THE EFFECTS OF HYDROSTATIC PRESSURE ON PROTEIN SYNTHESIS IN *ESCHERICHIA COLI*

by

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ABSTRACT

Hydrostatic pressure has been shown to inhibit protein synthesis in Escherichia coli by reducing the active transport of amino acid into whole cells and to inhibit amino acid activation and polypeptide synthesis in cell-free systems. Pressure decreases translational ambiguity by suppressing the non-specific activity of phenylalanyl-tRNA synthetase and by preferentially decreasing the stability of leucyl-tRNA.

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INTRODUCTION

Hydrostatic pressure is an important physical feature of the marine environment, where pressure increases from 1 atm at the ocean surface to approximately 1150 atm in the greatest depths (7). The effects of this range of pressures on the growth of marine and terrestrial microbes have been determined, and these organisms have been classified according to their tolerances (34, 35). Firstly, there are the barophobes, which are organisms unable to grow at pressures greater than 400 to 600 atm. Secondly, there are the barophiles, organisms which require pressures of 500 to 1100 atm for growth and survival. Thirdly, a very few species of baroduric bacteria have been reported to grow over the entire pressure range of 1 to 1100 atm.

Hydrostatic pressure is believed to suppress the growth of barophobic bacteria in a variety of ways. The data presently available suggest that the most important of these are the following. Firstly, pressure appears to reduce the active transport of nutrients and to diminish cell permeability (26). Secondly, pressure is known to inhibit macromolecular synthesis (1, 2, 16-18, 28, 32), and in particular, to inhibit protein synthesis to a greater extent than either DNA or RNA synthesis (1, 2, 18, 33). Thirdly, through its influence on molecular volume changes, pressure interferes with cellular metabolism by altering the rates of activity of various anabolic and catabolic enzymes (9, 12, 13, 15, 22-24, 27). The studies reported here deal with aspects of the first and second processes as they relate to protein synthesis. The experimental organism was the

terrestrial barophobic bacterium Escherichia coli B/r, which grows at pressures of 1 to 500 atm.

Protein synthesis by intact bacterial cells is dependent, at least in part, on exogenous sources of amino acids. Evidence of significant inhibition at the level of active transport could provide, therefore, an important segment of the total explanation of the effects of pressure on protein synthesis. Moreover, if pressure were demonstrated to inactivate totally the transport mechanisms required by the cell to obtain polypeptide precursors, there would be less justification to proceed further into an investigation of pressure effects on subsequent events in protein synthesis. Accordingly, active uptake of L-phenylalanine by whole cells of E. coli was studied, at pressures of 1 to 600 atm.

Recent studies have shown, nonetheless, that pressure inhibits cell-free protein synthesis during the translation stages (6). The binding of aminoacyl-tRNA to ribosomes, the formation of the peptide bond, and the elongation of the peptide chain appear, in particular, to be affected. These data suggested two ways in which the effects of pressure on translation might be even more extensive than was previously supposed. Firstly, the other stages in the synthetic process might also be affected, and secondly, the fidelity of translation of the genetic code could be reduced. The products of such ambiguous translation would likely be partially-functional or non-functional polypeptides. This could explain, at least in part, the decrease in cell viability known to occur under elevated pressures (1, 35).

In order to explore these possibilities, various cell-free systems

for protein synthesis were prepared, for the study of individual steps. Pressure was applied to determine the extent to which each such process was inhibited, and to estimate the extent of pressure-induced translational ambiguity, if any. The processes so studied were the following: (1) synthesis of polypeptides from amino acids, (2) synthesis of polypeptides from aminoacyl-tRNA's, and (3) formation of aminoacyl-tRNA. The results of these studies suggested a concluding set of experiments in which the stability of aminoacyl-tRNA under pressure was investigated.

MATERIALS AND METHODS

Cultures and Media

Stock cultures of Escherichia coli B/r were maintained on Trypticase-soy (BBL) agar slants at 17C and were transferred monthly.

In all cases, cells were grown in the medium of Littauer and Eisenberg (19), having the following composition: yeast extract (Fisher), 1%; K_2HPO_4, 2.8%; KH_2PO_4, 1.7%; glucose, 1%; pH 6.8.

Growth and Preparation of Cells for Uptake Studies

A 30 ml overnight culture grown at 20C was inoculated into 300 ml of fresh medium and grown at the same temperature for an additional two hours. Cells were harvested by centrifugation at 10,000 x g for 10 min, resuspended in Buffer A (0.22 M potassium phosphate, pH 6.8) and sedimented again. After one additional wash, the final resuspension was made in the same buffer. Cell concentration was determined directly with a Petroff-Hausser counting chamber and a final dilution of 6.0×10^5 cells/ml was prepared using additional buffer. This result was verified by a viable cell count using the spread plate technique.

Growth and Preparation of Bacteria for Cell-Free Extracts

Cells were grown at 33C by inoculation of 11 litres of fresh medium with 1 litre of an overnight culture. The 12 litre culture was vigorously stirred and generously aerated and was harvested in early log phase by

continuous-flow centrifugation. Cells were washed twice in Buffer B (0.01 M Tris-HCl, pH 7.5; 0.01 M $Mg(C_2H_3O_2)_2$; 0.006 M $HSCH_2CH_2OH$) and stored as a pellet at -20C for a period not exceeding three days.

All biochemicals used in the following procedures were purchased from Sigma unless otherwise specified, and all procedures were performed at 4C unless otherwise indicated.

Preparation of Incubated S-30 Extract

Freshly thawed cells were disrupted by grinding with alumina by the method of Allende et al (3) and extracted in Buffer C (0.01 M Tris-HCl, pH 7.8; 0.014 M $Mg(C_2H_3O_2)_2$; 0.06 M KCl; 0.006 M $HSCH_2CH_2OH$). The homogenate was centrifuged at 20,000 x g for 20 min and an incubated S-30 extract was prepared from the S-20 supernatant by the method of Nirenberg (25). DNase-treated S-20 supernatant was centrifuged twice at 30,000 x g for 30 min. The upper four-fifths of the final S-30 supernatant was removed by aspiration and incubated at 37C with GTP, ATP, phosphoenolpyruvate, pyruvate kinase and 20 amino acids, to deplete endogenous mRNA. After overnight dialysis against 120 vol Buffer C (with one change of buffer midway), the extract was frozen in liquid nitrogen.

Isolation of Ribosomes and S-150 Supernatant

Ribosomes were isolated by centrifugation of freshly prepared incubated S-30 extract at 150,000 x g for 120 min and the supernatant (S-150) was removed and frozen separately at -20C. The ribosomes were resuspended in Buffer D (0.1 M Tris-HCl, pH 7.8; 0.5 M NH_4Cl; 0.01 M $MgCl_2$; 0.006 M

$HSCH_2CH_2OH$) and then sedimented and resuspended two additional times before a final resuspension in Buffer C. Ribosomes were stored in liquid nitrogen.

The concentration of protein in the incubated S-30 extract, the ribosomal suspension, and the S-150 supernatant was determined in each case by the method of Lowry et al (20), with bovine serum albumin as the standard.

Preparation of Phenylalanyl-tRNA Synthetase

Cells were disrupted as above and a partially purified preparation of phenylalanyl-tRNA synthetase was made essentially by the method of Stulberg (30). Alumina-ground cells were resuspended in 2 ml Buffer E (0.01 M Tris-HCl, pH 7.5; 0.01 M $Mg(C_2H_3O_2)_2$; 0.001 M $HSCH_2CH_2OH$) per g of cells (wet weight) and centrifuged at 20,000 x g for 20 min. The pellet was discarded and the supernatant was diluted by further addition of 1.0 ml of Buffer E per g of cells (original weight). The resulting suspension was centrifuged at 14,600 x g for 120 min and the pellet was discarded. The supernatant was centrifuged at 105,000 x g for 120 min and the pellet was discarded. The S-105 supernatant was adjusted to 20% glycerol (v/v) before storage at -30C.

Aliquots of frozen S-150 supernatant were thawed and treated in the following manner. Nucleic acids were removed by dropwise addition of 0.1 vol of 10% streptomycin sulfate with constant stirring. The suspension was centrifuged at 30,000 x g for 30 min and the precipitate was discarded. The supernatant was dialyzed overnight against 100 vol of Buffer F (0.05 M

potassium phosphate, pH 7.5; 0.01 M $HSCH_2CH_2OH$; 0.001 M EDTA), with one change of buffer midway.

A 41-51% ammonium sulfate fraction was prepared from the dialyzed streptomycin sulfate fraction and the final precipitate, dissolved in a minimum volume of Buffer F, was dialyzed overnight against the same buffer in the manner indicated above.

