on the other hand concluded that several steps in the protein synthesizing process were sufficiently sensitive to high pressure to be considered responsible, in part, for the overall inhibition of protein synthesis by high pressure. These included: amino acid transport, aa-tRNA formation, peptide bond formation, aa-tRNA stability, aa-tRNA-ribosome complex formation, stability of the polysomal complex, and fidelity of translation. Recent work (D. H. Pope, N. T. Conners, and J. V. Landau, submitted for publication), however, indicates that a pressure of 670 atm, which totally inhibits protein synthesis, prevents the dissociation of polysomes in actively growing cells of *E. coli*. The degree of polysomal stability at this pressure was shown to surpass even the stability conferred by the addition of chloramphenicol. The data led to the conclusion that polysomes do not dissociate at high pressure, and therefore, that polysomal stability cannot be considered a primary factor in the inhibition

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of protein synthesis by high pressure.

The studies reported here were done to determine whether the various other reactions, as discussed above, were of primary importance to the overall inhibition of protein synthesis by high pressure. Earlier work comparing the effects of pressure on protein synthesis in a bacterium recovered from the Marianas trench (*Pseudomonas bathycetes*), a pseudomonad of terrestrial origin (*Pseudomonas fluorescens*), and *E. coli* indicated that protein synthesis in the pseudomonads was quantitatively less sensitive to pressure than in *E. coli*. (15).

Our approach, therefore, was initially to prepare polyuridylate-directed cell free protein synthesizing systems for each of the aforementioned organisms and compare the effects of pressure on these with the effects observed utilizing whole cells. After this, hybrid cell free systems were prepared, combining isolated ribosomes from a relatively pressure-resistant organism and supernatant factors from a relatively pressure-sensitive organism. Since the supernatant fraction (S-100) contained tRNA, soluble factors G and T, termination factors, amino acyl-tRNA synthetases, and other factors (9), a determination of the role of the ribosome and of the supernatant factors in the resistance or sensitivity of protein synthesis to increased pressure could readily be made.

## MATERIALS AND METHODS

**Bacterial strains.** *P. bathycetes* $C_3M_2$ (ATCC no. 23597) was obtained from R. Colwell, Univ. of Maryland. *P. fluorescens* (ATCC no. 13525) was obtained from the American Type Culture Collection. *E. coli* K-12 (streptomycin sensitive, β-galactosidase and permease constitutive) was obtained from C. Hurwitz, Veterans Administration Hospital, Albany, N.Y.

**Growth conditions.** Stock cultures of *E. coli* and *P. fluorescens* were maintained on nutrient agar (Difco) slants. *P. bathycetes* was maintained on marine agar (Difco) slants. *P. fluorescens* and *E. coli* were grown in nutrient broth (Difco) at 25 and 37 C, respectively, and *P. bathycetes* was grown in a medium (at 25 C) consisting of (in grams per liter): yeast extract (Difco), 3; proteose peptone (Difco), 10; NaCl, 24; KCl, 0.7; $MgCl_2 \cdot 6H_2O$, 5.3; $MgSO_4 \cdot 7H_2O$, 7. In preparation for testing, 300 ml of the appropriate medium contained in 1-liter flasks was inoculated from fresh slants and allowed to grow overnight on a rotary shaker at 125 rpm and at the appropriate growth temperature. Fifty milliliters of this overnight culture was then transferred to fresh flasks of broth and allowed to grow to mid-log phase (at least two generations). Growth of the cultures was measured spectrophotometrically at 600 nm with a Spectronic 20 spectrophotometer.

**Preparation of cell free extracts.** Cells were harvested by centrifugation at 9,000 rpm for 15 min at 3 C in a refrigerated Sorvall centrifuge. The cells were then suspended in standard buffer (9) and recentrifuged as above. Cells were ruptured, and S-30, S-100, and ribosome fractions were prepared according to Modolell's modification of the Nirenberg method (9) with the following exceptions: (i) *P. fluorescens* and *P. bathycetes* cells were ruptured at 7,500 psi in a prechilled French pressure cell (Fred S. Carver Inc., Summit, N.J.). (ii) Treatment with deoxyribonuclease and subsequent dialysis steps were omitted, as these steps had no effect on the activity of the preparations or their sensitivity to pressure.