A 0.5 ml aliquot of the dialyzed solution (containing 10 mg/ml protein) was loaded on a 1.6 x 20 cm column containing DEAE-Sephadex (A-25) previously equilibrated in Buffer F, and the sample was eluted with a 200 ml linear gradient of increasing potassium phosphate concentration (0.05 M to 0.25 M) and decreasing pH (7.5 to 6.5) containing 0.01 M $HSCH_2CH_2OH$ and 0.001 M EDTA. All chromatographic fractions were adjusted to 20% glycerol (v/v) and 10 µmoles of $HSCH_2CH_2OH$ were added per ml before storage at 4C. Active fractions were pooled and dialyzed against Buffer G (0.05 M potassium phosphate, pH 7.0; 0.01 M $HSCH_2CH_2OH$; 0.001 M EDTA). The dialyzed solution was adjusted to 75% $(NH_4)_2SO_4$ by slow addition of the crystalline salt. After centrifugation at 30,000 x g for 30 min, the precipitate was dissolved in a minimum volume of Buffer H (0.05 M potassium phosphate, pH 7.0; 0.01 M $HSCH_2CH_2OH$), and dialyzed against the same buffer. The dialyzed solution was then stored at 4C as the source of phenylalanyl-tRNA synthetase. Protein concentration was determined by measurement of absorbance at 280 nm and reference to a standard curve for bovine serum albumin.

Assay for Uptake of Phenylalanine into Whole Cells Under Pressure

Uptake of ^{14}C-phenylalanine was determined by a modification of the method of Paul and Morita (26). L-Phenylalanine-^{14}C (U) (Amersham/Searle,

492 mC/mM) was added to a final concentration of 0.02 μC/ml and 0.0074 μg/ml to the suspension of washed cells. Duplicate 5 ml samples were drawn into sterile plastic syringes, pressurized in an apparatus similar to that described by ZoBell and Oppenheimer (36), and incubated under pressure for 60 min at 20C. At the end of this period, samples were transferred into 25 ml Erlenmeyer flasks stoppered with rubber serum caps, by injection through the rubber septum. Each flask contained 0.2 ml 2N H_2SO_4 to fix the cells and release CO_2, and each serum cap was equipped with a plastic bucket containing a piece of fluted Whatman No. 1 filter paper (25 mm x 50 mm). Shortly after addition of the sample, the filter paper was soaked with 0.2 ml phenethylamine introduced through the cap by means of a syringe. The flasks were incubated with reciprocal shaking for 60 min in a water bath at 20C. The filter papers were removed and transferred without delay to 12 ml of a toluene-based scintillation fluid (LSF-1) containing 2,5-diphenyloxazole (0.4%) and 1,4-bis-(5-phenyloxazolyl-2)-benzene (0.01%). Vial contents were filtered across HA Millipore filters (0.45 μ pore size, 25 mm diameter) and the filters were washed with two 10 ml aliquots of Buffer A, dried at 60-65C for 20 min and then immersed in 12 ml of LSF-1.

Control samples were prepared by fixing the cells with 2 ml 2N H_2SO_4 prior to addition of labelled phenylalanine. These were treated thereafter in a manner identical to that for the experimental samples, with the exception that further addition of H_2SO_4 was not required. Although the radioactivity observed in these samples indicated a low level of non-metabolic uptake (less than 3% of the value for active uptake),

the experimental data were corrected accordingly.

Assay for Cell-Free Polypeptide Synthesis from Amino Acid

Incorporation of ^{14}C-phenylalanine or ^{14}C-leucine was measured by a modification of the method of Nirenberg (25). The composition of the reaction mixture (0.87 ml in volume) was the following (in micromoles per ml unless otherwise specified): Tris-HCl, pH 7.8, 100; $Mg(C_2H_3O_2)_2$, 13; KCl, 55; $HSCH_2CH_2OH$, 5.8; either L-Phenylalanine-^{14}C (U) or L-Leucine-^{14}C (U) (both Amersham/Searle, 10 mC/mM), 0.058; a mixture of nineteen ^{12}C-amino acids, omitting the amino acid present in the radioactive form, 0.058 of each amino acid; ATP, 0.29; GTP, 0.0086; PEP, 2.2; pyruvate kinase, 5.0 μg; poly U, 20 μg; incubated S-30 protein, 2.0 mg..

Reaction tubes were kept on ice and poly U was added last to initiate the reaction. All experiments were performed at 20C unless otherwise specified using a uniform 20 min incubation period. Reactions were terminated by transferring the mixtures to 3 ml ice cold 10% TCA (containing 4 mg/ml of the unlabelled amino acid corresponding to the one present in the radioactive form) and then heated at 90-95C for 25 min to hydrolyze aminoacyl-tRNA. After 30 min on ice, the precipitates were collected on Whatman GF/C glass fibre filters. These were washed with five aliquots of ice cold 5% TCA, dried at 60-65C for 15 min, and then immersed in 5 ml of LSF-2, a commercially prepared xylene-based scintillation fluid (Aquasol, New England Nuclear).

Assay for Cell-Free Polypeptide Synthesis from Aminoacyl-tRNA

The reaction mixture (total volume 0.87 ml) contained the following (in micromoles per ml unless otherwise specified): Tris-HCl, pH 7.7, 100; $MgCl_2$, 12; KCl, 80; NH_4Cl, 80; $HSCH_2CH_2OH$, 12; GTP, 0.1; either L-Phenylalanine-^{14}C (U)-tRNA (New England Nuclear, 0.171 μC/mg) or L-Leucyl-^{14}C (U)-tRNA (New England Nuclear, 0.355 μC/mg), 50 μg; poly U, 5.0 μg; S-150 supernatant protein, 50 μg; ribosomes, 100 μg.

Reaction mixtures were treated exactly as were those in the assay for incorporation of amino acid, with the exception that in this case filters were immersed in 15 ml of LSF-1.

Assay for Formation of Aminoacyl-tRNA

Formation of aminoacyl-tRNA was measured by a modification of the method of Stulberg (30). The reaction mixture (0.87 ml) contained the following (in micromoles per ml unless otherwise noted): Tris-HCl, pH 8.0, 100; $Mg(C_2H_3O_2)_2$, 10; KCl, 5; ATP, 2; $HSCH_2CH_2OH$, 2; either L-Phenylalanine-^{14}C (U) or L-Leucine-^{14}C (U) (both Amersham/Searle, 10 mC/mM), 10; E. coli B transfer RNA (Calbiochem, Grade B), 1.0 mg; enzyme protein, 10 μg.

Mixtures were kept on ice and the reaction was initiated by addition of the enzyme. After 5 min incubation at 20C, reactions were terminated by transferring tube contents to 3 ml ice cold 10% TCA containing 4 mg/ml unlabelled amino acid in the manner described above. These mixtures were kept on ice for 10-20 min before precipitates were collected on Whatman

GF/C filters. These were washed with 3 aliquots of ice cold 5% TCA, dried at 60-65C for 15 min, and then immersed in 5 ml of LSF-2.

Assay for Stability of Aminoacyl-tRNA

The reaction mixture (0.87 ml) had the following composition (in micromoles per ml unless otherwise noted): Tris-HCl, pH 8.0, 100; $Mg(C_2H_3O_2)_2$, 10; KCl, 5; either L-Phenylalanyl-^{14}C (U)-tRNA (New England Nuclear, 0.156 μC/mg) or L-Leucyl-^{14}C (U)-tRNA (New England Nuclear, 0.355 μC/mg), 50 μg; ^{12}C-phenylalanine or ^{12}C-leucine in an amount equivalent to the amount of radioactive amino acid present as ^{14}C-aminoacyl-tRNA, unless otherwise noted.

Reaction mixtures were kept on ice and aminoacyl-tRNA was added just prior to commencement of the incubation period. Samples were incubated at 20C for 20 min unless otherwise specified, and then transferred to 3 ml ice cold 10% TCA containing unlabelled amino acid in the manner indicated above. After 10-20 min on ice, these samples were treated in exactly the same way as those for the assay of formation of ^{14}C-aminoacyl-tRNA.

Assay for Polypeptide Synthesis, Aminoacyl-tRNA Formation, and Stability of Aminoacyl-tRNA Under Pressure

Reaction mixtures were contained in 6 x 15 mm stoppered culture tubes and were pressurized in the manner described above. Since reactions could neither be initiated nor terminated under pressure, the appropriate corrections for incorporation of amino acid, for incorporation of aminoacyl-

tRNA, for synthesis of aminoacyl-tRNA, or for the degradation of aminoacyl-tRNA which took place before and after pressurization, were made in each experiment.

Radioactivity was determined in all instances with a Beckman LS-250 spectrometer. Quench corrections were made by reference to an external standard. All data reported are the mean values of two to six replicates.

RESULTS AND DISCUSSION

Effects of Pressure on Uptake of ^{14}C-Phenylalanine

The effects of hydrostatic pressure on uptake of ^{14}C-phenylalanine by E. coli are illustrated in Fig. 1, which shows that a marked inhibition of active transport occurred even at moderate pressures (100 to 300 atm). Beyond 400 atm, the uptake process proceeded at a minimal level and was totally inhibited at 600 atm. Since no respiration of amino acid took place at any pressure (as evidenced by the absence of radioactivity in the Whatman filter samples) these data are based upon the radioactivity incorporated into and retained by the cells during the incubation period.

Similar results have been published by Paul and Morita (26), who demonstrated that uptake activity in the marine psychrophile MP-38 at 500 atm was less than 10% of the value observed at 1 atm, for four amino acids. Conflicting data have been reported by Schwarz and Landau (29), who claimed that no inhibition of transport of amino acids or reduction in cellular permeability occurred at 670 atm. However, it is difficult to view their work as conclusive, for two reasons. Firstly, uptake was measured at only one elevated pressure. Secondly, and much more importantly, their conclusions are based upon observations in which radioactive substrate incorporated into cells apparently represented no more than 0.00004% of the total labelled material originally added.