**Assay for protein synthesis in growing cells.** A 9-ml sample of a mid-log phase culture was rapidly equilibrated to the temperature at which the reaction was to be run. One milliliter of pre-equilibrated $^{14}C$-labeled amino acid mixture was added and thoroughly mixed with the cells. At the times indicated for each experiment, 0.2-ml samples, in duplicate, were removed by using a biopette (Schwartz-Mann, Orangeburg, N.Y.), to 2 ml of ice-cold 5% trichloroacetic acid. Samples of the test mixtures to be pressurized were drawn into a 1-ml syringe; the syringe was capped with a serum stopper and put into a pressure cylinder filled with water at the appropriate temperature, and the pressure cylinder was capped. Pressure was applied 1 min after the addition of the $^{14}C$-labeled amino acid mixture to the cell suspension and released after 2 min incubation under pressure. Upon release of pressure, the sample was removed from the syringe to a vial at the same temperature. Samples (0.2 ml) were taken to 2 ml of 5% trichloroacetic acid at 15 and 60 s after release of pressure. All samples were left in ice cold 5% trichloroacetic acid for at least 30 min, heated to 85 C for 30 min, and returned to an ice bath for 30 min. The samples were filtered onto 0.45-μm membrane filters (Millipore Corp.), washed with two portions of ice cold 5% trichloroacetic acid, and dried at 80 C in scintillation vials. Ten milliliters of scintillation fluid (Aquasol, New England Nuclear Corp., Boston Mass.) was added to each vial and the samples were counted using an Intertechnique SL-30 liquid scintillation counter at approximately 60% efficiency.

**Assay for protein synthesis by cell free systems.** [$^{14}C$]phenylalanine incorporation into protein was tested by methods similar to those given above for growing cells, with the following modifications: the volume of the reaction mixture was 3 ml and contained 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.8), 0.01 mg of [$^{12}C$]phenylalanine, 1.5 μCi of [$^{14}C$]phenylalanine, 0.6 mg of polyuridylic acid, 6 mM 2-mercaptoethanol, 1 mM Tris-adenosine 5'-triphosphate, 5 mM potassium phosphoenol pyruvate, 0.025 mM guanosine 5'-triphosphate, 0.065 mg of pyruvate kinase, 37.5 mM $NH_4Cl$, and 12.5 mM $Mg(OAc)_2$. To initiate the reaction, preincubated S-30, or S-100 plus ribosome preparation, was added to the above mixture. The final concentration of ribosomes was in all cases 1 mg/ml.

**Chemicals.** Polyuridylic acid, phosphoenol pyruvate (trisodium salt), adenosine 5'-triphosphate (Tris salt), guanosine 5'-triphosphate (Tris salt, type VI), and pyruvate kinase were obtained from Sigma

Chemical Co., St. Louis, Mo. Uniformly labeled [$^{14}$C]phenylalanine (2.7 mCi/mg) and $^{14}$C-labeled amino acid mixes (100 $\mu$Ci/ml) were obtained from New England Nuclear Corp., Boston, Mass. All other chemicals used were of reagent grade.

## RESULTS

Figure 1 presents the results of experiments to determine the effect of high hydrostatic pressure on the incorporation of $^{14}$C-labeled amino acids into protein by actively growing cells of *P. bathycetes*, *P. fluorescens*, and *E. coli* at several temperatures. The data of Swartz and Landau (15) for *P. bathycetes* at 25 C were normalized and are included for comparison. The data are expressed as the rate of protein synthesis relative to the rate at 1 atm for each temperature and organism, i.e., as the precent of the rate at 1 atm, to facilitate their comparison. It should be noted that the absolute rates of protein synthesis decreased with decreased temperatures (Table 1). In all cases, $^{14}$C-labeled amino acid incorportation into protein at 1 atm was linear for the duration of the experiment. After release of pressure, the rate of incorporation resumed at the 1-atm rate. As can be seen, protein synthesis in whole cells of *P. bathycetes* and *P. fluorescens* is more resistant to pressures greater than 400 atm than synthesis in whole cells in *E. coli*. *E. coli* appears to be slightly more sensitive to these pressures at 15 C than at 25 C. At the lower pressures, synthesis in *E. coli* is stimulated at 15 and 37 C but such stimulation is not evident at 25 C.

**Effect of high pressure on polyphenylalanine synthesis by cell preparations.** The effect of high pressure on polyuridylate-directed polyphenylalanine synthesis by cell free preparations (S-30) from *E. coli*, *P. fluorescens*, and *P. bathycetes* at various temperatures is shown in Fig. 2. Experiments not shown demonstrated that the rate of protein synthesis was a linear function of ribosome concentration to at least 1.6 mg of ribosomes per ml. Therefore, a ribosome concentration of 1 mg/ml was used throughout these experiments. Again, the data are expressed relative to the rate of protein synthesis at 1 atm. The absolute rates of protein synthesis for each cell-free system at various temperatures and 1 atm are given in Table 1. There is essentially no change for *P. fluorescens* from the results obtained using whole cells. The pattern of inhibition for *E. coli* is similar to that for whole cells, i.e., at 15 C the cell free preparations showed a slight stimulation at lower pressures (up to 350 atm), but unlike the whole cells, the cell free preparations showed incomplete inhibition of polyphenylalanine synthesis even at 800 atm. It should be noted that the degree of inhibition by pressures in the range 200 to 600 atm is much less than at 15 and 37 C. The most intriguing, and surprising, results are those with *P. bathycetes*. The pattern for inhibition of protein synthesis by pressure has changed drastically from that seen with whole cells of this organism; polyphenylalanine synthesis in cell preparations of *P. bathycetes* is quite sensitive to pressure at all temperatures tested. The pattern of inhibition is quite similar to that seen for *E. coli*, i.e.,

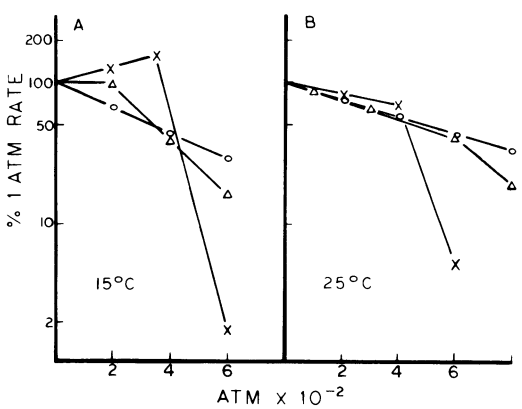


FIG. 1. *Effect of hydrostatic pressure on the rate of $^{14}$C-labeled-amino acids incorporation into protein at 15 and 25 C. Exponentially growing cells were used in all cases. P. fluorescens* (O), *P. bathycetes* (Δ), *and E. coli* (×).

TABLE 1. *Rates of $^{14}$C-labeled amino acid and [$^{14}$C]phenylalanine incorporation into protein by whole cells and cell free preparations, respectively, at 1 atm and various temperatures*[a]

| System | 15 C | 25 C | 37 C |
|---|---|---|---|
| *E. coli* | | | |
| Whole cells | ND | ND | 0.2106 |
| Cell free | 0.0112 | 0.0421 | 0.0702 |
| *P. fluorescens* | | | |
| Whole cells | ND | 0.1986 | ND |
| Cell free | 0.0151 | 0.0662 | 0.0720 |
| *P. bathycetes* | | | |
| Whole cells | ND | 0.0660 | ND |
| Cell free | 0.0115 | 0.0172 | 0.0230 |
| *E. coli* ribosomes + *P. fluorescens* S-100 | 0.0046 | 0.0138 | 0.0260 |
| *P. fluorescens* ribosomes + *E. coli* S-100 | 0.0174 | 0.0232 | 0.0290 |

[a] Micromoles of $^{14}$C-labeled amino acids incorporated into protein per minute for whole cells; micromoles of [$^{14}$C]phenylalanine incorporated into protein per minute for cell free. ND, No data obtained.

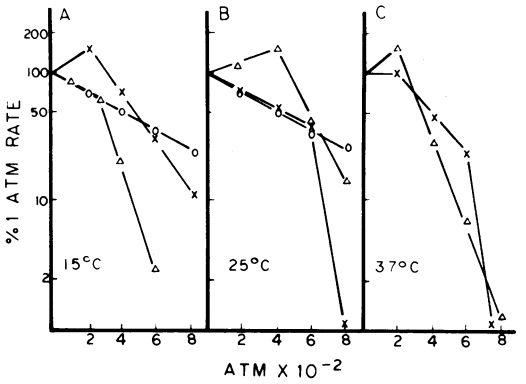


FIG. 2. *Effect of hydrostatic pressure on the rate of [$^{14}$C]phenylalanine incorporation into polyphenylalanine by polyuridylate-directed cell free preparations at 15, 25, and 37 C. P. fluorescens* (O), *P. bathycetes* (Δ), *and E. coli* (×).

inhibition at 25 C is less than that at 15 and 37 C, and synthesis is completely inhibited by approximately 700 to 800 atm. However, *P. bathycetes* seems to be even more sensitive to pressure than *E. coli* over the range from 200 to 800 atm. These results, however, clearly rule out the possibility of testing a *P. bathycetes* + *E. coli* hybrid system at high pressure. A hybrid system between *E. coli* and *P. fluorescens* would, however, serve as a test of the original hypothesis. Therefore, it was used in the following experiments.