Hence the initial effect of pressure on bacterial cells appears to be an inhibition of the transport of nutrients to the intracellular environment. The extent of inhibition is such that the reduction of macromolecular synthesis observed with increasing pressures is likely the result, at

least in part, of decreasing availability of the necessary substrates and precursors. Nonetheless, transport is not inhibited completely until fairly severe pressures (600 atm and greater) are applied. Therefore the synthetic processes within the cell may continue to function, although at reduced rates. Pressure may exert an effect on these independent of its influence on transport. Several such effects on the synthesis of protein in cell-free systems are discussed below.

Effects of Pressure on Polypeptide Synthesis from Amino Acids

The characteristics of this cell-free system for protein synthesis were investigated first, by monitoring incorporation of ^{14}C-phenylalanine and incorporation of ^{14}C-leucine in separate poly U-directed systems at 37C and 20C at 1 atm. The results of these experiments are shown in Fig. 2 and Fig. 3, respectively. Both amino acids were incorporated at both temperatures, a result which was expected, since the insertion of leucine in place of phenylalanine in poly U-directed polypeptide synthesis has been identified as the most common example of ambiguity in cell-free protein synthesis (31). Levels of leucine incorporation are obviously lower than levels of phenylalanine incorporation, also an expected result. Although the rate of incorporation at 37C exceeds the rate at 20C, the latter temperature was selected so that results could be correlated more readily with closely related studies already completed in this laboratory (6). An incubation period of 20 min was chosen to approximate the initial rate of protein synthesis.

The effects of pressure on incorporation of ^{14}C-phenylalanine and

^{14}C-leucine are shown in Fig. 4. Application of moderate pressures (100 to 300 atm) effected an immediate reduction in incorporation of phenylalanine, with no synthesis of poly phenylalanine occurring at 600 atm or greater. The effects of pressure on incorporation of ^{14}C-leucine indicated that pressures of 100-300 atm inhibited leucine incorporation even more extensively than phenylalanine incorporation, such that no synthesis of poly leucine could be detected at pressures of 400 atm or greater. At this point it was decided that a comparison between the effects of pressure and those of temperature should be made. The results of the temperature experiment (Fig. 5) indicated that an increase in temperature from 20C to 37C resulted in a marked stimulation of phenylalanine incorporation; but only a moderate stimulation of leucine incorporation. Conversely, it might be said that a reduction in temperature appeared to increase the frequency of coding errors, an effect which has been observed previously (8, 31). Temperature and pressure data are summarized in Fig. 6, where the graph of the ratio of leucine:phenylalanine incorporated under increasing pressure can be compared with the graph of the same ratio for increasing temperature. The observed similarity seems to indicate that although hydrostatic pressure progressively inhibits net amino acid incorporation, it may also function to reduce the frequency of coding errors (as evidenced by a reduction in the ratio of leucine:phenylalanine), in much the same way that an increase in temperature does.

Pressure Effects on Polypeptide Synthesis from Aminoacyl-tRNA

Moderate pressures of 100 to 300 atm exerted little inhibitory effect

on the incorporation of phenylalanyl-tRNA (Fig. 7). At 400 atm, however, incorporation was sharply reduced and was totally inhibited at 600 atm or greater. These results are in contrast with those from the amino acid system, and the differences are summarized in Table I. The amino acid system responded immediately to the application of pressure, through a sharp reduction in incorporation. The aminoacyl-tRNA system appears to have been more resistant in the same initial range of pressures, although both systems eventually displayed minimum values at 400 and 500 atm which were quite similar. These results suggested that the final steps in protein synthesis, namely the binding of aminoacyl-tRNA to the ribosome and the subsequent formation of the peptide bond are, in fact, more pressure-resistant than is the first step, namely the activation of amino acid to form aminoacyl-tRNA. This appeared to be the case, since amino acid incorporation under pressure proceeded more readily when the amino acid units were presented to the system in the form of aminoacyl-tRNA, rather than in the unactivated form. Consequently, this possibility was explored further in the next section of this work, by testing the effects of pressure on the activity of an aminoacyl-tRNA synthetase in vitro.

No evidence of coding ambiguity could be detected in this experiment (Fig. 7) since it was not possible to detect poly U-directed insertion of leucine into polypeptide from leucyl-tRNA in place of phenylalanine from phenylalanyl-tRNA. Such was the case at 1 atm, and incubation under elevated pressures produced the same results.

Pressure Effects on Formation of Aminoacyl-tRNA

The effects of pressure on the formation of phenylalanyl-tRNA and

leucyl-tRNA by a partially purified preparation of phenylalanyl-tRNA synthetase were examined, and the results are presented in Fig. 8.

Pressure exerted a strongly inhibitory effect on the formation of phenylalanyl-tRNA: synthesis of this intermediate decreased rapidly with application of pressure, and did not take place at 800 atm or greater. Therefore the differences previously observed between the incorporation of phenylalanine into polypeptide and the incorporation of phenylalanyl-tRNA may be explained, at least to some extent, on the basis of the sensitivity of the aminoacylation process to pressure. Polypeptide synthesis proceeds more rapidly from phenylalanyl-tRNA than from phenylalanine since in the former case the inhibitory effect of pressure on phenylalanyl-tRNA synthetase is avoided.

It is noted that the opposite result was obtained by Schwarz and Landau (29), using an in vivo system to assay for formation of aminoacyl-tRNA. Correlation of their results with those presented here is difficult, for the following reasons. Their experiments were done at 37C, they worked with only a single elevated pressure (670 atm) and again, obtained very low incorporated radioactivity. These factors may account, at least in part, for the discrepancy between the two sets of data.

The formation of small amounts of leucyl-tRNA in the presence of this enzyme preparation appears to indicate that the specificity of the synthetase reaction is such that the formation of more than one species of aminoacyl-tRNA is permitted, at least in the cell-free state. This phenomenon has been reported previously (5). (Alternately, it is possible that the preparation used here was contaminated with small amounts of leucyl-tRNA synthetase). In any event, pressure did not promote any further

non-specific enzyme action (as measured by an increase in leucyl-tRNA formation). On the contrary, the application of pressure inhibited the formation of leucyl-tRNA even more effectively than it limited the formation of phenylalanyl-tRNA, such that no leucyl-tRNA was synthesized at pressures of 400 atm or greater.

At pressures in excess of 800 atm, it was noted in some cases that the amount of phenylalanyl-tRNA recovered was less than the amount expected to be produced during the periods preceding and following pressurization. This suggested that pressure induces dissociation or degradation of aminoacyl-tRNA. This hypothesis was tested experimentally and the results are presented in the following section.

Pressure Effects on Stability of Aminoacyl-tRNA

It was established through the following experiments that pressure reduces the stability of ^{14}C-aminoacyl-tRNA. This was measured as the loss of radioactivity in the aminoacyl-tRNA recovered from reaction mixtures after pressurization, indicating a dissociation of the labelled amino acid from the tRNA molecule.

In the initial study, dissociation of ^{14}C-phenylalanyl-tRNA and ^{14}C-leucyl-tRNA under pressure of 600 atm was observed as a function of time (Fig. 9). The results indicated that the dissociation of aminoacyl-tRNA is a progressive, time-dependent process, rather than a single, instantaneous event occurring upon the application of pressure. On the basis of these results, an incubation period of 20 min was selected for the following experiment in which the pressure-dependent dissociation of aminoacyl-tRNA was examined.

The results are shown in Fig. 10, where it is seen that the stability of both phenylalanyl-tRNA and leucyl-tRNA decreases as a function of increasing pressure. Phenylalanyl-tRNA appeared to reach a minimum level around 600 to 700 atm, but the stability of leucyl-tRNA was evidently more sensitive, as revealed by a progressive decrease in recovery up to 1000 atm.

The degree to which this dissociation is reversible was investigated by incubating ^{14}C-phenylalanyl-tRNA under pressure in the presence of several concentrations of ^{12}C-phenylalanine, as indicated in Table II. If the process were reversible, then the labelled and unlabelled species would compete for reassociation with the tRNA upon depressurization. By increasing the concentration of unlabelled amino acid, one could then presumably decrease the yield of labelled aminoacyl-tRNA. Such was probably not the case, since the recovery of labelled aminoacyl-tRNA was essentially constant, irrespective of the concentration of unlabelled phenylalanine present. The small decrease in recovery observed when the higher concentrations of ^{12}C-phenylalanine were added may indicate that the process is reversible, but only to a very slight extent.

The dissociation process was investigated further by studying the effects of ATP and phenylalanyl-tRNA synthetase, as shown in Table III. Dissociation of phenylalanyl-tRNA was in no way facilitated by the presence of ATP, the synthetase enzyme, or the two in combination. The higher rate of recovery observed when ATP and enzyme were included is likely due to aminoacylation of tRNA occurring in the period after pressurization but before termination of the reaction.

The finding that aminoacyl-tRNA is unstable under pressure has several important implications, both for the present work and for previous observations on pressure effects. The reduced rates of amino acid incorporation, aminoacyl-tRNA incorporation and formation of aminoacyl-tRNA known to occur under elevated pressures are likely due, at least in some part, to the pressure-induced dissociation of aminoacyl-tRNA. The differences in rates of incorporation and activity of phenylalanine and leucine under pressure reflect the greater vulnerability of leucyl-tRNA to this dissociation. The instability of aminoacyl-tRNA under pressure may also account for, in some degree, the results of studies of binding of aminoacyl-tRNA to ribosomes (6). The apparent reduction in binding, is probably due, in fact, to the additive effects of pressure on the binding process and on the stability of aminoacyl-tRNA itself.

TABLE I

A comparison of the incorporation of ^{14}C-phenylalanine and the incorporation of ^{14}C-phenylalanyl-tRNA into polypeptide as a function of pressure. Results are expressed as a percentage of the incorporation at 1 atm, and are derived from the data of Fig. 4 and Fig. 7.

PRESSURE (atm)	% Amino Acid Incorporated	% Aminoacyl-tRNA Incorporated
100	46.5	97.2
200	25.5	93.0
300	13.5	82.6
400	6.0	16.5
500	2.1	3.0

TABLE II

Recovery of ^{14}C-aminoacyl-tRNA after pressurization at 600 atm for 5 min as a function of the concentration of unlabelled phenylalanine present in the reaction mixture. Unlabelled amino acid was added in an amount equivalent to the labelled amino acid present, multiplied by the factor shown below. The data are expressed as a percentage of recovery relative to a control maintained at 1 atm.

Concentration of unlabelled phenylalanine (equivalents)	% Recovery of ^{14}C-aminoacyl-tRNA
0	97.8
1	97.9
2	93.8
10	94.6
50	94.0

TABLE III

Effect of ATP and phenylalanyl-tRNA synthetase on dissociation of phenylalanyl-tRNA. ATP and the enzyme were added in the same concentration as in the reaction mixture for the formation of phenylalanyl-tRNA. Incubation was for 5 min at 600 atm. The recovery of ^{14}C-phenylalanyl-tRNA from the pressurized mixture is reported as a percentage of the recovery from a control held at 1 atm.

Reaction Mixture Components	% Recovery of ^{14}C - aminoacyl-tRNA
No Additions	93.6
ATP	92.8
Synthetase Enzyme	94.7
ATP + Synthetase Enzyme	96.8

Figure 1 The effect of hydrostatic pressure on uptake of ^{14}C-phenylalanine by intact cells of *Escherichia coli* at 20C.

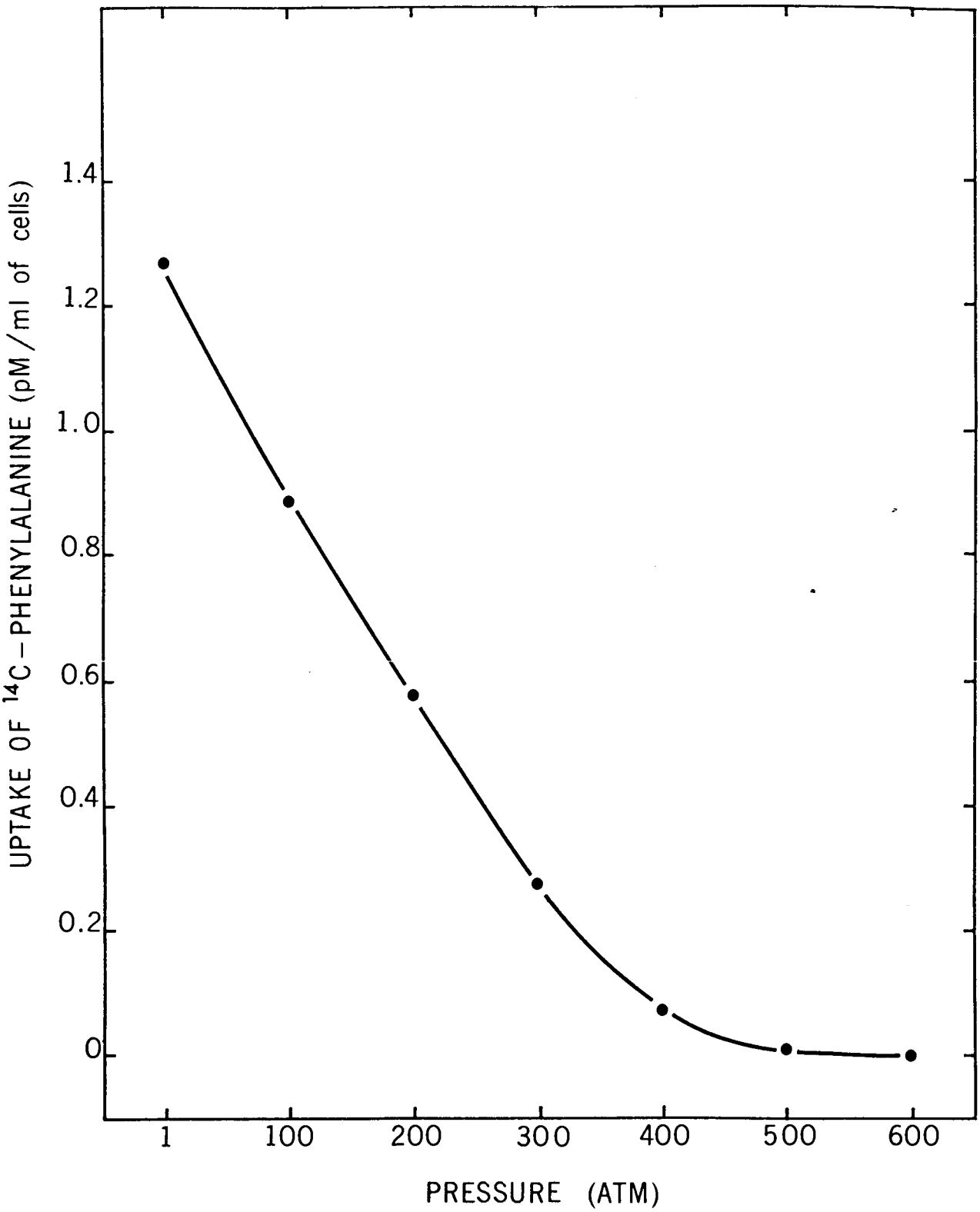

UPTAKE OF ^{14}C-PHENYLALANINE (pM/ml of cells)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1
100
200
300
400
500
600
PRESSURE (ATM)

Figure 2 Incorporation of ^{14}C-phenylalanine (●) and ^{14}C-leucine (▲) into polypeptide per reaction mixture as a function of time at 37C and 1 atm.

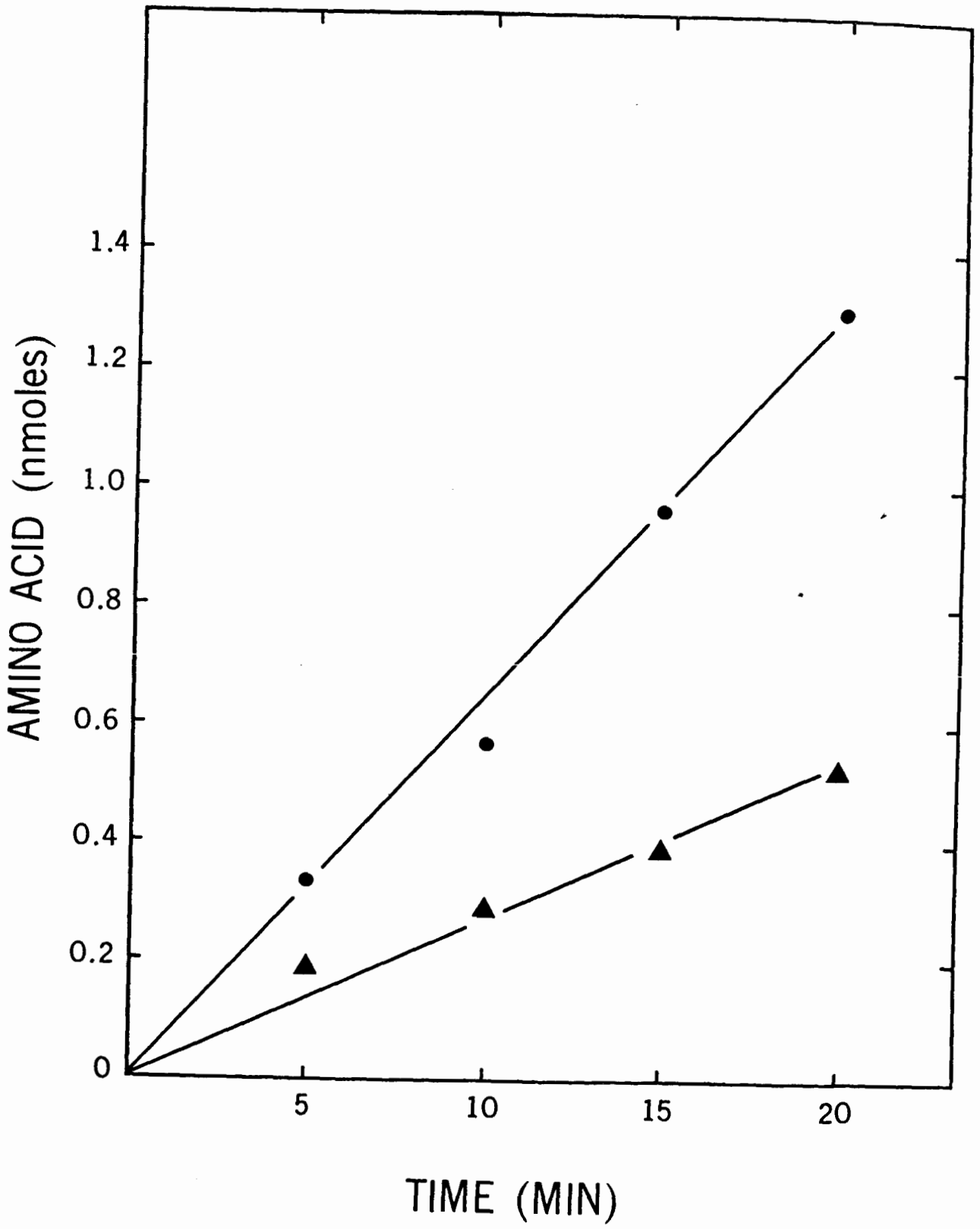

AMINO ACID (nmoles)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
5
10
15
20
TIME (MIN)

Figure 3 Incorporation of ^{14}C-phenylalanine (●) and ^{14}C-leucine (▲) into polypeptide per reaction mixture as a function of time at 20C and 1 atm.