**Protein synthesis by hybrid cell free systems at high pressures.** Figure 3 gives the results of the experiments done to determine the rates of polyphenylalanine synthesis by homologous (both ribosomes and S-100 from the same organism) and heterologous (the ribosomes are from one species and the S-100 from another species) cell free preparations at various pressures. Each component, e.g., ribosomes or S-100, was tested individually to determine completeness of the separation. Only negligible rates of [$^{14}$C]phenylalanine incorporation were found in these control experiments.

Homologous and hybrid systems were tested at both 25 and 15 C. Similar results were obtained at both temperatures; only the results at 15 C are shown in Fig. 3. These results reveal that the pressure sensitivity of each hybrid system is quantitatively identical to that of the homologous system from which the ribosomes were derived. This clearly indicates that the sensitivity of protein synthesis to high pressure is a direct function of the ribosome, and that inhibition of interactions between other components involved in protein synthesis, if it occurs, may be of secondary importance to the overall inhibition of protein synthesis by pressure.

The Arrhenius plots (Fig. 4) show that the effect of temperature on protein synthesis is, as with pressure, correlated with the origin of the ribosomes. That is, the curves for *E. coli* homologous and *E. coli* ribosomes + *P. fluorescens* S-100 preparations parallel each other over the entire range of temperature tested (5 to 37 C). A good correlation is also seen for the *P. fluorescens* homologous and *P. fluorescens* ribosomes + *E. coli* S-100 preparations over the range 25 to 37 C, whereas the data indicate a marked difference in the range 5 to 25 C.

Table 2 presents the $\Delta V^*$ values calculated from the inhibition data of Fig. 1, 2, and 3. The $\Delta V^*$ values are used to indicate the slopes of the of the lines obtained on log rate versus pressure plots, and to facilitate comparisons between responses of the various organisms.

## DISCUSSION

Our results show that the response of polyphenylalanine synthesis to high pressure is determined by the ribosomal component of the

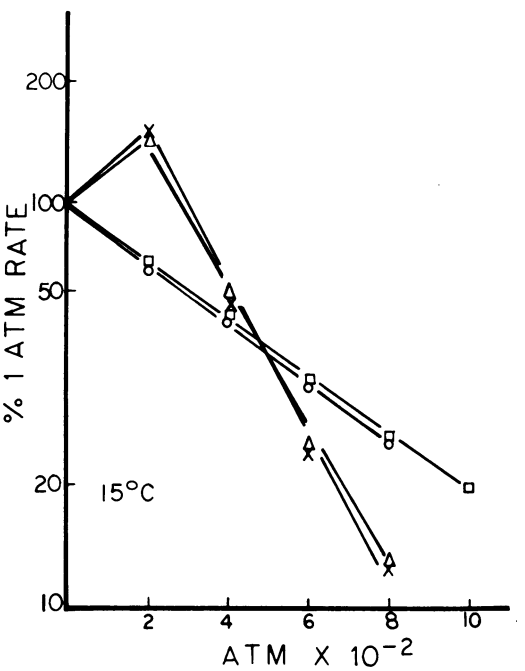


FIG. 3. *Effect of hydrostatic pressure on the rate of [$^{14}$C]phenylalanine incorporation into polyphenylalanine by polyuridylate-directed homologous and hybrid cell free preparations at 15 C. P. fluorescens ribosomes + P. fluorescens S-100* (O), *E. coli ribosomes + E. coli S-100* (×), *E. coli ribosomes + P. fluorescens S-100* (Δ), *and P. fluorescens ribosomes + E. coli S-100* (□).