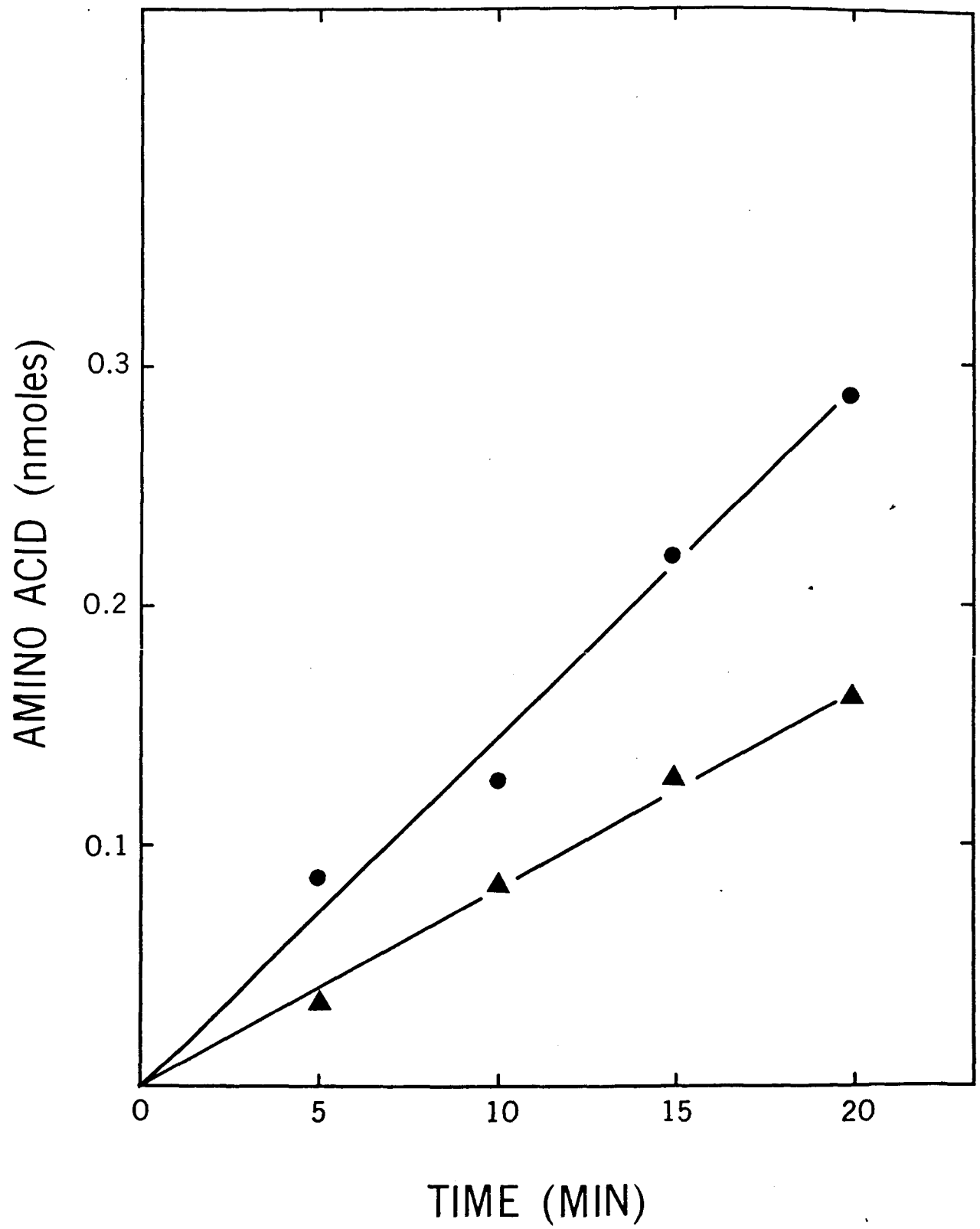

AMINO ACID (nmoles)
0.3
0.2
0.1
0
5
10
15
20
TIME (MIN)

Figure 4 Incorporation of ^{14}C-phenylalanine (●) and ^{14}C-leucine (▲) into polypeptide per reaction mixture as a function of pressure from 1 to 1000 atm.

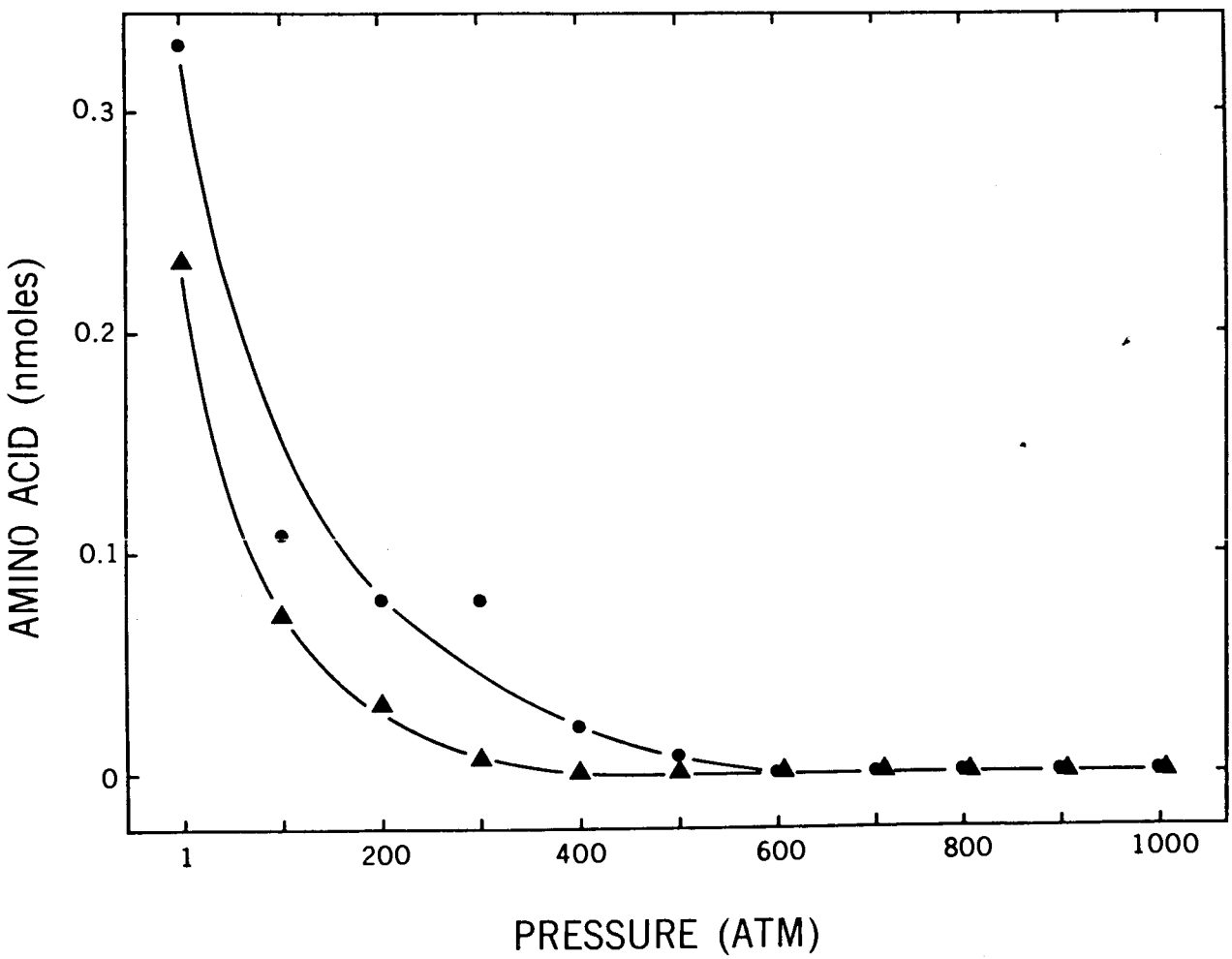

AMINO ACID (nmoles)
0.3
0.2
0.1
0
1
200
400
600
800
1000
PRESSURE (ATM)

Figure 5 Incorporation of ^{14}C-phenylalanine (●) and ^{14}C-leucine (▲) into polypeptide per reaction mixture as a function of temperature from 20C to 37C at 1 atm.

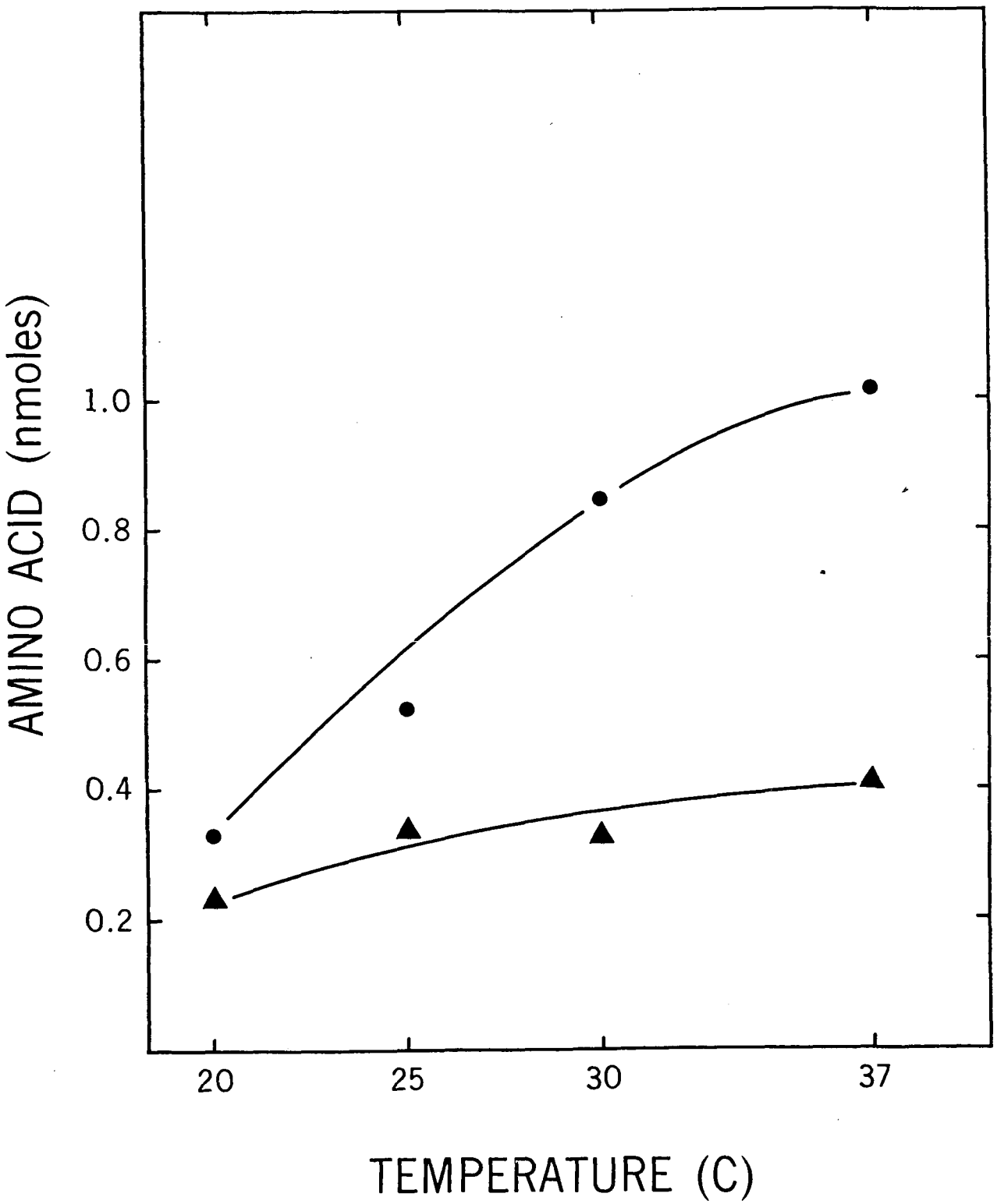

AMINO ACID (nmoles)
1.0
0.8
0.6
0.4
0.2
20
25
30
37
TEMPERATURE (C)

Figure 6 The ratio of leucine:phenylalanine incorporated as a function of temperature at 1 atm, and the ratio of leucine:phenylalanine incorporated as a function of pressure at 20C. (Data are derived from Fig. 4 and Fig. 6).

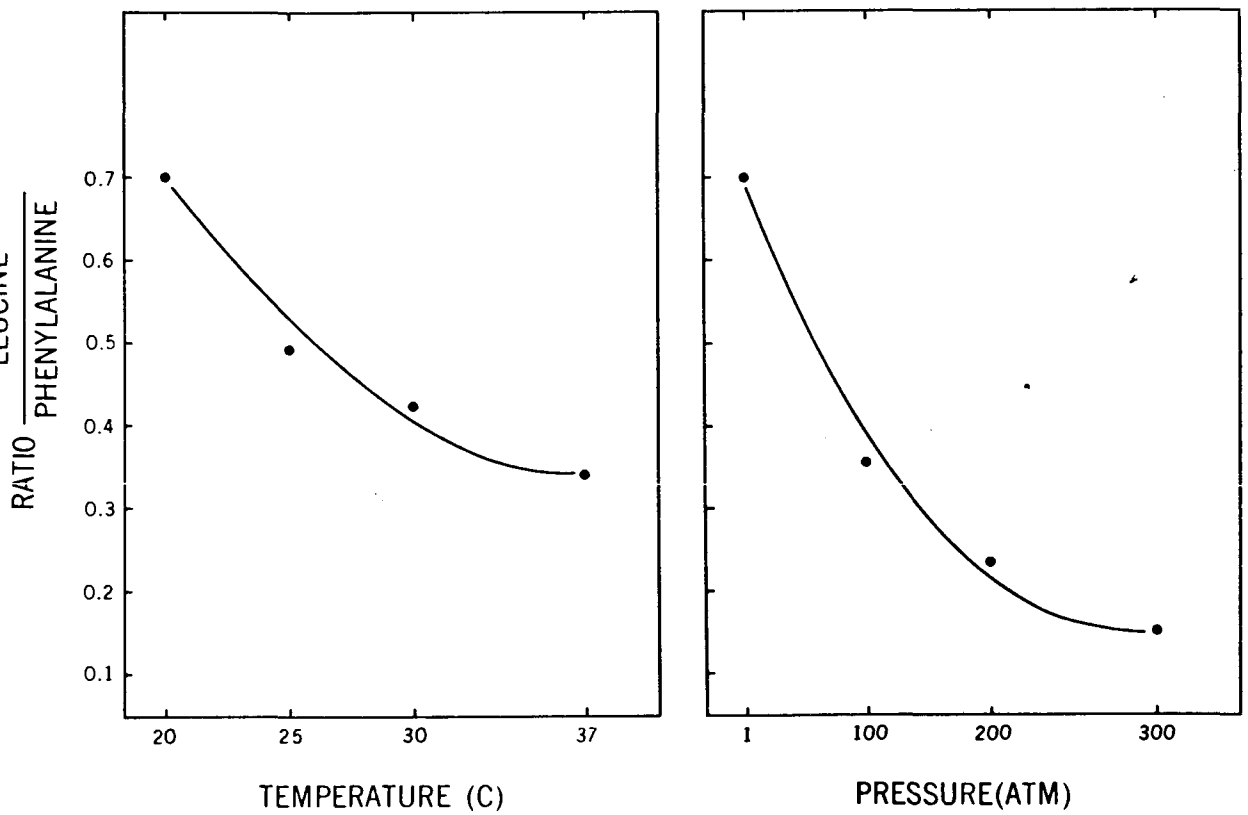

RATIO LEUCINE / PHENYLALANINE
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20
25
30
37
TEMPERATURE (C)
1
100
200
300
PRESSURE(ATM)

Figure 7 Incorporation of ^{14}C-phenylalanyl-tRNA (●) and ^{14}C-leucyl-tRNA (▲) into polypeptide per reaction mixture as a function of pressure from 1 to 1000 atm.