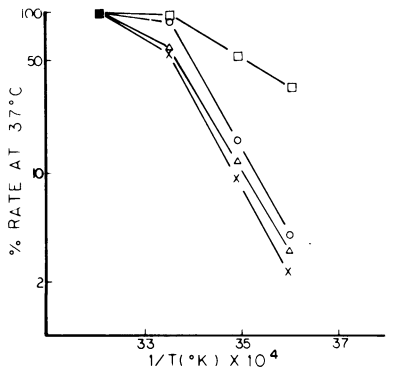


FIG. 4. *Effect of temperature (over the range 5 to 37 C) on the rate of [$^{14}$C]phenylalanine incorporation into polyphenylalanine by polyuridylate-directed homologous and hybrid cell free preparations. All experiments were done at 1 atm. P. fluorescens ribosomes + P. fluorescens S-100* (○), *E. coli ribosomes + E. coli S-100* (×), *E. coli ribosomes + P. fluorescens S-100* (△), *and P. fluorescens ribosomes + E. coli S-100* (□).

system and not by the soluble factors. The degree of sensitivity to pressure would therefore seem to be dependent primarily on some aspect of ribosomal function. The close quantitative correlation between the inhibition of polypeptide synthesis in cell free preparations and the inhibition of protein synthesis in whole cells suggests that the latter also occurs as the result of a direct pressure effect on the ribosome. This had been postulated earlier (12–14; Pope et al., submitted for publication), though a different possibility had been raised by other authors (1, 3, 4). Although each of the reactions involved in translation may exhibit a response to pressure, it remains our contention that the primary pressure-sensitive, rate limiting reaction of functional synthesizing system is one which directly involves the ribosome.

A rather striking parallel between the effects of temperature and pressure on protein synthesis is seen by a comparison of the data in Fig. 2, 3 and 4. Protein synthesizing systems containing *E. coli* ribosomes show breaks in both the protein synthesis versus temperature and protein synthesis versus pressure curves, whereas systems containing *P. fluorescens* ribosomes show no such breaks, i.e., there is no increase in the rate of protein synthesis at temperatures above 25 C and no stimulation by low pressures as seen with *E. coli* systems.

The stimulation of synthesis in *E. coli* systems at low pressures (up to 300 atm) is difficult to explain without invoking the possibility of an effect on at least two simultaneously occurring reactions (6). The $\Delta V^*$ values (Table 2) are calculated from the data above 300 atm where one may consider possible first order kinetics. These data would seem to indicate a basic distinction between *E. coli* and *P. fluorescens* ribosomes which allows for different functional capability under varied conditions of temperature and pressure. If the application of reaction rate theory to the data is valid on the assumption of a pressure effect on a single rate limiting reaction, the $\Delta V^*$ calculations indicate that formation of some activated complex involving the *P. fluorescens* ribosome entails a distinctly smaller volume increase than that involving the *E. coli* ribosome. In theory, the binding of aa-tRNA to the ribosome-messenger RNA complex or the process of translocation could entail configurational changes resulting in the $\Delta V^*$ indicated.

One of our most interesting observations was that the pressure tolerance of protein synthesis exhibited by whole cells of the deep sea organism, *P. bathycetes*, decreased markedly in polyuridylic acid-directed cell free synthesizing systems. It should be noted, however, that the preparation procedure for this system was very much like that for *E. coli*. The concentrations of various ions ($Mg^{2+}$, $Ca^{2+}$, $Na^+$, $K^+$) reflected the levels present in *E. coli* cell free preparation medium rather than those significantly higher levels of *P. bathycetes* growth medium. The effects of higher specific ion concentrations on the pressure sensitivity of this system are currently being investigated.

Finally, the results with *P. fluorescens* indicate that resistance to high pressure is not a characteristic limited to deep sea bacteria.

TABLE 2. *Approximate volume change of activation values (cm³/mol) for whole cell, cell free, and hybrid protein synthesizing systems at various temperatures*[a]

| System | 15 C | 25 C | 37 C |
|---|---|---|---|
| *E. coli* | | | |
| Whole cells | 110 | 50 | 100 |
| Cell free | 100 | 50 | 100 |
| *P. fluorescens* | | | |
| Whole cells | 40 | 40 | |
| Cell free | 40 | 40 | |
| *P. bathycetes* | | | |
| Whole cells | 60 | 50 | |
| Cell free | 85 | 150 | 200 |
| Hybrid systems | | | |
| *E. coli* ribosomes + *P. fluorescens* S-100 | 100 | 55 | |
| *E. coli* S-100 + *P. fluorescens* ribosomes | 40 | 40 | |

[a] Calculated from the data in Fig. 1, 2, and 3.

Such resistance could be a characteristic of pseudomonads as a class or be associated with bacteria capable of growth at lower temperatures, since both of the pseudomonads examined are pressure resistant and both are capable of growth at lower temperatures than *E. coli*.

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