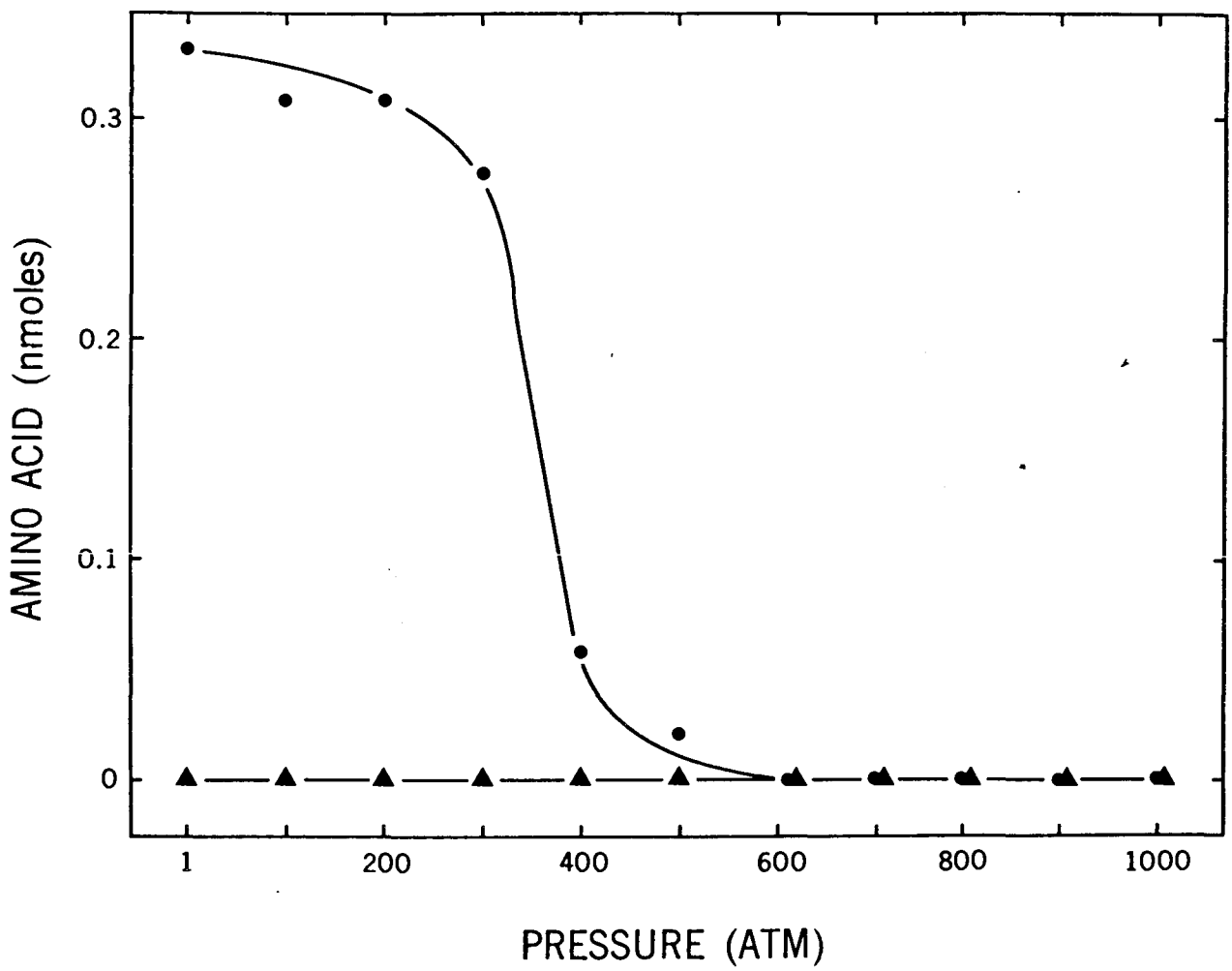

AMINO ACID (nmoles)
0.3
0.2
0.1
0
1
200
400
600
800
1000
PRESSURE (ATM)

Figure 8 Formation of ^{14}C-phenylalanyl-tRNA (●) and ^{14}C-leucyl-tRNA (▲) by partially purified phenylalanyl-tRNA synthetase as a function of pressure from 1 to 1000 atm.

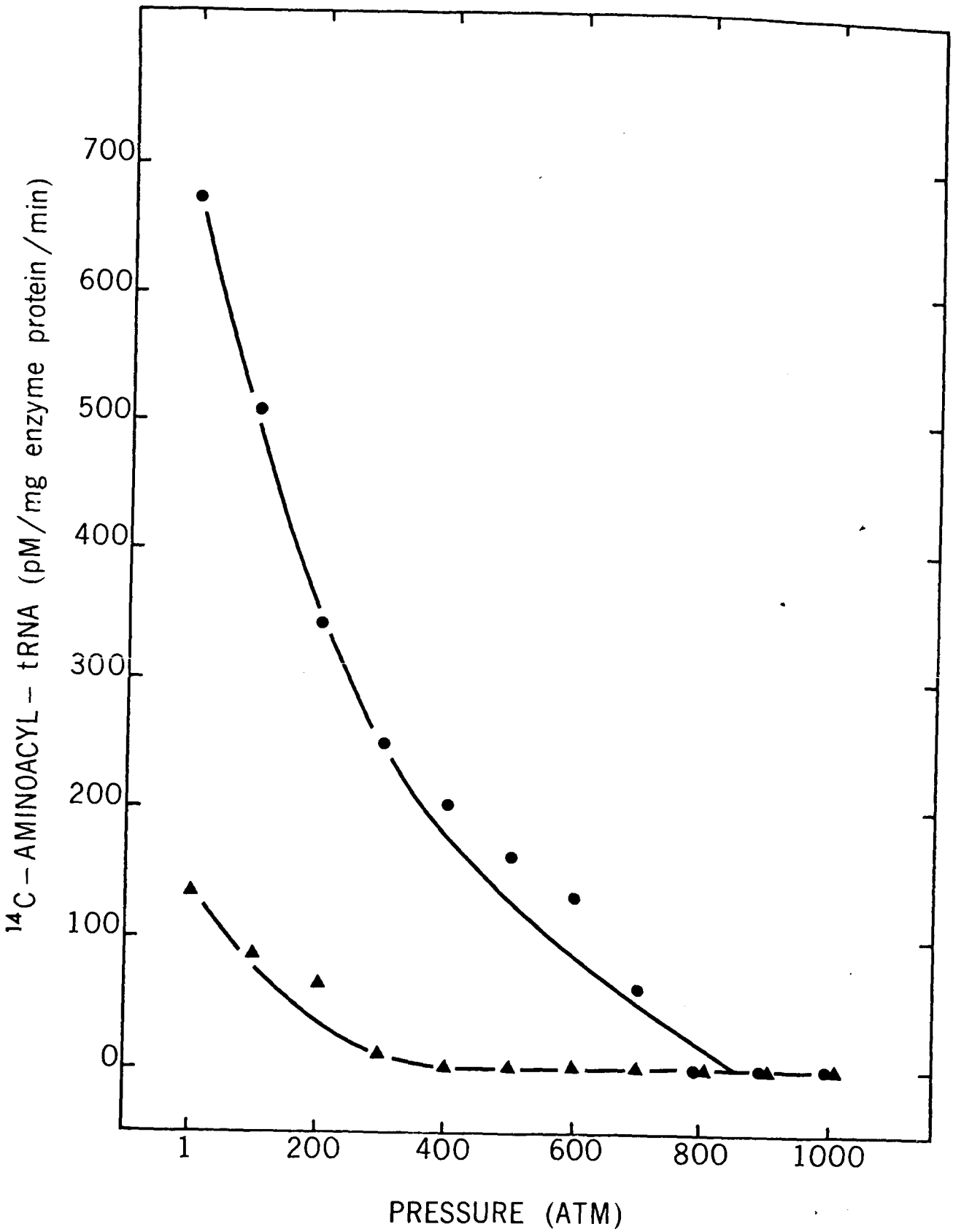

700
600
500
400
300
200
100
0
^{14}C – AMINOACYL – tRNA (pM/mg enzyme protein/min)
1
200
400
600
800
1000
PRESSURE (ATM)

Figure 9 Dissociation of ^{14}C-phenylalanyl-tRNA (●) and ^{14}C-leucyl-tRNA (▲) as a function of time at 600 atm. Values under pressure are expressed as a percentage of the value of the control held at 1 atm.

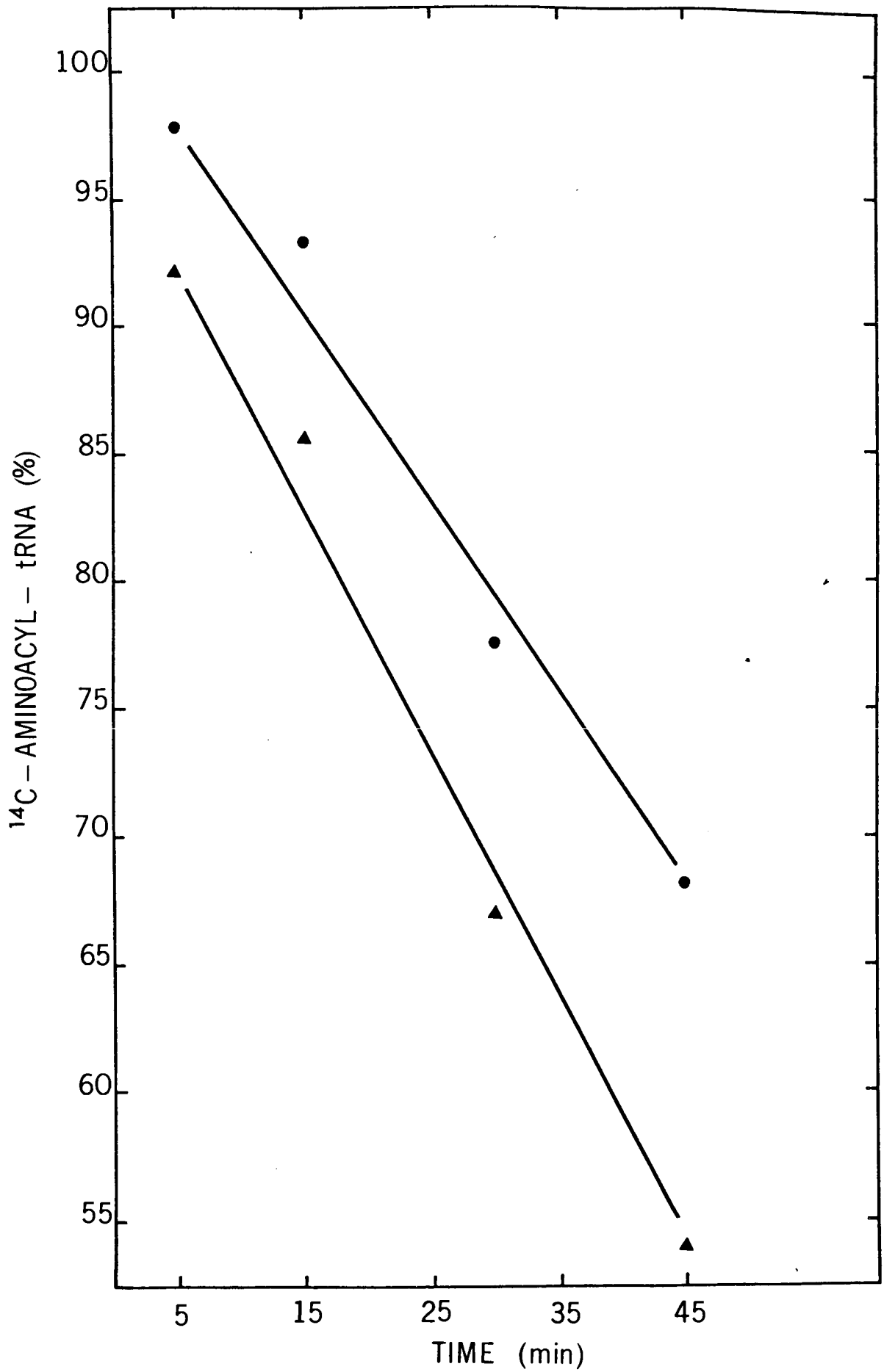
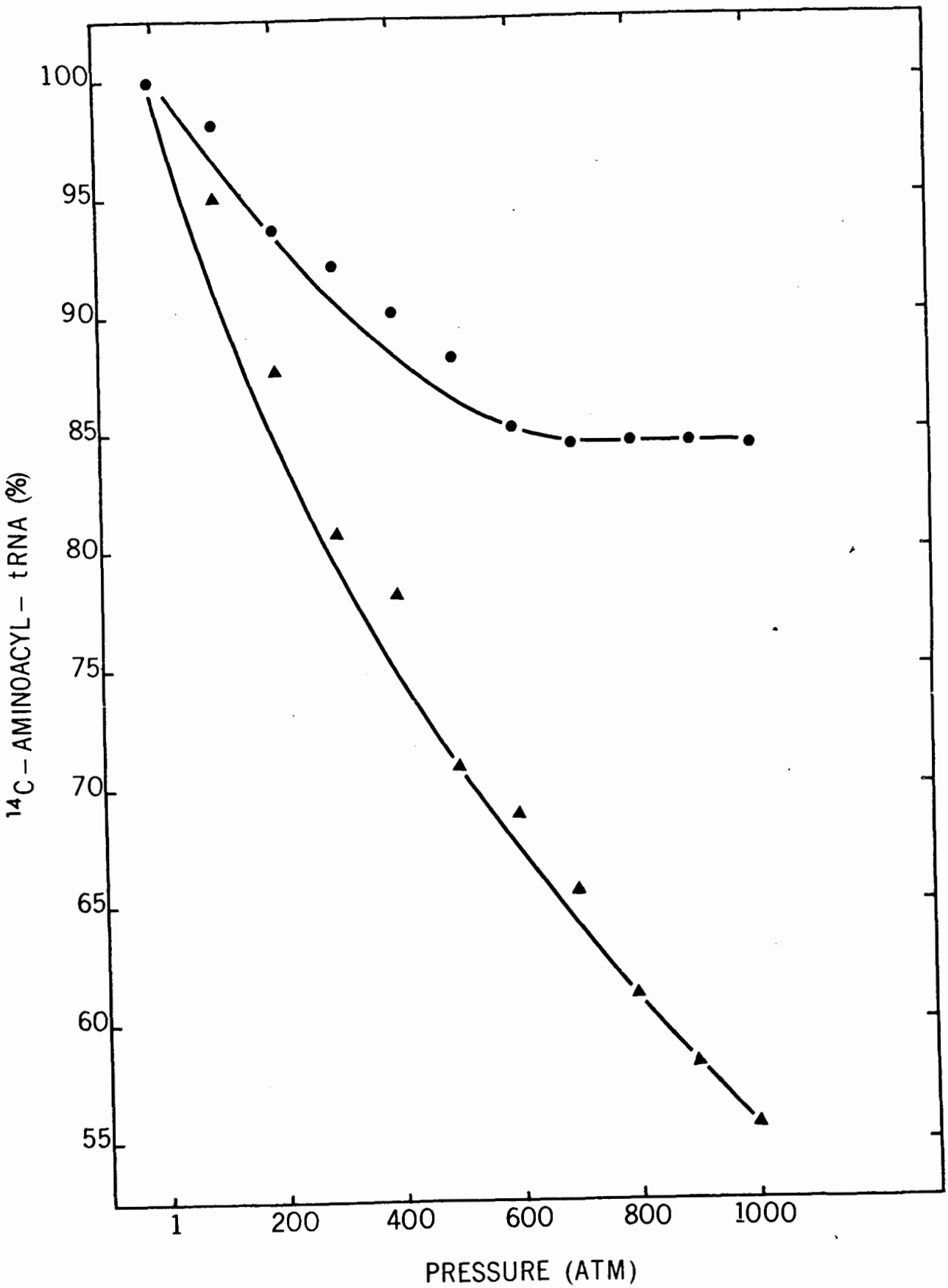

100
95
90
85
80
75
70
65
60
55
^{14}C – AMINOACYL – tRNA (%)
5
15
25
35
45
TIME (min)

Figure 10 Dissociation of ^{14}C-phenylalanyl-tRNA (●) and ^{14}C-leucyl-tRNA (▲) as a function of pressure from 1 to 1000 atm. Values under pressure are expressed as a percentage of the value of a control held at 1 atm.


100
95
90
85
80
75
70
65
60
55
^{14}C-AMINOACYL-tRNA (%)
1
200
400
600
800
1000
PRESSURE (ATM)

CONCLUSIONS

The results of these studies indicate that the known inhibitory effect of hydrostatic pressure on net protein synthesis applies in some way to each and every step in this process. Initially, the transport of amino acids into the cell is significantly reduced. The rate at which this reaction proceeds determines the rate of flow of amino acids through the subsequent steps in protein synthesis, and in that sense, limits the rate of the overall process. Nevertheless, the events which follow, namely the activation of amino acid to form aminoacyl-tRNA and the incorporation of aminoacyl-tRNA into polypeptide, are likewise inhibited. At moderately elevated pressures (100-300 atm), aminoacylation appears to be the rate-limiting step. At greater pressures (400-600 atm), pressure effects on binding of aminoacyl-tRNA to the ribosome and formation of the peptide bond (6) become manifest, and protein synthesis ceases. Therefore, at these pressures, the sensitive element in the synthetic sequence is likely the ribosome. Recent studies (10, 11, 32) have indicated that hydrostatic pressures up to 1200 atm encountered by sea urchin ribosomes during ultracentrifugation induced dissociation of 75S particles into 56S and 35S subunits. In view of the cooperative nature of ribosomal function (4, 14, 21), such a disruption would very likely preclude the possibility of any further normal ribosomal operation, and protein synthesis would cease.

Hydrostatic pressure appears to effect a decrease in coding ambiguity in the leucine-phenylalanine system in two ways. Firstly, pressure suppresses

the apparent non-specific activity of phenylalanyl-tRNA synthetase, with the result that less leucyl-tRNA is formed. Secondly, pressure preferentially decreases the stability of leucyl-tRNA, and may prevent the incorporation of the amino acid into protein in this way also.

Finally, a total understanding of the inhibitory influence of hydrostatic pressure on protein synthesis is yet to be achieved. Given the fact that the translation stage alone involves the orderly interaction of more than one hundred different macromolecules (14), it is not surprising that this is the case. But as the elucidation of the overall mechanism of protein synthesis proceeds, new techniques and new research alternatives will become available. By utilizing these, our understanding of how pressure affects protein synthesis and cell viability will be expanded, and ultimately, our knowledge of how life processes function in the deep sea will be increased.

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1. Hydrostatic pressure effects upon protein synthesis and coding ambiguity in a cell-free system from Escherichia coli B/r (with L.J. Albright). Proc. Canad. Fed. Biol. Soc. 15: 503.

2. Protein synthesis and coding ambiguity in a cell-free system from Escherichia coli B/r: a hydrostatic pressure study. (with L.J. Albright). Presented at the 32nd annual meeting of the Northwest Branch, American Society for Microbiology, June 1972, Vancouver, B.C.